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Evaluation of ion pairing reversed-phase liquid chromatography for the separation of large RNA molecules

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ABSTRACT

The rapid development of mRNA-based therapeutics, especially post-COVID-19, has necessitated the precise characterization of mRNA quality attributes, including sequence integrity. Ion-pairing reversed-phase liquid chromatography (IP-RPLC) has been widely accepted as a reference method for the characterization of small oligonucleotides. Some studies have already investigated the use of IP-RPLC for RNA, but no systematic approach has been developed to assess the impact of ion-pairing agents (IPAs) on the separation of large RNA molecules. This study addresses this gap by investigating the potential of IP-RPLC for the separation and characterization of large RNA molecules, with a specific focus on optimizing the use of IPAs to enhance retention and selectivity. Thirteen different IPAs, varying in hydrophobicity, were systematically tested using a supermacroporous polymeric (divinylbenzene) column with a very broad pore size range under various conditions, including different temperatures, pH, and IPA concentrations. The results demonstrate that moderately hydrophobic IPAs provide superior resolution for RNA species up to 6000 nucleotides. An optimized combination of 100 mM butylammonium acetate and 50 mM tripropylammonium acetate achieved the best overall separation, significantly improving resolution by 35% compared to individual IPAs. The study also identifies optimal conditions for RNA separation, including a mobile phase pH of 7.0, acetonitrile as the organic solvent, and a column temperature of 65 °C. In a second step, a solution to increase the retention of small nucleotides and thereby separate nucleic acids ranging from 1 to 6000 nucleotides allowing to characterize IVT-mRNA differing in length and study their integrity and fragmentation or monitor the presence of in-process impurities (nucleotides) was investigated by combining two different LC columns. These findings enhance the analytical toolbox for evaluating the critical quality attributes of RNA, supporting the development of reliable and efficient RNA-based therapeutics.

1. Introduction

The COVID-19 pandemic shed new lights on the use of mRNA as effective vaccines, thus accelerating new developments for prophylactic and therapeutic applications [1]. With the growing number of mRNA-based medicines in development – several hundred mRNA drugs are in the clinical pipeline worldwide – there is an urgent need to fully characterize the mRNA produced by in vitro transcription (IVT) by evaluating specific critical quality attributes (CQAs) [2].

Some of these CQAs are directly related to the sequence itself, such as integrity of mRNA (% of fragments versus intact sequence, 3′-poly(A) tail length, 5′-capping efficiency), sequence conformity, and mRNA content. Capillary gel electrophoresis (CGE) under denaturing conditions, to disrupt base-pairing interactions to improve retention and peak shapes, is commonly employed to assess these attributes [3,4]. With the aim of a more precise characterization, alternative strategies such as size exclusion chromatography (SEC), anion exchange chromatography (AEX), or ion-pairing reversed phase liquid chromatography (IP-RPLC) are being developed [5–7].

The effectiveness of these methods is often significantly influenced by advancements in column technology. Indeed, large RNAs require low-binding materials like high-performance surface (HPS) coating, PEEK, or any other kind of bioinert columns due to their sensitivity to adsorption. Moreover, pore size plays a critical role for proper analysis,

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since large RNA can reach up to 3000 kDa, and are not adequately analyzed with columns having traditional pore sizes of 100–300 Å, not matching RNA hydrodynamic radii. This results in suboptimal performance, because the accessibility of the stationary phase and the diffusion are reduced, limiting the interactions and negatively impacting the mass transfer and kinetic efficiency [8–10]. Finally, newly developed stationary phases, such as divinylbenzene-based columns and supermacroporous polymer resin offer enhanced performance for large RNA and exhibit exceptional resistance to harsh pH and temperature conditions. The integration of these innovations is essential for achieving effective separation and developing robust methods for large RNA analysis [11].

In this context, ultra-wide pore SEC columns were suggested as a good alternative to CGE [9,12]. However, SEC suffers several drawbacks, such as susceptibility to unwanted physicochemical interactions requiring low-adsorption columns, its filtering-only mechanism, and other technical and physical limitations due to filtration effects and shear forces [13]. As an alternative, AEX utilizes the negatively charged phosphate groups in the RNA structure to retain RNA on positively charged stationary phases. Accordingly, AEX appears as a very good choice for the separation of large RNA. Despite this, only few applications are reported, mainly on small oligonucleotides (ONs), as larger RNA often displaying broad peaks, poor resolution and non-negligible carry-over effects. This could be due to AEX's non-denaturing conditions, which allow RNA to retain its secondary structure conformation, thus decreasing accessibility to the phosphate groups. Additionally, AEX, as SEC, are poorly compatible with mass spectrometry (MS), compared to other techniques due to the presence of non-volatile salts in the mobile phase [14]. However, this is not a real issue for intact mRNA analysis, since MS analysis of such molecules is not readily feasible with electrospray ionization due to the large size of mRNA molecules and the multiple charging states and adducts that generate highly complex convoluted spectra and very limited sensitivity.

On the other hand, IP-RPLC offers a great potential, since it takes profit of the RNA negatively charged backbone that complexes with the positively charged alkylamine ion-pairing agent (IPA) added to the mobile phase [15]. The RNA-ion pair complex displays an increased affinity for the stationary phase compared to the naked RNA, enhancing retention on a hydrophobic column, which is further improved by harsh denaturing conditions. This makes possible the separation of RNA species based on size and sequence, with the possibility to tune the retention by varying the IPA used. While IP-RPLC has been extensively studied for small ONs analysis [16–23], its application for large RNA analysis remains largely underexplored [14]. Only a few applications have been reported, mostly using triethylammonium acetate (TEA) as IPA [15, 24–29]. Other authors advocate for the use of a mixture of IPAs, such as the combination of TEA with propylamine [30], or with dibutylammonium acetate [31]. While combining IPAs appears to be a promising strategy for enhancing resolution, there is currently no systematic published study to validate this approach, and there has been no comprehensive study on the behavior of large RNAs in IP-RPLC conditions.

To address this gap, we systematically explored the effect of thirteen IPA with different hydrophobicities on the retention and selectivity of RNAs, using the recently commercialized Thermo DNAPac RP column. The ion-pairing agents were evaluated alone or in combination, at temperatures varying from 45 to 85 °C, with pH from 6 to 8, and concentrations from 25 mM to 200 mM. In a second step, we further investigated a solution to increase the retention of small nucleotides. Finally, our optimized method successfully separated oligonucleotides containing between 1 and 6000 units, expanding the toolbox to comprehensively evaluate CQAs of newly produced RNA.

