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Ion-pair reversed-phase chromatography analysis of oligonucleotides using ultra-short (20 x 2.1 mm) columns. Tutorial

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ABSTRACT

With this work, we present a comprehensive tutorial for the analysis of oligonucleotides (ONs, 5 to 100 mer) using ion-pair reversed-phase liquid chromatography (IP-RPLC) on ultra-short columns (20 × 2.1 mm). We explore the impact of ion-pairing (IP) agents on ON retention and demonstrate that while IP agents significantly influence absolute retention, their effect on selectivity is often minimal. Our findings emphasize the utility of systematic method development, including software-assisted retention modeling, to optimize gradient steepness and temperature such that resolution can be optimized for both sequence and length variants. We recommend the use of low-adsorption column hardware to minimize nonspecific interactions, which is to the benefit of improving method robustness, peak shapes and recovery (especially of shortmer impurities). Our results confirm the utility of so-called ultra-short column formats as is demonstrated by way of the example, quick run time separations and high-throughput ON analyses. The study establishes practical guidelines for developing robust, reproducible, and high-efficiency IP-RPLC methods for ONs which will be of assistance to analysts working on new therapeutics and new sequencing and diagnostic reagents alike.

1. Introduction

Ion-pair reversed-phase liquid chromatography (IP-RPLC) is considered to be the reference method for analytical scale separations of oligonucleotides (ONs) [1]. A major advantage of RPLC over other chromatographic modes is that it allows direct liquid chromatography-mass spectrometry (LC-MS) analysis of both ONs and nucleic acids [1]. RPLC, in general, results in high separation efficiency (plate count or peak capacity) compared to any other chromatographic mode (note that anion exchange (AEX) and hydrophilic interaction chromatography (HILIC) are also often considered as alternative separation modes.). In current practice, IP mobile phases consist of volatile components: alkylamines buffered with acids (acetic acid (AcOH) or hexafluoroisopropanol (HFIP)). Although the adsorption and elution mechanisms are quite complex because of combined hydrophobic and electrostatic interactions, IP-RP measurement are known to be reproducible and robust [2]. In addition, common retention models (such as the Linear Solvent Strength (LSS) model) can be effectively applied to describe the experimentally observed retention times, making it possible to predict selectivity and resolution and to optimize a separation with just two or three initial calibration experiments [3].

Large molecule separations (i.e. proteins, nucleic acids) have been historically developed by trial-and-error. Arbitrary variables have been studied, usually one factor at a time (OFAT), which is a low efficiency and time-consuming approach. Moreover, such a strategy does not guarantee finding the global optimum of a method. In contrast, computer (or software) assisted method development approaches have been routinely used for the optimization of small molecule separations, where there has been more of a concerted aim to improve throughput and provide detailed information about method specificity and robustness [4,5]. These approaches are now being applied to the development of peptide and therapeutic protein separations [6,7]. Illustrating this trend, a recent paper reported a fully automated, systematic modeling process to rapidly optimize high-throughput RP, ion exchange (IEX), hydrophilic interaction liquid chromatography (HILIC), and size exclusion chromatography (SEC) separations for two COVID-19-related monoclonal antibodies [8]. Although the retention behavior and selectivity of IP-RPLC separations of ONs have been studied in detail [9,10,11,12], a systematic and rapid method development approach has not yet been proposed. Gilar et al. reported that the retention behavior of hetero-oligonucleotides can be extrapolated from the retention of homo-oligonucleotide ladders [13]. Their model has been very useful for

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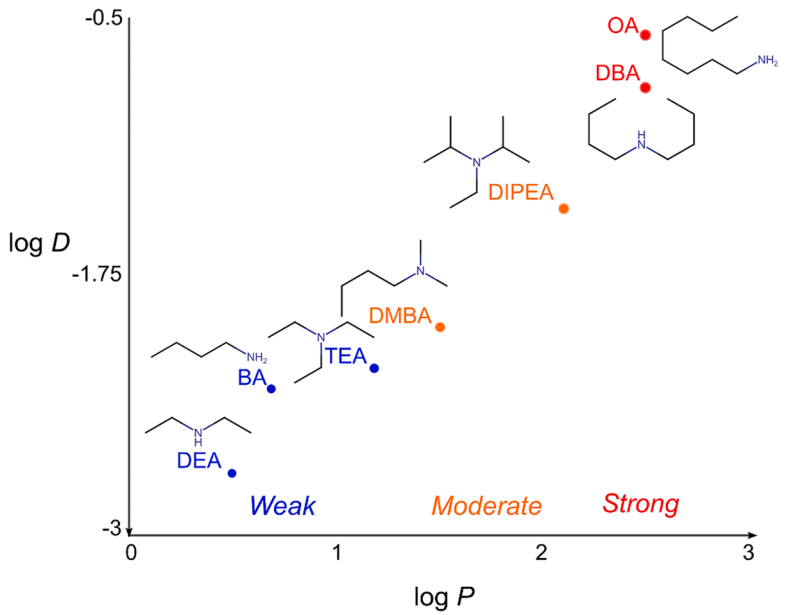

Fig. 1. Map of log *D* and log *P* values for selected IP agents categorized as weak, moderate or strong (blue, orange, red) with their corresponding chemical structures and abbreviation.

the rational selection of initial mobile phase compositions. This early work also suggested an on-off (bind and elute) like elution behavior of ONs and confirmed that length variants of ONs follow the so-called homologs rule, wherein the retention time of larger ONs increases logarithmically rather than linearly with the elution strength of the mobile phase. These important findings provided a very good basis for retention modeling and systematic method development. Gilar and Zampa proposed a workflow that helps to select the most appropriate ion pairing system ("weak" or "strong") for a given purpose (depending on the size of the ONs) [14].

