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# Hydrophilic additives enhancing electrospray ionization of oligonucleotides in hexafluoro-2-propanol-free reversed-phase ion-pair liquid chromatography/mass spectrometry

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ABSTRACT

The characterization of oligonucleotide therapeutics is commonly performed by liquid chromatography/mass spectrometry (LC/MS) using reversed-phase ion-pair (RP-IP) chromatography with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and triethylamine (TEA) due to its efficient electrospray ionization (ESI) performance. However, HFIP poses several challenges, including environmental concerns as a per- and polyfluoroalkyl substance (PFAS), limited mobile phase stability, and high reagent costs. Traditional HFIP-free alternatives, such as acetic acid (AA) and TEA, generally result in lower MS sensitivity. This study aimed to develop an HFIP-free analytical method for oligonucleotides and explored the use of AA/TEA-based mobile phase additives to improve MS sensitivity. An AA/glycine/TEA-based mobile phase prepared by adding glycine to an AA/TEA-based mobile phase, exhibited significantly enhanced MS sensitivity compared to the original AA/TEA system, reaching levels comparable to those achieved with HFIP/TEA. Comprehensive screening of amino acids revealed that aliphatic amino acids, particularly those with high hydrophilicity, contributed to enhanced ionization. These findings suggest that hydrophilic additives may promote the localization of oligonucleotides at the droplet surface and facilitate ion release, thereby enhancing ionization efficiency. Moreover, the AA/glycine/TEA system maintained MS sensitivity for a longer duration than the HFIP/TEA system. Although glycine is typically avoided in LC/MS due to its non-volatile nature, its use at sufficiently low concentrations did not lead to contamination or performance degradation. These findings support the potential of hydrophilic additives like glycine to enhance ionization efficiency in HFIP-free RP-IP-LC/MS for oligonucleotide analysis.

## 1. Introduction

Oligonucleotide therapeutics, such as antisense oligonucleotides (ASOs) and small-interfering RNAs (siRNAs), have emerged as novel modalities for treating diseases that are difficult to address using conventional approaches. In recent years, several oligonucleotide-based drugs have received regulatory approval, reflecting significant progress in this field. ASOs are typically single-stranded oligonucleotides of approximately 20 nucleotides in length and are chemically modified at the sugar, nucleobase, and phosphate moieties to improve their efficacy and safety [1–4]. However, due to their multi-step solid-support synthesis and limited purification after cleavage from the solid support, oligonucleotide therapeutics contain impurities that closely resemble the desired product. These impurities can compromise the efficacy and safety of the final formulation and may hinder regulatory approval and commercialization [5–7]. Therefore, establishing reliable analytical methods for detecting and quantifying such impurities is important throughout development and production. Furthermore, as manufacturing scales up, analytical methods must also emphasize simplicity and sustainability.

The characterization and identification of synthetic oligonucleotides and their impurities are generally performed using liquid chromatography/mass spectrometry (LC/MS). High-performance liquid chromatography (HPLC) modes used for oligonucleotides include ion-exchange

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chromatography (IEC), reversed-phase chromatography (RPC), size-exclusion chromatography (SEC), and hydrophilic interaction liquid chromatography (HILIC). Notably, reversed-phase ion-pair (RP-IP) chromatography is the most widely used due to its high separation efficiency and MS compatibility. Oligonucleotides are typically analyzed using electrospray ionization (ESI) in the negative ionization mode, owing to their negatively charged phosphate backbones [6–8]. Historically, mobile phases for RP-IP chromatography have combined alkylamines with carboxylic acids. However, conventional acids such as acetic acid (AA) or formic acid suppress oligonucleotide ionization. In contrast, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), with its appropriate $pK_a$ and high volatility, has proven well-suited for oligonucleotides analysis [9,10]. The combination of alkylamines, such as triethylamine (TEA), and HFIP has since become a standard in oligonucleotide LC/MS analysis [11–14]. Nevertheless, HFIP presents several challenges, including environmental concerns due to its classification as a per- and polyfluoroalkyl substance (PFAS) [15], instability in mobile phase composition over time [16,17], and high costs. These issues underscore the need for HFIP-free methods that ensure efficient ionization and robust MS sensitivity in oligonucleotide analysis. Alternative approaches have been explored, such as the use of different mobile phase systems [18] or switching to positive ionization mode [19]. HILIC has also shown promise as an alternative separation mode and demonstrated superior performance under certain conditions [20,21], although RP-IP chromatography generally provides higher MS sensitivity [22,23]. However, these approaches have not yet been widely adopted as general alternatives to the fluoroalcohol/alkylamine system.

