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Weak to strong ion-pair gradients to expand the selectivity of oligonucleotide separations in reversed phase liquid chromatography - A proof of concept

Szabolcs Fekete [a,*], Mateusz Imiolek [a], Matthew Lauber [b]

[a] Waters Corporation, Geneva, Switzerland
[b] Waters Corporation, Milford MA, USA

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ABSTRACT

This study introduces a novel ion-pair reversed-phase liquid chromatography (IP-RPLC) method using a dual gradient approach to enhance the separation of oligonucleotides. By combining a weak ion-pairing (IP) agent in the starting (weaker) mobile phase with a strong IP agent in the final (stronger) mobile phase, this method widens the elution window, improving the resolution and selectivity for complex ON mixtures. The approach outperforms traditional single IP systems, particularly in separating sequence and size variants of ONs. Experimental results demonstrate that the weak-to-strong gradient yields better peak separation and higher selectivity compared to conventional methods. This innovative gradient strategy, combined with concave gradients, offers significant potential for optimizing ON separations in the development of oligonucleotide-based therapeutics.

1. Introduction

Ion-pair reversed-phase liquid chromatography (IP-RPLC) is considered to be the method of choice for analytical scale separations of oligonucleotides (ONs) [1–8]. The main advantage of IP-RPLC over other chromatographic modes (such as ion-exchange or size-exclusion) is that it can be readily coupled with mass spectrometry, and it provides high kinetic performance (peak capacity) for ONs as well as for smaller nucleic acids [9–14].

Most commonly, alkylamines are used as ion-pairing (IP) agents, and the mobile phase is buffered with weak volatile acids (i.e. acetic acid (AcOH) or hexafluoroisopropanol (HFIP)). Based on the retention observed in the presence of an IP system, alkylamines can be classified as (1) weak, (2) moderate and (3) strong IP agents [1,15,16]. The elution strength of an IP agent is determined by its (1) hydrophobicity, (2) basicity, (3) charge density and (4) solubility. Therefore, the strength of an IP system is often correlated with its log*P* or log*D* values and boiling point [1].

In brief, oligonucleotide retention in IP-RPLC involves several processes. The cationic group of the IP agent forms pairs (complexes) with the negatively charged phosphate groups of the ONs. The IP agent can also adsorb to the stationary phase ligands via its hydrophobic moieties. The ion pairs are then adsorbed onto the surface of the stationary phase. Finally, the adsorbed ion pairs can be eluted by a gradient of organic co-solvent, typically acetonitrile or methanol. In reality, this is a complex process involving a multi-step, mixed-mode mechanism; both hydrophobic and electrostatic interactions are involved.

It is thought that strong (hydrophobic) IP agents lead to improved retention of ONs due to a greater contribution from electrostatic (ion-exchange) interactions. Strong IP reagents can bind strongly to the stationary phase ligands (hydrophobic ligands) and thus the alkyl ligands of the stationary phase are masked by the charges of the adsorbed IP reagent, as the charges are likely to be directed towards the aqueous mobile phase at the top of the stationary phase-ligand - IP complex. In other words, strong IP agents are immobilized on the surface of the stationary phase ligands. On the other hand, (weak) hydrophilic IP agents are likely to favor a more hydrophobic interaction dominated retention mechanism. The stationary phase ligands are likely to be less covered by the IP reagents due to the weaker hydrophobic interaction strength (between the IP reagent and stationary phase ligands), leaving more stationary phase ligands (hydrophobic alkyl chains) accessible to the analytes. Therefore, practicing chromatographers often select a weak IP system where the oligonucleotides to be separated have different hydrophobicity (i.e. length or size variants), while strong IP systems are preferred for sequence or charge variant (modifications) separations [4,8,9]. Another reason to use strong IP systems is to

* Corresponding author.
E-mail address: Szabolcs_Fekete@waters.com (S. Fekete).

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suppress diastereomer selectivity for PS oligonucleotides and thus elute them in apparently sharper peaks [8]. However, in reality, it is reasonable to assume that electrostatic and hydrophobic interactions do occur at the same time and therefore the retention mechanism is a mixed mode mechanism [16].

It is also plausible to consider that incorporating both weak and moderate (or strong) IP agents into the mobile phase will enhance separation efficiency by leveraging both hydrophobic and electrostatic interactions. The application of multiple IP agents in the mobile phase has been utilized for the purity assessment of oligonucleotides (ONs) [17]. The authors indicated that employing a combination of ion-pairing agents facilitated establishing a 'generic' analytical method. Additionally, a dual ion-pairing agent system has been employed for analyzing mRNA isolated from lipid nanoparticles [18]. It is important to note that in all the studies mentioned, the concentrations of the two ion-pairing agents were kept constant throughout the separation, while only the proportion of the organic co-solvent was adjusted during gradient elution.