In this article, we attempt to provide a general tutorial for practicing chromatographers so they can efficiently optimize high throughput IP-RPLC methods for ONs. We discuss the important method variables, including the retention behavior of ON analytes, the influence of column length, and the possibility of applying retention modeling and *in silico* robustness studies. Recommendations for establishing ultra-fast generic (platform) methods are also provided. Note that throughout the paper we refer to the 20 × 2.1 mm format (ultra short) 1.7 µm 300 Å ACQUITY™ Premier Oligonucleotide BEH^{TM} C18 Column. Based on our batch material test data, the total carbon content of this packing material is 12.8 %, the C18 surface coverage is 3.53 µmol/m^2, the average pore diameter is 278 Å and the pore volume is 0.64 cm^3/g. In terms of column efficiency, a small test analyte (acenaphthene) resulted in plate number of $N = 3900$ at $k = 5.27$. Other short columns with similar properties can also be used.

2. General considerations on IP agents and mobile phase systems

Ion pairing systems (alkylamines buffered with volatile acids) have been classified as (1) weak, (2) moderate, and (3) strong. The attributed strength of an IP agent is determined by its hydrophobic/hydrophilic characteristics, sometimes driven even just by the number of carbon atoms it contains.

More hydrophilic IP agents (or less hydrophobic i.e. those with lower log *D* or log *P* values) are considered weak IP agents because they provide lower retention of ONs than hydrophobic IP agents. It is assumed that weak IP agents form a weaker interaction with the hydrophobic ligands of the stationary phase. Thus, analyte retention is mainly driven by hydrophobic interactions between the analytes (in this case, ion-paired ONs or nucleic acids) and the stationary phase ligands. For these reasons, weak IP systems have been preferred in applications where the ONs or nucleic acids differ in their hydrophobicity (sequence variants or diastereomers) [9,10,15].

More hydrophobic alkylamines act as strong IP agents. It is reasonable to assume that such agents can adsorb strongly onto the stationary phase and thus the hydrophobic ligands of the stationary phase are masked by the charges of the bound IP agents, with their charges likely directed towards the aqueous mobile phase forming a stagnant ionic layer on its surface. Therefore, a strong ionic (ion-exchange-like) interaction could occur between the negatively charged ONs or nucleic acids and the surface of the stationary phase (covered by the positively charged layer of IP agent molecules). For these reasons, strong IP agents are usually used for length-based (size-based) ON separations. The retention behavior observed in IP-RPLC with strong IP agents is often described by the so-called electrostatic retention model (ERM) which predicts the retention increase for analytes with opposite charge with the increase of the IP reagent concentration [16,17]. The strength of the electrostatic interaction depends on the number of charges on the ON, hence on its length.

The strength of an IP system can be estimated by some common physicochemical descriptors such as log *P*, log *D* or boiling point. However, the observed chromatographic retention is probably the most relevant descriptor of an alkylamine's hydrophobicity [1,18]. Based on experimentally observed retention values of common alkylamines, Gilar et al. classified a wide range of IP reagents. Butylamine (BA), diethylamine (DEA) and triethylamine (TEA) are considered as weak IP agents, while hexylamine (HA), octylamine (OA), dibutylamine (DBA), dicyclohexylamine (DcHA), dihexylamine (DHA), tripropylamine (TPA) and tributylamine (TBA) can be considered as strong IP agents. Diisopropylethylamine (DIPEA) and dimethylbutylamine (DMBA) might be considered as moderate IP agents (Fig. 1). Similar retention order was obtained for both methanol and acetonitrile as organic eluents [1]. Note that the apparent strength of an IP system may also depend on the buffer system's counter ion (acetate or HFIP) [1]. It has been reported that HFIP in the mobile phase promotes adsorption of IP reagents on the RP stationary phase and increases the apparent strength of TEA, TPA and TBA [1,2,10,19] compared to mobile phases buffered with acetate. The observed retention gains were smaller for secondary amines, and minimal for primary alkylamines.

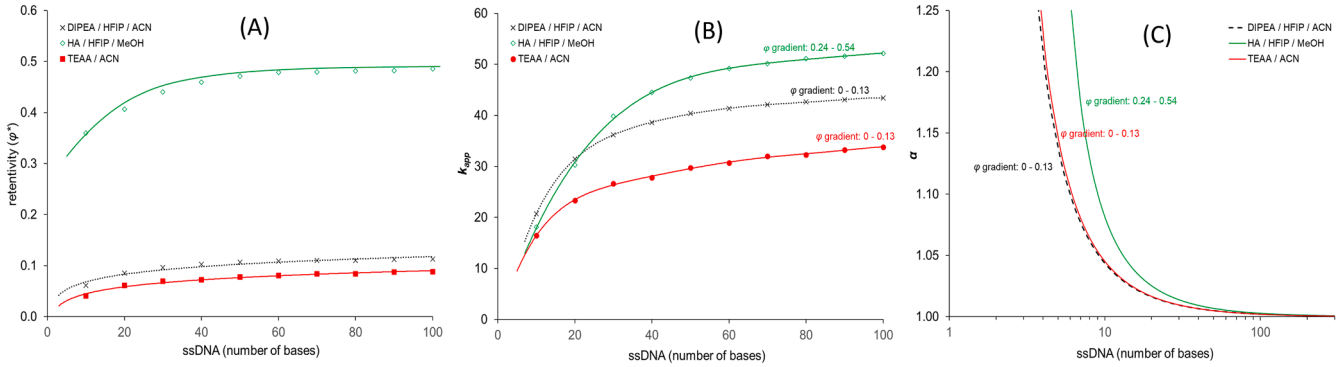

Fig. 2. Absolute retentivity (A), apparent retention (B) and selectivity (C) as a function of base number (length / size) derived for ssDNA ladder.

Acetate and bicarbonate have been commonly used as mobile phase buffering counter ions for ON separations with UV detection [20]. In 1997, Apffel first proposed the use of a fluoroalcohol - HFIP in combination with TEA for improved sensitivity mass spectrometry (MS) detection [21]. Since then, HFIP and other fluorinated, weakly acidic alcohols have been adopted as routine IP buffer acids for superior MS detection [22,23,24].

Regarding organic co-solvents, both AcOH and HFIP are miscible with water, methanol and acetonitrile. However, in the presence of some strong IP agents, a non-miscible two-phase system may be observed in some cases. For example, ambient temperature 100 mM HFIP with 10 mM HA solution separates into a two-phase system when adding 10–30 % acetonitrile into the aqueous solution. Interestingly, a higher concentration of acetonitrile (i.e. 50 %) is not critical as a clear solution (one-phase system) is observed with increased organic solvent amount. In most cases the miscibility of a given mobile phase composition has to be verified experimentally.