For developing high-sensitivity methods, understanding the ESI mechanism is important. In ESI, charged droplets are formed from the mobile phase using high voltage and nebulizing gas. As the solvent evaporates and Coulombic repulsion overcomes surface tension, the droplets undergo fission, producing smaller charged droplets [24,25]. Gas-phase ions are subsequently generated *via* either charge residue model (CRM) or ion evaporation model (IEM). In CRM, ions are produced when neutral molecules completely evaporate from a droplet containing a single analyte molecule [26,27], while IEM involves direct emission of ions from the droplet surface once a critical radius is reached [28,29]. While CRM predominates for large biomolecules [27], IEM is thought to be more relevant for oligonucleotides in the presence of ion-pair reagents [8]. The total gas-phase ion yield in ESI $J_i$ depends on the ionization efficiency in liquid-phase $I$ and the vaporization rate of the analyte from liquid-phase to gas-phase $J_V$. Ionization efficiency $I$ is associated with thermochemical parameters such as proton affinity, gas-phase basicity, ionization energy, or electron affinity. The vaporization rate $J_V$, which reflects the ability of analytes to transition from condensed to gas-phase, is more difficult to predict but equally critical [30,31]. HFIP's effectiveness likely stems from its ability to generate a high proportion of ionized oligonucleotides in the droplet, improving $I$. However, no non-fluorinated acids have been found to achieve similar effects. An approach to enhance $J_V$ has been reported utilizing ammonium bicarbonate in the mobile phase, which may generate gas upon decomposition and facilitate droplet fission, thereby improving MS sensitivity [18]. While not applied to oligonucleotides, glycine has been shown to enhance MS sensitivity for flavonoid glycosides analysis [31], probably due to its strong hydration capability. Glycine interacts strongly with water molecules and releases the analytes from solvation, thereby enhancing efficient gas-phase ion generation. Similarly, glycine has been reported to improve peptide detection in positive ionization mode [32]. Although the primary mechanisms may differ, these findings suggest that non-volatile compounds concentrated during droplet atomization can enhance ionization, a concept that merits further exploration. To our knowledge, this strategy has not been applied to oligonucleotide analysis.

In this study, we aimed to develop an HFIP-free, high-sensitivity analytical method for oligonucleotides by exploring additives that enhance MS sensitivity in typical AA/TEA-based mobile phase. Glycine was initially investigated, followed by a comprehensive evaluation of the potential of glycine derivatives, amino acids, and other low-molecular-weight compounds. The long-term stability of glycine-containing mobile phases was also assessed.

## 2. Materials and methods

### 2.1. Chemicals and solvents

Methanol, AA, alanine, d-alanine, arginine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine, d-valine, $\gamma$-aminobutyric acid (GABA), *N*-methylglycine (sarcosine), urea, thiourea, formamide, acetamide, formaldehyde, glucose, and glycerol were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). TEA was purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Honeywell (Charlotte, NC, USA). HFIP was purchased from Nacalai Tesque (Kyoto, Japan). Asparagine and *N, N*-dimethylglycine were purchased from Sigma-Aldrich. Serine was purchased from Kanto Chemical (Tokyo, Japan). $\alpha$-Aminobutyric acid (AABA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Diglycine and triglycine were purchased from Peptide Institute (Osaka, Japan). A Milli-Q water purification system, purchased from Merck Millipore (Burlington, MA, USA) was used to prepare mobile phases and sample solutions. Synthetic oligonucleotides were purchased from Hokkaido System Science (Hokkaido, Japan), Ajinomoto Bio-Pharma Services GeneDesign (Osaka, Japan), and Juzen Chemical Corporation (Toyama, Japan) as HPLC-purified grade. The sequences used in this study are listed in Table S1. Oligonucleotides were dissolved in water to a concentration of 1 or 5 μM and used for analysis.

