**Evaluation of Hydrophilic Interaction Liquid Chromatography, Capillary Zone Electrophoresis and Drift Tube Ion-Mobility Quadrupole Time Of Flight mass spectrometry for the characterization of phosphodiester and phosphorothioate oligonucleotides**

Alice Demelenne\*, Marie-Jia Gou\*, Gwenaël Nys, Chloé Parulski, Jacques Crommen, Anne-Catherine Servais, Marianne Fillet

Laboratory for the Analysis of Medicines, Center for Interdisciplinary Research on Medicines (CIRM), University of Liege, Quartier hopital, Avenue Hippocrate 15, 4000 Liege, Belgium

\* Equally contributed to the work

Corresponding author: Professor Marianne Fillet, Laboratory for the Analysis of Medicines, Department of Pharmacy, CIRM, University of Liege, CHU, B36, Quartier Hopital, Avenue Hippocrate 15, 4000 Liege, Belgium.

**Email:** marianne.fillet@uliege.be

**Fax:** +32-4-366-4347

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**Highlights:**

* Optimization of two complementary separation techniques to analyze oligonucleotides
* Successful coupling of HILIC and CZE to drift tube ion mobility-QTOF
* Use of collision cross section measurements to determine the number of nucleotides

**Abstract**

Oligonucleotide-based medicines that can modulate gene expression have numerous potential applications in targeted therapies. Most of the commercialized therapeutic oligonucleotides are chemically modified to increase their *in vivo* lifetime. In this work, we studied poly-deoxy(thymidylic) acids (dT) and modified phosphorothioate oligonucleotides (PS). Several analytical techniques, including ion-pair reverse phase liquid chromatography, are described in the literature to assess their quality but most of them present significant drawbacks.

In the present study, dT and PS mixtures were analyzed by hydrophilic interaction liquid chromatography (HILIC) and capillary zone electrophoresis (CZE) coupled to ultraviolet detection. In HILIC, the selectivities of three types of stationary phases (dihydroxypropane, phosphorylcholine and amide) were compared. Optimal conditions were determined and consisted of an amide stationary phase with a mobile phase made up of water, acetonitrile and 15 mM ammonium acetate (pH 5.5). In those conditions, high resolving power and good repeatability were achieved. In CZE, the effect of the background electrolyte (BGE), its pH and concentration were evaluated. A BGE made up of 300 mM ammonium acetate adjusted to pH 6.0 was selected. Finally, the two techniques were compared in terms of selectivity, repeatability and peak efficiency.

In the second part of the study, HILIC and CZE were both coupled to a drift-tube ion-mobility quadrupole time-of-flight MS detector (DTIMS-QTOF) to assess the added value of this coupling for oligonucleotide characterization. Indeed, by using the measured collision cross section (CCS), the evaluation of the number of nucleotides was performed. Looking across the results, HILIC and CZE coupled to DTIMS-QTOF can be considered as promising tools for the quality control of oligonucleotides.

1. **Introduction**

For more than 30 years, oligonucleotides have been studied as an attractive new category of medicines to modulate gene expression [1]. The first therapeutic oligonucleotide commercialized was Fomiversen, an antisense oligonucleotide approved in 1998 by the Food and Drug Administration (FDA) and in 1999 by the European Medicines Agency (EMA). It was intended to treat cytomegalovirus retinitis [1]. Since then, six oligonucleotides have been approved either by the FDA and/or the EMA. Among them, Pegaptanib, an aptamer commercialized in 2004, was introduced to treat age-related macular degeneration. More recently, Patisiran, a small interfering RNA (siRNA), was commercialized to treat familial amyloidosis [1,2].

Since nucleic acids suffer from rapid degradation by nucleases, the most crucial step during the development of therapeutic oligonucleotides is the modification of their structure to increase their *in vivo* lifetime. This is commonly realized by replacing the oxygen of the phosphodiester (PO) group by a sulfur to create a phosphorothioate (PS) linkage. This modification also creates an asymmetrical center on the phosphorous atom. As a consequence, two diastereoisomers will appear for each PS linkage created. These diastereoisomers present interesting features. Indeed, the Rp (right-handed configuration) presents better binding activity towards the RNA polymerases and ribonuclease H and thus induces a stimulation of the synthesis of RNA and the duplication of DNA. The Sp (left-handed configuration) shows better exonuclease resistance and thus increases oligonucleotide stability [3-5].