The current practice of IP-RPLC of ONs is either (a) to use the same concentration of IP agent(s) and buffer in both mobile phases 'A' and 'B' and only change the proportion of water and organic co-solvent - by running an organic solvent gradient [1,18,19] - or (b) very often mobile phase 'B' is a dilution of 'A' in organic co-solvent. Note that with the latter approach, the later eluting solutes will experience a lower concentration of IP agents. In other words, a positive organic co-solvent gradient is combined with a negative (decreasing) IP concentration gradient [1,3,7]. The choice between maintaining the IP concentration or diluting the IP system is usually based on practical considerations, like the simplicity of making the mobile phases. It should be noted, however, that the dilution approach is likely to result in a compressed elution window for the analytes, since in addition to the organic co-solvent gradient, the temporal decrease in the concentration of the IP agent will result in reduced retention for the later eluting peaks (as the mobile phase "B" increases). This approach may therefore have practical advantages but is likely to result in reduced selectivity through a narrower elution window.

It is worth mentioning that recently hydrophilic interaction liquid chromatography (HILIC) has gained increasing interest for oligonucleotide (ON) separation. It offers an alternative to the most commonly used IP-RPLC methods with the advantage of using ion-pair free mobile phases [20–22].

In this work, we propose an innovative "dual gradient" approach for IP-RPLC, which consists of combining a negative (decreasing) weak IP gradient with a positive (increasing) strong IP gradient, in order to widen the elution window of complex ON mixtures. Theoretically, this approach should result in new separation options for oligonucleotide therapeutics, where there is a need to better resolve increasingly diverse modified residues and their related impurities.

2. Experimental

2.1. Chemicals and reagents

HPLC grade water and methanol (MeOH) were obtained from Fisher Scientific (Dublin, Ireland). Triethylamine (TEA), hexylamine (HA) and hexafluoroisopropanol (HFIP) were purchased from Sigma- Aldrich (Buchs, Switzerland). An oligo dT (oligodeoxythymidines) ladder, ssDNA ladders (10 to 60 and 20 to 100 mers) and an siRNA standard (mixture of annealed 25-mer and 27-mer RNA strands) were obtained as certified reference materials from Waters Corporation (Milford, USA).

2.2. Instrumentation, software and column

All experiments were performed on an ACQUITY™ UPLC™ H—Class Bio System with Binary Solvent Manager (Waters) equipped with a flow-through needle (FTN) sample manager and UV (TUV) detector. The system dwell volume and extra-column volume were measured as V_d = 0.105 mL and V_{EC} = 8.5 µL, respectively. Instrument control and data acquisition were performed with Empower™ Pro 3 Software. Data processing and retention modelling was performed using DryLab™ 4.4 Software (Molnar Institute, Berlin, Germany).

An ultra-short 20 × 2.1 mm 1.7 µm 300 Å ACQUITY Premier Oligonucleotide BEH™ C18 Column was used (Waters).

2.3. Mobile phase compositions and sample preparation

To investigate the retentivity of the mobile phase systems, three systems (with different IP strengths) were compared. The first (weak) mobile phase system was 10 mM TEA + 100 mM HFIP in water as "A1″ and 10 mM TEA + 100 mM HFIP in MeOH/water 7/3 as "B1". The second system (strong) consisted of 10 mM HA + 100 mM HFIP in water as "A2″ and 10 mM HA + 100 mM HFIP in MeOH/water 7/3 as "B2". Note that in these two mobile phase systems the concentration of the IP agent does not change over time when running a gradient from "A" to "B", only the MeOH fraction changes. The third system was a mixture of the weak and strong IP systems, namely a "weak to strong IP gradient" (dual opposed gradient) consisting of the use of "A1" and "B2". In this system, the total concentration of the IP agent is kept at 10 mM, but the proportions of TEA and HA change in time accompanied with an increase of MeOH.

To mimic the extreme of the current practice of diluting mobile phase "A" with an organic solvent mixture as "B" solvent, a "B3" mobile phase consisting of MeOH/water 7/3 (no IP agent and no HFIP) was considered.

Samples were prepared by reconstituting the contents of the ON standards in 100 µL water (corresponding to ~0.1 mg/mL) in low adsorption, low volume (300 µL) vials. To mimic a complex ON sample, a mixture of the two ssDNA ladders and the oligo dT ladder was prepared.

2.4. Measuring the retentivity of the different IP systems

Calibration input experiments were performed by running generic linear gradients of different gradient steepness. Retention model parameters were then derived for all phase systems and the retentivity of the different IP-systems (fraction of the two model parameters "logk_0″ and "S") was plotted as a function of oligonucleotide length. (Model parameter log k_0 refers to logarithmic retention factor observed in pure aqueous mobile phase while parameter S is the solvent strength parameter.) Please note that the ratio logk_0/S corresponds to a mobile phase composition φ^*, (φ is the volume fraction of the B mobile phase in the eluent, normalized between 0 and 1) which results in a retention factor of $k = 1$. Since ONs follow an on-off like elution mechanism [3,15,16], 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) while a stronger mobile phase than φ^* (i.e. $\varphi > \varphi^*$) results in the complete release of the solute (no physico-chemical retention). Therefore, comparing the φ^* values is an appropriate method to study and compare the absolute retentivity observed in different phase systems. For more details, please refer to previously published work [15,16,23,24]. The following conditions were set for the input experiments. The flow rate was set to F = 0.4 mL/min. Six gradient conditions were set: "A1" to "B1", "A2" to "B2" and "A1" to "B2" in 10 and 20 min (0 – 100 %). Temperature was set to T = 50 °C, detection was carried out at 260 nm. The injection volume was 0.5 µL across all samples and standards. Retention model parameters (logk_0, S and φ^*) were derived from the experimentally observed retention time values, and φ^* was plotted as the function of ON length (nucleotide number - nt). The first derivative of φ^* ($\varphi^{*\prime}$) was also plotted against the nt number which illustrates the change of selectivity with ON length for the different IP systems (i.e. ratio of retention factors between n and n-1 mers). (To obtain the derivative of the φ^* plot, a polynomial fit was used to the experimentally

measured data and the first derivative of the polynomial was calculated and used to illustrate the selectivity changes as a function of nt across different IP systems.)