Generally speaking, methanol results in slightly higher selectivity than acetonitrile. The likeliest reason for this is that methanol often results in lower solvent strength parameters (S) for ONs, so a longer column segment is utilized to control retention and gain selectivity. (See Section 3 and 5 for more details on solvent strength parameters). However, due to the higher viscosity of methanol, eluted peaks are somewhat broader and therefore the overall resolution is very similar when using both solvents.

In conclusion, when selecting the mobile phase system, the most appropriate IP system depends on the analytical objective and the nature of the analytes. Specific requirements such as LC-MS compatibility and sensitivity, length-based or sequence-based separations, and the solubility (miscibility) of IP agents and buffer components in the organic co-solvent will dictate the choice of IP system.

3. The impact of IP system on selectivity

Despite the fact that various IP systems have been studied in detail for a long time, some misconceptions can be found in the literature, commonly related to the selectivity of the IP system. The basis of these misunderstandings probably stems from the fact that, when studying the selectivity of different IP systems, the elution windows of the analytes changes and so does the intrinsic gradient steepness. These must be systematically adjusted. Here, we try to accurately explain the effect of the IP system on the absolute and relative retention of ONs appropriately considering equivalent elution conditions.

The intrinsic gradient steepness (b) is the absolute measure of the gradient steepness of the entire phase system and it takes into account column dimension (spatial), time (temporal), and solute-specific properties (i.e., how solute retention is sensitive to changes in mobile phase composition). Maintaining the same rate of change of mobile phase composition over time (ΔB%/min) does not necessarily result in the same intrinsic gradient steepness (since the solvent strength parameter depends on the strength of the ion pairing agent).

$$b = S \cdot \Delta\varphi \cdot \frac{t_0}{t_G} \tag{1}$$

The above equation helps to define gradient steepness as a function of S - a retention model parameter (solvent strength parameter), φ - the volume fraction of the stronger eluent in the mobile phase ($\varphi = B$ %/100), t_0 - the column dead-time (determined by the column length and flow rate), and t_G - the duration of the gradient program. The $\Delta\varphi$ and t_G are set by the analyst, t_0 is measured directly from the chromatogram and b (and therefore S) can be determined from two linear gradient experiments [25], considering that ONs retention can be described by the semi-empirical linear solvent strength (LSS) model [3,13]:

$$k = k_0 10^{-S\varphi} \tag{2}$$

where k is the retention factor and k_0 is the limit of the solute retention factor observed in the weakest possible eluent (100 % aqueous mobile phase in the case of IP-RP). By performing two or more linear gradient experiments (with different t_G), the two model parameters (S and k_0) can be derived from the experimentally observed retention times (t_r):

$$t_r = \frac{t_0}{b} \cdot \log(2.3 k_0 b + 1) + t_0 + t_d \tag{3}$$

Where t_d is the gradient delay time of the chromatographic system. Note that Eq. (3) contains two unknown variables (b and k_0). The b and k_0 can be determined by iteration or fitting processes [7,26,27,28].

When running gradient experiments, there is no guarantee that the same S parameter will be observed with different IP systems despite the fact that ΔB%/min is maintained. Therefore, the best approximation is to consider the ratio of the two model parameters ($\varphi^* = \frac{\log k_0}{S}$). The φ^* value defines the mobile phase composition which results in retention $k = 1$ for a given solute. Since ONs follow an on-off like elution mechanism, it is reasonable to assume that a weaker mobile phase than φ^* (i.e. $\varphi < \varphi^*$) results in a very strong retention of the analytes (no migration at all) while a stronger mobile phase than φ^* (i.e. $\varphi > \varphi^*$) results in the complete release of the solutes (elution without any physico-chemical retention). Therefore, comparing the φ^* values is an appropriate method to study and compare the absolute retentivity observed in different IP systems.

The above-described method has been used to compare the absolute retentivity (φ^*), the apparent retention (gradient retention factors observed via running linear gradients) and the selectivity (α) obtained with different IP systems (TEA, DIPEA and HA) for a ssDNA oligonucleotide ladder (Fig. 2). Figs. 2A and B clearly show that, as the number of bases increases, retentivity and retention converge to a limit, no matter which IP system is employed. Fig. 2C illustrates that the selectivity of the separation decreases with increasing ON size (length) and converges to $\alpha = 1$. For these ssDNAs, beyond 40–50 bases, the selectivity plots completely overlap, indicating that the IP system has no