2. Experimental

2.1. Materials and reagents

RNase-free water, acetic acid (AA, ≥ 99.8%), butylamine (BA, 99.5%), dibutylamine (DBA, ≥ 99.5%), dicyclohexylamine (DcHA, ≥ 99%), diethylamine (DEA, ≥ 99.5%), dihexylamine (DHA, ≥ 97%), diisopropylamine (DIPA, ≥ 99.5%), diisopropylethylamine (DIPEA, ≥ 99%), dimethylbutylamine (DMBA, ≥ 98%), hexylamine (HA, ≥ 99%), octylamine (OA, ≥ 99%), tributylamine (TBA, ≥ 99.5%), tripropylamine (TPA, ≥ 98%), adenosine 5′-triphosphate disodium salt, guanosine 5′-triphosphate sodium salt, cytidine 5′-triphosphate disodium salt, uridine 5′-triphosphate trisodium salt, and pentafluoropropionic acid (PFPA, ≥ 97%) were all purchased from Sigma-Aldrich (Buchs, Switzerland). LC-MS grade acetonitrile and methanol were purchased from Thermo Fischer Scientific (Reinach, Switzerland). A Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to prepare HPLC mobile phases.

2.2. Samples

The method was developed using the RiboRuler High Range RNA Ladder (200 – 6000 nt) purchased from Thermo Fischer Scientific and diluted in RNAse-free water (1:5, v/v). Oligodeoxythymidines ranging from 10 to 100 deoxythymidine units and purchased from Eurogentec (Seraing, Belgium) and Integrated DNA Technologies (IDT, Leuven, Belgium) were also analyzed. Finally, a mixture of adenosine, guanosine, cytidine, and uridine triphosphate (ATP, GTP, CTP, and UTP, respectively) purchased from Sigma-Aldrich (Buchs, Switzerland) were also considered. To evaluate the final separation, analysis was performed using a combination of the RNA ladder, the oligodeoxythymidine ladder, and a mixture of nucleotide triphosphates, thus constituting the following extended ladder: ATP, UTP, CTP, and GTP; 10, 15, 20, 25, 30, 35, 40, 60, 80, and 100 dT; 200, 500, 1000, 1500, 2000, 3000, 4000, and 6000 nt. The extended ladder sample was diluted in RNAse-free water to a concentration of 0.1 mg/ml and the injection volume was 4 µL.

2.3. UHPLC instrumentation, columns and experimental conditions

The chromatographic measurements were performed on an ACQUITY UPLC H-class system (Waters, Milford, MA, USA), equipped with a 10 µl flow through needle injector, a quaternary solvent manager (QSM) equipped with a 380 µl mixing chamber, a column manager module, and an ACQUITY photodiode array detector. UV was set at a wavelength of 260 nm. Data acquisition and instrument control were performed by Empower 3 software (Waters). Resolution (Rs) was calculated using the European Pharmacopeia equation using peak widths at half height (1).

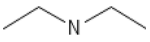
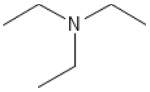
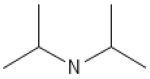
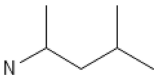
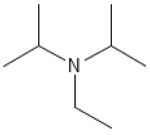
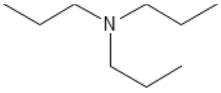
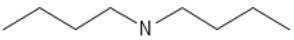
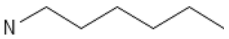
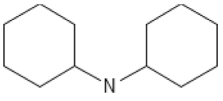
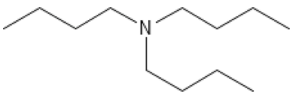
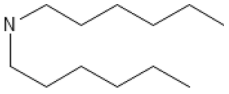
$$Rs = \frac{1.18(Rt_2 - Rt_1)}{(W_2 + W_1)} \quad (1)$$

Where Rt_2 and Rt_1 are the retention times of the peak 2 and 1, respectively, and $W_2 + W_1$ the sum of peak widths at 50% peak height.

Several chromatographic columns were employed in this work, namely Thermo DNAPac RP 2.1×100 mm 4 µm column, which is a supermacroporous column made of 4µm divinylbenzene polymer resin with a broad range of pore sizes, specifically designed for the separation of small and large oligonucleotides [11], and Waters ACQUITY BEH C18 300 Å columns of 2.1×5, 20, and 100 mm.

The physico-chemical properties of IPAs used in this study are summarized in Table 1. The mobile phases were always composed of an equimolar concentration of IPA and acetic acid. In this study, hexafluoro-2-propanol (HFIP) was not considered as an alternative to acetic acid, since HFIP is typically used to boost ESI-MS sensitivity and it is not appropriate to analyze large mRNA. Mobile phase A was

Table 1
summary of the IPA used in this study, classified according to their hydrophobicity (from weakly to strongly hydrophobic). IPAs are classified based on their effects on Health and Environment using letters: A: Not harmful; B: Harmful; C: Toxic; D: Deadly. This is based on Globally Harmonized System of Classification and Labelling of Chemicals, obtained on the provider's website.

Name	Abb.	Structure	MW (g/mol)	Health	Environment
Diethylamine	DEA		73.14	C	A
Triethylamine	TEA		101.19	C	A
Diisopropylamine	DIPA		101.19	C	B
Butylamine	BA		73.14	C	A
Dimethylbutylamine	DMBA		101.19	C	A
Diisopropylethylamine	DIPEA		129.24	C	C
Tripropylamine	TPA		143.27	C	B
Dibutylamine	DBA		129.24	D	A
Hexylamine	HA		101.19	C	C
Dicyclohexylamine	DcHA		181.32	C	D
Octylamine	OA		129.24	C	D
Tributylamine	TBA		185.35	D	C
Dihexylamine	DHA		185.35	D	D

composed of pure water or 30% acetonitrile, depending on the solubility of the IPAs in the mobile phase. The pH was measured in the mobile phase A (in absence of organic solvent). For the initial screening, pH was adjusted to 7.0 ($\pm$ 0.1) by adding acetic acid. The mobile phase B was composed of 75% to 95% acetonitrile, depending on the IPAs hydrophobicity. The mobile phase B was supplemented with the same concentrations of IPA and acetic acid as in mobile phase A, to prevent the formation of an IPA gradient. The flow rate was set at 0.4 mL/min and column temperature at 65 °C. A generic gradient having a composition range of 45% ACN in 20 minutes was always used to have reliable comparison of retention. In a second instance, the gradient conditions (initial and final compositions) were optimized for each IPA, while the gradient time was kept at 20 minutes.