2.5. Comparing single and dual opposed (weak to strong) IP gradients

The peak patterns and elution windows obtained by running a simple weak, a simple strong and the combined weak to strong IP gradients have been compared for all samples. For that, a 15 min linear gradient was considered (0–100 %; "A1" to "B1", "A2" to "B2" and "A1" to "B2") at $F = 0.4$ mL/min. To illustrate the practice of diluting mobile phase "A" with an organic solvent mixture as "B" solvent, an additional gradient of "A2" to "B3" was also run. For these comparative experiments, column temperature was set to $T = 50$ °C and chromatograms were recorded at 260 nm.

To improve the separation of complex ON samples, the dual IP gradient method was further optimized. It has been shown recently that a logarithmic-like (concave) mobile phase gradient overcomes the inherent problem of separating homologous compounds (e.g.: oligonucleotides and their shortmer impurities) and affords the most uniform peak pattern distribution possible [15,16]. This concave platform gradient approach has been combined with the dual IP gradient method. A multi-linear (3-segment) gradient was programmed from "A1" to "B2" with the following segment points: 6 % "B2" initial composition, 37 % "B2" at 1.2 min, 63 % "B2" at 6.1 min and 72 %B (final composition) at 10 min.

2.6. Temperature effects: simultaneous optimization of temperature and gradient program

The effect of mobile phase temperature on selectivity has been studied for dual IP gradients. It has been reported that temperature is one of the most important method variables for the separation of ON sequence variants [15]. Most likely, the best practice is to apply a retention model combining gradient time (tG) and temperature (T) to map a large design space and optimize peak resolution (Rs) [15]. Four input experiments have been conducted (2 x tG + 2 x T) to build up the model. Linear gradients (0–100 %B) from "A1" to "B2" have been performed with $tG = 10$ and 20 min, at $T = 40$ and 70 °C ($F = 0.4$ mL/min). Then Rs map was created and studied.

3. Results and discussion

3.1. Generic considerations

It has been reported that selectivity decreases with increasing ON size (due to smaller retention differences between large ONs) [3,15,16]. With increasing ON size, the selectivity converges to $\alpha = 1$ (co-elution). These observations appear to be independent of IP agent or stationary phase, as the same trends have been reported for different IP systems and on different columns [1,7,15,16]. To overcome this inherent problem of homologous separations, the use of concave gradients has recently been proposed as a general solution and improved separations have been reported [15,16].

To further expand selectivity, one can consider the fact that the absolute retentivity of a weak IP system is significantly lower than that of a strong IP system. It is then reasonable to assume that decreasing the concentration of a weaker IP agent over time while increasing the concentration of another stronger IP agent may result in a widened (stretched) elution window and thus improved selectivity. In other words, mobile phase 'A' should contain only (or mostly) a weak IP agent, while mobile phase 'B' should contain only (or mostly) a strong IP agent. Thus, combining a negative weak IP agent gradient with a positive strong IP agent gradient is likely to increase selectivity and resolution simply because the earlier eluting solutes (shorter ONs) will experience an inherently weaker retaining phase system, while the later eluting peaks (longer ONs) will experience a stronger retaining system. Theoretical considerations also suggest that this opposed dual gradient approach allows the elution window to be widened while maintaining an appropriate intrinsic gradient steepness (and thus an effective gradient band compression effect). It is planned to confirm and report this effect in a future paper (i.e. deriving band compression factors for various dual IP systems or considering not only dual but ternary gradient IP systems). In conclusion, it seems to be promising to run a weak to strong IP gradient rather than a conventional single IP system or a multi-IP agent phase system where the concentration of the IP agents is maintained throughout the duration of solvent mobile phase gradient.

3.2. Comparing the selectivity of different IP systems

The absolute retentivity (φ^*) and selectivity ($\varphi^{*\prime}$) of a weak (TEA), a strong (HA) and a weak to strong (TEA to HA) phase systems were compared for oligo dT and ssDNA ladders. The same trends were observed. Fig. 1A shows the experimentally measured retentivity for the oligo dT ladder while Fig. 1B shows the derived selectivity for an extrapolated nt range (up to 110 nt) to illustrate the phenomenon. As the number of bases increases, the retentivity shows a concave trend, suggesting a plateau (convergence to a limiting retention), regardless of whether a weak or strong IP system is used. Note, however, that the weak to strong system gives the widest range of $\Delta\varphi^*$ and the highest initial slope (Fig. 1A), and thus the highest overall selectivity. Note that the $\varphi^{*\prime}$ curve of the weak to strong system lies above the curves of the individual single IP systems (Fig. 1B). The difference in selectivity between 10 and 40 nt oligo dTs is $\Delta\varphi^* = 0.34$ for the weak to strong system, while the single strong and weak IP systems yielded only $\Delta\varphi^* = 0.23$ and 0.08, respectively. Fig. 1B also shows that the selectivity of all IP systems decreases with increasing ON size, but the absolute selectivity of the weak to strong system is by far the highest. At 100 nt ON size, the weak to strong IP system is predicted to provide 1.9 and 5 times higher selectivity than the single strong and single weak IP systems, respectively. These calculations suggest that a weak to strong IP system does indeed result in the highest achievable selectivity compared to a single IP system (weak or strong).