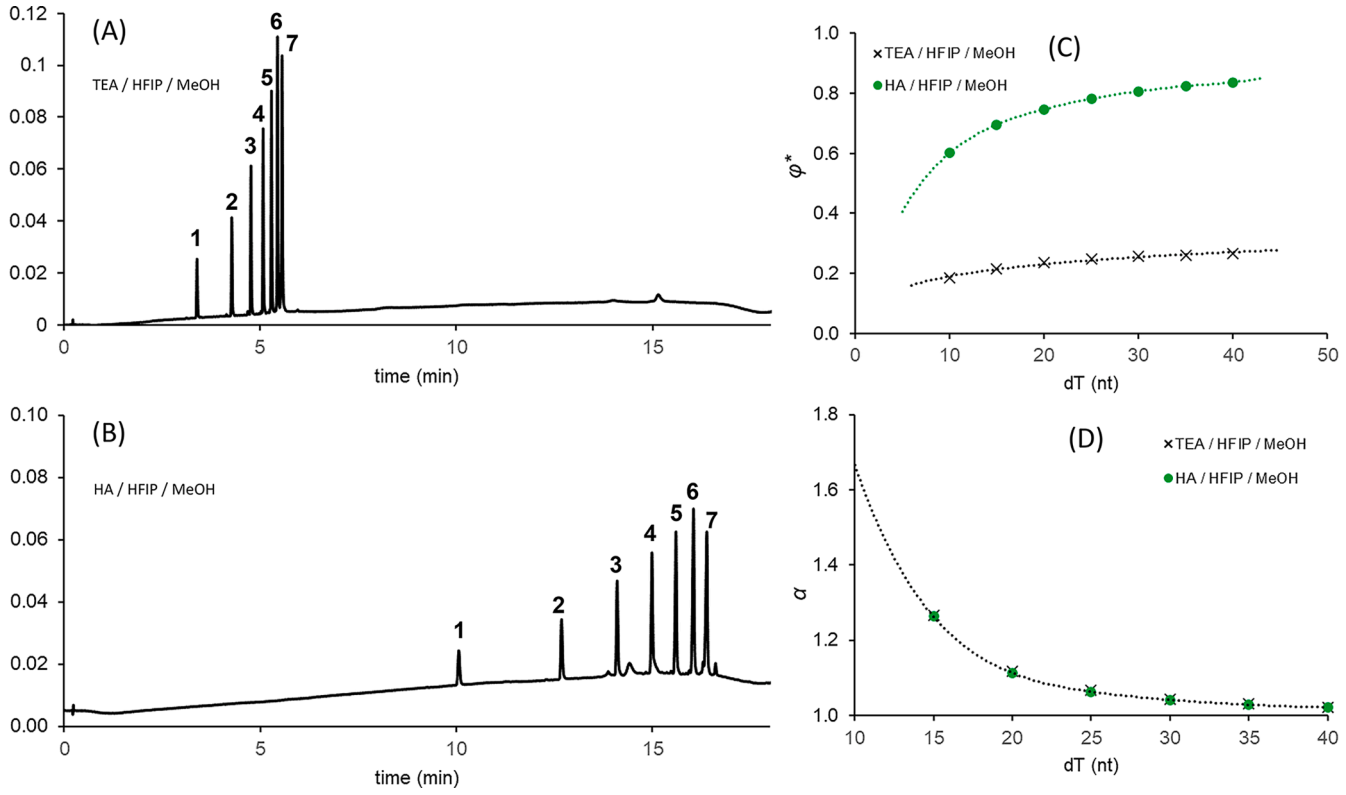

Fig. 3. Separation of oligodeoxythymidine ladder with weak (A) and strong (B) IP systems, and the corresponding absolute retentivity (C) and selectivity (D) plots. Conditions: 20 × 2.1 mm, 1.7 µm 300 Å ACQUITY Premier Oligonucleotide BEH C18 Column, Mobile phase A: 10 mM IP agent + 100 mM HFIP in water, Mobile phase B: 10 mM IP agent + 100 mM HFIP in methanol/water 70/30. The IP agents are TEA (on panel A) and HA (on panel B). Flow rate: 0.4 mL/min, Gradient: 0 – 100 %B in 20 min, Temperature: 55 °C. Peaks dT-10 (1), dT-15 (2), dT-20 (3), dT-25 (4), dT-30 (5), dT-35 (6) and dT-40 (7).

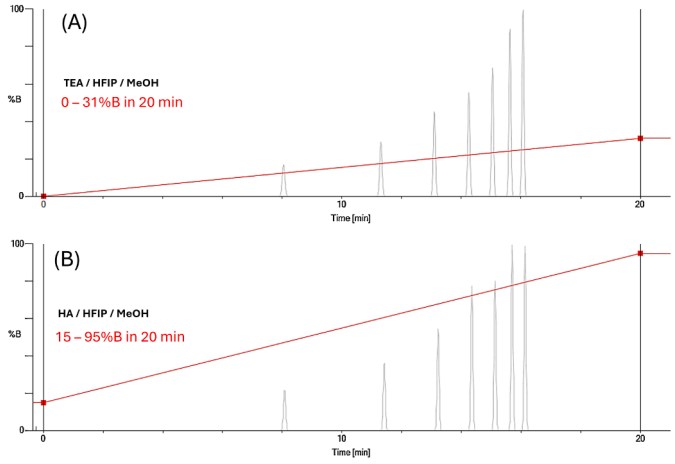

Fig. 4. Separation of oligodeoxythymidine ladder with weak (A) and strong (B) IP systems. The conditions and peaks are the same as in the caption of Fig. 3, except for the initial and final composition of the gradient. The gradient program is adjusted to match the peak distribution.

effect on the separation of > 50 bases ssDNAs. It is believed that stronger IP agents are advantageous for the separation of large ONs, but this is not necessarily true. Strong IP agents resulted in slightly higher selectivity only for the smaller ONs (< 50 bases) compared to weak IP systems (in this example). However, for this sequence length (< 50 bases), the weak IP agents already provide high enough selectivity. Therefore, from selectivity point of view, it is indifferent to use weak or strong IP agents. The characteristics of the IP system mainly affect the absolute retention of ONs, but the relative retention (selectivity) changes only to a very small extent and only for the smaller (shorter) ssDNAs.

Another example is shown in Fig. 3. It illustrates the separation of an oligodeoxythymidine homopolymer ladder. Fig. 3A and B show experimentally measured chromatograms using 10 mM TEA and 10 mM HA as IP agents. As expected, HA results in significantly higher retention than TEA. Fig. 3C shows the absolute retention of the two IP systems. Note, however, that the relative retention of the peaks (α between sequentially eluted peaks) is identical (Fig. 3D).

By adjusting the linear gradient conditions between two (or more) IP systems to set the same retention time for the first and last eluting peaks, an identical separation is expected. This behavior is illustrated in Fig. 4, as the peak retention times are completely matched only by changing the initial and final mobile phase compositions. The same rules apply to any other oligonucleotide homolog.

The above discussion supports the idea that the IP agent does not have a significant effect on the selectivity of ON homologs, but on absolute retention. Therefore, varying the IP agent (i.e., replacing a weak IP agent with a stronger one) is only beneficial if a weak IP system has not resulted in sufficiently high retention. Otherwise, very similar (or identical) selectivity and separation can be achieved with any IP system by only adjusting the initial and final gradient composition (setting the same intrinsic gradient steepness).

