Development of Multiple Heartcutting Two-Dimensional Liquid Chromatography with Ion-Pairing Reversed-Phase Separations in Both Dimensions for Analysis of Impurities in Therapeutic Oligonucleotides

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Highlights

- 2D-LC separation of oligonucleotides using IPRP separations in both dimensions
- Elution conditions are systematically discovered through iterative retention modeling
- 2D-LC separations provide both the selectivity and sensitivity needed for impurity profiling
- Application to therapeutic siRNA single strands from solid phase oligonucleotide synthesis

Abstract

Oligonucleotides constitute an emerging and highly complex bioanalytical challenge and it is becoming increasingly clear that ^{1}D methodologies are unable to fully resolve all possible impurities present in these samples. 2D-LC therefore constitutes a perfect solution wherein, critical pairs can be sampled from a steep gradient ^{1}D and separated in a shallower gradient ^{2}D. Herein, we provide a facile 2D-LC method development approach to quickly generate high selectivity gradients utilizing ion pairing reverse phase (IPRP-IPRP). In particular we demonstrate how to iteratively generate a 12% gradient from two training runs and then to utilize that data to predict retentions of analytes with a 2% gradient with retention prediction errors as low as 3 and 11% respectively. This iterative method development workflow was applied to impurity profiling down to 1:1000 for the full-length product and phosphorothioate modified impurities. Additionally, we demonstrated the elucidation of critical pairs in complex crude pharmaceutical oligonucleotide samples by applying tailored high selectivity gradients in the ^{2}D. It was found that the iterative retention modeling approach allows fast and facile 2D-LC method development for complex oligonucleotide separations.

Keywords: Method Development, Two-dimensional liquid chromatography, Ion pairing reverse phase, oligonucleotides, impurity profiling

1. Introduction

Therapeutic oligonucleotides (ONs) are emerging as a promising modality to treat a wide range of diseases in a precise manner by targeting specific genes of interest. Small interfering RNA (siRNA) is a subset of ON therapeutics composed of a double stranded RNA duplex with each strand typically 20 – 30 nucleotides long. Measuring quality attributes of siRNA such as purity, potency, and sequence is challenging due to the relatively large size, polyanionic nature, and large number of synthetic modifications [1]. Continuous improvements in analytical capabilities used to characterize ONs will help support the growing therapeutic market and ensure safe and efficacious medicine is delivered to patients.

Although formal regulatory guidelines are currently not defined for impurity reporting thresholds for synthetic ONs, many investigators follow the levels (0.1-0.3%) proposed by Capaldi, et al. or the European Pharmacopoeia (Ph. Eur.) substances for pharmaceutical use (2034) recommendation for synthetic peptides [2]. Ion pairing-reversed phase (IP-RP) high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) is a powerful technique capable of determining many impurities with the required sensitivity [3,4]. Although other techniques such as anion exchange [5] and hydrophilic interaction liquid chromatography [6] have been reported and may be beneficial in certain areas, IP-RP is the most common and arguably the highest resolving mode of LC for ONs [7]. IP-RP HPLC can often separate common impurities such as deletion and truncation products (e.g., n-1, n-2), additions (e.g., n+1), minor structural changes such as de-fluorination and oxidation (e.g., of phosphorothioate (PS) group to a phosphodiester (PO) group), as well as major structural changes such as loss of GalNAc or lipid moieties. Moreover, efficient online coupling with MS allows identification and sequencing of impurities and the full length product [8,9].

A wide array of literature is available focused on the effects of different types of IP reagents [10,11], acid modifiers [12,13], and column stationary phases [14–16] on ON separations and MS response. The method of choice depends on the type of ON and desired application. For example, impurity profiling while suppressing diastereomer separations due to phosphorthioate (PS) groups is possible using very hydrophobic IP reagents for fully thiolated ONs or mildly hydrophobic IP reagents for partially thiolated ONs such as siRNAs. Small IP reagents are used when diastereomer separation is desired or with non-thiolated ONs where diastereomers are not present. When using mass spectrometric (MS) detection, use of fluorinated alcohols greatly improves MS response without sacrificing chromatographic resolution.

