Published in final edited form as:

J Chromatogr A. 2019 June 21; 1595: 39–48. doi:10.1016/j.chroma.2019.02.016.

Oligonucleotide Analysis by Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry in the Absence of Ion-Pair Reagents

Peter A Lobue, Manasses Jora, Balasubrahmanyam Addepalli, and Patrick A Limbach*

Rieveschl Laboratories for Mass Spectrometry, Department of Chemistry, University of Cincinnati, PO Box 210172, Cincinnati, Ohio 45221-0172, United States

Abstract

Improving our understanding of nucleic acids, both in biological and synthetic applications, remains a bustling area of research for both academic and industrial laboratories. As nucleic acids research evolves, so must the analytical techniques used to characterize nucleic acids. One powerful analytical technique has been coupled liquid chromatography – tandem mass spectrometry (LC-MS/MS). To date, the most successful chromatographic mode has been ion-pairing reversed-phase liquid chromatography. Hydrophilic interaction liquid chromatography (HILIC), in the absence of ion-pair reagents, has been investigated here as an alternative chromatographic approach to the analysis of oligonucleotides. By combining a mobile phase system using commonly employed in liquid chromatography-mass spectrometry (LC-MS) - i.e., water, acetonitrile, and ammonium acetate - and a new, commercially available diol-based HILIC column, high chromatographic and mass spectrometric performance for a wide range of oligonucleotides is demonstrated. Particular applications of HILIC-MS for the analysis of deoxynucleic acid (DNA) oligomers, modified and unmodified oligoribonucleotides, and phosphorothioate DNA oligonucleotides are presented. Based on the LC-MS performance, this HILIC-based approach provides an attractive, sensitive and robust alternative to prior ion-pairing dependent methods with potential utility for both qualitative and quantitative analyses of oligonucleotides without compromising chromatographic or mass spectrometric performance.

Keywords

HILIC-MS; ribonucleotides; DNA; RNA; phosphorothioate; modified nucleosides

1. Introduction

There remains an interest in the characterization of nucleic acids and their modifications in both academic and industrial laboratories. Both the natural (enzymatic) and synthetic

*To whom correspondence should be addressed. Phone (513) 556-1871, Fax (513) 556-9239, Pat.Limbach@uc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

modifications of nucleic acids continue to receive attention as details into their roles in biological systems have continued to increase [1, 2]. Two important and active areas of research include the characterization of post-transcriptional modifications to ribonucleic acid (RNA) [3] and the development of synthetic antisense and aptamer-based nucleic acids for gene silencing experiments and therapeutics [4, 5]. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been the primary technique for the direct detection of unmodified and modified oligonucleotides with various applications including the characterization of endogenous modifications [6, 7], xenobiotic modifications [8], and synthetic modifications [9, 10] of the base, ribose, or phosphodiester backbone.

Although LC-MS/MS of oligonucleotides has been a primary tool for characterization of oligonucleotides of all types, many technical hurdles persist. The gold-standard method for the chromatographic separation of oligonucleotides, developed over two decades ago [11], requires the use of ion-pair reagents (e.g., triethylamine (TEA), dibutylamine (DBA), diisopropylethylamine (DIPEA)) [12, 13] and electrospray modifier (e.g., 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP)) [14] to obtain optimal chromatographic performance and ionization [15, 16]. Ion-pairing reversed-phase liquid chromatography (IP-RP-LC) of oligonucleotides has been coupled with both ultraviolet (UV) and mass spectrometry (MS) detection [7, 17–19]. Although the use of ion-pair reagents can deliver high chromatographic performance [20, 21], MS charge state reduction [22, 23], and reduction in alkali metal adduct formation [24, 25], these advantages do not come without compromises. The use of a modifier, like HFIP, is required to overcome the reduced MS sensitivity for electrospray ionization typical with ion-pair reagents [11, 26], although high concentrations of HFIP can also lead to ion suppression [27]. It has also been shown that the choice of ion-pair reagent can affect the degree of ion suppression and that the optimal ion-pair reagent and modifier system can depend on the type and content of the oligonucleotide [28], requiring extensive screening for optimized chromatographic and ionization conditions. It is also common practice to have dedicated systems for IP-RP-LC due to contamination concerns that can result in the suppression of ionization for samples analyzed in positive polarity.

Alternatives to the traditional IP-RP-LC of oligonucleotides have been explored over the years, including capillary gel electrophoresis [29, 30], size exclusion [31, 32], anion exchange [30, 33, 34], mixed-mode [35], monolithic columns [36, 37], non-porous polymeric particles [38–40], phenyl, fluorophenyl and cyano stationary phases [13, 41, 42], and various combinations of these techniques in multi-dimensional approaches [34, 43]. A promising alternative to IP-RP for LC-MS analysis is hydrophilic interaction liquid chromatography (HILIC) [44]. Instead of the hydrophobic stationary phase employed in IP-RP-LC, HILIC stationary phases consist of a polar (hydrophilic) stationary phase where compounds are eluted in order of increasing polarity. A number of studies on the nature of the analyte and stationary phase interactions in HILIC have been performed [45–47]. The consensus is that retention occurs primarily due to the partitioning of the analyte between the organic-rich mobile phase and aqueous layer on the surface of the stationary phase; however, electrostatic and hydrogen-bonding interactions may also be present [46].

Stationary phases in HILIC include those as simple as bare silica (Waters Atlantis), or can consist of more complex bonded phases that contain neutral or ionizable functional groups. Examples of neutral stationary phases include diols (YMC Diol, Phenomenex Luna HILIC), cyano groups (YMC Cyano, Ascentis Cyano), and amides (Waters BEH Amide, TSKgel Amide). Those containing ionizable/zwitterionic groups include poly(2-sulfoethyl) (PolyLC), and sulfobetaine (ZIC-HILIC) and other proprietary structures (SIELC Obelisc N). Unlike most RP separations, there is no one HILIC stationary phase that can be universally applied to the separation of polar compounds. The HILIC separation of oligonucleotides was first demonstrated by Alpert on a poly-hydroxyethyl aspartamide stationary phase paired with triethylamine phosphate containing mobile phase and UV detection [44]. In addition to the HILIC-based separation nucleosides and nucleotides [48, 49], investigation into the application of HILIC and HILIC-MS to oligonucleotides has been pursued due to the potential for sensitive detection of oligonucleotides given the typical mobile phase compositions for HILIC. Those studies have focused on a variety of mobile and stationary phases, including zwitterionic columns [50], monolithic columns [51], the use of ion-pair reagents [52], and inclusion in multi-dimensional analysis (HILICxIP-RP-LC) [43]. The application of HILIC to the quantification of phosphorothioates in serum has also been shown [53]. However, poor column stability [50], poor retention due to silanol activity [53], and a need for improved resolution of multicomponent mixtures with HILIC have been reported [50, 53, 54]. The separation of oligonucleotides on a diol stationary phase has been limited to silica-based particles. However, those columns exhibited poor retention time reproducibility [54] and poor retention and peak symmetry compared to other HILIC stationary phases [53].