With the emergence of oligonucleotides on the market, it is of increasing importance to develop analytical methods to study modified phosphorothioate oligonucleotides and their impurities. Common impurities are created by deletion or addition of one or more nucleotide(s) to the sequence of interest. Impurities are called “shortmers” when there is a failure in the addition of nucleotides during the elongation phase. On the other hand, the addition of nucleotide(s) by inefficient cleavage will generate longmers.

To analyze oligonucleotides, several methods have been described in the literature [6-9]. Due to the negatively charged phosphate backbone, anion-exchange chromatography has historically been used to study oligonucleotides. However, this technique requires high concentrations of non-volatile salts or high ionic strength buffers that impair the coupling to mass spectrometry (MS) detection. Ion-pair reverse phase liquid-chromatography (IP-RP-LC) has thus been described as an alternative more compatible with mass spectrometry [6]. This technique makes use of ion-pairing agents to increase the retention and improve the separation of oligonucleotides but MS sensitivity is reduced due to ion suppression phenomena [7].

Hydrophilic interaction liquid chromatography (HILIC) seems to be an interesting alternative technique to analyze oligonucleotides. Indeed, this approach is natively compatible with MS and does not require the use of ion-pairing agents. Moreover, it is well suited for the analysis of polar compounds since analytes distribute between an immobilized layer of water on the stationary phase and the organic solvent in the mobile phase. Only a few articles can be found using HILIC for the analysis of oligonucleotides in the literature [7,10-14]. HILIC was coupled to ultraviolet detection (UV) [10] and MS [9, 13-16]. With ESI-MS as detection method, Gong *et al.* used HILIC with a zwitterionic stationary phase to analyze polydeoxythymidylic acids (dT), PO oligonucleotides and their methylated forms [12]. More recently, Studzińska *et al.* and Lobue *et al.* employed HILIC for the analysis of phosphorothioate oligonucleotides [13,14]. Studzińska *et al.* demonstrated the influence of the stationary phase type by comparing three columns (carbamoyl, diol and acylamide stationary phases) and applied them to serum analysis [14]. Lobue *et al.* optimized an HILIC-MS method using a diol stationary phase and compared it with IP-RP-LC-MS for a wide variety of modified oligonucleotides including PS [13].

Besides chromatographic technniques, electrophoretic approaches have also been used in the early years of oligonucleotide drug development. Among them, capillary gel electrophoresis (CGE) is a technique with a high resolving power for the separation of parent oligonucleotides from shortened metabolites [8]. However, its coupling to MS is hampered by the presence of gels and surfactants. On the contrary, capillary zone electrophoresis (CZE) seems to be optimal to analyze oligonucleotides if a MS-compatible background electrolyte (BGE) is employed. Already in 1996, Gelfi *et al.* compared capillary electrophoresis with a highly concentrated polymer as background electrolyte, RP-HPLC using UV detection and polyacrylamide gel-slab electrophoresis [15]*.* They showed the advantages of capillary electrophoresis for the analysis of the impurities of modified and unmodified oligonucleotides, due to the flexibility of the polymer network used and the small amount of sample required.Li *et al.* used CZE in acidic media to separate PS and PO oligonucleotides and pointed out the repeatability of the method [16]. Willems *et al.* coupled CZE to MS for the analysis of PO oligonucleotides and highlighted the advantages of this technique for quality control using an innovative stacking procedure [9]. Feng *et al.* also employed CZE-MS for the analysis of oligonucleotides and their adducts. They developed a pressure assisted electrokinetic injection method for sample enrichment [17].

Mass spectrometers could be coupled with ion mobility spectrometry (IMS) to provide insights into the conformation of the ions. In the drift-time ion mobility spectrometer (DTIMS), ions are separated in a tube filled with an inert gas (like nitrogen or helium) at low pressure and kept under a low electric field. The separation takes place based on mobility differences, which depends on their shapes and charges. Larger ions will spend more time to drift through the gas since they will encounter collisions more frequently. On the contrary, compact ions will drift faster. A drift time corresponding to the time spent in the tube is calculated from the total time spent in the system and can subsequently be converted in a collision cross-section (CCS) value which represents the rotationally-averaged cross section of the ion [18]. These CCS values have already been evaluated by Hoaglund *et al.* [19] for oligothymine samples. Arcella *et al.* [20] also studied the behaviour of nucleic acids in the gas phase. To the best of our knowledge, HILIC or CZE coupled to IMS for the analysis of oligonucleotides has not been described yet.