### 2.2. Instruments and analytical conditions

The analytical system consisted of a Nexera XS HPLC system coupled to a LCMS-9030 quadrupole time-of-flight (QTOF) mass spectrometer (Shimadzu Corporation, Kyoto, Japan). LabSolutions LCMS software (Shimadzu Corporation) was used for system control, data acquisition, and data processing. Chromatographic separation was performed on a Shim-pack Scepter C18–300 column (2.1 mm I.D. × 50 mm, 1.9 μm; Shimadzu Corporation). For AA-based analysis, mobile phase A contained 7.5 mM AA and 10 mM TEA in water. For HFIP-based analysis, mobile phase A consisted of 100 mM HFIP and 10 mM TEA in water. For all analyses, mobile phase B was methanol. Detailed HPLC parameters are presented in Table S2. Samples were monitored at a wavelength of 260 nm using a photodiode array (PDA) detector and analyzed by ESI-MS in negative ionization mode over the $m/z$ range of 500–2600. Deconvolution of multiply charged signals was performed using LabSolutions Insight Biologics software (Shimadzu Corporation). Details of the condition of the MS ionization interface and the software settings for deconvolution are also listed in Table S2.

## 3. Results and discussion

### 3.1. Electrospray ionization enhancement of oligonucleotides via amino acid addition

We hypothesized that hydrophilic compounds could promote electrospray ionization of oligonucleotides under HFIP-free conditions by influencing droplet behavior and ion formation. As representative hydrophilic additives, we focused on simple amino acids, particularly glycine, alanine, and valine, due to their structure simplicity. Glycine, the simplest amino acid, was first evaluated under AA/TEA-based conditions. To assess its effect, 1 mM glycine was added to the mobile phase, and ASO1–1, a model ASO with the same sequence and modifications as nusinersen (see Table S1), was analyzed under gradient elution. MS deconvolution was performed by summing all charge state signals of the

analyte within the acquired *m/z* range (500–2600). As a result, the AA/glycine/TEA-based mobile phase enhanced MS sensitivity by over tenfold compared to the AA/TEA system and achieved a sensitivity level comparable to that of the HFIP/TEA system (Fig. 1). The UV peak areas were comparable across all conditions, confirming consistent sample injection amounts. The MS spectra varied depending on the mobile phase conditions (Fig. S1). Increase in the predominant charge state from –4 to –5 upon glycine addition suggested more rapid ionization of the oligonucleotides, as the charge-state distribution dependent on desolvation efficiency [33]. Although the mobile phases had different pH values (pH 9.7 for AA/glycine/TEA, pH 10.1 for AA/TEA, and pH 8.4 for HFIP/TEA), which are known to influence charge-state distributions [34,35], adjusting the pH of the AA/glycine/TEA mobile phase by adding AA had little impact (Fig. S2), supporting the idea that the sift of charge state from –4 to –5 results from enhanced desolvation rather than pH effect. We next examined the effect of glycine concentration (0.1–10 mM) in the AA/glycine/TEA-based mobile phase (Fig. S3). The MS peak area increased with rising glycine concentration but began to plateau above 1 mM. Therefore, 1 mM glycine was selected as the optimal additive to minimize potential MS contamination.

To ensure that the addition of glycine did not compromise chromatographic performance, we examined its effect using a one-nucleotide deletion (n–1) impurity, a representative impurity with physicochemical properties similar to those of the full-length product (FLP). A mixture of the FLP and n–1 impurity of ASO1–1 was analyzed under gradient conditions where the peak tops were distinguishable (Fig. S4, Table S3). Replacing the AA/TEA mobile phase with the AA/glycine/TEA system had little influence on separation; the elution order and resolution remained comparable. However, the AA/glycine/TEA-based mobile phase exhibited slightly longer retention times (a 0.4 min difference) compared with the glycine-free conditions. The slight pH decrease caused by glycine likely strengthened retention by promoting alkylamine (TEA) protonation [36]. The HFIP/TEA system yielded slightly sharper peaks than AA-containing systems, likely due to suppression of diastereomer separation in phosphorothioate-modified oligonucleotides [37]. Importantly, the addition of glycine did not affect the resolution between the FLP and n–1 impurity.