(In the supplementary information, Supplementary Figure 1 - SF1 - shows the same plots of φ^* and $\varphi^{*\prime}$ derived for the ssDNA ladder.)

3.3. Comparison of single and dual opposed IP gradients

To investigate the effect of a weak to strong IP gradient on the elution window and selectivity, chromatograms obtained by injecting an ssDNA ladder (10 to 100 mers; a mixture of the 10 - 60 and the 20 - 100 mer ladders) were examined. Fig. 2 shows a direct comparison between (A) a single weak IP system with constant ionic strength, (B) a single strong IP system with constant ionic strength, (C) a single strong IP system with decreasing ionic strength (mobile phase "B" is a dilution of "A") and (D) a weak to strong IP system with a constant overall concentration of ion pairing agent. The steepness of the organic co-solvent (methanol) gradient is the same for all four IP systems. The narrowest elution window was observed with the single weak IP system. Peaks eluted within a time interval of 13.5 % of the total gradient time. The single strong IP system resulted in a significantly wider elution window (the peaks occupied 41.5 % of the total gradient time). When mimicking the current practice of diluting mobile phase 'A' with organic solvent to prepare eluent 'B', the elution window became narrower, decreasing from 41.5 % to 36.3 %. This is expected due to the decrease in IP agent concentration over time. Later eluting peaks will experience less IP agent. Therefore, the ion-paired complex of the ONs may not be complete and/or less IP agent will be adsorbed onto the stationary phase, resulting in a lower charge density covering the stationary phase ligands. All this suggests that the dilution approach is not advantageous from a chromatographic selectivity point of view (though it may be advantageous from a practical point of view to avoid precipitation or

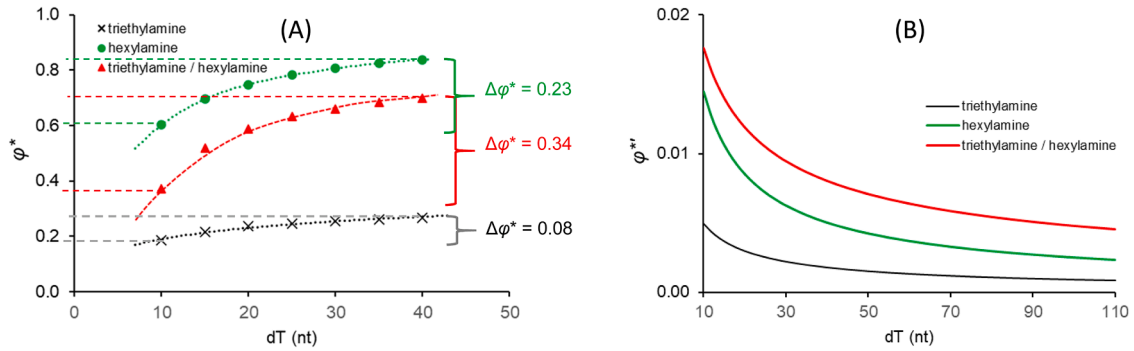

Fig. 1. Absolute retentivity (A), and selectivity (B) as a function of nucleotide number (nt) derived for oligo dT ladder (homopolymers) for a single weak (triethylamine), a single strong (hexylamine) and a weak to strong (triethylamine/hexylamine) IP systems.