In this work, MS-compatible HILIC and CZE methods for the analysis of unmodified (dT and PO) and modified (PS) oligonucleotides coupled to UV detection were first developed. HILIC and CZE methods were compared in terms of selectivity, resolution, repeatability and separation, in particularly for the separation of closely related compounds. Afterwards, these techniques were coupled to a drift-tube ion mobility quadrupole time-of-flight (DTIMS-QTOF) mass spectrometer. CCS values of phosphodiester and phosphorothioate oligonucleotides were calculated and used to retrieve the number of nucleotides in PS oligonucleotide sequences.

1. **Material and methods** 
   1. Chemicals

Ammonium hydroxide 25% of EMSURE® ISO, Reag. Ph Eur grade, acetic acid (glacial) 100% of EMPARTA® ACS grade and hydrochloric acid (HCl) (37% wt.) of EMSURE® ACS, ISO, Reag. Ph Eur gade were purchased from Merck (Damstadt, Germany). Ultra gradient HPLC grade acetonitrile (ACN) and methanol (MeOH) were obtained from JT Baker (Deventer, The Netherlands). Sodium hydroxide 97.0-100.5% pellets (NaOH) of Ph. Eur., NF, BP, JP grade was obtained from VWR chemicals (Leuven, Belgium). ULC/MS-CC/SFC grade acetonitrile and water were acquired from Biosolve (Valkenswaard, the Netherlands). ESI-L Low Concentration Tuning Mix and biopolymer kit HP-0321 solutions were acquired from Agilent (Waldbronn, Germany). Purine and hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine were acquired from Sigma (St. Louis, MO, USA). Ultrapure water was provided by a Milli-Q equipment (Millipore, Bedford, MA, USA) and Chromafil syringe filters (0.22 µm) were purchased from Macherey-Nagel (Duren, Germany). All lyophilized oligonucleotide samples were purchased from Integrated DNA Technologies (Coralville, IA, United States).

* 1. Oligonucleotides

Firstly, a mixture comprising of six poly-deoxy(thymidylic) acids (dT) of different lengths was used. It was composed of oligonucleotides of 5, 10, 15, 20, 30 and 50 units (dT5, dT10, dT15, dT20, dT30, dT50, in Table 1, dT mix).

Secondly, a mixture of 20 phosphorothioate oligonucleotides varying from 5 to 24 nucleotides and all linked through PS linkage was tested (PS 5 to PS 24, in Table 1, PS mix). The sequence for this mix was inspired from ref. [13], as it was shown that it was challenging to separate the full-length product of long nucleotides from their shortmer impurities.

* 1. Sample preparation

Lyophilized single oligonucleotides were individually resuspended in ultrapure water at a concentration of 1 mM. For HILIC-UV experiments, all samples were diluted to prepare a 10 µM solution made up of a mixture of mobile phases A and B (5/95; v/v), which consisted in a final mixture of water/ACN/ammonium acetate 150 mM (pH 5.5), 3.5/86.5/10 (v/v/v). For HILIC-DTIMS-QTOF, the dT mix was diluted to a concentration of 50 µM in the same mixture. The PS samples were injected individually and were diluted to a concentration of 50 µM as well. For CZE-UV experiments, ultrapure water was used to dilute the samples to 50 µM. For CZE-DTIMS-QTOF experiments, the dT mix at 50 µM was used and the PS samples were diluted in ultrapure water to 100 µM.