Here we have examined the performance of a new, commercially available HILIC stationary phase containing modified diol groups on a polymer-based particle (Shodex VN-50 2D) for the separation and analysis of various oligonucleotides. We were intrigued by the vendor's report of high quality separation of DNA oligomers without the need for ion-pair reagents using this column [55]. A thorough evaluation of the performance of the stationary phase is presented here with demonstration samples that include oligodeoxynucleotides, oligoribonucleotides, and phosphorothioates. We identified mobile phase compositions and gradient conditions that enabled repeatable and robust retention and appropriate resolution of the sample components with high quality MS detection. Additionally, a direct comparison of the sensitivity of a HILIC-MS and IP-RPLC-MS method for a subset of oligonucleotides was performed to compare method performance for oligonucleotide analysis by LC-MS. These findings demonstrate that HILIC-based LC-MS of various oligonucleotide sample classes using this particular column yields analytical performance that is comparable to existing IP-RP-LC methods. Moreover, the broader findings in this work can provide the basis for future studies to identify appropriate chromatographic and mass spectrometric conditions for sensitive, reproducible and quantitative analysis of oligonucleotides without the need for ion-pair reagents.

2. Experimental

2.1 Materials and Reagents

DNA oligonucleotides consisting of polythymidylic acids (dT₅, dT₁₀, dT₁₅, dT₂₀, dT₃₀ and dT₅₀) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). RNA oligonucleotides were purchased from Dharmacon, Inc. (Lafayette, CO) and de-protected, as needed, according to the manufacturer's protocol. The phosphorothioate oligonucleotide sample and corresponding shortmer failure sequences (n-1, n-2, and n-3) were purchased from Biosearch Technologies (Petaluma, CA). All oligonucleotides used in this study are summarized in Table 1. Oligonucleotide concentrations were measured on an IMPLIN Pearl nanophotometer (Westlake Village, CA).

LC-MS grade ammonium acetate and ammonium formate, 1,1,13,3,3-hexafluoro-2-propanol (HFIP, >99%), and triethylamine (TEA, HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO). LC-MS grade formic acid, methanol, and acetonitrile were purchased from Fisher Scientific (Waltham, MA). Acetic acid was purchased from VRW International (Radnor, PA). High-purity water (18.2 MΩ.cm) was produced in-house with Barnstead Nanopure system (Thermo Scientific, Waltham, MA).

2.2 Sample Preparation

Stock solutions of oligonucleotides were prepared in purified water and stored at -20 °C until needed. A mixture of oligodeoxynucleotides was prepared by combining stock solutions of dT₅, dT₁₀, dT₁₅, dT₂₀, dT₃₀, and dT₅₀ and diluting to 20 pmol/μL in mobile phase A. An oligoribonucleotide mixture was prepared in a similar fashion, but diluted to 5 pmol/μL in mobile phase A. The phosphorothioate sample was prepared as other oligonucleotides mixtures and then diluted to 5 pmol/μL in mobile phase A. For the impurity analysis of the phosphorothioate fulllength product and its related impurities, the preparation was diluted to a higher concentration (75 pmol/μL) in mobile phase A. The model compounds used in this study were selected because they represent a diverse and relevant set of oligonucleotides for different types of research, they could be used to examine the selectivity and retention characteristics for both modified and unmodified oligonucleotides of varying lengths, and they have been previously used within the laboratory [56, 57].

For sensitivity determination of the HILIC and IP-RP methods, a dilution series of oligoribonucleotides (AAAUCCUUG (R7), AAA[m³U]UCCUUG (mR7), and GCAAGCUGACCCUGAAGUUC AU (R10)) and a phosphorothioate oligonucleotide (T*C*G*T*G*C*T*T*T*T*G*T*T*G*T*T*T*T*C*G*C*G*T*T (FLP)) was prepared to generate response curves by using a common aqueous stock of each oligonucleotide, with final dilutions being made in the appropriate corresponding weak mobile phase – H₂O/ acetonitrile/ammonium acetate for HILIC and H₂O/TEA/HFIP for IP-RP. Each dilution series consisted of six points over the following concentration ranges; 0.14–29 μM for AAAUCCUUG and AAA[m³U]UCCUUG; 0.32–0.46 μM for GCAAGCUGACCCUGAAGUUC AU; and 0.33–0.48 μM for the T*C*G*T*G*C*T*T*T*T*G*T*T*G*T*T*T*T*C*G*C*G*T*T. An additional limit of quantification (LOQ) solution was prepared that provided an average S:N of approximately

10 (n=3 injections) for an extracted-ion chromatogram (EIC) generated from the most abundant charge state for each method.

2.3 LC-MS Analysis

HILIC separations were performed on an ultra-high performance liquid chromatography (UHPLC) system (Ultimate 3000, Thermo Scientific) with a 2.0×150 mm (5 μ m particle size, 100 Å pore size) PEEK Shodex HILICpak VN-50 column. Mobile phases were prepared by pre-mixing aqueous and organic components to improve gradient reproducibility. Ammonium acetate and ammonium formate aqueous solutions were pH adjusted using acetic acid or formic acid, respectively, prior to mixing with acetonitrile. Concentrations of ammonium acetate and ammonium formate reported in this study are the final concentrations in the premixed mobile phase. Mobile phase A (MPA, strong solvent) consisted of 70/30 (v/v) H₂O/acetonitrile with various concentrations of ammonium acetate or ammonium formate and mobile phase B (MPB, weak solvent) consisted of 30/70 (v/v) H₂O/acetonitrile with varying concentrations of ammonium acetate or ammonium formate. For oligo(deoxy)nucleotide analysis, a gradient consisting of a 1.5 min hold at 30% MPA, then ramping to 56% MPA at 30 min was used before 20 min re-equilibration (~20 column volumes). Flow rate and column temperature were 200 μ L/min and 40 °C. For phosphorothioate sample analysis, a gradient consisting of a 1.5 min hold at 30% MPA, then ramping to 50% MPA at 32 min was used before 20 min re-equilibration (~20 column volumes). Flow rate and column temperature were 220 μ L/min and 50°C. These same separation conditions were used for the sensitivity determination. An injection volume of 4 μ L was used for all injections.