4. Need for low-adsorption column hardware

IP-RP analysis of oligonucleotides has been demonstrated to suffer from nonspecific adsorption of the highly charged analytes to the exposed metallic surfaces (column hardware, flow paths, flow cell) leading to reduced recovery and tailing of peaks [29]. For successful method development, it is imperative to minimize these sorts of unwanted interactions. In turn, an analyst can study the true effect of IP systems and their method conditions. It has been concluded that column

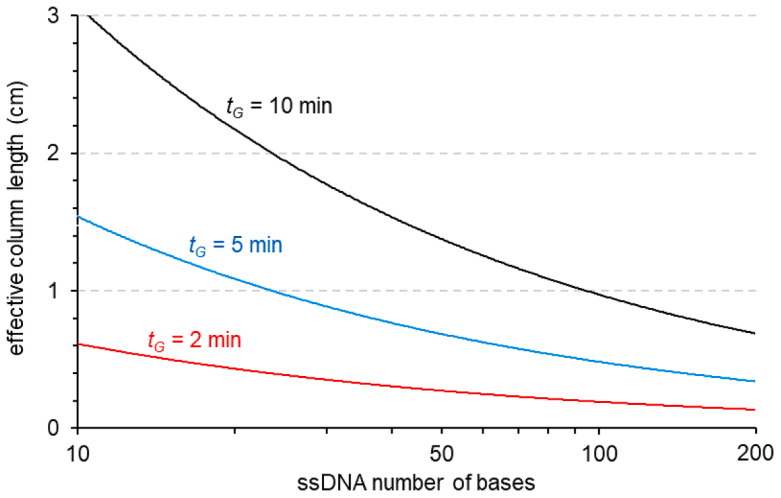

Fig. 5. Effective column length as a function of ssDNA homolog base numbers. Conditions: Mobile phase system: 10 mM DIPEA, 100 mM HFIP with acetonitrile as co-solvent, $F = 0.4$ mL/min, column internal diameter: 2.1 mm, gradient $\Delta\varphi = 0.25$.

hardware can have a very significant impact on observed recoveries, as well as flow cells and system components [30]. Various mitigation strategies have been explored with the replacement of stainless-steel surfaces with lower adsorption surfaces, with titanium, PEEK, and hybrid surface technology (HST) being the most effective alternatives [31]. Current state-of-the-art practice is to use a column with bioinert hardware which removes the need for lengthy conditioning processes and ensures high recovery and reproducible separations [32]. The material of the UV flow cell can play an important role on the observed peak shape too. PTFE and stainless-steel flow cell parts might be suitable for many different types of analyses, but there are times when it may become critical to consider alternative flow cells and LC flow path components.

5. Column format: no need for long columns

Previous studies have shown that ON separations benefit from shallow gradients and that ON species elute in relatively sharp peaks despite the shallow gradients that have been applied [1,3,13,14]. This suggests that ON retention is highly sensitive to small changes in mobile phase composition, resulting in a large gradient band compression effect. Retention sensitivity and strong band compression effects are consequences of very high solvent strength parameter values ($S > 50$) and the so-called "on-off" (often called "bind and elute" or "trap and shoot") elution behavior [3]. An important consequence of the on-off-like behavior is that very short columns can be used without loss of chromatographic resolution. Very short columns and disk-like formats have been investigated in the past [33]. Monolithic disks (3 × 16, 2 × 25 and 3 × 12 mm formats), perfusion chromatography and "chromatographic cakes" (with 1 - 50 mm length and 5 to 500 mm i.d.) have been applied in different chromatographic modes such as RPLC, ion exchange (IEX), hydrophobic interaction chromatography (HIC) and affinity chromatography [34-38]. Podgornik et al. have already reported a 3.5 min separation of oligomers using a 12 mm ion exchange monolithic column [39].

Since ONs tend to exhibit an on-off elution mechanism, only a short inlet segment of a column bed is involved in their retention [3,40]. Recently, a calculation method was developed to estimate the length of the column bed required to achieve an effective separation [40]. The procedure requires only the S model parameter and a set retention factor at the column outlet. The required length (L_{eff}) can then be calculated for a given column diameter, flow rate and gradient steepness.

This procedure was used to determine the effective column length for ssDNA separations. Fig. 5 shows the effective length for three different

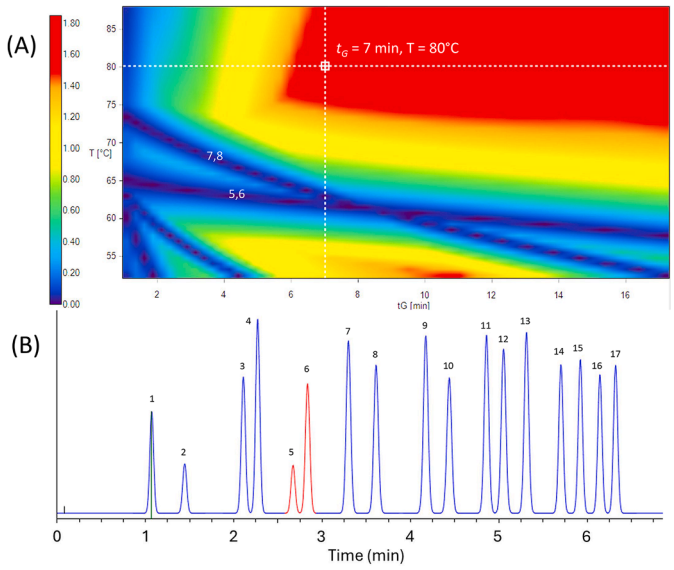

Fig. 6. 2D resolution map (A) and corresponding chromatogram (B) obtained for a complex ON mixture. Conditions: 20 × 2.1 mm, 1.7 µm 300 Å ACQUITY Premier Oligonucleotide BEH C18 Column, Mobile phase A: 10 mM DIPEA + 100 mM HFIP in water, Mobile phase B: 10 mM DIPEA + 100 mM HFIP in acetonitrile/water 50/50. Flow rate: 0.5 mL/min, Gradient: 3 – 23 %B in 7 min, Temperature: 80 °C. Peaks: ssDNA-10 (1), ssDNA-15 (2), dT-15 (3), ssDNA-20 (4), ssDNA-25 (5), dT-20 (6), ssDNA-30 (7), dT-25 (8), ssDNA-40 (9), dT-30 (10), ssDNA-50 (11), dT-35 (12), ssDNA-60 (13), ssDNA-70 (14), ssDNA-80 (15), ssDNA-90 (16), ssDNA-100 (17). On panel (A), on the color-coded resolution map, the red area indicates high resolution conditions while dark blue areas correspond to low resolution conditions.