* 1. Equipments

UV separations were performed on an Agilent 1200 HPLC system and an Agilent Infinity II 1290 UHPLC for the 1.7 µm column (Agilent Technologies, Waldbronn, Germany). Detection was performed with a diode-array detector. CZE-UV separations were carried out on a G7100 CE system (Agilent Technologies, Waldbronn, Germany) coupled to a diode-array detector. For MS detection and ion mobility measurements, the UHPLC and the CE were coupled to a DTIMS-QTOF mass spectrometer 6560 (Agilent Technologies, Waldbronn, Germany). A Dual Agilent Jet Stream ESI (Agilent Technologies, Waldbronn, Germany) was used as electrospray ionization source. To connect CE to MS, a coaxial sheath liquid ESI-MS interface (Agilent Technologies, Waldbronn, Germany) was employed. Additionally, the reference masses and the sheath liquid were infused using an Infinity II isocratic pump (Agilent Technologies).

* 1. Operational conditions
     1. *HILIC-UV*

Four columns were selected: Luna HILIC® column (dihydroxypropane, 3 µm particle size) (Phenomenex, Torrance, CA, USA), ZIC-cHILIC® (phosphorylcholine, 3 µm particle size) (Merck, Darmstadt, Germany) and BEH amide® (amide, 1.7 and 3.5 µm particle size) (Waters, Milford, MA, USA) (cf. Supplemental Table 1). The column compartment was thermostated at 40 °C. The wavelength was set at 260 nm. The flow rate and injection volume were respectively set at 0.3 mL/min and 10 µL. Agilent OpenLab CDS C.01.08 (210) software was used for system control and data acquisition.

Mobile phase A consisted of water/ACN/150 mM ammonium acetate (pH 5.5), 70/20/10 (v/v), while mobile phase B was composed of ACN/150 mM ammonium acetate (pH 5.5), 90/10 (v/v). The 150 mM ammonium acetate solution was prepared by dissolving ammonium acetate in water at a concentration of 150 mM and then was brought to pH 5.5 with acetic acid.

A gradient elution was used for all separations and was optimized for each column. For Luna® HILIC column (dihydroxypropane), the final gradient started at 85% B and was performed as follows: 0-15 min, from 85 to 70% B; 15-20 min, from 70 to 65% B; 20-30 min, from 65 to 20% B. Concerning ZIC-cHILIC® column (phosphorylcholine), the optimized gradient started at 70% B and was carried out as follows: 0-10 min, from 70 to 50% B; 10-30 min, from 50 to 30% B. It remained at 30% B for 10 minutes. Regarding BEH amide® columns (amide), the final gradient started at 70% B and was performed as follows: 0-15 min, from 70 to 50% B; 15-40 min, from 50 to 30% B. A 20 min column equilibration time was observed before each injection for all columns.

* + 1. *CZE-UV*

An uncoated fused-silica capillary of 48.5 cm long (50 µm ID, 40 cm effective length) was used to perform all separations (Polymicro Technologies, Phoenix, AZ, USA). Hydrodynamic injection was performed for all samples by applying a pressure of 1.45 psi for 2 s. The separation voltage was set at + 20 kV and the UV-detection wavelength was set at 260 nm. Instrument control and data acquisition were achieved by using the Agilent OpenLab CDS C.01.07 (27) software.

A new capillary was conditioned first by flushing water, followed by 1 M NaOH, then 0.1 M NaOH and with water again, for 5 min each. At the beginning of each day, the capillary was rinsed with 0.1 M NaOH and water, both for 10 min, and then with BGE for 15 min. In addition, before each run, 0.1 M NaOH, water and BGE were successively flushed for 3, 3 and 5 min, respectively. The BGE consisted of ammonium acetate at different concentrations (50, 100, 150, 200, 300 and 400 mM) and adjusted at various pH (6.0, 8.0 and 10.0). For each condition, a solution of ammonia and a solution of acetic acid at the desired concentrations were prepared and various volumes of each solution were mixed to achieve the required pH.

* + 1. *CCS measurements and data analysis*

The mobility cell consisted of a 78 cm drift tube filled with high purity nitrogen. The drift field was set at 18.6 V/cm. Ions were gated for 20 ms with a voltage of 150 V and released in the tube in 150 µs. They were allowed to drift for 60 ms before the next pulse was released. The pressure in the tube was between 3.95 and 4 Torr. CCS measurements were carried out in single-field after daily calibration with well-known calibrants (Agilent Tuning mix). References masses (purine and hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine) were constantly infused at concentrations of 10 and 2 µM (in ACN/water 95:5, v/v), respectively. These masses were used to correct flight times using the IM-MS Reprocessor utility (Agilent technlogies). Oligonucleotides were analyzed in the negative ESI mode in the m/z range of 100-3000 m/z.