Despite these improvements, co-elution of structurally related impurities is still challenging. Interest in two-dimensional HPLC (2D-LC) has grown due to the potential for improved resolving power of closely related ON impurities, and for coupling separations involving non-volatile buffers with MS detection [17]. Recent applications include HILIC x IPRP [18], SAX × HILIC, IP-RP × HILIC [19], SEC × IP-RP[20], IP-RP × SAX [21], as well as IPRP × IPRP with different IPs [22] and unique stationary phases such as terephthalate [23] and carbamate-based stationary phases [24]. Studies of the complementarity of different separation modes for oligonucleotides have also been published recently which could aid in further 2D-LC implementations [21,23,25]. However, the use of 2D-LC as means to sample and transfer co-eluting analytes into a separation that uses more selective/shallower gradient is also a promising tool to enhance selectivity without impacting the ^{1}D separation.

As such, the goal of this work was to develop an IP-RP × IP-RP 2D-LC-MS workflow for impurity profiling of ONs. We have chosen IP-RP in both dimensions as the technique offers the highest resolving power for most ON impurities and is MS compatible. Although counterintuitive based on traditional principles of complementarity in 2D-LC separations [17], we took advantage of the high sensitivity of ONs to mobile phase composition [26] to implement unique gradient conditions in each dimension of the 2D system. Separation of a wide range of impurities was obtained using a broad gradient range (e.g., 1-20 %B) in the first dimension, followed by implementation of a very narrow gradient in the second dimension with selective comprehensive sampling to separate closely related impurities that co-elute in the first dimension. The work expands on our previous workflow recently implemented for therapeutic peptides [27], where ^{2}D gradient elution conditions are developed iteratively, ultimately enabling highly selective, shallow gradient slopes to separate the impurities of interest. The model is first developed with impurity standards representative of typical ONs, and then further demonstrated using therapeutic siRNA ONs made using solid-phase oligonucleotide synthesis.

2. **Materials and methods**

2.1 Chemicals and reagents

Oligonucleotide standards (LR sequences) were purchased from Biosynthesis Inc. (Lewisville, TX). Crude single stranded oligonucleotides used in Figs. 5 and 6 were synthesized by Eli Lilly and Company using solid-phase oligonucleotide synthesis (Indianapolis, IN, USA). Ultra-pure water (18.2 MΩ) was prepared in-house using a Milli-Q Advantage A10 water purification system (Z00Q0V0T0) purchased from Millipore (Burlington, MA). Dibutylamine (DBA, >99%) and dimethylbutylamine (DMBA) were purchased from TCI (Portland, OR, USA). Acetonitrile (ACN, HPLC-grade), diisopropylethylamine

(DIPEA), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, ≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Sample preparation

Table 1 lists all ONs and their respective sequences and modifications. All samples were prepared by reconstituting lyophilized material to 1 mg/mL in MilliQ water and stored at -20 °C until use. Analytical samples were prepared by dilution to desired concentrations using MilliQ water.

2.3 Mobile Phase Preparation

For the work shown in Figs. 1, 3, and 4, solvent A was prepared by dissolving 0.65g of DBA and 2.61 mL of HFIP in 1 L of Milli-Q water (MQ), which produced a solution with formal concentrations of 10 mM and 50 mM for DBA and HFIP, respectively. For the work shown in Fig. 5, solvent A containing 10 mM DIPEA and 100 mM HFIP was prepared by dissolving 435 µL of DIPEA and 2.63 mL of HFIP in 250 mL of MQ water. For the work shown in Fig. 6, solvent A containing 5 mM DMBA and 50 mM HFIP was prepared by dissolving 169 µL of DMBA and 1.31 mL of HFIP in 250 mL of MQ water.

Solvent B consisted of 30/70 (v/v) ACN/Water (Figs 1, 3, 4) or pure ACN (Figs 5 and 6).

2.4 Instrumentation and columns

Separations were carried out using two different 2D-LC systems, both from Agilent Technologies.