Ion-pair reversed phase separations were performed using a Phenomenex Luna C-18 column with identical dimensions and particle size (i.e., 2.0×150 mm, 5 μ m) as the HILIC column to attempt to obtain similar chromatographic efficiency between the two methods. Mobile phase A was 8 mM TEA and 200 mM HFIP (pH=7.8) in H₂O, and mobile phase B was 8 mM TEA and 200 mM HFIP in 1:1 H₂O:methanol. The gradient conditions of the IP-RP separation were adjusted to obtain as similar retention times as possible for the oligonucleotides as those obtained under HILIC conditions. A gradient consisting of a 1.5 min hold at 8%B ramping to 65%B at 32 min was used before a 20 min re-equilibration. Flow rate and column temperature were identical to that of the HILIC method (220 μ L/min and 50 °C, respectively).

Mass spectrometric detection was performed on a Waters Synapt G2-S time-of-flight (TOF) mass spectrometer with an electrospray ionization source (ESI) in negative ion polarity. The ESI source was operated with source voltage of 2.7 kV, sample cone 30 V, source and desolvation temperatures of 120 °C and 350 °C, and cone and desolvation gas flow rates of 10 and 800 L/h, respectively. LC-MS data was acquired in full scan (m/z 400 to 2000) and sensitivity mode (V-mode) with 1.0 s scan time. LC-MS data was processed using MassLynx v4.1 (Waters Corporation). Identical acquisition parameters were used for sensitivity experiments for both HILIC and IP-RP LC-MS analyses.

Peak widths were calculated at full width at half maximum (FWHM) and reported in minutes. Peak resolution was calculated using $R_s = 2 (t_{R2} - t_{R1}) / (w_{b1} + w_{b2})$, where the

retention times (t_{R2} and t_{R1}) and peak widths (w_{b1} and w_{b2}) are also in minutes. Phosphorothioate impurities (Supplemental Table 1) detected during analysis of full-length product were identified by comparing measured and theoretical monoisotopic mass of the most abundant charge state for each chromatographic peak observed in the total ion chromatogram (TIC). Extracted ion chromatograms (EICs) were created for each identified impurity for replicate injections (n=5) to calculate retention time and peak area reproducibility (Supplemental Table 2).

To generate response curves for oligonucleotides analyzed by HILIC and IP-RP methods for the sensitivity comparison, EICs for sensitivity and LOQ determination were generated for each standard injection from the most abundant charge state with an m/z extraction window of 30 ppm. The peak areas of the EICS were plotted versus concentration to generate linear regression results (slope and R^2). The LOQ of each method was determined based on a target S:N of 10 as calculated in the MassLynx software by selecting both an appropriate signal and noise region within the EIC.

3. Results and Discussion

3.1 LC-MS of DNA Oligonucleotides

Initial investigations were conducted using a mixture of oligodeoxynucleotides (dT₅, dT₁₀, dT₁₅, dT₂₀, dT₃₀ and dT₅₀) with various mobile phase compositions. The TICs resulting from these analyses are provided in Figure 1. All components of the mixture eluted within 30 min. Consistent with a HILIC retention mechanism, the oligodeoxynucleotides eluted in order of increasing length (and increasing water content in the gradient). While some retention was observed at all ammonium acetate concentrations for all components, the lowest concentration used (2.5 mM) provided poor chromatographic performance.

At ammonium acetate concentrations of 5 mM and higher, very little change in retention was observed. However, an increase in ammonium acetate concentration lead to an improvement in peak shape, peak width, and resolution with the greatest effect observed for the longest oligodeoxynucleotides. For example, the peak width (FWHM) for dT₅₀ was reduced from approximately 1.5 min to 0.6 min as the concentration of ammonium acetate increased from 5 to 15 mM. The resolution between dT₃₀ and dT₅₀ increased from 2.6 to 5.1 for the 5 and 15 mM ammonium acetate mobile phases, respectively.

The importance of sufficient ionic strength of the mobile phase in HILIC separations has been previously reported [44, 58] and is also observed here for the separation of oligonucleotides on the diol stationary phase based on polymeric particles. Additional dT_n oligonucleotides, present as synthetic or degradation impurities in the purchased oligonucleotides, were also observed in the TIC (Supplemental Figure 1). The chromatographic performance obtained under these conditions is similar to that reported by Gilar et al. [21] for a mixture of dT lengths (dT₂ to dT₃₀) analyzed using a triethylammonium acetate (TEAA) based mobile phase on an Xterra C18 column with the same 5 μ m particle size.

In addition to ammonium acetate as a mobile phase modifier, the use of ammonium formate was also evaluated for the mixtures of oligodeoxynucleotides. Using a similar concentration to that used in the ammonium acetate experiments (10 mM), but at a pH within the buffer range of ammonium formate (pH 4.5), excessive retention of the mixture under identical gradient conditions was observed (Supplemental Figure 2). The longer the oligonucleotide, the more of an increase in retention was observed between the two mobile phases. In fact, dT₅₀ did not elute using 10 mM ammonium formate at pH 4.5. Previous reports have shown little change in retention behavior switching from ammonium acetate to ammonium formate when no ionic interactions are present in HILIC [59].

As the diol stationary phase used in this study is neutral, the change in retention observed for the oligonucleotides is attributed to the pH change of the mobile phase and not due a change in ionic strength of the buffer. As a confirmation, similar retention was observed when ammonium acetate and ammonium formate were both prepared at pH 4.5 (10 mM) using an increased aqueous content gradient (Supplemental Figure 2). A reduction in MS response for the mixture was also observed using 10 mM ammonium formate pH 4.5, which is attributed to a combination of the lower pH, wider chromatographic peak, and increased water content required to elute each oligonucleotide. The mass spectra obtained (same y-axis scale) for dT₃₀ under the ammonium acetate pH 5.5 and ammonium formate pH 4.5 conditions are shown in Supplemental Figure 3. Given these results, the use of ammonium formate was not pursued further in this study.

3.2 LC-MS Analysis of RNA Oligonucleotides

We first evaluated the retention and resolution of a mixture of oligoribonucleotides under the same separation conditions that provided the best separation for the oligodeoxynucleotides (i.e., 15 mM ammonium acetate pH 5.5). The mixture of synthetic oligonucleotides listed in Table 1 was selected due to its variation of oligomer lengths and the presence (or absence) of commonly observed modified nucleosides. The unmodified/modified sets (e.g., ACACAG (R4) and ACA[Cm]AG (R4m)) also represent much smaller chemical differences than those used in the oligodeoxynucleotide mixture.