CCS values have already been reported to study gas phase structure of precursor ions [19–21]. In our article, CCS values were used for characterization and more particularly for the determination of the number of nucleotides in a given oligonucleotide. To achieve this goal, two calculation methods were developed. Firstly, the number of nucleotides was estimated by establishing a linear relationship between the measured CCS values of the dT oligonucleotides (used as calibrants) and their number of nucleotides. The number of nucleotides in the unknown oligonucleotide was subsequently estimated by reporting its measured CCS value in the established calibration curve (method 1).

Since the previously described calibration curve was established using compounds with an increasing number of dT whereas the controlled oligonucleotide may contain thymine (T) but also adenine (A), cytosine (C) and guanine (G) as well as PS links instead of PO links, the mass differences between the bases and the linkages (PO and PS) should be considered. In method 2, the theoretical CCS value of the controlled oligonucleotide was calculated (CCStheoretical) taking into account the proportion of each nucleobase and the PS links. By dividing the CCS value experimentally obtained (CCSexperimental) by the CCStheoretical,a correction factor was obtained. The number of nucleotides of the controlled oligonucleotides was calculated using a linear regression curve drawn by plotting the number of nucleotides against the measured dT CCS values and by applying the previously calculated correction factor.

* + - 1. *HILIC DTIMS-QTOF*

he separation was carried out on a 1.7 µm BEH amide column (Waters, Milford, MA, USA). The same conditions as for the UV optimization (2.5.1) were used for the MS separation. The reference masses were infused at 0.1 mL/min. The Dual Agilent Jet Stream ESI source was set at a drying gas temperature of 200 °C and at a drying gas flow of 10 L/min. The sheath gas temperature was set at 200 °C and the corresponding flow at 12 L/min. The nebulizer was fixed at 35 psi. The capillary voltage was set at -2500 V and the nozzle voltage at -1000 V.

* + - 1. *CZE DTIMS-QTOF*

As described in CZE optimization (2.5.2.), an uncoated fused-silica capillary was used but the capillary length had to be adapted for MS coupling. Thus, a commercialized pre-cut capillary of 50 µm ID and 125 cm long was used (Agilent Technologies, Waldbronn, Germany). A BGE consisting of 100 mM ammonium acetate (pH 8.0) was selected for CZE-MS experiments. The concentration was decreased compared to optimal conditions in CZE-UV due to the resulting high current that might be detrimental for MS detection. In addition, an electrolyte solution of pH 8.0 was chosen instead of pH 6.0 to increase the electroosmotic flow and thus speed up the separation. The new capillary was conditioned by flushing with water first, followed by 1 M ammonium hydroxide and then water again, for 5 min each. At the beginning of each day, the capillary was rinsed with 1 M ammonium hydroxide and water, both for 10 min and then BGE for 15 min. In addition, before each run, BGE was flushed for 5 min.

Ionization was performed in the negative mode. The coaxial sheath liquid ESI-MS interface provides a conductive make-up liquid and a nebulizing gas to the system to close the electrical circuit at the capillary outlet as well as to assist in the desolvation process. The injection method was inspired from Willems *et al.* [9] since a stacking method was found to be mandatory to achieve sufficient sensitivity and satisfactory peak efficiency. Firstly, samples were hydrodynamically injected for 30 seconds at 1.45 psi. Then, a negative separation voltage of -20 kV was applied for 0.95 min, followed by a decrease to -5 kV for 0.45 min. During the injection procedure, the nebulizer gas flow, the sheath liquid flow and the capillary voltage were turned off. An internal pressure of 0.87 psi was applied at the inlet during injection and separation. After the injection program was performed, the nebulizer gas flow, the sheath liquid flow and the capillary voltage were turned on to respectively 4 psi, 3 µl/min and 4000 V. The separation voltage was set at +30 kV. The sheath liquid consisted of 80% MeOH and 20% 5 mM ammonium acetate (pH 8.0) and was enriched with the reference masses for permanent infusion during the analysis. The gas temperature was set at 300 °C.