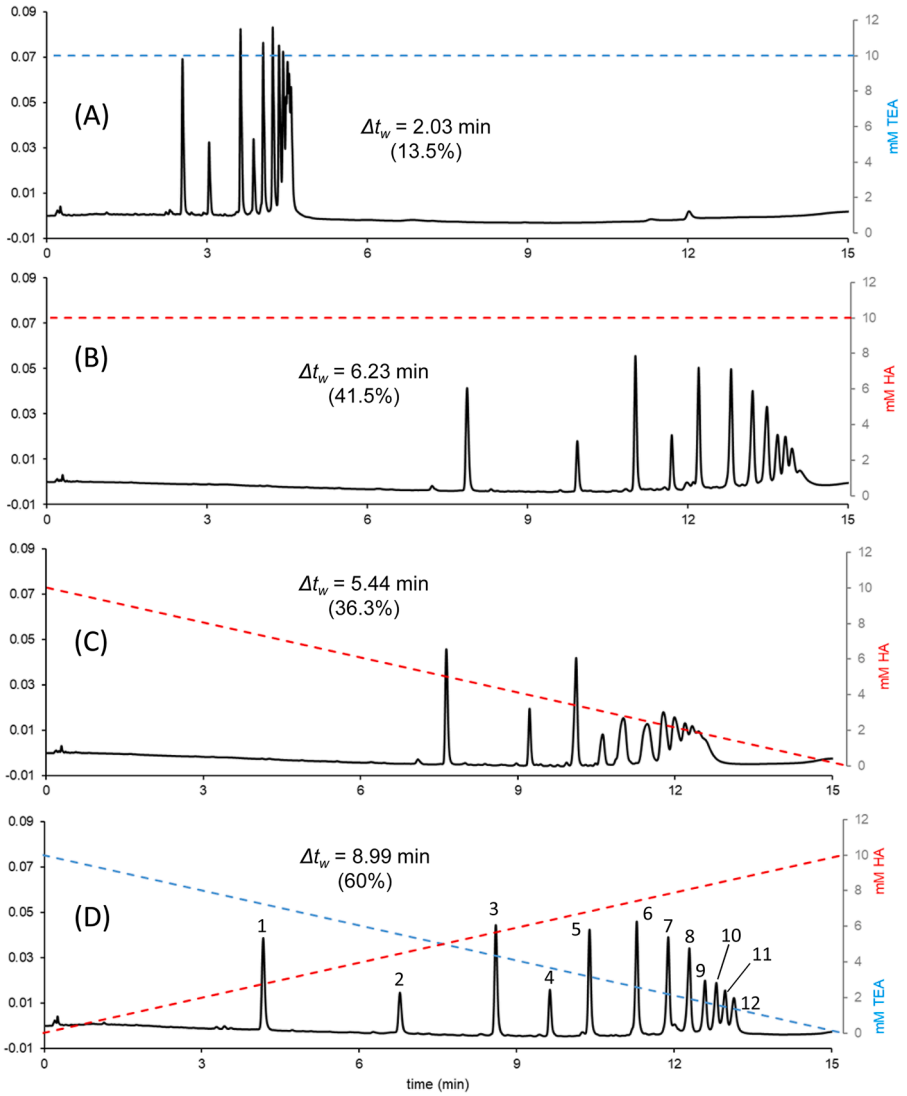

Fig. 2. Separation of ssDNAs (10 – 100 mer) with a single weak (triethylamine) IP system (A), a single strong (hexylamine) IP system (B), a diluted strong (hexylamine) IP gradient (C) and a weak to strong – triethylamine/hexylamine – IP gradient (D). Detailed chromatographic conditions are provided in Section 2.5. For all separations, the organic co-solvent gradient was 0 to 70 % MeOH in 15 min (linear gradient). Peaks: ssDNA-10 (1), ssDNA-15 (2), ssDNA-20 (3), ssDNA-25 (4), ssDNA-30 (5), ssDNA-40 (6), ssDNA-50 (7), ssDNA-60 (8), ssDNA-70 (9), ssDNA-80 (10), ssDNA-90 (11) and ssDNA-100 (12).

mobile phase miscibility issues). Finally, the weak to strong dual IP gradient method resulted in the widest elution window, with peaks eluting over a time interval of 60 % of the total gradient. This observation proves that a weak to strong IP gradient does indeed significantly extend the elution window for a wide range of ON size variants (10 to 100 mers). Not only was the elution window extended, but both selectivity and resolution were the highest with the weak to strong IP gradient. The average resolution (Rs_{av}) considering all peak pairs was

Rs_{av} = 1.39 with the weak IP system, Rs_{av} = 5.16 with the strong IP system and Rs_{av} = 7.09 with the weak to strong gradient IP system.

In addition to separating a wide range of ON size variants, the weak to strong IP gradient method is also advantageous for improving the resolution of a closely eluting critical peak pair. To illustrate this, the chromatograms obtained by injecting the siRNA sample were examined. Under the denaturing conditions applied, this particular siRNA dissociates into its single strands (25-mer and 27-mer). Fig. 3 shows their separations with a single weak, single strong and weak to strong IP gradient. The single weak IP system gave the lowest resolution (Rs = 0.81), the single strong IP system gave a separation of Rs = 1.43 and the dual opposed IP gradient resulted in a higher than baseline resolution, Rs = 2.10. Note that this separation can probably be further improved by adjusting the gradient conditions, but our purpose here was to make a direct comparison with the same organic solvent gradient steepness.

(In the supplementary information, Supplementary Figure 2 – SF2 - shows the chromatograms obtained for the oligo dT ladder.)

Note that there is a potential risk that the strong ion-pairing agent may not be completely removed by the time the next injection is started with the weak ion-pairing agent (incomplete removal of the strong ion-pairing agent). In our study, hexylamine was found to be a reversible adsorbing agent and therefore does not require special (long) washing procedures. However, a stronger ion-pairing agent than hexylamine may require a specific removal procedure.

3.4. Concave dual opposed IP gradients

To further investigate the potential of the weak to strong IP gradient approach, a complex ON mixture (mixture of oligo dT ladder and the two ssDNA ladders: 17 peaks) was injected and separated with single IP systems and with the dual opposed IP gradient system. Traditional linear and concave gradient profiles were compared. It has been reported that when separating a wide range of ON length variants (i.e. 10 to 100 mers), a concave (logarithmic) eluent strength gradient results in the most uniform peak distribution [15,16]. Therefore, we believed that combining the weak to strong IP gradient method with the concave gradient approach would result in unseen opportunities to further improve ON separations.