The base peak chromatogram (BPC) generated for this mixture using 15 mM ammonium acetate pH 5.5 is shown in Figure 2. Similar to the oligodeoxynucleotides, good retention and peak shape were obtained for all components. The shortest components in the mixture, [m⁵U]UCG (R1) and [m⁵U]ΨCG (R1Y), eluted around 7 min, while the longest component (a 22-mer) eluted at 24 min. The retention time of the 22-mer was similar to dT₅₀ (~25 min), consistent with a general trend of increased retention of oligoribonucleotides compared to oligodeoxynucleotides. This change in retention may be attributed to the relative increase in polarity/hydrophilicity of oligoribonucleotides compared to oligodeoxynucleotides by the presence of the additional 2'-OH on the ribose [60].

The selectivity of the HILIC separation is best illustrated by the resolution of unmodified/modified component mixtures. For example, baseline resolution is observed for ACACAG/ACA[Cm]AG (R4/R4m) and AAAUCCUUG/AAA[m³U]UCCUUG (R7/mR7). At least partial resolution of all methylated oligoribonucleotides from their unmethylated counterparts was observed. In all cases, the methylated component eluted before the

matching unmethylated component. This outcome is consistent with a relative decrease in polarity/hydrophilicity of the oligonucleotide base with the methyl group present [61, 62].

Importantly, the HILIC conditions used here could enable separation of more challenging mixtures (e.g., UG[m²A]ΨΨC and UG[m⁶A]ΨΨC (m²R5/m⁶R5)), where only the location of the methyl group on an internal nucleobase varies. These two oligonucleotides have the same mass and cannot be distinguished by MS or MS/MS directly. It was found that the m⁶A containing oligonucleotide eluted prior to the m²A containing oligonucleotide consistent with a relative decrease in polarity/hydrophilicity when the methyl group is present on the NH₂ group of adenosine [61, 62]. In addition, the separation of uridine and pseudouridine containing oligonucleotides was also possible, with the uridine containing oligonucleotides eluting before the pseudouridine containing oligonucleotide due to the relative increase in polarity/hydrophilicity [61, 62]. The one exception was a set of 11-mers (UAACUAUAACG/UAACΨAUAACG/UAACΨAΨAACG (R8/R8Y/R8YY)) where co-elution occurred for all three sequence isomers. This suggests that the resolution of oligonucleotide pairs with uridine/pseudouridine is limited to less than 11-mers, which is similar to the behavior on IP-RP-LC-MS reported previously [56].

3.3 LC-MS Analysis of Phosphorothioate Sample

The solid-state synthesis commonly used in the production of oligonucleotides, though optimized and well-controlled, is still capable of generating synthesis-related impurities. A large number of different types of impurities may be present [10]. The most common impurities are short-mer failure sequences (e.g., n-1, n-2, etc.) and long-mer (n+1) sequences that have retained the 5'-O-DMTr (4,4'-dimethoxytrityl) protecting group. The control and characterization of impurities is important in both the production and therapeutic development of synthetic oligonucleotides. The chromatographic resolution of these failure sequences can be challenging, especially in the case of phosphorothioates, where the presence of the diastereomers can contribute to broad peaks and poor resolution [20]. The ability of the developed HILIC conditions to resolve a full-length phosphorothioate from closely related failure sequences was evaluated.

The separation of a mixture of the 24-mer phosphorothioate and its failure sequences (n-1, n-2, and n-3, Table 1) using the developed HILIC conditions (15 mM ammonium acetate pH 5.5) is shown in Supplemental Figure 4A. Symmetric peak shape and adequate retention (approximately 23 min) are observed for all four oligonucleotides, with an average peak width of 0.4 min. However, only partial chromatographic resolution was achieved using the separation conditions suitable for DNA and RNA oligonucleotide mixtures. Further investigation into additional separation parameters (i.e., flow rate and column temperature) showed only modest improvement in peak widths for phosphorothioate peaks using elevated flow rate (220 μL/min) and column temperature (50 °C). Complete resolution of the phosphorothioates could not be achieved under any of the flow rate and column temperatures evaluated (Supplemental Figures 4 and 5). Because mass spectrometric detection of oligonucleotides does not necessarily require chromatographic resolution of impurities for detection and/or quantification like other detection techniques (e.g., UV

detection), the ability of the HILIC separation to detect synthetic impurities in a phosphorothioate full-length product was assessed.

Using the increased flow rate and column temperature, impurity analysis of the phosphorothioate full-length product was performed and the resulting BPC is provided in Figure 3. A slight change in the gradient (1.56 %B/min versus 1.87 %B/min) was used to increase retention and enhance separation. All detected short-mer sequences, consisting of oligonucleotides of length from 2 to 21 nucleotides, were retained and well-separated from the main peak. The full-length product with an intact 5'-O-dimethoxytrityl protecting group was also detected prior to the main peak. Figure 3 (**inset**) shows an overlay of the EICs for three partially resolved impurity peaks that were detected by MS, including the n-2 and n-1 failure sequences along with an impurity arising from the base depurination of the full-length product. The chromatographic performance for the analysis of the phosphorothioate is similar to previous reports using IP-RP-LC-MS [57]. The mass spectrum obtained for the full-length product is provided in Figure 4. The corresponding single and double PO impurities, due to non-quantitative sulfurization of the oligonucleotide during synthesis, were also detected (Figure 4 (**inset**)). A complete summary of all impurities detected and identified is provided in Supplemental Table 2.

The challenge of completely resolving synthetic impurities for phosphorothioates, even under fully-optimized IP-RP-LC conditions, has been previously reported [57]. The results obtained here, without the use of ion-pair reagents, are similar to that reported using IP-RP-LC-MS [57]. Close et al. [20] recently reported the resolution of a phosphorothioate full length product from its n-1 and single PO impurities on a C18 column using superficially porous particles and a strong-ion pair reagent (tetrabutylammonium bromide, TBAB) with UV detection. Additionally, Studzinska et al. [9] showed that by fine tuning ion-pair concentration, the separation of closely related oligonucleotide sequences can also be obtained with UV detection. Though this degree of chromatographic resolution for specifically developed IP-RP-LC-UV methods was not achieved using the HILIC-MS method here, the resolution provided in the absence of ion-pair reagents with MS detection still represents an attractive alternative to previously developed methods. While it is important to resolve chromatographic impurities of interest using UV detection, the advantage of using MS for detection is that impurities can still be detected and quantified even when no/partial resolution is present [27].

In addition to the qualitative detection and identification of synthesis-related impurities, the quantification of these impurities is also of analytical importance. To assess the precision (repeatability) of the developed HILIC-MS method for quantitative applications, five technical replicate injections of the sample were performed. EICs were generated for each impurity detected and the retention time and peak area repeatability calculated. Retention time repeatability was 0.5% RSD (n=5) for all peaks, including the main peak, which illustrates the repeatable retention behavior for oligonucleotides on this column. The peak area repeatability ranged from 3.3–11% RSD (n=5) over the wide range of abundances present for impurities and main peak in the sample, showing suitable peak area repeatability for quantitative applications. A summary of the repeatability results is provided in Supplemental Table 2. Moreover, the conditions have shown suitable intermediate precision,

with similar chromatographic retention and resolution being obtained for a wide range of oligonucleotides over the course of 6 months and 300+ injections on the column. A comparison of the chromatographic impurity profile generated for the phosphorothioate sample generated 6 months apart is provided in Supplemental Figure 6.