gradient times as a function of ssDNA base number (size). As can be seen, the effective length decreases as the size of the ON species increases. When running very fast gradients ($t_G = 2$ min), no more than $L_{eff} = 0.6$ cm is required for the analysis of 10 to 200 mer ssDNAs. For a $t_G = 5$ min gradient, $L_{eff} = 0.5$ - 1.5 cm is required. Finally, when considering a $t_G =$ 10 min separation, $L_{eff} = 0.8$ - 3 cm is required. Note that for >25 base species, there is no need for a $L > 2$ cm column. For most fast linear gradient separations, a column length between 0.5 and 2 cm is appropriate. Longer columns do not improve selectivity or separation efficiency. We encourage chromatographers to use 2 cm column formats (ultra-short columns, i.e. 20 × 2.1 mm) for analytical scale ON

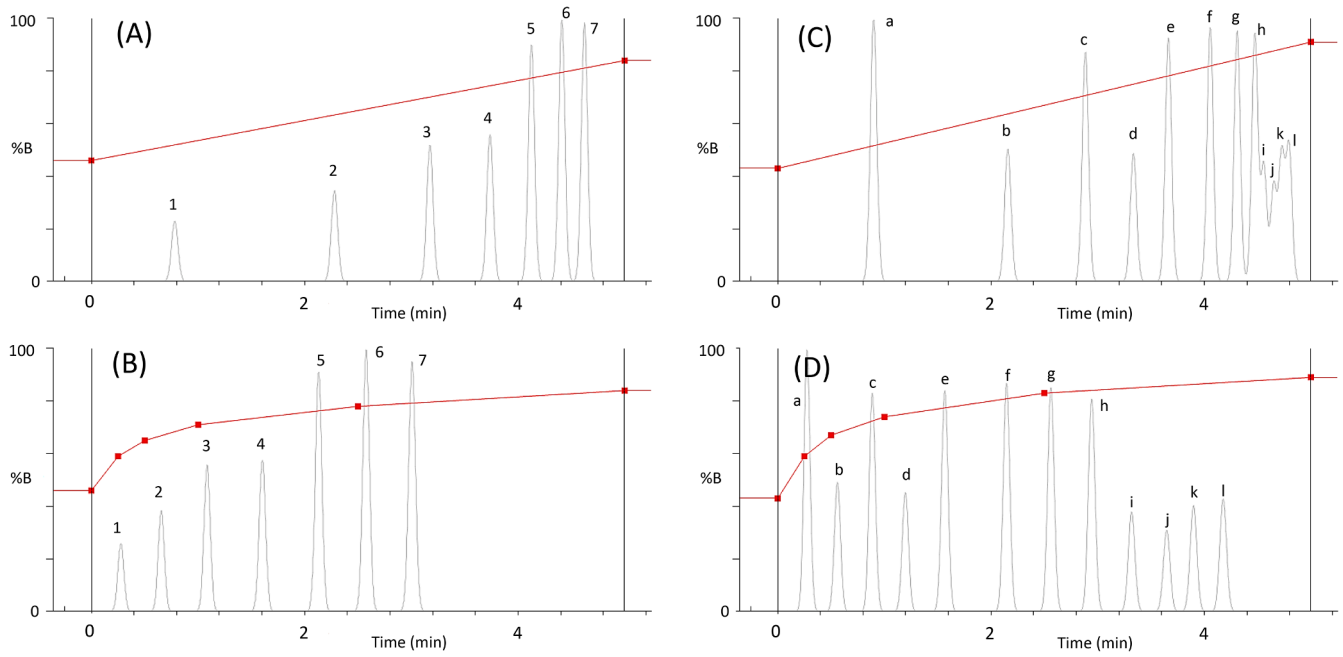

Fig. 7. Comparison of linear (A,C) and logarithmic (B,D) gradients and the retention patterns of dT ladder (A,B) and ssDNA ladder (C,D) separations. Conditions: 20 × 2.1 mm, 1.7 µm 300 Å ACQUITY Premier Oligonucleotide BEH C18 Column, Mobile phase A: 10 mM DIPEA + 100 mM HFIP in water, Mobile phase B: 10 mM HA + 100 mM HFIP in methanol/water 70/30. Flow rate: 0.4 mL/min, Temperature: 55 °C. Gradient: 46 – 83 %B in 5 min for the dT ladder and 43 – 91 %B in 5 min for the ssDNA ladder. Peaks: dT-10 (1), dT-15 (2), dT-20 (3), dT-25 (4), dT-30 (5), dT-35 (6), dT-40 (7), ssDNA-10 (a), ssDNA-15 (b), ssDNA-20 (c), ssDNA-25 (d), ssDNA-30 (e), ssDNA-40 (f), ssDNA-50 (g), ssDNA-60 (h), ssDNA-70 (i), ssDNA-80 (j), ssDNA-90 (k) and ssDNA-100 (l).

separations, instead of the 5 - 15 cm columns routinely used.

6. Method development for sequence variants (optimization of resolution)

When optimizing the separation of complex ON mixtures, the two most important method variables are gradient steepness (gradient time, t_G) and mobile phase temperature (T). First, an appropriate mobile phase system (IP and % co-solvent in mobile phase B) must be selected that allows elution of all species in the sample mixture. This may require some screening experiments. It is then best practice to apply a retention model combining t_G and T and optimize the peak resolution (Rs). As described in Section 3, the LSS model can be used, which requires studying the effect of t_G at two levels (two sets of t_G, i.e. a short (steep) and a long (shallow) gradient). To model the effect of temperature, the common Van't Hoff model can be safely used (the logarithmic retention factor is a linear function of the reciprocal temperature in RP conditions). Temperature also needs to be studied at two levels (low and high temperature). Therefore, the entire optimization process requires only four experiments (2 x t_G + 2 x T). By adjusting the t_G, the entire elution window of all peaks and the Rs between short and long ONs (size variants) can be adjusted. Meanwhile, by changing the temperature, the selectivity between sequence variants of similar ON lengths can be improved. The retention of length variants is primarily sensitive to gradient steepness, while the relative retention of sequence variants is mainly determined by temperature.