Ion-pairing agents, particularly those with higher hydrophobicity, can exhibit poor solubility in water. To enhance solubility, it is advisable to add a small percentage of an organic solvent to the mobile phase, with sonication further helping dissolution. Also, a thorough column equilibration is needed prior to analysis, allowing sufficient time for the ion-pairing agent to bind to the stationary phase. We used a minimum equilibration time of 10 column volumes. Finally, mobile phases containing IPAs are stable for several weeks, but to maintain homogeneity, we shook the bottles briefly each day.

IPAs are persistent and can remain in columns and tubing for weeks. To avoid cross-contamination, it is advisable to dedicate columns solely for use with IPAs. Additionally, traditional washes are ineffective at removing IPAs; therefore, we used a specific wash protocol between each change of mobile phase conditions: a 45-minute wash at 65 °C, at 0.2 mL/min using a 1:1:1:1 mixture of isopropanol, methanol, acetonitrile, and water, with 0.1% pentafluoropropionic acid, followed by a 10-minute wash with 100% acetonitrile. This cleaning method efficiently removes positively charged IPAs, as demonstrated in Figures S1 and S2, where a ladder and a surrogate analyte (ibuprofen) were used, respectively. It resulted in clean chromatograms and reliable analyses. When this cleaning procedure was implemented, we did not experience

anymore loss of sensitivity and resolution, or baseline drift when changing the nature of IPA. While we cannot prove that our washing procedure completely removes the remaining IPAs out of the system, we can assume with high confidence that their impact on RNA retention is extremely limited. This wash mixture is also highly effective for cleaning IPAs from mobile phase bottles, particularly for hydrophobic agents like dihexylamine that tend to adsorb onto glass surfaces.

3. Results and discussion

3.1. Behavior of RNA samples in generic ion-pairing systems

With the use of a ladder composed of 8 RNA products having a size comprised between 200 and 6000 nt (200, 500, 1000, 1500, 2000, 3000, 4000, and 6000 nt), we evaluated 13 different ion pairing agents to systematically investigated the influence of IPA on retention and selectivity of large RNA. In Fig. 1, each dot represents the average elution composition for the ladder, with the two ends of the bar indicating the elution points for the smallest (200-nt) and largest (6000-nt) RNAs. Importantly, the four most hydrophobic IPAs (DHA, TBA, OA, and DcHA) of the list were used at a lower concentration of 25 mM instead of 100 mM, since they were poorly soluble in water and induced very high retention, as highlighted by the high percentage of ACN needed to elute the RNA ladder. Elution compositions were calculated using the following equation:

$$C_e = C_i + \frac{C_f - C_i}{t_{grad}} \times \left(t_{average} - t_0 - t_D\right) \quad (2)$$

Where C_i and C_f are the initial and final compositions of the gradient, respectively. t_{grad} is the gradient time, $t_{average}$ is the average retention time of the analyzed RNA ladder, t_0 is the column dead time measured by injecting uracil on the column, and t_D is the gradient dwell time.

We observed that all RNAs, ranging from 200 to 6000 nucleotides, were consistently eluted within a narrow range of less than 10% ACN, suggesting an on-off retention mechanism similar to that observed for smaller ONs [32]. However, the organic proportion required for elution varied significantly depending on the IPA used. For instance, with 100 mM DEA, RNAs were eluted as a single peak at 10% ACN, while only 25 mM DHA required up to 71% ACN.

Based on this series of experiments, we were able to classify the IPAs based on their hydrophobicity -from weakest to strongest - in Table 1, considering their elution composition. Based on this classification, the IPAs can be grouped into three distinct populations, highlighted in green, blue, and orange, representing weak, moderate, and strong hydrophobic agents, respectively. In their study, Donegan et al. observed a positive correlation between retention time of alkylamines and their boiling points and used the latter as surrogate for hydrophobicity [17]. Our results support these findings, as we observed a very strong positive correlation between the mean elution composition of the RNA ladder and the IPA boiling point, Log P, and Log D ($r = 0.95$, 0.89, and 0.94, respectively), when DcHA (atypical cyclic alkylamine) was excluded. It is noteworthy, however, that the retention order observed in this experiment (DIPEA > DMBA > BA) differed from that described by Donegan et al. (DMBA > BA > DIPEA). Moreover, none of the surrogates for hydrophobicity can accurately predict the % ACN at elution for RNA molecules (see Table S1). This underscores the difference of behavior between small and large oligonucleotides and the need to explore in depth the effect of IPAs on specific RNA sequences.

In a second instance, the gradient profile of each IPA was optimized, based on the values reported in Fig. 1, to enhance selectivity and resolution. Importantly, the composition window to elute the ladder was comprised between 5 and 10 % ACN, to have comparable chromatographic profiles between all IPAs. Fig. 2 displays the elution composition for the ladder using the optimized gradient across all IPAs, the wider the bars, the better the separation. Firstly, DEA was found to be insufficiently hydrophobic to effectively retain RNA molecules, resulting in complete elution of the whole ladder very early on the chromatogram with poor selectivity. Another issue arose with DHA as it was observed to strongly adsorbed onto the mobile phase glass bottles. Consequently, only a minimal number of tests were conducted with this IPA and it was not included in further investigations. For other IPAs, we observed that separation improved with increasing hydrophobicity. On the one hand, the weakly hydrophobic IPAs lacked sufficient hydrophobicity to effectively retain large RNAs. On the other hand, optimal separation was achieved with moderately hydrophobic IPAs, while strongly hydrophobic IPAs did not yield additional improvements. Consequently, moderately hydrophobic IPAs, such as TPA, DBA, and HA, emerged as the most promising candidates for enhancing separation.