3.4 HILIC and IP-RP Sensitivity Analysis

To assess how the sensitivity of HILIC-MS for oligonucleotide analysis compares to that found when using IP-RP-LC-MS, the slopes of standard response curves and LOQ were generated and compared for a set of oligonucleotides under similar HILIC and IP-RP separation and detection conditions. To minimize the effect of chromatographic efficiency on peak shape and width, which can influence LOQ determination, an IP-RP column with identical dimensions and particle size was selected. As noted in the experimental section, retention times for the oligonucleotides were also adjusted to be as similar as possible to those obtained in the HILIC separation.

The sensitivity (slope of the response curve) and LOQ results obtained for both the HILIC and IPRP methods are provided in Table 2. A linear response was obtained for all oligonucleotides over the concentration ranges examined. For all oligonucleotides analyzed here, the HILIC-MS method provided a larger slope than that obtained during IP-RP-LC-MS analysis of the same oligonucleotide. This indicates that, for these oligonucleotides under the stated acquisition conditions, the HILIC-MS method provides more sensitive mass spectrometric detection than IPRP-LC-MS. It should be noted that the HILIC and IP-RP conditions did produce different charge state distributions, however for this analysis only the most abundant charge state was used to generate EICs and peak area responses. The most abundant charge state was a single charge higher for each oligonucleotide under HILIC conditions as compared to IP-RP conditions. Representative mass spectra, shown on the same absolute y-axis scale, for each oligonucleotide under both HILIC and IP-RP conditions are given in Supplemental Figure 7.

The higher organic content of the mobile phase under HILIC conditions, leading to more efficient desolvation, is expected to be a primary reason for the improved sensitivity. However, the sensitivity differences between the two methods increased as the hydrophobicity of the oligonucleotide decreased. For example, the sample mR7 yielded similar slopes for both methods; however significantly different slopes were seen for the increasingly less hydrophobic samples R7, R10, and FLP – with the FLP resulting in the largest difference in slopes. It will be informative to follow up on these initial results to better understand oligonucleotide and chromatographic properties that lead to enhanced responses in HILIC-MS.

Although the sensitivity was greater for the HILIC method for all oligonucleotides studied, this did not necessarily lead to an improvement in LOQ when using the HILIC method. For example, the R7 and mR7 samples yielded LOQs that ranged from ~30 to 60 nM for both methods (Table 2) consistent with the similar sensitivities of these methods for the two samples. In contrast, while the HILIC method was significantly more sensitive than the IP-RP method for both the R10 and FLP samples, a better LOQ was found with IP-RP for R10

while the converse was true for the FLP sample. These results suggest a further examination of the chromatographic performance of the two methods must also be considered.

LOQ values were determined based on a signal-to-noise measurement, which depends on the intensity of the signal in the EIC (i.e., the chromatographic peak height). The peak heights obtained for the same oligonucleotide concentration were significantly different for R10 and the 24-mer FLP (but similar for R7 and mR7). The IP-RP method provided improved peak shape for R10, leading to the improved signal-to-noise measurement. Although the IP-RP method also provided better peak shape for the phosphorothioate sample, it was not enough to overcome the significantly increased response of the HILIC method for this oligonucleotide. The trade-off between peak shape and mass spectrometric response is illustrated in the EIC overlays provided in Supplemental Figure 8. The results found here for longer oligonucleotides demonstrate the need for further development and optimization of HILIC separation conditions to improve chromatographic peak shape as compared to IP-RP.

4. Conclusion

A HILIC-MS method that balances high chromatographic and MS performance without the use of ion-pair reagents has been developed for the analysis of a wide range of oligonucleotides. Suitable retention, selectivity, and resolution for structurally similar multicomponent oligonucleotide mixtures was achieved using a commonly employed LC-MS mobile phase system containing only water, acetonitrile, and ammonium acetate and a commercially available HILIC column. In the case of oligodeoxynucleotide analysis, a similar level of performance was shown for HILIC to that obtained for the same particle size under IP-RP-LC-UV conditions. Additional applications of HILIC-MS for analysis of modified oligoribonucleotides and phosphorothioates were shown. The method also demonstrated suitable reproducibility for quantitative applications.

Although the same chromatographic resolution of closely related phosphorothioate impurities did not match that of finely optimized IP-RP-LC-UV methods, the HILIC-MS method still presents an attractive alternative to current methodology. The availability of smaller particle sizes, additional column size configurations and additional investigation into mobile phase systems could lead to similar or better chromatographic performance, with increased sensitivity of MS detection. Though the HILIC-MS conditions presented here provided an improvement in sensitivity compared IP-RP for the oligonucleotides tested, additional work is warranted to define the reproducibility of HILIC and improve chromatographic performance to achieve additional gains in LOQs for quantitative applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Financial support for this work is provided by the National Institutes of Health (NIH GM058843). The generous support of the Rieveschl Eminent Scholar Endowment and the University of Cincinnati for these studies is also appreciated.