To verify broader applicability, additional ASOs with different sequences and modifications were analyzed (Fig. S5). Regardless of sequences or modifications, the AA/glycine/TEA-based mobile phase improved MS sensitivity without adversely affecting chromatographic behavior. These findings demonstrate that adding glycine to the AA/TEA-based mobile phase enhances MS sensitivity by alleviating ionization suppression caused by carboxylic acids while maintaining chromatographic performance This supports the general utility of glycine as an additive for HFIP-free analysis.

Alanine and valine were subsequently examined (Fig. S6). Similar to glycine, these amino acids enhanced ionization without affecting retention times. The retention times did not differ between l- and d-enantiomers. These findings suggest that amino acids are not directly involved in the formation of ion pairs between oligonucleotides and TEA and do not impact their separation on the column. Furthermore, no differences were observed in terms of MS intensity or spectral features between the enantiomers. This observation indicates that the stereochemistry of amino acids does not significantly affect the ionization of oligonucleotides and that the chemical properties of amino acids are responsible for enhancing ionization.

According to the ion evaporation model (IEM) in ESI, analytes located at the droplet surface are more efficiently released into the gas-phase [38]. Oligonucleotides and glycine, being highly hydrophilic, tend to localize at the droplet interior. However, the presence of glycine may promote redistribution of some oligonucleotide molecules toward the droplet surface, thereby facilitating ion release and enhancing ionization efficiency (Fig. 2).

To investigate the relationship between hydrophilicity and ionization enhancement, glycine derivatives with lower hydrophilicity were evaluated (Fig. S7). *N*-Methylglycine, in which the amino group is substituted with a methyl group, showed a moderate enhancement effect, whereas *N,N*-dimethylglycine had no effect. Similarly, diglycine and triglycine, which are less hydrophilic than glycine [39], showed a decreasing trend in ESI enhancement with increasing chain length. Substitution of hydrogen atoms on the amino group also alters the p$K_a$; however, the p$K_a$ values of glycine (9.62), *N*-methylglycine (10.01), and *N,N*-dimethylglycine (9.80) [40] do not correlate with the observed ionization enhancement effects. These results suggest that the hydrophilicity of the additives is a crucial factor in promoting efficient ionization of oligonucleotides in ESI.

### 3.2. Evaluation of other amino acids

A comprehensive screening of amino acids was conducted to identify additives that could further enhance MS sensitivity, by flow injection