In addition to their effect on retention, we also examined the influence of IPAs on the resolution of large RNA molecules ranging from 200 to 6000 nt. Some representative chromatograms are shown in Fig. 3. When using IPAs with low hydrophobicity such as TEA, poor separation of the RNA species was observed, particularly for those over 1500 nt, indicating their limited ability to effectively distinguish larger RNA. The separation improved as IPA hydrophobicity increased, with BA, TPA and HA offering progressively better resolution. Notably, HA achieved good

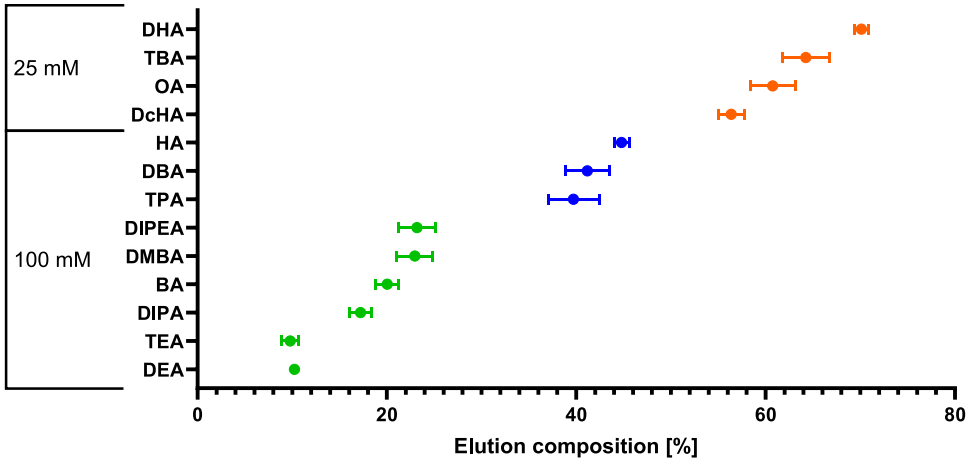


Fig. 1. Elution composition for the 8 RNA from the RiboRuler High Range RNA Ladder during a 20 min linear gradient run from 50 to 95% ACN for DcHA, OAA, TBA, and DHA, and 5% to 50% ACN for the other IPAs. The concentrations of IPAs in the mobile phases were 25 mM for DHA, TBA, OAA, and DcHA, and 100 mM for the other IPAs. The left error bar indicates the first eluting peak (200 nt); the dot indicates the mean elution composition; the right error bar indicates the last eluting peak (6000 nt). Weak, moderate and strong hydrophobic IPAs are highlighted in green, blue, and orange, respectively.

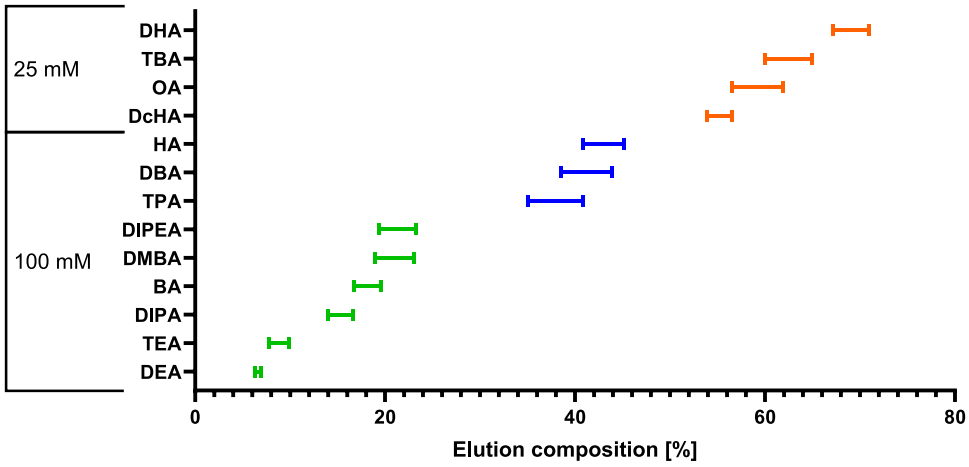


Fig. 2. Elution composition for the 8 RNA from the RiboRuler High Range RNA Ladder using a 20 min optimized linear gradient run for each IPA. The concentrations of IPAs in the mobile phases were 25 mM for DHA, TBA, OAA, and DcHA, and 100 mM for the other IPAs. The error bars indicate the window of elution. Weak, moderate and strong hydrophobic IPAs are highlighted in green, blue, and orange, respectively.

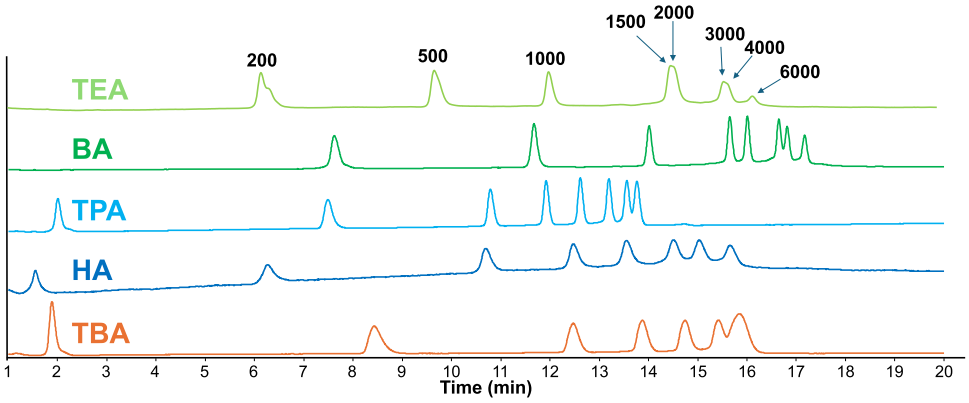


Fig. 3. Chromatograms obtained for the 8 RNA from the RiboRuler High Range RNA Ladder during a 20 min linear optimized gradient run at a temperature of 65 °C and a flow rate of 0.4 ml/min. Mobile phases are composed of 25 mM ion pairing agent for TBA, and 100 mM ion pairing agent for the other IPAs. Peaks have been labelled with the corresponding number of nucleotides. Weak, moderate and strong hydrophobic IPAs are highlighted in green, blue, and orange, respectively.

separation across all ladder species. However, under these conditions, the peaks became broader, likely due to the very shallow gradient slope (0.3%/min). The strongest hydrophobic IPAs (OA, DHA, DcHA, and TBA) were also tested, but their lower concentration (25 mM) compared to the other IPAs significantly reduced their separation power. Consequently, only the moderately hydrophobic IPAs provided effective separation across the ladder.