References

- [1]. Agris PF, Narendran A, Sarachan K, Vare VYP, Eruysal E, The Importance of Being Modified: The Role of RNA Modifications in Translational Fidelity, *The Enzymes*, 41 (2017) 1–50. [PubMed: 28601219]
- [2]. Roundtree IA, Evans ME, Pan T, He C, Dynamic RNA Modifications in Gene Expression Regulation, *Cell*, 169 (2017) 1187–1200. [PubMed: 28622506]
- [3]. Bohnsack MT, Sloan KE, The mitochondrial epitranscriptome: the roles of RNA modifications in mitochondrial translation and human disease, *Cellular and molecular life sciences : CMLS*, 75 (2018) 241–260. [PubMed: 28752201]
- [4]. Kole R, Krainer AR, Altman S, RNA therapeutics: beyond RNA interference and antisense oligonucleotides, *Nat Rev Drug Discov*, 11 (2012) 125–140. [PubMed: 22262036]
- [5]. Mingardi J, Musazzi L, De Petro G, Barbon A, miRNA Editing: New Insights into the Fast Control of Gene Expression in Health and Disease, *Molecular neurobiology*, 55 (2018) 7717–7727. [PubMed: 29460265]
- [6]. Suzuki T, Suzuki T, A complete landscape of post-transcriptional modifications in mammalian mitochondrial tRNAs, *Nucleic Acids Res*, 42 (2014) 7346–7357. [PubMed: 24831542]
- [7]. Wetzel C, Limbach PA, Mass spectrometry of modified RNAs: recent developments, *Analyst*, 141 (2016) 16–23. [PubMed: 26501195]
- [8]. Sun C, Jora M, Solivio B, Limbach PA, Addepalli B, The Effects of Ultraviolet Radiation on Nucleoside Modifications in RNA, *ACS Chemical Biology*, 13 (2018) 567–572. [PubMed: 29400945]
- [9]. Studzinska S, Buszewski B, Evaluation of ultra high-performance [corrected] liquid chromatography columns for the analysis of unmodified and antisense oligonucleotides, *Anal Bioanal Chem*, 406 (2014) 7127–7136. [PubMed: 24939137]
- [10]. El Zahar NM, Magdy N, El-Kosasy AM, Bartlett MG, Chromatographic approaches for the characterization and quality control of therapeutic oligonucleotide impurities, *Biomed Chromatogr*, 32 (2018).
- [11]. Apffel A, Chakel JA, Fischer S, Lichtenwalter K, Hancock WS, Analysis of Oligonucleotides by HPLC-Electrospray Ionization Mass Spectrometry, *Anal Chem*, 69 (1997) 1320–1325. [PubMed: 21639339]
- [12]. Gong L, McCullagh JS, Comparing ion-pairing reagents and sample dissolution solvents for ion-pairing reversed-phase liquid chromatography/electrospray ionization mass spectrometry analysis of oligonucleotides, *Rapid Commun Mass Spectrom*, 28 (2014) 339–350. [PubMed: 24395501]
- [13]. Studzinska S, Rola R, Buszewski B, The impact of ion-pairing reagents on the selectivity and sensitivity in the analysis of modified oligonucleotides in serum samples by liquid chromatography coupled with tandem mass spectrometry, *J Pharm Biomed Anal*, 138 (2017) 146–152. [PubMed: 28208106]
- [14]. Basiri B, van Hattum H, van Dongen WD, Murph MM, Bartlett MG, The Role of Fluorinated Alcohols as Mobile Phase Modifiers for LC-MS Analysis of Oligonucleotides, *J Am Soc Mass Spectrom*, 28 (2017) 190–199. [PubMed: 27644940]
- [15]. Gilar M, Analysis and purification of synthetic oligonucleotides by reversed-phase high-performance liquid chromatography with photodiode array and mass spectrometry detection, *Anal Biochem*, 298 (2001) 196–206. [PubMed: 11700974]
- [16]. Fountain KJ, Gilar M, Gebler JC, Analysis of native and chemically modified oligonucleotides by tandem ion-pair reversed-phase high-performance liquid chromatography/electrospray ionization mass spectrometry, *Rapid Commun Mass Spectrom*, 17 (2003) 646–653. [PubMed: 12661016]

- [17]. Erb R, Leithner K, Bernkop-Schnurch A, Oberacher H, Phosphorothioate oligonucleotide quantification by mu-liquid chromatography-mass spectrometry, *AAPS J*, 14 (2012) 728–737. [PubMed: 22806756]
- [18]. Elzahar NM, Magdy N, El-Kosasy AM, Bartlett MG, Degradation product characterization of therapeutic oligonucleotides using liquid chromatography mass spectrometry, *Anal Bioanal Chem*, 410 (2018) 3375–3384. [PubMed: 29654340]
- [19]. Popova AM, Williamson JR, Quantitative analysis of rRNA modifications using stable isotope labeling and mass spectrometry, *J Am Chem Soc*, 136 (2014) 2058–2069. [PubMed: 24422502]
- [20]. Close ED, Nwokeoji AO, Milton D, Cook K, Hindocha DM, Hook EC, Wood H, Dickman MJ, Nucleic acid separations using superficially porous silica particles, *Journal of Chromatography A*, 1440 (2016) 135–144. [PubMed: 26948761]
- [21]. Gilar M, Fountain KJ, Budman Y, Neue UD, Yardley KR, Rainville PD, Russell RJ 2nd, Gebler JC, Ion-pair reversed-phase high-performance liquid chromatography analysis of oligonucleotides: retention prediction, *J Chromatogr A*, 958 (2002) 167–182. [PubMed: 12134814]
- [22]. Chen B, Mason SF, Bartlett MG, The effect of organic modifiers on electrospray ionization charge-state distribution and desorption efficiency for oligonucleotides, *J Am Soc Mass Spectrom*, 24 (2013) 257–264. [PubMed: 23325666]
- [23]. Ganisl B, Taucher M, Riml C, Breuker K, Charge as you like! Efficient manipulation of negative ion net charge in electrospray ionization of proteins and nucleic acids, *Eur J Mass Spectrom (Chichester)*, 17 (2011) 333–343. [PubMed: 22006635]
- [24]. Muddiman DC, Cheng X, Udseth HR, Smith RD, Charge-state reduction with improved signal intensity of oligonucleotides in electrospray ionization mass spectrometry, *J Am Soc Mass Spectrom*, 7 (1996) 697–706. [PubMed: 24203563]
- [25]. Greig M, Griffey RH, Utility of organic bases for improved electrospray mass spectrometry of oligonucleotides, *Rapid Commun Mass Spectrom*, 9 (1995) 97–102. [PubMed: 7888709]
- [26]. Apffel A, Chakel JA, Fischer S, Lichtenwalter K, Hancock WS, New procedure for the use of high-performance liquid chromatography electrospray ionization mass spectrometry for the analysis of nucleotides and oligonucleotides, *Journal of Chromatography A*, 777 (1997) 3–21.
- [27]. Chen B, Bartlett MG, Evaluation of mobile phase composition for enhancing sensitivity of targeted quantification of oligonucleotides using ultra-high performance liquid chromatography and mass spectrometry: application to phosphorothioate deoxyribonucleic acid, *J Chromatogr A*, 1288 (2013) 73–81. [PubMed: 23528868]
- [28]. Basiri B, Murph MM, Bartlett MG, Assessing the Interplay between the Physicochemical Parameters of Ion-Pairing Reagents and the Analyte Sequence on the Electrospray Desorption Process for Oligonucleotides, *J Am Soc Mass Spectrom*, 28 (2017) 1647–1656. [PubMed: 28405940]
- [29]. Gilar M, Belenky A, Smisek DL, Bourque A, Cohen AS, Kinetics of phosphorothioate oligonucleotide metabolism in biological fluids, *Nucleic Acids Res*, 25 (1997) 3615–3620. [PubMed: 9278481]
- [30]. Chen SH, Qian M, Brennan JM, Gallo JM, Determination of antisense phosphorothioate oligonucleotides and catabolites in biological fluids and tissue extracts using anion-exchange high-performance liquid chromatography and capillary gel electrophoresis, *J Chromatogr B Biomed Sci Appl*, 692 (1997) 43–51. [PubMed: 9187382]
- [31]. Shimoyama A, Fujisaka A, Obika S, Evaluation of size-exclusion chromatography for the analysis of phosphorothioate oligonucleotides, *J Pharm Biomed Anal*, 136 (2017) 55–65. [PubMed: 28063336]
- [32]. Chionh YH, Ho CH, Pruksakorn D, Ramesh Babu I, Ng CS, Hia F, McBee ME, Su D, Pang YL, Gu C, Dong H, Prestwich EG, Shi PY, Preiser PR, Alonso S, Dedon PC, A multidimensional platform for the purification of non-coding RNA species, *Nucleic Acids Res*, 41 (2013) e168. [PubMed: 23907385]
- [33]. Thayer JR, Wu Y, Hansen E, Angelino MD, Rao S, Separation of oligonucleotide phosphorothioate diastereoisomers by pellicular anion-exchange chromatography, *J Chromatogr A*, 1218 (2011) 802–808. [PubMed: 21216401]