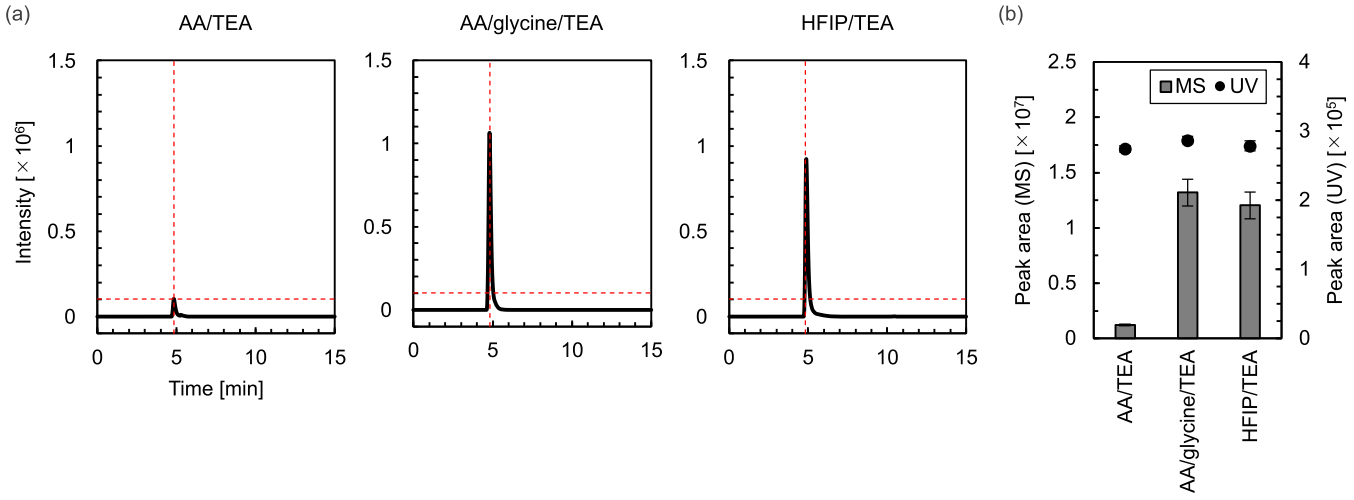


**Fig. 1.** (a) Deconvoluted chromatograms of ASO1–1 obtained under the AA/TEA-based, the AA/glycine/TEA-based, and the HFIP/TEA-based mobile phase conditions. Red dotted lines along the X-axis indicate signal intensity, and those along the Y-axis represent the retention time of ASO1–1, both under the AA/TEA conditions. (b) Evaluation of glycine as a potential ionization-enhancing additive. Data are expressed as mean ± SD ($n = 9$; $n = 3$ on each of three days). The AA/glycine/TEA-based mobile phase enhanced MS sensitivity by more than tenfold compared to the AA/TEA system, which was comparable to that of the HFIP/TEA system.

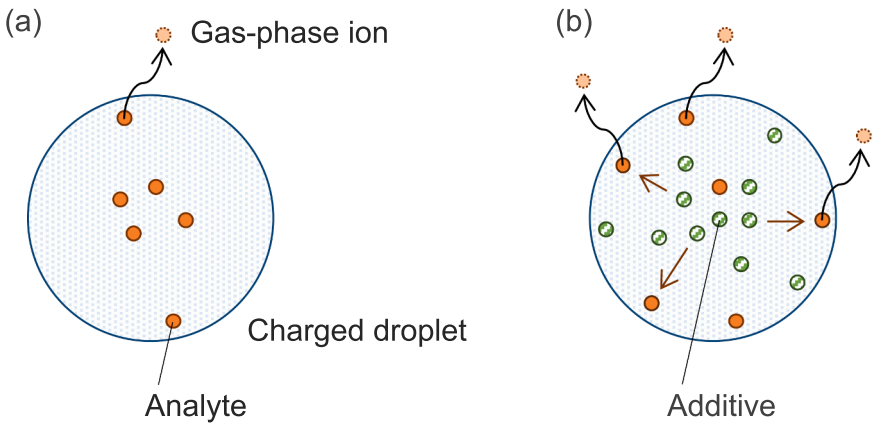


**Fig. 2.** Schematic electrospray ionization model. (a) Analytes are released from the surface of charged droplets. (b) Hydrophilic additives may promote the localization of analytes at the droplet surface, thereby facilitating their release as gas-phase ions.

analysis (FIA) of ASO1–1. Among the twenty-two amino acids tested, alanine exhibited enhancement comparable to that of glycine, while most others showed reduced effectiveness (Fig. 3a). AABA and GABA exhibited moderate enhancement, and valine, isoleucine, and leucine, which have larger side chains, also showed some enhancement effects. In contrast, many other amino acids had minimal to no impact for ionization.

To better understanding these differences, the results were analyzed in relation to hydrophilicity, represented by the calculated logP (ClogP) values (Fig. 3b). ClogP values for each amino acid were calculated using a ChemDraw software (Revvity, Waltham, MA, USA) (Fig. S8). Although no overall correlation was observed between MS sensitivity and hydrophilicity, a positive trend was noted among structurally similar aliphatic amino acids. More hydrophilic compounds in aliphatic amino acids tend to enhance the ionization of oligonucleotide. This observation suggests that hydrophilicity may serve as a useful criterion for assessing additive potential among structurally similar compounds. Since the p$K_a$ values of alanine, glycine, AABA, valine, isoleucine, and leucine are similar (ranging from 9.59 to 9.92 for the amino group) [41,42], no clear correlation was found between p$K_a$ and ionization enhancement. These findings further support the proposed mechanism of ionization enhancement by hydrophilic additives shown in Fig. 2. In contrast, acidic and basic amino acids such as glutamic acid and arginine exhibited little enhancement despite their low ClogP values. Similarly, neutral but non-aliphatic amino acids such as glutamine and serine also failed to enhance ionization. These amino acids, characterized by diverse functional groups and physicochemical properties, likely interact with the ionization process in more complex ways. The factors such as the ionization propensity of the additives themselves, pH changes within the droplets, and surface tension of the droplets may affect the ionization of oligonucleotides, potentially leading to decreased MS sensitivity.