System A (Figs. 1, 3, and 4)

All separations were performed on an Agilent Technologies 1290 Infinity UHPLC system (Waldbronn, Germany). The first dimension consisted of an autosampler with a flow through needle configuration (G4226A), a binary pump with a 35 µL Jet Weaver mixer (G4220A), a thermostated column compartment (G1316C), and a DAD detector (G7117B) with a 10 mm Max-Light flow cell. The second dimension included a binary pump with a 35 µL Jet Weaver mixer, a thermostated column compartment, and a DAD UV absorbance detector (G4212A) with a 10 mm Max-Light flow cell. A multiple heartcutting interface (ASM valve + 2 x Deck valves) (5067-6585) was used to collect fractions of ^{1}D effluent and transfer them to the second dimension for further separation. The interface was equipped with 80 µL loops (5067-5426) and an 85 mm ASM bypass capillary (1 µL; ASM factor - 5). A pressure relief kit (G4212-68001) was used between the outlet of the ^{1}D detector and the interface. Instrument control and data acquisition was

performed using MassHunter (Agilent Technologies, Version 11.0, Build 11.0.203) or ChemStation Method and Run Control software (Agilent Technologies, Rev.C.01.10[201]). Agilent Qualitative Analysis (Version 10.0, Build 10.0.10305.0) and ChemStation Data Analysis software (Agilent Technologies, Rev.C.01.10[201]) software were used for data analysis.

System B (Figs. 5 and 6)

All separations were performed on an Agilent Technologies 1290 Infinity II UHPLC system. The first dimension consisted of an autosampler with a flow through needle configuration (G7129B), a flexible pump (G7104A), a thermostated column compartment (G7116B), and a VWD UV absorbance detector (G7114B) with a 2 µL microflow cell (G1314-60187). A multiple heartcutting interface (ASM valve + 2 x Deck valves) (5067-6585) was used for modulation between dimensions. The interface was equipped with 40 µL loops (G4242-64000) and a 340 mm ASM bypass capillary (PN 5500-1302; 3.8 µL; ASM factor – 2). A pressure relief kit (G4212-68001) was used between the outlet of the ^{1}D detector and the modulation interface. The second dimension consisted of a 1290 high speed pump (G7120A) with a 35 µL Jet Weaver mixer, a thermostated column compartment, and a DAD UV absorbance detector (G7117A) with a 10 mm, 0.6 µL flow cell. A pressure release kit was used between the outlet of the ^{2}D DAD detector and the inlet of the mass spectrometer. The mass spectrometer was a 6545XT AdvanceBio quadrupole time-of-flight system (Agilent Technologies, Santa Clara, CA, USA). Instrument control and data acquisition was performed using MassHunter 11.0 (Agilent Technologies). Data analysis was performed using MassHunter qualitative analysis 10.0 (Agilent Technologies)

Columns

The separations shown in Figs. 1, 3, and 4 used InfinityLab Poroshell HPH-C18 columns in both dimensions. The ^{1}D column was 50 mm x 2.1 mm i.d. (2.7 µm), and the ^{2}D column was 150 mm x 2.1 mm i.d. (2.7 µm).

The separations shown in Figs. 5 and 6 used Acquity BEH C18 (Waters Corporation, Milford, MA, USA) columns in both dimensions. The ^{1}D column was a 50 x 2.1 mm i.d. (1.7 µm) and the ^{2}D column was a 100 x 2.1 mm i.d. (1.7 µm).

2.5 Chromatographic Conditions

The 2D-LC instrument control software was configured with a 10 µL loop, even though 80 µL physical loops were installed. This enabled the use of very narrow cuts in multiple heartcutting mode, when desired for development purposes. The ASM valve was plumbed in the First-In / Last-Out (FILO) configuration.

General method parameters are summarized in table 2, specific gradient conditions are included in the respective figure captions.

2.6 Mass spectrometric conditions

Online mass spectrometry measurements were performed using a 6545XT AdvanceBio quadrupole time-of-flight mass spectrometer (Agilent Technologies) operated in negative ionization mode. The capillary voltage was 4 kV, nozzle voltage was 1 kV, gas and sheath gas temperatures were 325 C, drying gas was 10 L/min, nebulizer was 28 psi, sheath gas flow of 10 L/min, fragmentor was 110 V, skimmer was 65 V, and mass range was 500-3000 *m/z* collected at 2 Hz.