1. **Results and discussion**

In this study, hydrophilic interaction liquid chromatography and capillary zone electrophoresis were used as complementary techniques for the analysis of oligonucleotides. Firstly, both techniques were optimized using UV detection. Secondly, they were coupled to DTIMS-QTOF. This detector was evaluated as a tool for oligonucleotide quality control. Indeed, the high-resolution detector (QTOF) provided the exact mass of the oligonucleotides while the DTIMS detector gave information about the number of nucleotides in the oligonucleotides through CCS measurements.

* 1. Optimization of HILIC separation

In this study, acetonitrile was chosen as an aprotic organic solvent and water as stronger eluent. As previously reported, the addition of salts such as ammonium acetate was found to improve the separation and peak shape [22]. Lobue *et al.* compared peak resolution at 2.5 mM, 5 mM, 10 mM and 15 mM of ammonium acetate and showed that the best chromatographic performance was obtained with the highest concentration [13]. Preliminary experiments also showed that 15 mM of ammonium acetate (pH 5.5) was the most appropriate conditions and it was added to mobile phase components A and B for all separations.

The dT mix was first injected on three stationary phases (dihydroxypropane, phosphorylcholine and amide stationary phases) and mobile phase gradients were optimized for each one. These columns exhibited various interactions with the analytes depending on the nature of the stationary phase. The more hydrophilic compounds will be more strongly retained due to partition between the immobilized aqueous layer on the polar stationary phase and the acetonitrile-rich mobile phase. Electrostatic interactions and/or hydrogen bonding to the stationary phase also play an important role in the separation [22]. As expected, the shortest oligonucleotides eluted with the lowest percentage of water (cf. Figure 1). When the percentage of water in the mobile phase was increased, longer oligonucleotides and thus more polar compounds eluted. As can be seen in Figure 1A, the five dTs were successfully separated using phosphorylcholine and amide stationary phases. However, the best resolution and peak shape was obtained with the amide stationary phase. To further improve peak efficiency, a column with 1.7 µm particle size was used instead of the 3.5 µm column. As expected, peak efficiency was significantly improved (cf. Figure 1). It is interesting to note that all impurities between dT5 and dT20 (shortmers) were easily detected as they were well separated from each other even though they only differed by one nucleotide.

The dT mix was injected three times on the four stationary phases in HILIC with an equilibration time of 20 min between each injection. Very good values for repeatability of retention times and peak areas were obtained as shown in Supplemental Table 2. RSD values for retention times and peak areas were below 0.4% for the amide column with 1.7 µm particle size.

The PS mix was then injected under the same conditions as the dT mix (cf. Figure 1B). On the phosphorylcholine and the amide stationary phases, 17 out of the 20 oligonucleotides with phosphorothioate linkages could be resolved. It could be noted that the peak shape was better with the amide stationary phase. Figure 1B demonstrates thus that an oligonucleotide of 20 units could be easily separated from its 19 units shortmer impurity or its 21 unit longmer impurity using the developed method with an amide or a phosphorylcholine stationary phase.

As already discussed by Lobue *et al.* [13] the high molecular weight oligonucleotides are the most difficult to separate. In this work, we succeeded to resolve PS 21 from PS 22, 23 and 24 using the amide or phosphorylcholine stationary phase. Moreover, with the 1.7 µm amide stationary phase the separation of these compounds was significantly improved compared to that obtained by Lobue *et al*.

The peak width at half-height was calculated for the oligonucleotides of 5 units linked through PO (dT 5) and PS (PS 5) linkages. In Figure 1, it can be seen that the peak width was broader for the PS compound (0.126 min) compared to the dT one (0.078 min); this is probably due to the fact that PS compounds are a mix of diastereoisomers as discussed in the introduction.

In conclusion, the amide and the zwitterionic phosphorylcholine stationary phases gave interesting results while the dihydroxypropane stationary phase did not show enough resolving power to separate the compounds of interest. The main difference between the columns in terms of selectivity is the possibility of ion exchange on the zwitterionic stationary phase. However, this is obviously not the main factor [23]. Indeed, as classified by Kawachi *et al.,* due to their composition, amide and zwitterionic stationary phases can be defined as hydrogen-bond acceptors, while the dihydroxypropane stationary phase is considered as hydrogen-bond donor*.* It was shown that hydrogen-bond acceptors offer higher retention and selectivity, due to hydrophilic interactions, than hydrogen-bond donors, which is in good accordance with our observations [24]. The amide phosphorylcholine stationary phase with the 1.7 µm particle size was selected since it gave the best results in terms of peak shape and analysis time.