### 3.3. Investigation of other low molecular weight compounds

In addition to amino acids, other low molecular weight compounds, such as amides, aldehydes, and monosaccharides, were evaluated as potential additives through gradient analysis of ASO1–1. Among these, urea, thiourea, and glucose exhibited MS sensitivity enhancement comparable to that of glycine (Fig. 4), without affecting the retention time of ASO1–1 (Fig. S9). Formamide, acetamide, formaldehyde, and glycerol, however, did not improve ionization efficiency. ClogP values for these compounds were also calculated (Fig. S8), but no clear relationship was observed between MS sensitivity and hydrophilicity. Given the structural and functional diversity of the compounds tested, it appears difficult to predict additive performance based on ClogPs, as was possible for aliphatic amino acids. It is highly probable that the ionization of oligonucleotides in PR-IP-LC/MS involves a combination of mechanisms [8]. The introduction of additives may further complicate these mechanisms. The ionization mechanism in ESI appears to be a

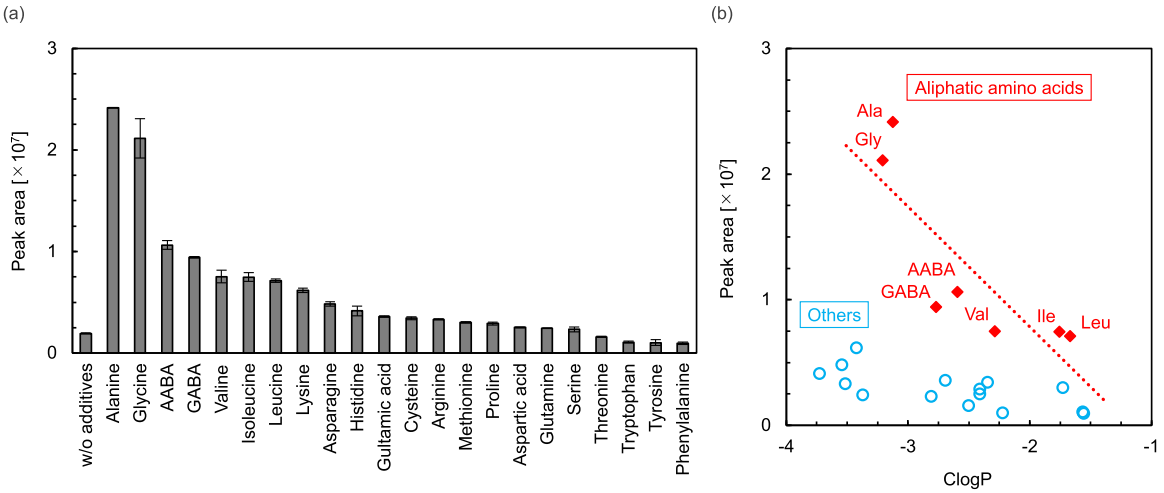


**Fig. 3.** (a) Evaluation of various amino acids as potential ionization-enhancing additives. Data are expressed as mean ± SD ($n$ = 3). (b) Correlation between the enhancement rate and ClogP, a calculated parameter reflecting the hydrophilicity of each amino acid. A correlation was observed among structurally similar aliphatic amino acids, such as alanine, glycine, AABA, GABA, valine, leucine, and isoleucine, between enhancement rate and hydrophilicity.

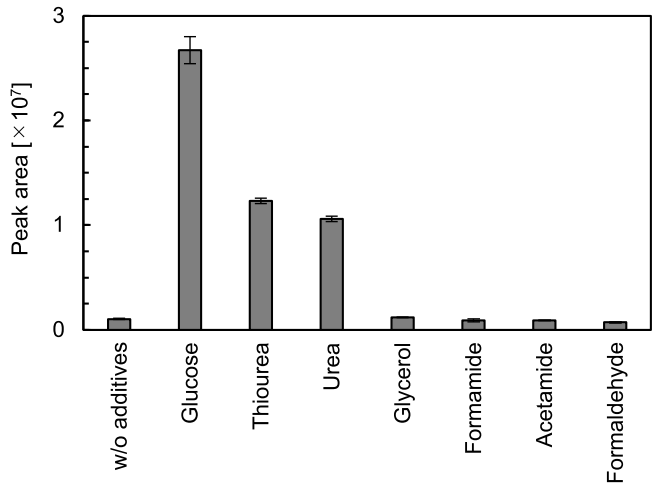


**Fig. 4.** Evaluation of low molecular weight compounds as potential ionization-enhancing additives. Data are expressed as mean ± SD ($n = 3$). Urea, thiourea, and glucose exhibited notable enhancement of MS sensitivity.