3. Results and Discussion

3.1 1D separations are inadequate for the complete resolution of some oligonucleotide samples

When working with ON mixtures containing truncated or elongated (i.e., shortmers or longmers) sequences that are variants of the full-length product (FLP), gradient elution methods are necessary to ensure both adequate retention of all possible truncations and elution of longer variants within a reasonable analysis time [28]. Figure 1 shows the separation of a mixture of the FLP LR1 (23-mer) and several shortmers. Additionally, several variants with different degrees of phosphorothioation (PS) (0, 1 and 2; LR8, LR10 and LR3, respectively) are included. These impurities were selected as they are common impurities generated during solid phase oligonucleotide synthesis. When considering the separation of a mixture of these impurities, a relatively steep gradient (23.5-98.5 %B) was employed to ensure a short analysis time while maintaining resolution of the shortmers; however, the PS variants co-elute with the main FLP peak under these conditions. If a substantially shallower gradient were used to provide better resolution of the PS variants, either the shortmers would not be sufficiently retained due to a gradient that starts too high in ACN, or the analysis time would be unreasonably long due to the high retention of the FLP in a mobile phase that is sufficiently weak to retain the shortmers. Herein lies the challenge of separating complex ON mixtures with a single chromatographic method - we must prioritize either resolution or analysis time. With current technology it is impossible to both accommodate a heterogenous mix of ONs in terms of length, and resolve closely related species, with a single method in a reasonable analysis time. In this paper we demonstrate that heartcutting 2D-LC provides a practical and approachable solution to this problem. A first-

dimension separation with a relatively steep gradient is used to provide a coarse separation of the sample, while the second dimension is used to provide the additional separation needed to resolve closely-related critical pairs that co-elute in the first dimension.

3.2 Iterative retention modeling for discovery of ^{2}D elution conditions

In previous work we developed a workflow for the systematic discovery of ^{2}D elution conditions needed to resolve closely-related species in 2D-LC separations of peptides [27]. Neue has shown that ONs are more sensitive to changing solvent composition than peptides and proteins (indicated by large *S* values) [29]. More recently, Guillarme and coworkers have demonstrated the high sensitivity of ONs to increasing solvent strength [26], suggesting that method development based on pure trial and error is particularly unforgiving. The work shown in the current paper builds upon this previously established foundation. The workflow is summarized schematically in Figure 2. It is an iterative approach that relies on the well-established linear solvent strength (LSS) theory of gradient elution [30,31] to predict elution conditions for shallow gradients that both provide the best possible change of resolving closely-related species, and have the right compositions needed to place the peaks of interest roughly in the middle of the ^{2}D separation window. Readers interested in a complete description of the development of the workflow and its limitations are again referred to our prior work [27]; here only the critical aspects relevant to the current work are repeated.

Ultimately, we want to know what starting (ϕ_i) and ending (ϕ_f) compositions are needed in a shallow (e.g., 1-2% B change during the gradient) ^{2}D gradient. Our approach is to begin with two generic model training methods that cover the full range of mobile phase composition (5-95% B in this case) but use different gradient times to produce different gradient steepness parameters. Using the observed retention times (t_r) under these conditions, we calculate effective gradient elution retention factors (k_{eff}) as shown in Eq. 1, where t_m and t_{ex} are the column dead time and the extra-column time, respectively.

$$k_{eff} = \frac{t_r - t_m}{t_m - t_{ex}} \quad (1)$$

LSS theory asserts that the relationship between the logarithm of retention factor and mobile phase composition is linear, and that they are linked between the analyte/condition-specific factors k_w and *S*, as shown in Eq. 2, where k_w is the hypothetical retention factor in 100% weak solvent (solvent A in this case) and *S* is related to the dependence of the analyte on organic concentration in the mobile phase (calculated by measuring the ln(*k*) under increasing organic fraction).

$$\ln(k) = \ln(k_w) - S \cdot \phi \quad (2)$$

With two k_{eff} values in hand following the first two training methods, we can solve for the analyte-specific k_w and S values using the gradient LSS equation shown in Eq. 3, where t_d is the gradient delay time, k_i is the retention factor of the analyte in the initial mobile phase composition used in the gradient (ϕ_i).