Fig. 4 shows the chromatograms obtained. With the single weak IP system (Fig. 4A), two critical peak pairs co-elute completely (ssDNA 15 mer with oligo dT 15 mer and ssDNA 25 mer with oligo dT 25 mer). The single strong IP system (Fig. 4B) results in four co-eluting peak pairs, although the overall elution window is wider. Note that sequence variants of the same length systematically co-elute (ssDNA 15 mer with oligo dT 15 mer, ssDNA 20 mer with oligo dT 20 mer, ssDNA 25 mer with oligo dT 25 mer, ssDNA 30 mer with oligo dT 30 mer). This contradicts current practices that frequently assume strong IP systems are universally advantageous for separating sequence variants. In our recent works, we have explained why this is not necessarily true [15,16]. However, when running the linear gradient from weak to strong IP (Fig. 4C), the number of co-eluting peak pairs is reduced to only one (ssDNA 30 mer and oligo dT 30 mer). Also note that for some peaks there is a change in elution order compared to single IP systems. For example, with the weak IP system, the 20 mer oligo dT elutes before the 20 mer ssDNA. Whereas, with the weak to strong IP gradient. it is reversed. Finally, when the generic concave gradient approach is combined with the dual opposed IP gradient method (Fig. 4D), all peaks are baseline separated, and high selectivity is achieved over the entire gradient span. This proves our hypothesis that a general weak to strong IP gradient would outperform any single IP method and should be used for routine separations when dealing with complex ON mixtures so long as precise mobile phase and pump mixing can be assured.

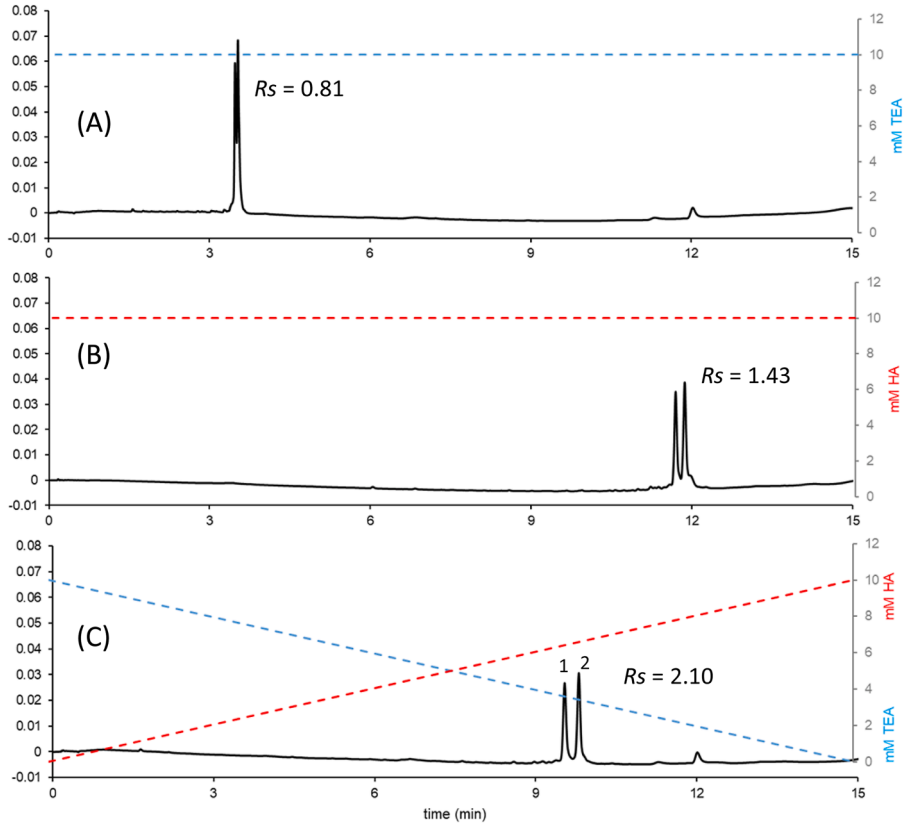

Fig. 3. Separation of siRNA single strands (25-mer and 27-mer) with a single weak (triethylamine) IP system (A), a single strong (hexylamine) IP system (B) and a weak to strong – triethylamine/hexylamine – IP gradient (C). Detailed chromatographic conditions are provided in Section 2.5. For all separations, the organic co-solvent gradient was 0 to 70 % MeOH in 15 min (linear gradient). Peaks: 25-mer (1) and 27-mer (2).