* 1. Optimization of CZE separation

In CZE, the composition of the BGE has a huge impact on the separation. In oligonucleotides, each nucleotide of the sequence contains a phosphate group that is negatively charged at any pH (pKa=1) [25].

In this study, the influence of the electrolyte concentration was evaluated by testing the BGE at several concentrations: 50, 100, 150, 200, 300 and 400 mM ammonium acetate (adjusted to pH 8.0). Those concentrations corresponded to ionic strength of 48, 96, 144, 192, 288 and 384 mM, respectively. At pH 8.0, oligonucleotides migrate as anions towards the cathode due to the strong electroosmotic flow (EOF). As can be seen in Supplemental Figure S1, higher concentrations of ammonium acetate led to higher ionic strength of the solution and thus diminished the EOF. Consequently, the migration of analytes was slowed down, which induced an increase in resolution. The results in Figure S1A show that a complete separation was achieved for dT mix. For the PS mix (cf. Figure S1B), the increase in electrolyte concentration could not lead to a better separation of PS 11 to 24.

The pH of the electrolyte solution is also a critical parameter in CZE analysis as it determines the EOF. We investigated the effect of the pH using a BGE composed of 300 mM ammonium acetate adjusted to pH 6.0, 8.0 and 10.0. The ionic strength of those electrolytes were calculated as well and corresponded respectively to ionic strength of 300, 288 and 53 mM. No pH values below 6.0 were selected as a minimum EOF is needed to detect the anions. Figure 2 shows the results obtained for the dT mix and PS mix under these conditions.

For the dT mix (cf. Figure 2A), the resolution decreased due to the higher EOF caused by the high pH value (10.0). Baseline separation and satisfactory resolution was achieved for the dT mix using pH 6.0.

For the PS mix, as can be seen in Figure 2B, only few oligonucleotides were separated. The peak efficiency was higher at high pH values while at the lower pH the amount of resolved oligonucleotides increased (6 vs 4 at pH 10.0). This method could be applied to detect shortmer impurities up to PS 10 (vs PS 21 in HILIC).

The final BGE composition selected in CZE was 300 mM ammonium acetate adjusted to pH 6.0. The dT mix was injected in triplicate and the RSD values for migration times for all compounds were below 1.2% (cf. Supplemental Table 3). The RSD values for peak areas were calculated after correction of the peak areas with dT 10 peak area (used as internal standard). All RSD values were below 6.7%. The number of plates was calculated for the oligonucleotides of 5 units linked through PO (dT 5) and PS (PS 5) linkages. Higher values were obtained for the 5 units dT compound compared to the 5 units PS, with 207296 and 25654 plates, respectively (cf. Figure 2). This could be explained by the coexistence of various configurations for the PS compound due to the formation of diastereoisomers for each PS linkage.

* 1. Comparison of HILIC and CZE

The HILIC method was a little more repeatable than the CZE method. However, the total analysis time in HILIC (20 minutes for the equilibration of the column + 35 minutes for the analysis) was longer compared to that in CZE (11 minutes for the preconditioning of the capillary + 20 minutes for the analysis). For the dT mix, both techniques were able to resolve the six compounds. Moreover, it should be noted that the shortmer impurities of the compounds present in the sample could also be detected and separated using both techniques. However, for the PS mix, a significantly better separation was obtained using HILIC.

* 1. Coupling to DTIMS-QTOF

3.4.1. Establishment of a CCS calibration curve

The CCS values were used to provide additional information about the oligonucleotides under study in an attempt to further confirm their identity. Indeed, while the exact mass can be obtained by a high-resolution mass spectrometer such as a QTOF, CCS values measured by a DTIMS can be employed to retrieve the number of nucleotides in the sequence.

Firstly, the CCS values of dT 5, 10, 15, 20 and 30 were measured. Extracted features corresponding to the monoisotopic mass of the injected oligonucleotides were considered. Figure 3 represents the CCS values corresponding to the charge state of each dT. Interestingly, for each compound, when the charge state increased, the CCS value increased as well. Hoaglund *et al.* described this phenomenom when they analyzed a dT 10 [19]. They showed that lower charge states favor globular conformations for the ions while higher charge states favor extended conformations. Consequently, different CCS values were obtained for the different charge states of the same compound. As shown in Figure 3A and 3B, species with charge states between 2 and 7 were produced in both HILIC and CZE.