- [34]. Roussis SG, Cedillo I, Rentel C, Two-dimensional liquid chromatography-mass spectrometry for the characterization of modified oligonucleotide impurities, *Anal Biochem*, 556 (2018) 45–52. [PubMed: 29936097]
- [35]. Zimmermann A, Greco R, Walker I, Horak J, Cavazzini A, Lammerhofer M, Synthetic oligonucleotide separations by mixed-mode reversed-phase/weak anion-exchange liquid chromatography, *J Chromatogr A*, 1354 (2014) 43–55. [PubMed: 24929908]
- [36]. Eeltink S, Wouters S, Does-Sousa JL, Svec F, Advances in organic polymer-based monolithic column technology for high-resolution liquid chromatography-mass spectrometry profiling of antibodies, intact proteins, oligonucleotides, and peptides, *J Chromatogr A*, 1498 (2017) 8–21. [PubMed: 28069168]
- [37]. Sharma VK, Glick J, Vouros P, Reversed-phase ion-pair liquid chromatography electrospray ionization tandem mass spectrometry for separation, sequencing and mapping of sites of base modification of isomeric oligonucleotide adducts using monolithic column, *J Chromatogr A*, 1245 (2012) 65–74. [PubMed: 22652552]
- [38]. Huber CG, Oefner PJ, Bonn GK, High-resolution liquid chromatography of oligonucleotides on nonporous alkylated styrene-divinylbenzene copolymers, *Anal Biochem*, 212 (1993) 351–358. [PubMed: 8214576]
- [39]. Oefner PJ, Huber CG, A decade of high-resolution liquid chromatography of nucleic acids on styrene-divinylbenzene copolymers, *J Chromatogr B Analyt Technol Biomed Life Sci*, 782 (2002) 27–55.
- [40]. Yamauchi Y, Taoka M, Nobe Y, Izumikawa K, Takahashi N, Nakayama H, Isobe T, Denaturing reversed phase liquid chromatographic separation of non-coding ribonucleic acids on macro-porous polystyrene-divinylbenzene resins, *J Chromatogr A*, 1312 (2013) 87–92. [PubMed: 24044980]
- [41]. Studzinska S, Bocian S, Siecinska L, Buszewski B, Application of phenyl-based stationary phases for the study of retention and separation of oligonucleotides, *J Chromatogr B Analyt Technol Biomed Life Sci*, 1060 (2017) 36–43.
- [42]. Li L, Leone T, Foley JP, Welch CJ, Separation of small interfering RNA stereoisomers using reversed-phase ion-pairing chromatography, *J Chromatogr A*, 1500 (2017) 84–88. [PubMed: 28449879]
- [43]. Li Q, Lynen F, Wang J, Li H, Xu G, Sandra P, Comprehensive hydrophilic interaction and ion-pair reversed-phase liquid chromatography for analysis of di- to deca-oligonucleotides, *J Chromatogr A*, 1255 (2012) 237–243. [PubMed: 22204934]
- [44]. Alpert AJ, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds, *J Chromatogr*, 499 (1990) 177–196. [PubMed: 2324207]
- [45]. Jandera P, Stationary and mobile phases in hydrophilic interaction chromatography: a review, *Anal Chim Acta*, 692 (2011) 1–25. [PubMed: 21501708]
- [46]. Buszewski B, Noga S, Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique, *Analytical and Bioanalytical Chemistry*, 402 (2012) 231–247. [PubMed: 21879300]
- [47]. McCalley DV, Understanding and manipulating the separation in hydrophilic interaction liquid chromatography, *J Chromatogr A*, 1523 (2017) 49–71. [PubMed: 28668366]
- [48]. Garcia-Gomez D, Rodriguez-Gonzalo E, Carabias-Martinez R, Stationary phases for separation of nucleosides and nucleotides by hydrophilic interaction liquid chromatography, *Trac-Trend Anal Chem*, 47 (2013) 111–128.
- [49]. Mateos-Vivas M, Rodriguez-Gonzalo E, Garcia-Gomez D, Carabias-Martinez R, Hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents for the separation of nucleosides and nucleotide mono-, di- and triphosphates, *Journal of Chromatography A*, 1414 (2015) 129–137. [PubMed: 26341591]
- [50]. Gong L, McCullagh JS, Analysis of oligonucleotides by hydrophilic interaction liquid chromatography coupled to negative ion electrospray ionization mass spectrometry, *J Chromatogr A*, 1218 (2011) 5480–5486. [PubMed: 21741051]
- [51]. Holdsvendova P, Suchankova J, Buncek M, Backovska V, Coufal P, Hydroxymethyl methacrylate-based monolithic columns designed for separation of oligonucleotides in