Finally, to progress with the identification of IPA candidates that offer the best separation, we evaluated the resolution achieved with the 13 different IPAs, for two critical RNA peak pairs: 1500/2000 nt and 4000/6000 nt. A minimum resolution of 1.5 was set as the threshold for selecting the best candidates. As depicted in Fig. 4, no single IPA provided the highest possible resolution for both selected RNA pairs. While most IPAs effectively separated the 1500/2000 nt pair, achieving the

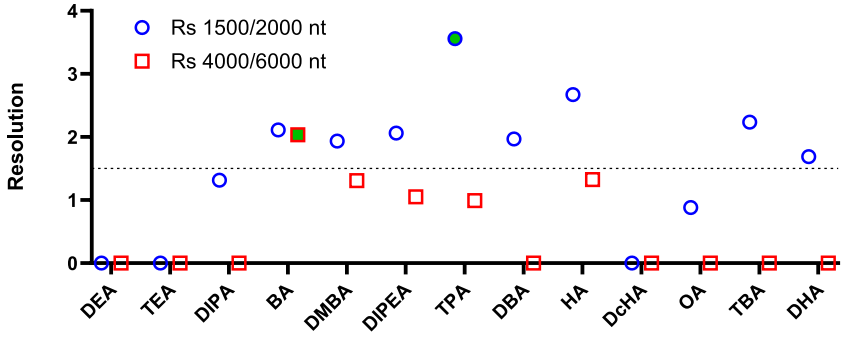


Fig. 4. Resolution calculated between 1500 – 2000 nt and 4000 – 6000 nt peak pairs from the RiboRuler High Range RNA Ladder during a 20 min linear optimized gradient run for each mobile phase. When resolution cannot be calculated because of bad peak shape or poor separation, the values were set to 0. The dotted line indicates the 1.5 resolution threshold. The best candidate for each resolution are highlighted in green. The concentrations of ion pairing agents in the mobile phases were 25 mM for DHA, TBA, OAA, and DcHA, and 100 mM for the other IPAs.

same level of separation for the larger RNA pairs was more challenging. BA was the only IPA able to adequately separating both RNA peak pairs, achieving a resolution greater than 1.5, and it was the only one that provided a resolution above 1.5 for the 4000/6000 nt peak pair. Interestingly, TPA delivered the highest resolution for the 1500/2000 nt peak pair, with a resolution exceeding 3. This highlights the complementary nature of these two IPAs, to achieve the best separation of RNA ladder.

3.2. Influence of IPA concentration, combination, pH, and temperature

To further enhance resolution, we tested various parameters related to the composition of the mobile phases, its pH, and temperature. A design of experiment (DoE) approach was not used in this case, due to the difficulty of reaching the extreme conditions required for DoE (harsh conditions, poor solubility of the IPAs, etc.). Instead, we employed a one factor at a time (OFAT) approach, starting with an exploration of IPA concentration, followed by evaluating combinations of IPAs. Once a suitable candidate was identified, we further evaluated the influence of pH, temperature, and solvent composition on resolution.

The resolution, especially for larger RNA, was greatly influenced by the IPA concentration. As illustrated in Fig. 5, a concentration of 25 mM DIPEA was insufficient to separate the 3000, 4000 and 6000 nt RNA species, which nearly coelute into a single peak. When the concentration was increased to 50 mM, the resolution improved, allowing the 3000 nt species to be separated from the 4000/6000 nt peak pair, although the resolution between the latter two remained below 1. At 100 mM, DIPEA successfully achieved separation of all RNA species, and no further improvement in resolution was observed at higher concentration. This indicates that when DIPEA concentration reaches 100 mM, it likely saturates the available binding sites in the column or fully covers the phosphate groups on the surface of the RNA molecules, leading to a plateau effect in resolution improvement. Data also showed that the increase in DIPEA concentration tends to decrease the retention. As reported elsewhere, this could be related to the fact that alkylamines at high concentration can form micelles. It has been shown that only a fraction of the alkylamines need to be neutral to initiate this aggregation process, leading to a drop in retention times, as the positive charges on the alkylamines are not anymore available to interact with RNA when there are forming micelles [33,34].

The concentration plateau effect was further examined using three different IPAs having different hydrophobicity (weak, moderate and high, as shown in Fig. 2). The corresponding resolutions for the 1500/2000 and 4000/6000 nt RNA pairs were calculated and are reported in Fig. 6. As shown, resolutions generally increased with IPA concentration, reaching a maximum at a concentration comprised between 100 and 200 mM, independently of the IPA hydrophobicity. However, the most hydrophobic IPA, TBA was not used at a sufficient concentration to fully saturate the available binding sites in the column or completely cover the phosphate groups on the RNA molecules, due to solubility limitations, resulting in poor resolution. In contrast, weakly and moderately hydrophobic IPAs provided much better resolution. These findings suggest that an optimal IPA concentration between 100 and 200 mM in the mobile phases is recommended to maximize resolution.

With an optimal concentration target of 100–200 mM, we explored combinations of IPAs with different hydrophobicities to further enhance resolution for the RNA ladder. Initially, we tested a combination of four moderately hydrophobic IPAs (25 mM each of TPA, DBA, HA, and DMBA), but observed no additional improvement in resolution. Next, we tested a combination of weak and moderately hydrophobic IPAs, specifically DIPEA and HA at varying concentrations. Significant resolution improvement was achieved when DIPEA concentration was higher that of HA. Building on this, we refined our optimal IPA formulation by incorporating a strongly hydrophobic IPA (TBA) into the mix, resulting in a final concentration of 100 mM DIPEA, 50 mM HA, and 12.5 mM TBA. Although this combination provided good resolution across all RNA samples, it also introduced notable drawbacks, such as high costs and the use of highly hydrophobic agents that are deadly when ingested or inhaled, toxic to aquatic life, and have long-lasting environmental impacts.

During our experiments, it became clear that IPAs do not uniformly affect the resolution of RNA pairs. To refine and simplify the method, we optimized resolution by pairing IPAs with complementary resolution. Specifically, we combined BA and TPA, as they were the most effective in separating 1500/2000 nt and 4000/6000 nt RNA pairs, respectively. To prevent oversaturation of the column binding sites or of the RNAs' phosphate groups, we used a higher concentration of the less hydrophobic agent and a lower concentration of the more hydrophobic one. The results showed that this combination of IPAs significantly improved resolution, achieving a resolution above 2 for all peaks while using less toxic IPAs (see Fig. 7 and Table S2). On average, the resolution of all the peak pairs increased by approximately 35% when comparing the use of either 100 mM BA or 50 mM TPA with the combination of 100 mM BA and 50 mM TPA.