$$k_{eff} = \frac{t_d}{t_m} + \frac{1}{b}\ln\left(\frac{b \cdot k_i\left(t_m - \frac{t_d}{k_i}\right)}{t_m} + 1\right) \quad (3)$$

The gradient slope b is given by Eq. 4, where t_g is the gradient time and $\Delta\phi$ is the change in mobile phase composition during the gradient (0-1 scale).

$$b = \frac{S \cdot \Delta\phi \cdot t_m}{t_g} \quad (4)$$

The gradients used in our application of the workflow for LR1 and related impurities, along with the corresponding chromatograms that resulted from their use, are shown in Fig. 3, and the quantitative results are shown in Table 3. The ^{2}D gradients used in the first iteration of the workflow are shown in panels A and B. The resulting retention times for LR1, and the k_w and S values determined using Eqs. 1-4 are shown in rows 1-3 of Table 2. Using these k_w and S values, elution conditions were then predicted that would place the LR1 peak at a retention time of 9 min. relative to the start of the ^{2}D separation. Using the k_w and S values from the training runs, the first iteration yielded gradient parameters with a starting %B of 65.4% for a gradient with a change in composition of 12% B. This gradient is shown in Fig. 3C (blue trace), along with the ^{2}D chromatogram where we see that the LR1 peak is quite close to the target retention time (3% prediction error). Under these conditions, the LR3 variant is quite nicely resolved from the FLP (LR1).

If additional ^{2}D resolution is desired, one can further decrease the gradient slope. In a second iteration of the workflow, the k_w and S values for LR1 were refined by fitting the retention data from the gradients shown in Figs. 3B/C instead of 3A/B. These refined k_w and S values were then used to predict the elution conditions needed to again place the LR1 peak at 9 min. relative to the start of the ^{2}D separation, but with a much shallower gradient with a change in composition of just 2% B. The resulting gradient, starting at 68.3% B, and the corresponding chromatogram are shown in Fig. 3D. Here again we see that the LR1 peak appears quite close to the target (11% prediction error), and the resolution of LR3 from LR1 is improved

relative to the result in Fig. 3C. There is an additional peak that appears between LR1 and LR3; this is a different impurity that is present in the LR1 sample. In cases where more accurate retention prediction in necessary, additional iterations can be performed to refine the retention model.

3.3 Iterative method development for phosphorothioate pharmaceuticals allows for high sensitivity impurity profiling

In the context of impurity profiling, it is important not only to be able to resolve impurities from the main product, but also to be able to detect and report impurity species at levels around 0.1% relative to the main component. To evaluate the sensitivity of the method developed using our workflow as discussed above, a series of samples was analyzed with LR3 spiked into LR1 at different levels. Here the concentration of LR1 was always 1 mg/mL, and LR3 was spiked in at concentrations corresponding to 0.1, 0.3, 1.0, 3.0, and 10% (w/w) of LR1. The resulting chromatograms are shown in Fig. 4, with results in panel A shown for samples containing only LR3 at different levels, and results in panel B for samples with LR3 spiked into LR1. These results show that sufficient signal is obtained for concentration levels down to ~ 0.1 – 0.3% relative to the main peak. This detection sensitivity is suitable for current recommendations from the industry which suggests reporting and identification thresholds of 0.1% and 0.5%.

3.4 IPRP-IPRP allows for targeted heartcut method development allowing MS identification of ON impurities in crude pharmaceutical samples

Following synthesis of oligonucleotides, there are many impurities ranging from large structural variants such as truncations and additions - which results in > 300 Da mass differences - to highly chemically similar impurities such as defluorinations, oxidations, or depurinations [2]. This large range in the types of impurities poses a difficult analytical challenge, and often requires implementing multiple separation modes or parameters to adequately characterize. Figure 5A exemplifies the type of sample that can be expected with crude ON synthesis; it is immediately apparent that a broader range of %B (2-13%) allows for the retention of all components in a reasonable amount of time (~10 minutes). However, the main peak has a co-eluting shoulder that cannot be resolved in this case. As such, heartcutting 2D-LC allows for the isolation of this critical pair into the second dimension wherein the method was iteratively modified from 7-12.5 %B (0.55 %B/min) (Figure 5B) to 9.6-10.1 %B (0.05 %B/min) to improve selectivity (Fig. 5C). The two

components are now well-separated and the impurity was identified *via* mass spectrometry as a -16 Da species, indicative of an oxidation of a phosphorothioate group (PS->PO).