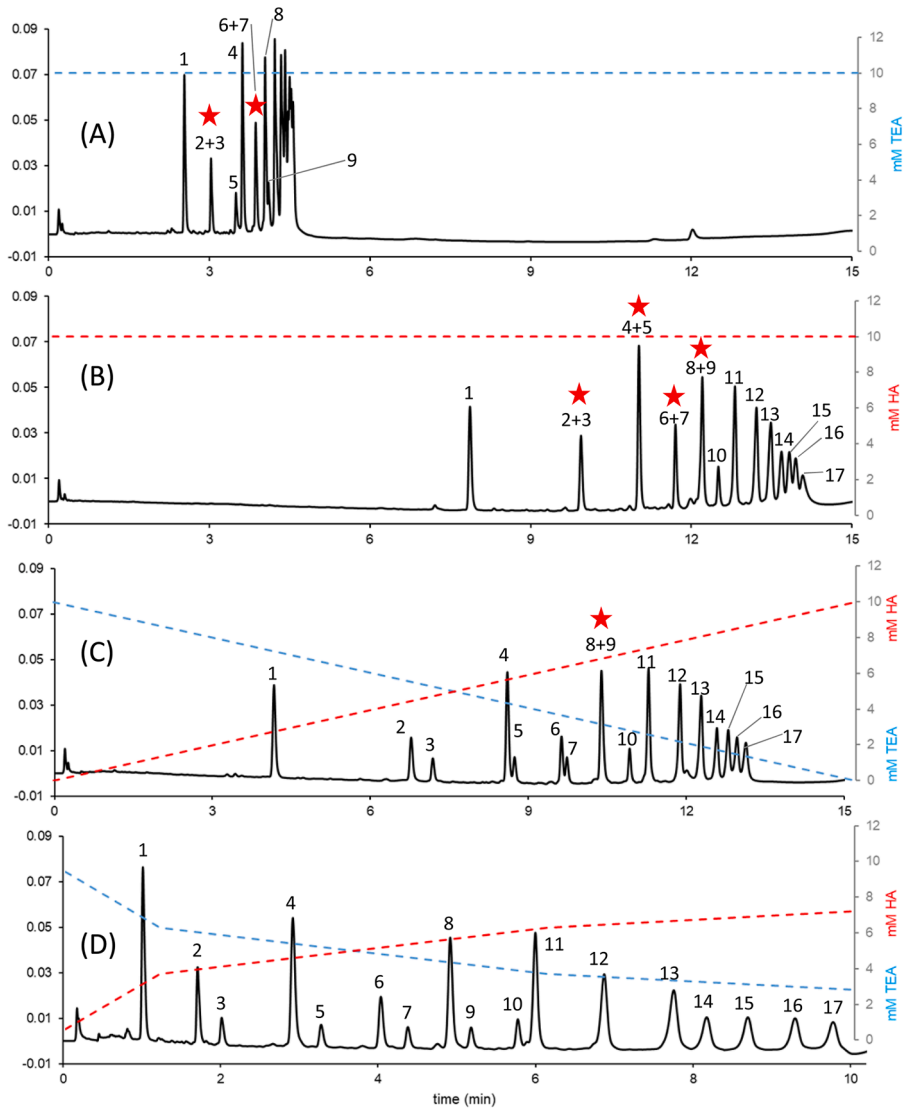

Fig. 4. Separation of a mixture of ssDNAs (10 – 100 mer) and oligo dTs (15 – 35 mer) with a single weak (triethylamine) IP system (A), a single strong (hexylamine) IP system (B), a weak to strong – triethylamine/hexylamine – IP linear gradient (C) and a weak to strong – triethylamine/hexylamine – IP concave gradient (D). Detailed chromatographic conditions are provided in Section 2.5. Red stars indicate co-eluting peak pairs. Peaks: ssDNA-10 (1), ssDNA-15 (2), oligo dT-15 (3), ssDNA-20 (4), oligo dT-20 (5), ssDNA-25 (6), oligo dT-25 (7), ssDNA-30 (8), oligo dT-30 (9), oligo dT-35 (10), ssDNA-40 (11), ssDNA-50 (12), ssDNA-60 (13), ssDNA-70 (14), ssDNA-80 (15), ssDNA-90 (16) and ssDNA-100 (17).

3.5. *Temperature effects*

It has recently been shown that the retention of length variants is primarily sensitive to gradient steepness (*tG*), whereas the relative retention of sequence variants is primarily determined by temperature (*T*) [15]. Therefore, based on these two method variables, a retention model was built for the new weak to strong IP gradient approach to see if temperature can further alter or improve selectivity. Fig. 5 shows the obtained resolution map, and two chromatograms observed at $T = 47$ and 65 °C, for the mixture of oligo dT ladder (oligo dT 15 – 35) and siRNA. The map considers the lowest (critical) resolution among all peak pairs including all the seven compounds. The resolution map allowed for the rapid identification of optimal method conditions. The red areas correspond to the highest resolution conditions, whereas the blue areas correspond to the lowest resolution conditions (The yellow and green sections correspond to moderate resolution conditions, see the resolution scale on Fig. 5A). Changes in elution order can be achieved by altering temperature, suggesting that the separation is indeed very sensitive to enthalpy effects. At $T = 65$ °C, the two siRNA strands eluted between the oligo dT 20 and 25 mer peaks, whereas at $T = 47$ °C the retention of the siRNA shifted drastically and eluted between the oligo dT 25 and 30 mer peaks. Different sequences responded differently to temperature changes. These observations suggest that, in general, temperature is a key variable to tune the selectivity of sequence variants, in addition to control the non-denaturing versus denaturing state of a double stranded oligonucleotide.