multifactorial process involving complex interactions among oligonucleotides, alkylamines, carboxylic acids, and the additives themselves. A comprehensive understanding of this process will require further elucidation of these interdependencies.

While several compounds were identified as effective additives for enhancing the ionization of oligonucleotides, practical considerations such as safety and applicability must also be taken into account. Thiourea demonstrated strong enhancement effects but may raise safety concerns when used in large quantities. Glucose, a non-toxic and environmentally friendly compound, is also a promising candidate; however, when MS spectra need to be acquired across a wide $m/z$ range, its relatively high molecular weight may cause spectral overlap with analytes, thereby complicating data analysis. In contrast, glycine, alanine, and urea are not only effective in enhancing ionization but are also widely regarded as safe, low-molecular-weight, and readily available. Considering their benign safety profiles, cost-effectiveness, and ease of handling, amino acids, particularly glycine, could represent a well-balanced and practical choice as additives for HFIP-free LC/MS analysis of oligonucleotides.

### 3.4. System stability with glycine-containing mobile phases

To evaluate mobile phase stability, ASO1–1 was continuously analyzed over a period of 72 h. With a run time of 15 min per cycle, a total of approximately 300 gradient runs were performed during this period. At 24 h, MS sensitivity in the HFIP/TEA system decreased by approximately 10 %, whereas the AA/glycine/TEA system exhibited no significant decline. At the end, the AA/glycine/TEA system retained MS sensitivity similar to that of the AA/TEA system, while the HFIP/TEA system showed a decrease to approximately 60 % of its initial sensitivity (Fig. 5). These results are consistent with previously reports of sensitivity loss over time in HFIP/TEA systems [16,17]. In contrast, the AA/glycine/TEA system—free of HFIP—showed fluctuations in MS sensitivity comparable to those observed in the AA/TEA system. Notably, the AA/glycine/TEA system maintained MS sensitivity for a longer duration than the HFIP/TEA system, suggesting improved robustness. Furthermore, in the AA/glycine/TEA system, chromatographic parameters, including retention time, theoretical plate number, and symmetry factor, remained stable throughout the 72-hour period (Table S4). Although C18 stationary phase can dissolve under basic mobile phase conditions, recent advances in the stationary phase materials enabled the AA/glycine/TEA-based mobile phase (pH 9.7) to show no significant degradation.

While glycine is generally avoided in LC/MS due to its non-volatile nature, its use at sufficiently low concentrations did not cause contamination or performance loss. In practice, after using the system under these conditions for over six months, no significant contamination was observed in the ion optical components within the MS. To further investigate the risk of contamination under more rigorous conditions, a mobile phase consisting of 25 mM glycine in water (mobile phase A) was continuously introduced into the ion source for 120 h. While deposition of glycine was observed inside the ion source, the low vacuum pressure in the MS remained unchanged, indicating that glycine did not block the sampling cone (Fig. S10). These observations suggest that glycine tends to deposit primarily within the ion source, rather than inside the MS itself, thus preventing contamination of internal components. Nevertheless, minimizing deposition in the ion source is still preferable to ensure long-term instrument performance. Given that glycine was effective at concentrations as low as 1 mM, and that no further enhancement in ESI was observed at higher concentrations (Fig. S3), excessive addition should be avoided.

## 4. Conclusions

This study established an HFIP-free RP-IP-LC/MS method for oligonucleotide analysis, with a particular focus on enhancing MS sensitivity through the use of hydrophilic additives. Among the tested compounds, glycine proved particularly effective: the AA/glycine/TEA mobile phase achieved an over tenfold enhancement in MS sensitivity compared to the AA/TEA system, with performance comparable to the HFIP/TEA system.