In a second example (Fig. 6), a crude sample of a 36-mer therapeutic oligonucleotide produced by solid phase synthesis was analyzed. It is again apparent that there are a number of shortmer impurities that are easily separated but require a broad gradient range to accommodate the large chemical and size differences of these impurities. Moreover, as the oligonucleotide length increases, the difficulty in separating n-1 or n+1 impurities also increases [32].A 36-mer oligonucleotide is on the upper size range for siRNA, which are typically ~20 nucleotides long for each strand. The ^{1}D chromatogram becomes quite crowded around the main peak at 16.5 min (Fig. 6A). Selective comprehensive sampling of the ^{1}D separation provides a means to characterize all of the co-eluting impurities by splitting the peak into fractions—in this way we can vastly improve the relative concentrations of the FLP and the impurities present in some of the fractions. Goyon and Zhang have employed similar methodologies to elucidate the antisense ONs which co-elute near the main peak [19], however, the authors utilized HILIC in the second dimension as a desalting step to improve MS detection. Focusing on cuts 1, 2, and 3, we see a number of species now resolved or partially resolved (Fig. 6B) that were previously co-eluting in the first dimension. Extracted ion chromatograms (EICs) were generated from cut 2 and are plotted in Fig. 6C. These EICs illustrate the benefit of second dimension separations in combination with mass spectrometry for in-depth impurity characterization of chemically-similar species, including a PS->PO oxidation, defluorination, and n-1 and n-2 truncations. The defluorination impurity in particular benefits from chromatographic resolution due to the large isotopic overlap of a Δ2 Da mass difference relative to the FLP when working with large molecules (~13 kDa for the 36-mer in this case), which is apparent in the EIC.

4. **Concluding remarks**

With the increasing popularity of ONs in the developmental pipeline of most major pharmaceutical companies it is becoming obvious that the separation of ON mixtures containing both very different sequence variants (e.g., shortmers/longmers) and very similar variants (e.g., phosphorthioate variants) using a single LC method is challenging. As such, 2D-LC provides an effective solution to preserve both separation performance and experimental throughput, as the co-eluting species in the ^{1}D are selectively taken for further separation in the ^{2}D. That said, method development in 2D-LC is still somewhat of an art. Herein, we have applied a workflow for the systematic determination of ^{2}D elution conditions that provide the best chance of resolving closely-related variants that coelute in the first dimension (i.e., shallow

gradients, or isocratic conditions). As a starting point we use IPRP conditions and the same stationary phase in both dimensions. In this case, the additional resolution realized in the second dimension is simply due to the use of shallower gradients. We find that a LSS model built from two or three training experiments for the second dimension is sufficiently accurate to guide choice of ^{2}D elution conditions. The resulting 2D methods not only provide good resolution of closely-related species, but are also quite sensitive; using UV detection, low concentration impurities can be detected down to 0.1% of the FLP (where the FLP is in the linear range of a UV detector). Utility of IPRP-IPRP methods have been demonstrated for several therapeutic ON materials. Crude single stranded siRNA ONs from solid phase synthesis were characterized using the IPRP-IPRP 2D-LC-MS iterative modeling approach. Several new impurities were resolved and identified that co-eluted in the first dimension. Reduction in co-elution allows for better implementation of MS detection as complex ON samples tend to contain many structurally related impurities with minimal mass differences leading to difficulties in the interpretation of MS spectra as well as considerable ion suppression effects from charge competition. As such, with the implementation of easily approachable method development tools for 2D-LC it is expected that many labs can take advantage of this methodology to spur the adoption of 2D-LC into the QC and QA lab environments.