Fig. 6 shows an example of the simultaneous optimization of t_G and T. To mimic a challenging sample, an oligodeoxythymidine ladder and an ssDNA ladder were mixed (different length variants of ONs from two different sequences). The method variables and their levels to calibrate a two-dimensional (2D) IP-RPLC model were selected as follows: t_{G1} = 5 and t_{G2} =15 min; T_1 = 55 and T_2 = 85 °C. Method optimization was performed on the basis of critical resolution (Rs,crit) maps generated by DryLab™ Retention Modeling Software. This allowed for the rapid identification of optimal method conditions based on the built-up model. Fig. 6A illustrates that elevated temperature ($T \geq 75$ °C) and $t_{G1} > 6$ min gradient duration are favorable as it results in Rs,crit > 1.5 (red area in the resolution map). The vertical blue lines indicate co-elution and elution order changes induced by temperature. The elution order of dT-25 (25mer deoxythymidine) and ssDNA-30 (30mer ssDNA) peak pair (peaks 5 and 6) and of dT-20 and ssDNA-25 (peaks 7 and 8) can be changed by adjusting mobile phase temperature. Fig. 6B shows the chromatogram obtained for a selected working point (t_G = 7 min and T = 80 °C). Note that elevated temperature in general results in sharper peaks for longer ONs due to improved solute diffusivity.

The combination of this 2-variable model and ultra-short columns (i.e. 20 × 2.1 mm) allows a very rapid method development process, requiring only four calibration experiments and a few hours of work. This approach is particularly advantageous when ON sequence variants or complex mixtures need to be separated.

7. Method development for length variants to yield an ultra-fast platform method

It is an empirical observation in liquid chromatography that the retention of compounds of a homolog series (size or length variants) increases in logarithmic steps with increasing number of homolog units (homolog rule). It has been reported for various homolog series and different elution modes [41,42]. As a result of the homolog rule, the selectivity between n-1-mers decreases significantly for longer ONs when running linear mobile phase gradients. Very often, unnecessarily high selectivity is obtained for the short ONs and very low (limited) selectivity for the long ONs. Therefore, a more homogeneous peak distribution (retention pattern) should be established for the separation of length variants to approach uniform selectivity across the entire elution window of ON homologs.

To set up a generic (platform) method, the best method to consider is one based on an "inverse function" or "inverse gradient" [7,43]. Briefly, if a linear mobile phase gradient (linear input function) results in a logarithmic ("concave") retention time distribution (logarithmic response function) for a set of compounds and the analytes elute by on-off mechanism (like ONs do), then programing a logarithmic concave

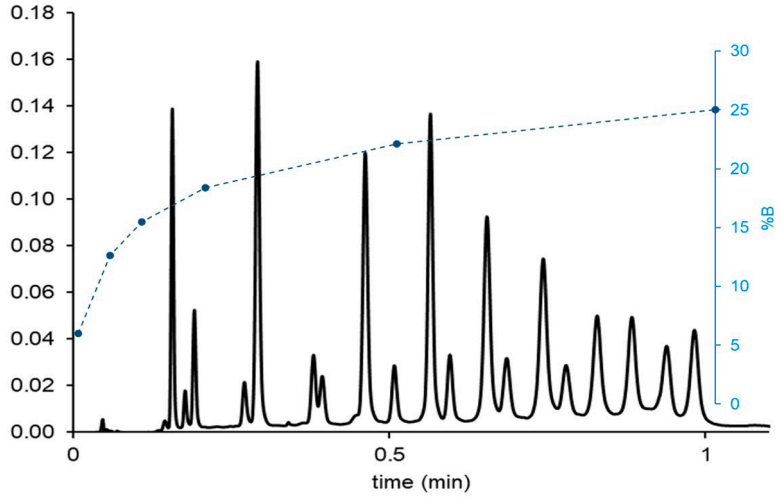

Fig. 8. Experimental chromatogram of a complex ON mixture observed with a 1-minute concave (logarithmic) gradient on a 20 × 2.1 mm ultra-short column and operated at $F = 2$ mL/min and $T = 70$ °C.

gradient program is predicted to yield a linear retention time distribution across the entire elution window (inverting the input and response functions) [44,45].

The proposed protocol to set a platform method is based on the following considerations and steps:

(1) Determine the eluting mobile phase compositions of the first (φ_{first}) and last (φ_{last}) peaks. Only one initial linear gradient experiment is required. Retention times need to be read and the corresponding eluting φ compositions can simply be calculated, though one must take into account the column dead time and the gradient delay time of the chromatographic system.
(2) Set $\varphi_{start} = \varphi_{first} - 0.05$ as the starting mobile phase composition and $\varphi_{final} = \varphi_{last} + 0.02$ for the final mobile phase strength.
(3) Program a five-segment multi linear gradient to approach a logarithmic function between φ_{start} and φ_{final}.

This gradient program should inherently result in a homogeneous retention pattern for ON size variants. Fig. 7 shows two examples comparing linear and logarithmic gradient profiles of dT and ssDNA ladders.

The logarithmic gradient approach can also be used to develop high-throughput (ultra-fast) platform methods by operating ultra-short columns (20 × 2.1 mm) at high flow rates ($F = 1$ - 2 mL/min). Fig. 8 shows an example for a complex ON mixture (19 peaks), running a 1-minute logarithmic gradient at 2 mL/min on a 20 × 2.1 mm column.

8. Practical considerations (mobile phase, repeatability, conditioning, troubleshooting)

While generally robust and reproducible, IP-RP analyses of oligonucleotides do require certain practical considerations. Mobile phase aging has been shown to lead to altered separation power and decreased MS sensitivity with the underlying mechanisms being poorly understood [46]. Consider using pre-mixed mobile phases and frequently replace the solutions (i.e. 24 h).