Utilizing the effective combination of BA and TPA, we investigated the influence of temperature, pH, and organic solvent nature on RNA retention/selectivity (Figure S3). Substituting methanol with acetonitrile resulted in reduced resolution for larger RNAs. We also assessed pH variations of ± 1 from the standard 7.0 and found no significant impact on retention, indicating that as long as the amine and phosphate groups remain charged, pH does not influence retention. The temperature was also tested between 45 °C and 85 °C. We observed that lower temperatures led to increased retention, while higher temperatures decreased retention without significantly affecting the overall selectivity. Based on these findings, we established the optimal conditions at pH 7.0, using

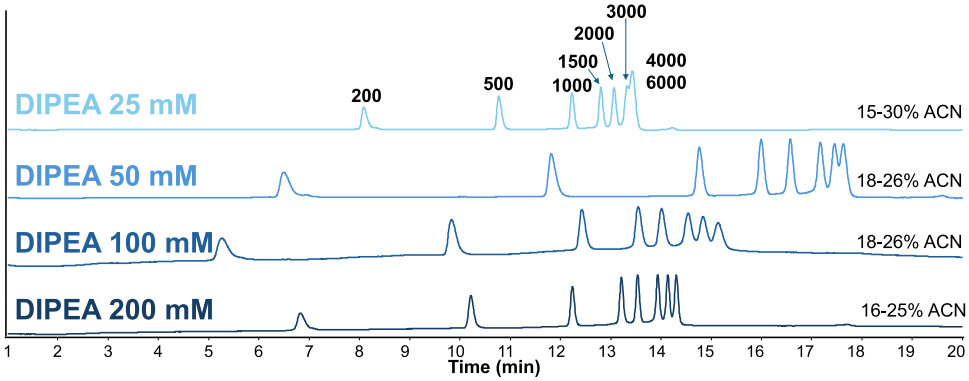


Fig. 5. Chromatograms obtained for the 8 RNA from the RiboRuler High Range RNA Ladder during a 20 min linear optimized gradient run at a temperature of 65 °C and a flow rate of 0.4 ml/min. Mobile phases contain an increasing concentration of DIPEA from 25 mM to 200 mM. Peaks have been labelled with the corresponding number of nucleotides and initial/final gradient compositions are also provided.

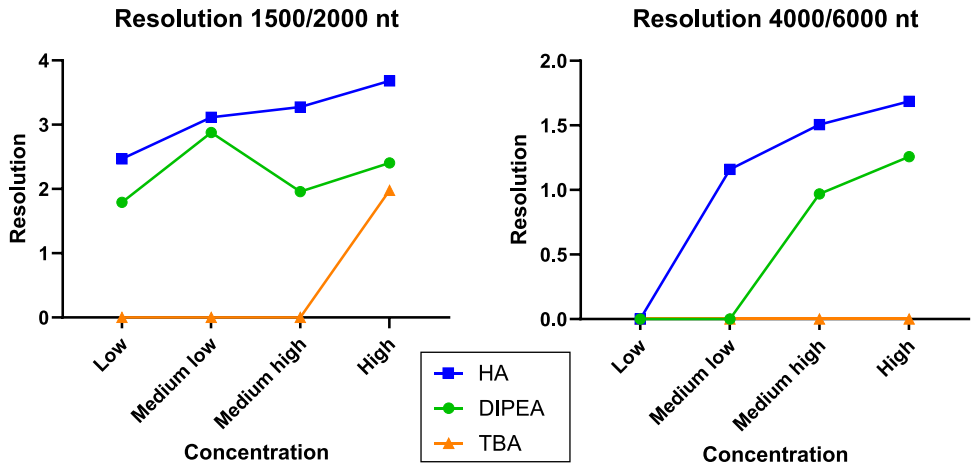


Fig. 6. Resolution calculated between 1500 – 2000 nt and 4000 – 6000 nt peak pairs from the RiboRuler High Range RNA Ladder during a 20 min linear optimized gradient run for each mobile phase condition. The concentrations tested were 2.5, 5, 10, and 25 mM for TBA; 25, 50, 100, and 150 mM for HA, and 25, 50, 100, and 200 mM for DIPEA. Weak, moderate and strong hydrophobic IPAs are highlighted in green, blue, and orange, respectively.

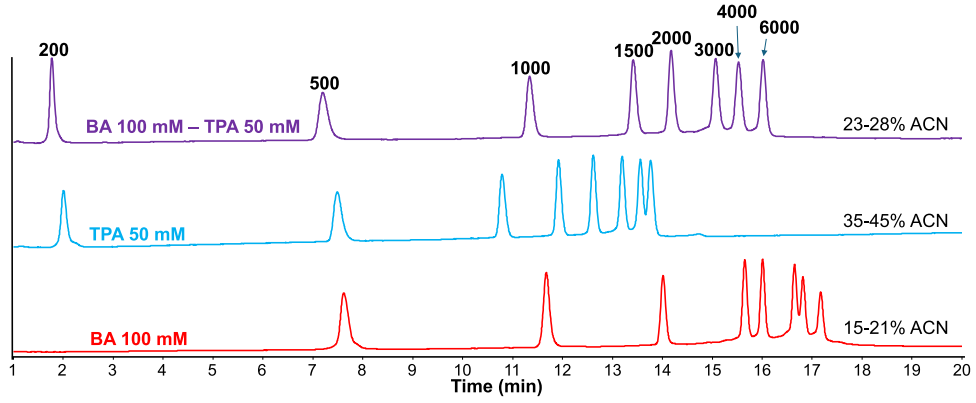


Fig. 7. Chromatograms obtained for the eight RNA from the RiboRuler High Range RNA Ladder during a 20 min linear optimized gradient run. Ion-pairing agents are added in both mobile phases. Peaks have been labelled with the corresponding number of nucleotides.

ACN as the organic solvent in the mobile phase, and an intermediate temperature of 65 °C.