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Conflict of interest

The authors declare no competing interests

Author contributions

Daniel Meston: Draft manuscript preparation, analysis and interpretation of results, review & editing;

Maria Sylvester: Data collection, draft manuscript preparation, analysis and interpretation of results;

Matt Sorensen: Sample production, draft manuscript preparation, analysis and interpretation of results;

Todd Maloney: Sample production, draft manuscript preparation, analysis and interpretation of results;

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Figure Captions

Figure 1. 1D IPRP separation of FLP LR1 and several shortmers and PS variants (LRs 3, 8, 10, 12, 13, 14) in water. The concentrations of LR1 and each of the variants were 1 and 0.1 µg/mL, respectively. Solvent gradient elution conditions were 23.5-98.5-100-100-23.5-23.5 %B from 0.0 - 6.0 - 6.01 - 6.5 – 7.0 – 8.0 min.

Figure 2. General workflow for iterative ^{2}D gradient method development. Reprinted with permission from [27].

Figure 3. Iterative modeling approach. The concentrations of LR1 and LR3 were 1 and 0.1 mg/mL, respectively. First dimension solvent gradient elution conditions were 23.5 - 98.5 – 100 – 100 - 23.5 - 23.5 %B from 0 - 6 - 6.01 - 6.5 - 6.51 - 8 min. Second dimension solvent gradient conditions were 5 - 95 – 100 – 100 - 5 - 5 %B from 0.0 - 10.0 - 10.01 - 11 – 12.0 – 15.0 min for training run A and the same gradient composition from 0.0 - 15.0 - 15.01 - 16 – 17.0 – 20.0 min for training run B. Subsequent shallower gradient conditions are outlined in Table 2.

Figure 4. Detection of a low-level impurity (LR3) in the presence of FLP at 1 mg/mL. A) Concentration series for LR3 only ranging from 1:1000 (LR3:LR1) (dark blue) to 1:10 (black); other concentrations are 1:300 (green), 1:100 (red), and 1:30 (blue). B) LR3 spiked into LR1 (1 mg/mL) at the same concentrations as in A. First dimension solvent gradient elution conditions were 23.5 - 98.5 – 100 – 100 - 23.5 - 23.5 %B from 0 - 6 - 6.01 - 6.5 - 6.51 - 8 min. Second dimension solvent gradient elution conditions were 5 - 68.3 - 70.3 – 100 – 100 - 5 - 5 %B from 0 - 0.25 - 10.25 - 10.26 - 11.25 - 11.5 - 13.5 min.

Figure 5: Crude oligonucleotide critical pair elucidation via heart cut 2D-LC. Full retention of all components was achieved with an 11% gradient, critical pair of co-eluted peaks sampled via UV-based heartcutting. First dimension gradient elution conditions were 2-13-2-2 %B from 0-7-7.01-10 min. (A). First iteration of second dimension separation conditions using 7-12.5% B over 6 min which partially

resolved co-eluting species (B). Final iteration of second dimension separation using 9.6-10.1% B over 10 min providing good resolution of co-eluting impurity with identification by high resolution mass spectrometry (C). Second dimension gradient elution conditions were 1-7-12.5-1-1% B from 0-0.5-6.5-6.51-7 min for the first iteration and 1-9.6-10.1-1-1 from 0-0.5-10.5-10.51-11 min for the final iteration.

Figure 6: Crude oligonucleotide impurity profiling via multiple heart cutting 2D-LC-MS. Full retention of all components possible with a 12% gradient, with selective comprehensive sampling of 5 cuts across the main peak (A). Second dimension UV chromatograms of cuts 1, 2, and 3 illustrating resolution improvements implementing a gradient span of 11.5 – 13.5% B following iterative modeling. (B). Extracted ion chromatograms from cut 2 allowing identification of impurities. The defluorination impurity is a 2 Da difference leading to overlap of the isotopic distribution with the main peak, thus the main peak is observed in the EIC (C). First dimension gradient elution conditions were 3-15-3-3% B from 0-18-18.01-20 min. Second dimension gradient elution conditions were 1-11.5-13.5-11.5-11.5% B from 0-0.5-10.5-10.51-11 min.