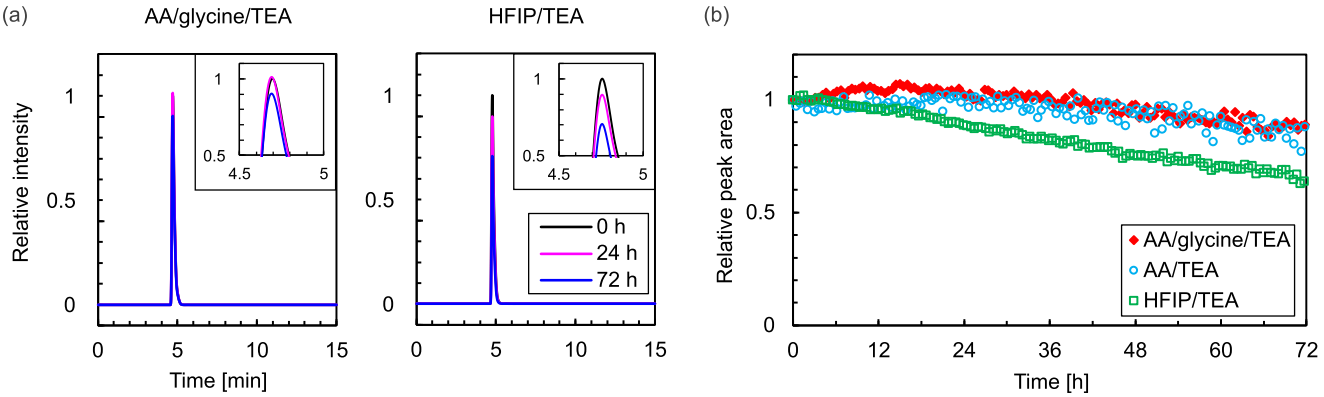


**Fig. 5.** (a) Deconvoluted chromatograms of ASO1–1 obtained under the AA/glycine/TEA-based and the HFIP/TEA-based mobile phase conditions. (b) Fluctuations in peak area during 72 h of continuous analysis. The AA/glycine/TEA system maintained MS sensitivity for a longer duration than the HFIP/TEA system. Glycine did not affect the stability of the AA-containing mobile phase.

Comprehensive screening of amino acids revealed that aliphatic amino acids, including AABA, GABA, and especially glycine and alanine, enhanced ionization, while amino acids with unique functional groups and distinct physicochemical properties showed reduced effectiveness. The results underscored the importance of hydrophilicity in promoting efficient electrospray ionization. It is hypothesized that the hydrophilic additives facilitate the localization of oligonucleotides to the droplet surface during electrospray, thereby enhancing gas-phase ion generation. Other low molecular weight compounds, such as urea, thiourea, and glucose, also enhanced MS sensitivity. Moreover, the AA/glycine/TEA-based mobile phase exhibited greater stability over time than the HFIP/TEA system, supporting its robustness for analytical applications.

The effective additives identified in this study are all non-volatile. Although they did not cause any noticeable instrument damage when used at low concentrations, the use of volatile analogs would be preferable to minimize long-term contamination risks. Volatile compounds such as acetamide and formamide, which are structurally similar to urea, showed no ionization enhancement effects; however, further investigation into volatile alternatives is warranted.

In conclusion, glycine represents a practical and effective additive for enhancing ESI-MS sensitivity in HFIP-free RP-IP-LC/MS analysis of oligonucleotides. As oligonucleotide therapeutics continue to gain traction as treatments for refractory diseases, it is increasingly important to consider the environmental impact of analytical processes—particularly those involving PFAS-containing reagents. The method developed in this study offers a sustainable, cost-effective, and accessible alternative, with robust performance suitable for both development and manufacturing settings. This approach not only facilitates the broader adoption of oligonucleotide therapeutics but also supports environmentally responsible and sustainable pharmaceutical development.

## Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve readability and language of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

## CRediT authorship contribution statement

**Takashi Miyazaki:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Natsuyo Asano:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Satoshi Yamaki:** Writing – review & editing, Supervision, Conceptualization. **Takao Yamaguchi:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Satoshi Obika:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.466485.

## Data availability

Data will be made available on request.

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