Use of low adsorption surfaces generally removes the need for conditioning, however, to ensure that the whole system provides maximum response consider first high mass load injection, especially when working with a new oligonucleotide sample.

In the event of insufficient MS sensitivity consider optimizing the composition of the IP system (IP reagent, concentration, pH) according to solutions proposed in literature [47]. Similarly, the presence of excessive salt adducts in MS spectra can be mitigated by careful preparation of the mobile phases and dedicated system cleaning procedures [48]. Consider using plastic ware, plastic bottle reservoirs instead of glass bottles to reduce the presence of sodium and other leachate effects [49].

The gradient delay volume of the chromatographic system can have a significant effect on the quality of the separation when using such low volume columns and fast gradients. A binary system with low delay volume and low system dispersion is preferred (i.e. ≤ 1 μL optical detector flow cell and the possible shortest and narrowest tubing between column outlet and the detector).

9. Conclusion

This tutorial article reviews the current practice of ONs IP-RPLC separations. Despite the fact that various IP systems have been studied over the past 20–30 years, practicing chromatographers often misunderstand the role of the IP agent. The IP agent alone has very little effect on the selectivity of ON separations. It mostly affects the absolute retention of the phase system, but not the selectivity of the separation. Nearly identical retention patterns can be obtained with any IP system by adjusting the strength of the initial and final mobile phase composition of a gradient. Strong IP agents are advantageous when a weak IP agent does not provide sufficient retention. If the ideal separation with a weak IP agent requires a very shallow gradient, it is worth changing to a stronger IP system that requires a steeper gradient (wider $\Delta\varphi$ range) to maintain the same elution window and fine-tuning control over the gradient.

Regarding columns, low adsorption column hardware and ultra-short column formats are highly recommended. Due to the elution behavior of ONs, long columns do not improve the separation quality, but only increase the analysis time. A good starting point is a 20 mm column that allows 1–5 min separations without compromising resolution.

Instead of tedious trial and error methods, we recommend a systematic method development approach based on four initial experiments to study the effect of gradient steepness and mobile phase temperature on separation. These two variables have the greatest impact on the selectivity of ON sequence variants. Software-assisted retention modeling allows very fast (1 day) method optimization.

When generic (platform) methods are to be set up in a laboratory, multi-segmented concave (logarithmic-like) mobile phase gradients overcome the inherent problem of homolog separations (e.g.: oligonucleotides and their shorter impurities) and a uniform peak pattern distribution is expected for the analysis of length variants. With the initial

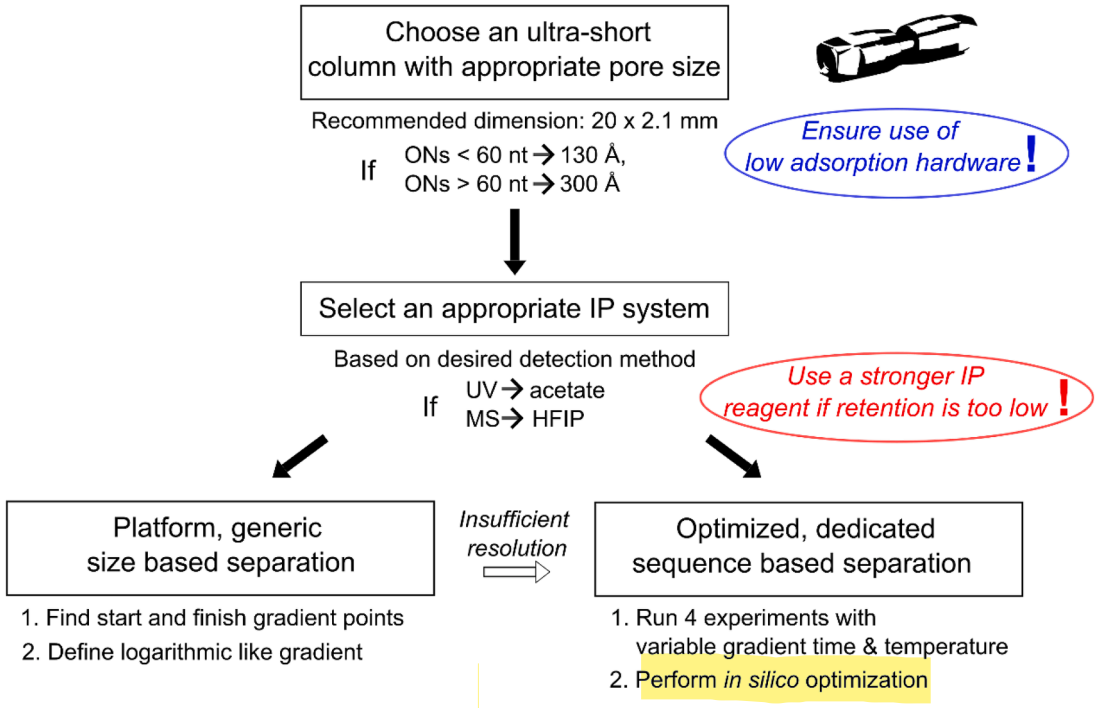

Fig. 9. General workflow diagram for ONs IP-RPLC method development.

and final mobile phase compositions determined from a screening experiment, a generic 5-segment multi-linear gradient is programmed to routinely perform high-throughput (1–3 min) ON separations. Fig. 9 summarizes the general workflow for ON IP-RPLC separations.

It should be noted that the results of this study were obtained with model oligonucleotides, which might differ from those in the clinic due to the presence of chemical modifications. Therefore, the mobile phase strength and gradient span may be different in other applications. Secondary structures may also affect the choice of the separation conditions.

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CRediT authorship contribution statement

Szabolcs Fekete: Methodology, Investigation, Conceptualization, Writing – original draft. **Mateusz Imiołek:** Methodology, Investigation, Conceptualization, Writing – original draft. **Matthew Lauber:** Writing – review & editing.

Declaration of competing interest

The authors are employed by Waters Corporation, a company that manufactures and sells chromatography columns.

Data availability

Data will be made available on request.

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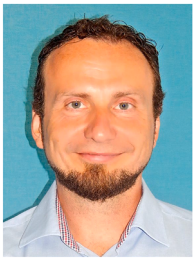

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