4. Conclusion

In this study, a novel IP-RPLC gradient approach has been proposed. The simultaneous application of an ion pairing gradient that transitions from a weak IP agent to a stronger one as the mobile phase's solvent eluotropic strength is changed results in a widened elution window of oligonucleotide compounds (more favorable retention pattern) and thus a higher selectivity. In all case studies, improved selectivity and resolution were observed compared to commonly used single IP system methods.

It is predicted that the combination of this novel weak to strong IP

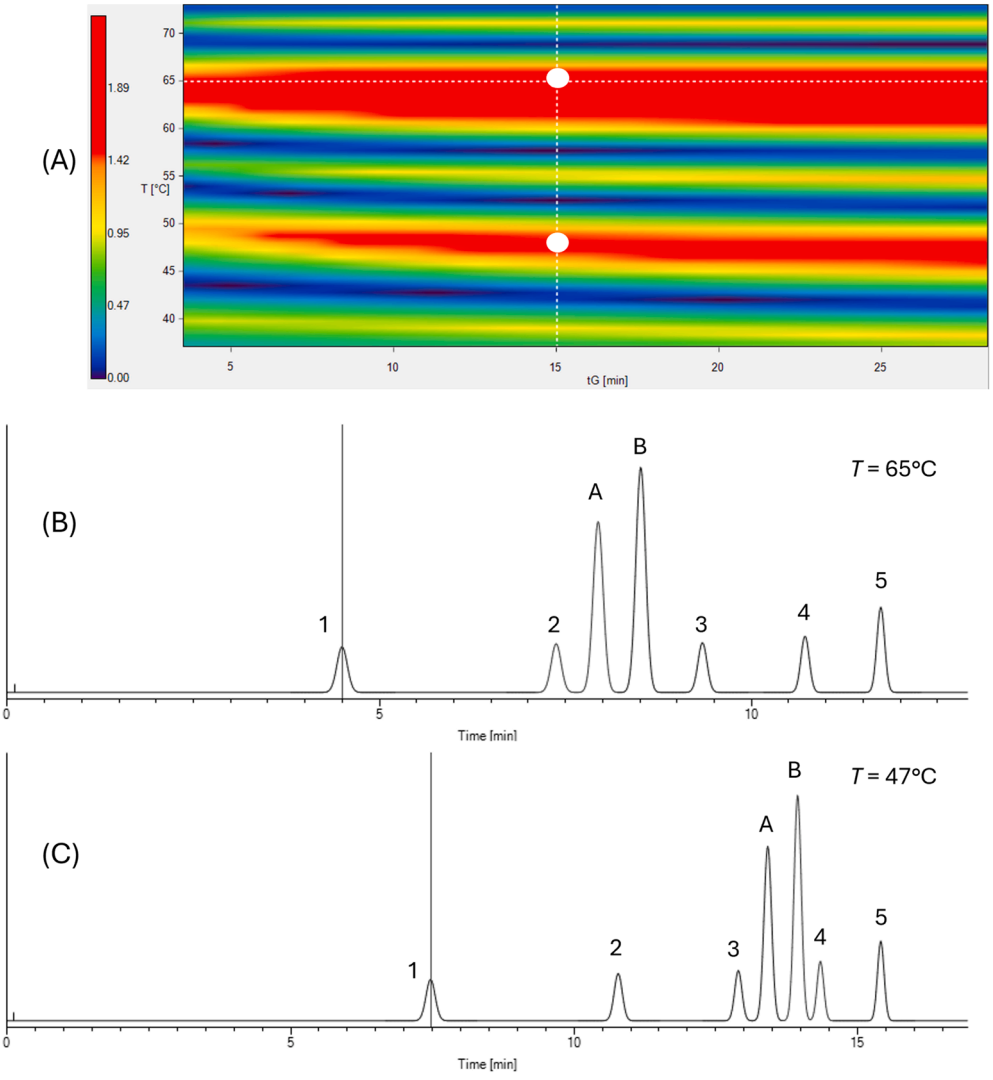

Fig. 5. 2D resolution map (A) and corresponding chromatograms observed at $T = 65$ °C (B) and at $T = 47$ °C (C) for a mixture of oligo dT ladder and siRNA single strands. Peaks: oligo dT-15 (1), oligo dT-20 (2), oligo dT-25 (3), oligo dT-30 (4), oligo dT-35 (5), siRNA 25-mer strand (A) and siRNA 27-mer strand (B). 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 method with a concave (logarithmic) gradient program will systematically improve the capabilities of platform oligonucleotide IP-RP methods.

Moreover, by adjusting the composition of the weak IP and strong IP mobile phases (i.e. the ratio or nature of the two IP agents), it is predicted that the degree of freedom can be significantly improved to map the selectivity to an even larger design space. The opposed dual IP gradient method offers unprecedented possibilities for tuning oligonucleotide separations, especially of complex ON mixtures. It is our hope that this method approach is helpful to the pharmaceutical industry as it looks to support a wave of new genetic medicines.

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CRediT authorship contribution statement

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

Declaration of competing interest

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

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcoa.2024.100200.

Data availability

Data will be made available on request.

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