3.3. Applicability of the method to separate species composed of 1 to 6000 nucleotides

To evaluate the applicability of the final method across a broader

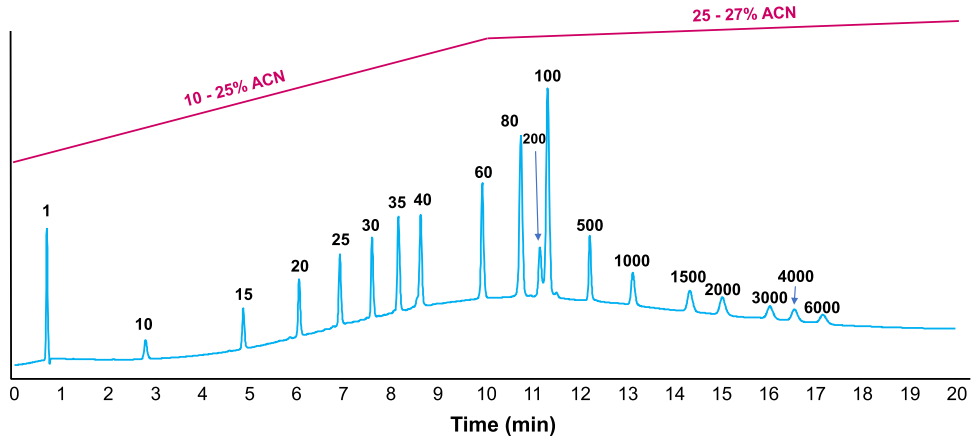


Fig. 8. Chromatogram obtained for a ladder composed of adenosine 5′-triphosphate, guanosine 5′-triphosphate, cytidine 5′-triphosphate, and uridine 5′-triphosphate as single nucleotide (1), ten oligodeoxythymidines ranging from 10 to 100 deoxythymidines, and eight RNA, ranging from 200 to 6000 nt, from the RiboRuler High Range RNA Ladder. The gradient is 10–25% ACN in 10 minutes followed by 25–27% ACN in 10 minutes for a total analysis time of 20 min, at a temperature of 65 °C and a flow rate of 0.4 ml/min, using a DNAPac RP column. IPAs used here come from the optimized mix (100 mM BA – 50 mM TPA). Peaks have been labelled with the corresponding number of nucleotides.

range of products, we used an extended ladder ranging from 1 to 6000 nt composed of four nucleotide triphosphate bases (commonly used as starting materials in IVT production of RNA), a 10 to 100 nt oligodeoxythymidine ladder, and a larger RNA ladder of 200 to 6000 nt. We optimized the gradient by reducing the initial proportion of ACN to better retain smaller ONs. However, as shown in Fig. 8, the retention of the four nucleotides was inadequate, and they were eluted within the dead time on the DNAPac RP column, due to the limited number of negative charges on their backbone (only three phosphate groups). Even with a gradient starting at 1 % ACN, these compounds still eluted in the dead time. On the other hand, we successfully achieved an efficient separation of all the other nucleotides, within an analysis time of 20 minutes. Oligodeoxythymidines ranging from 10 to 100 nt were well separated using a gradient from 10 to 25 % ACN, even when differing by just 5 nt. Interestingly, the 200-nucleotides RNA from the RiboRuler High Range RNA Ladder was less retained than the 100 dT. This is likely due to three factors: i) the absence of the 2′ OH functionality on the deoxythymidines, ii) the greater availability of the phosphate backbone in the 100 dT compared to the 200-nucleotide RNA, as the latter consist of random AUGC-nucleotide repeats that may form secondary structures, and iii) the relative hydrophobicity of nucleotides follows the order T>A>G>C, making thymidine more hydrophobic than the other nucleobases. Thus, the hydrophobicity of single stranded RNA and DNA are related to both their nucleic acid sequence and their secondary structure [35].

In the context of IVT RNA production, single nucleotides are highly informative, as they serve as the base material for synthesizing RNA molecules, in conjunction with polymerase enzyme and a DNA template. Their concentration reflects the proportion of nucleotides left unused during synthesis and helps verify whether the intended RNA product has been adequately produced [36]. To improve the retention of these single nucleotides, we evaluated their behavior using the optimal IPA formulation of 100 mM BA and 50 mM TPA on a Waters ACQUITY BEH C18 column with pore sizes of 300Å, and lengths ranging from 5 to 100 mm. It was important to test various column lengths, as we aim to use the shortest possible length to minimize its influence on the separation of other species eluted during the final gradient. If these single nucleotides can be effectively retained on this column, we could combine the BEH C18 and DNAPac RP columns in series to achieve suitable separation of all species.

As shown in Fig. 9, with an isocratic composition of 1 % ACN, the standard nucleoside triphosphates were adequately retained on the BEH C18 300Å column. However, shortening the column length significantly compromised resolution. The 100 mm column provided excellent separation, while the 20 mm column still achieved baseline separation of the nucleoside triphosphates. In contrast, the 5 mm column yielded inadequate performance. This behavior is expected, as the number of theoretical plates is directly proportional to column length, resulting in a 20-fold decrease in plate count between the 100 and 5 mm columns. Additionally, the UHPLC system used in this study was not optimized for very short columns (due to significant extra-column volume), further reducing the plate count for the 5 mm column. Notably, the chromatogram obtained with the 100 mm column revealed several additional peaks, corresponding to the mono- and di-phosphate forms of the nucleobases, which were confirmed by injecting standard molecules. Baseline separation of UTP and GTP was only achieved with the 100 mm column.

To take advantage of the suitable retention of the four nucleoside triphosphates on the Acquity BEH C18 column, we combined it with the DNAPac RP column, which is effective for separating larger RNA molecules, as described earlier. The order in which these columns are combined has minimal impact on the separation of small oligonucleotides, but significantly affects the separation of larger RNA molecules. As reported in Figure S4, when the BEH C18 is placed before the DNAPac RP column, the performance is notably reduced. This occurs because the BEH C18 columns is more retentive, requiring a higher elution composition. As a result, retention on the DNAPac RP column is reduced, leading to a final resolution for the coupled columns that is comparable to that of the BEH C18 alone. Conversely, positioning the DNAPac RP column before the BEH C18 enables the successful integration of both column properties, resulting in a separation of large RNA molecules similar to that achieved with the DNAPac RP column alone (Figure S4). In summary, it is absolutely essential to place the BEH C18 after the DNAPac RP column.

In the end, the optimized method serially combines a Thermo DNAPac RP 2.1×100 mm 4 µm column with a Waters Acquity BEH C18 2.1×20 mm 300Å column, at a temperature of 65 °C, a flow rate of 0.4 ml/min, and using a multi-steps gradient. This method efficiently separates nucleic acids ranging from 1 to 6000 nucleotides, as illustrated in Fig. 10. However, it is observed that resolution for large RNA is worse in this configuration. This is likely due to peak broadening caused by the additional dead volume introduced by column coupling, potential mismatches in flow dynamics between the two columns, and suboptimal ion-pairing interactions arising from changes in stationary phase characteristics or mobile phase behavior in the coupled system. Additionally, the smaller pore size of the BEH C18 column may further degrade resolution obtained with the large pore size DNAPac column.