- hydrophilic-interaction capillary liquid chromatography, *J Biochem Biophys Methods*, 70 (2007) 23–29. [PubMed: 17207534]
- [52]. Gong L, Analysis of oligonucleotides by ion-pairing hydrophilic interaction liquid chromatography/electrospray ionization mass spectrometry, *Rapid Commun Mass Spectrom*, 31 (2017) 2125–2134. [PubMed: 28972295]
- [53]. Studzinska S, Lobodzinski F, Buszewski B, Application of hydrophilic interaction liquid chromatography coupled with mass spectrometry in the analysis of phosphorothioate oligonucleotides in serum, *J Chromatogr B Analyt Technol Biomed Life Sci*, 1040 (2017) 282–288.
- [54]. Easter RN, Kroning KK, Caruso JA, Limbach PA, Separation and identification of oligonucleotides by hydrophilic interaction liquid chromatography (HILIC)-inductively coupled plasma mass spectrometry (ICPMS), *Analyst*, 135 (2010) 2560–2565. [PubMed: 20830328]
- [55]. Addepalli B, Limbach PA, Mass spectrometry-based quantification of pseudouridine in RNA, *J AmSoc Mass Spectrom*, 22 (2011) 1363–1372.
- [56]. Nikcevic I, Wyrzykiewicz TK, Limbach PA, Detecting Low-Level Synthesis Impurities in Modified Phosphorothioate Oligonucleotides Using Liquid Chromatography - High Resolution Mass Spectrometry, *Int J Mass Spectrom*, 304 (2011) 98–104. [PubMed: 21811394]
- [57]. Heaton JC, Russell JJ, Underwood T, Boughtflower R, McCalley DV, Comparison of peak shape in hydrophilic interaction chromatography using acidic salt buffers and simple acid solutions, *J Chromatogr A*, 1347 (2014) 39–48. [PubMed: 24813934]
- [58]. Guo Y, Gaiki S, Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography, *J Chromatogr A*, 1074 (2005) 71–80. [PubMed: 15941041]
- [59]. Sakaguchi Y, Miyauchi K, Kang BI, Suzuki T, Nucleoside Analysis by Hydrophilic Interaction Liquid Chromatography Coupled with Mass Spectrometry, *Methods in enzymology*, 560 (2015) 19–28. [PubMed: 26253964]
- [60]. Kellner S, Ochel A, Thuring K, Spenkuch F, Neumann J, Sharma S, Entian KD, Schneider D, Helm M, Absolute and relative quantification of RNA modifications via biosynthetic isotopomers, *Nucleic Acids Res*, 42 (2014) e142. [PubMed: 25129236]
- [61]. Russell SP, Limbach PA, Evaluating the reproducibility of quantifying modified nucleosides from ribonucleic acids by LC–UV–MS, *Journal of Chromatography B*, 923–924 (2013) 74–82.
- [62]. Null AP, Nepomuceno AI, Muddiman DC, Implications of hydrophobicity and free energy of solvation for characterization of nucleic acids by electrospray ionization mass spectrometry, *Anal Chem*, 75 (2003) 1331–1339. [PubMed: 12659193]

Highlights

- HILIC-MS analysis of oligonucleotides without the use of ion-pair reagents
- Applicable to oligodeoxy(ribo)nucleotides and phosphorothioates (PS)
- Improved LC-MS sensitivity over ion-pair reverse phase methods
- Additional chromatographic developments required to match UV-based PS methods

Figure 1.

Total ion chromatograms (TICs) of poly dT mix with ammonium acetate (pH=5.5) concentrations of (A) 2.5 mM (B) 5 mM (C) 10 mM and (D) 15 mM. Resolution (R_s) between dT₃₀ and dT₅₀ peaks and FWHM of dT₅₀ peak included. All TICs are shown on the same absolute (y-axis) scale.

Figure 2. Base peak chromatogram (BPC) of RNA oligonucleotide mix using 15 mM ammonium acetate pH=5.5. See Table 1 for sample composition.

Figure 3.

Base peak chromatogram (BPC) for phosphorothioate full length product using 15 mM ammonium acetate pH 5.5, column temperature 50 °C and flow rate 220 μ L/min. Failure sequence short-mers detected are labeled with 3' terminal nucleotide and oligomer length. (Inset) Extracted ion chromatogram (EIC) for peaks eluting near full-length product.

Figure 4. Mass spectrum of phosphorothioate full-length product. (Inset) Expanded view of -5 charge state with sodium and adducts and co-eluting process impurities containing one PO linkage and two PO linkages.

Table 1.

Oligonucleotides used in this study

Oligonucleotide Sequence	ID	DNA/RNA	MW (g mol ⁻¹)
dT ₅	T5	DNA	1459
dT ₁₀	T10	DNA	2980
dT ₁₅	T15	DNA	4500
dT ₂₀	T20	DNA	6023
dT ₃₀	T30	DNA	9064
dT ₅₀	T50	DNA	15148
[m ⁵ U]UCG	R1	RNA	1215
[m ⁵ U]ΨCG	R1Y	RNA	1215
ACCUG	R2	RNA	1528
A[Cm]CUG	R2m	RNA	1543
[m ⁵ U]UCAAG	R3	RNA	1873
[m ⁵ U]ΨCAAG	R3Y	RNA	1873
ACACAG	R4	RNA	1881
ACA[Cm]AG	R4m	RNA	1895
UG[m ² A]ΨΨCC	m2R5	RNA	2155
UG[m ⁶ A]ΨΨCC	m6R5	RNA	2155
[m ² A]AΨΨCCAG	m2R6	RNA	2508
[m ⁶ A]AΨΨCCAG	m6R6	RNA	2508
AAAUCCUUG	R7	RNA	3106
AAA[m ³ U]UCCUUG	mR7	RNA	3120
UAAUAUAACG	R8	RNA	3458
UAA[Cm]UAUAACG	R8m	RNA	3472
UAAΨUAUAACG	R8Y	RNA	3458
UAAΨAΨUAACG	R8YY	RNA	3458
AUCACCUCCΨΨUCU	R9	RNA	4265
GCAAGCUGACCCUGAAGUUCAU	R10	RNA	7001
T*C*G*T*G*C*T*T*T*G*T*G*T*T*T*C*G*C	n-3	PS-DNA	6692
T*C*G*T*G*C*T*T*T*G*T*G*T*T*T*C*G*C*G	n-2	PS-DNA	7036
T*C*G*T*G*C*T*T*T*G*T*G*T*T*T*T*C*G*C*G*T	n-1	PS-DNA	7356
T*C*G*T*G*C*T*T*T*G*T*G*T*T*T*T*C*G*C*G*T*T	FLP	PS-DNA	7675

Table 2.

HILIC-MS and IP-RP-LC-MS Sensitivity Comparison Results

Oligonucleotide	LC Method	Conc. Range (uM)	R ²	Slope	Charge	LOQ (nM)
mR7	HILIC	0.14–29	0.986	15075	–3	57 ± 12
	Ion-Pair	0.14–29	0.999	13071	–2	42 ± 4
R7	HILIC	0.14–29	0.996	22810	–3	41 ± 13
	Ion-Pair	0.14–29	0.998	15348	–2	28 ± 6
R10	HILIC	0.32–46	0.982	17582	–5	191 ± 70
	Ion-Pair	0.32–46	0.977	5881	–4	41 ± 3
FLP	HILIC	0.33–48	0.987	25267	–5	76 ± 14
	Ion-Pair	0.33–48	0.986	5149	–4	146 ± 26