4. Conclusion

The objective of this study was to assess the effects of different IPAs on RNA separation under IP-RPLC conditions and to develop a method capable of separating nucleic acids ranging from 1 to 6000 nucleotides. To identify the optimal conditions, 13 IPAs were tested using a Thermo

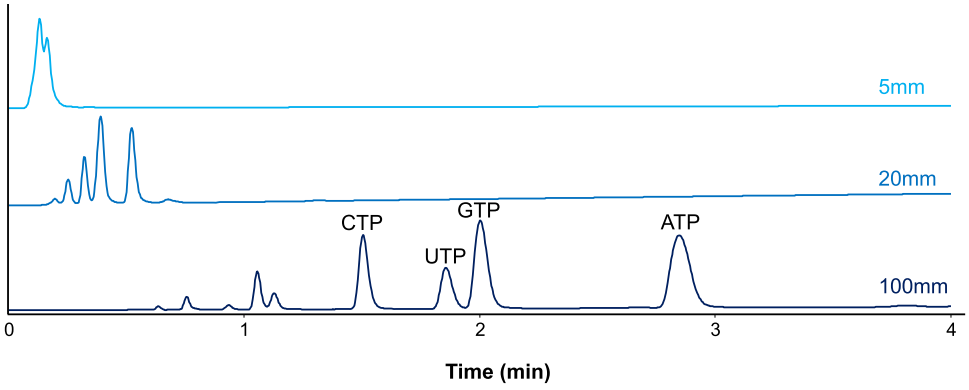


Fig. 9. Chromatograms obtained for adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), cytidine 5′-triphosphate (CTP), and uridine 5′-triphosphate (UTP) as single nucleotide, using Waters Acquity C18 300Å columns of different lengths. An isocratic mobile phase composed of 1% ACN was used, at a temperature of 65 °C and a flow rate of 0.4 ml/min. IPAs used here come from the optimized mix (100 mM BA – 50 mM TPA).

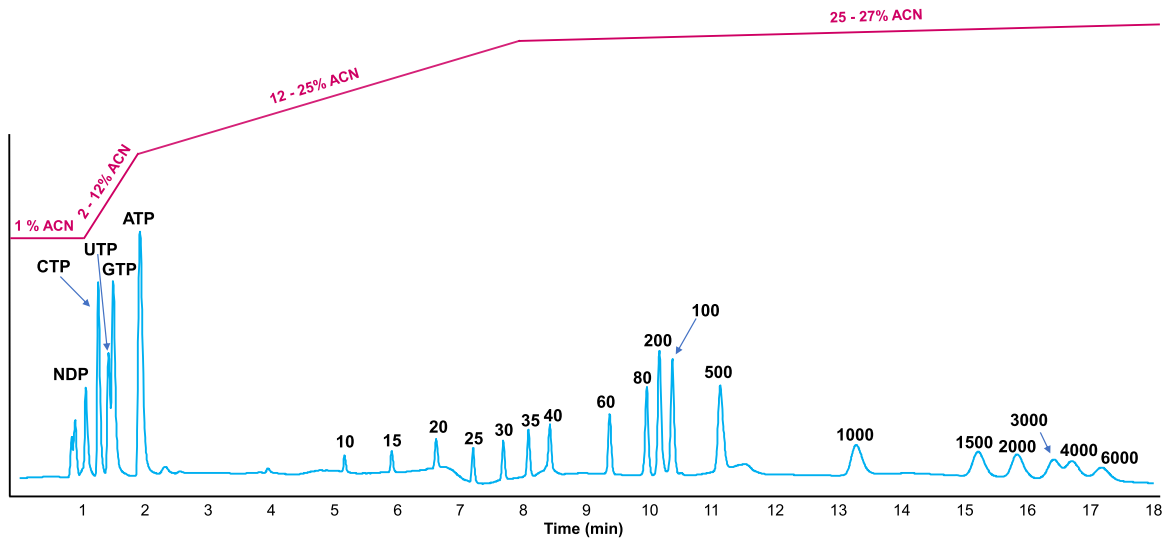


Fig. 10. Chromatogram obtained for a ladder composed of adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), cytidine 5′-triphosphate (CTP), and uridine 5′-triphosphate (UTP) as single nucleotide, ten oligodeoxythymidines ranging from 10 to 100 deoxythymidines, and eight RNA, ranging from 200 to 6000 nt, from the RiboRuler High Range RNA Ladder. This optimized method serially combines a 2.1×100mm DNAPac RP column and a 2.1×20mm BEH C18 300 Å The gradient is symbolized with the slopes. The analysis is made at a temperature of 65 °C and a flow rate of 0.4 ml/min. IPAs used in the mobile phases are 100 mM BA – 50 mM TPA. Peaks have been labelled with the corresponding number of nucleotides. NDP represents the di-phosphate nucleotides.

DNAPac RP column of 2.1×100mm. The results demonstrated that moderately hydrophobic IPAs at concentrations between 100 and 200 mM are the most effective additives for enhancing RNA retention and resolution. Additionally, combining a weakly hydrophobic IPA with a moderately hydrophobic one further improved resolution, by taking advantage of the complementary properties of the IPAs. The best resolution was achieved using 100 mM BA and 50 mM TPA in both mobile phases, allowing effective separation of RNA across a wide range of sizes. However, under these conditions, nucleoside triphosphates exhibited poor retention, which is problematic, since these molecules are used as starting material in IVT RNA production. To address this, we combined the Thermo DNAPac RP column of 2.1×100mm with a Waters Acquity BEH C18 300 Å of 2.1×20 mm as a post-column. This setup achieved good retention and resolution of a ladder composed of 22 nucleic acids ranging from 1 to 6000 nucleotides, resulting in an efficient and robust method for evaluating RNA integrity as well as for monitoring IVT reactions at the core of RNA-based therapeutics.

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CRediT authorship contribution statement

Jonathan Maurer: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Camille Malburet:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Marc François-Heude:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Davy Guillarme:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Camille Malburet and Marc Francois-Heude are Sanofi employees and may hold shares and/or stock options in the company. Jonathan Maurer and Davy Guillarme declare no competing interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.465574.

Data availability

The data that has been used is confidential.

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