Different CCS values were calculated for the same charge state of the same oligonucleotide, which could be explained by differences in ionization sites leading to various conformations [19]. Here, we considered the CCS values obtained from the most abundant produced ions for each charge state. For example, for [dT10]3-, two CCS values of 580 and 680 were obtained. However, the [dT10]3- corresponding to the CCS value of 580 was significantly more abundant and was thus considered to establish the calibration curve.

In Figure 3C, the CCS values of charge states 3, 4 and 5 were plotted against the dT number in HILIC and CZE. The calibration curves show that the CCS values obtained using both techniques are very similar. The coefficients of determination (r2) are higher than 0.99 for Z=3, higher than 0.97 for Z=4 and higher than 0.96 for Z=5.

These calibration curves were used to estimate the number of nucleotides in PS oligonucleotides. Two methods of calculation were applied. The first one aimed to estimate the number of nucleotides of an unknown oligonucleotide; the second method aimed to confirm the number of nucleotides of a known oligonucleotide in the case of a quality control process. These two methods of calculation are described in paragraph 2.5.3.

3.4.2. Assessment of the number of nucleotides using CCS values

To show the feasibility of the methods described above, oligonucleotides with 5 to 24 PS linkages were injected and their CCS values were measured in order to estimate (in the case of an unknown oligonucleotide, method 1) or to confirm (in the case of a known oligonucleotide, method 2) the number of nucleotides of the tested compounds (cf. 2.5.3.).

Figure 4A shows the values obtained with HILIC-DTIMS-QTOF. As can be seen in this figure, when method 1 was used, the number of nucleotides was estimated with rather good accuracy (between 98 and 106% of the expected value, except for PS 5 which was 90% accurate (cf. Supplemental Table 4)). This resulted in a correct determination of 10 oligonucleotides. When CCStheoretical values were calculated (method 2), the accuracy of the number of nucleotides improved (between 98 and 102% of the expected value, except for PS 5 which was 92% accurate (cf. Supplemental Table 4)). This resulted in the correct determination of the nucleotide number for all the tested compounds. It is worth noting that the RSDs for the CCS values were below 0.4%.

The same experiments were carried out with CZE-DTIMS-QTOF coupling (cf. Figure 4B). As can be seen, the accuracy was slightly lower compared to HILIC-DTIMS-QTOF (with a range from 86 to 107% (cf. Supplemental Table 5)). Nevertheless, the nucleotide numbers of eight compounds were correctly determined. By applying the correction factor (method 2), the accuracy values ranged from 87 to 101% and the number of nucleotides in all oligonucleotides up to PS 19 was accurately confirmed (cf. Supplemental Table 5). The electrophoretic mobilities for the analytes were calculated and can be found in Supplemental Table 5 as well.

It should be noted that, for CZE-DTIMS-QTOF, the only ion produced for PS 20 and above during the ionization process was [PS]5- . At this charge state, less accurate results were obtained. It may be explained by the presence of two populations of oligonucleotides with the same charge state but with charges located at different positions in the sequence resulting in two different spatial configurations and thus CCS values. One of them could be similar to the configuration of the dT with a charge state of 5 while the other one seems to be more globular. This hypothesis was confirmed for the PS 20, which presented two ions with a charge state of 5 having CCS values of 944 and 1007. When the CCS value of 944 was used, the calculation provided a sequence of 17 nucleotides, while a number of 20 nucleotides was accurately calculated when the CCS value of 1007 was used. However, the ion with a charge state of 5 and a CCS value around 1000 was only observed for one of the three injections. Except for PS 20, the RSDs for CCS values were below 0.8%. It is likely that further optimization of the BGE and the shealth liquid composition could promote the lower charge states and improve the separation of oligonucleotides, which would be benefical to the accuracy of the approach.

Finally, these approaches are original and orthogonal concepts that could be applied to the quality control of oligonucleotides.

1. **Conclusion**

HILIC and CZE were evaluated as separation techniques for the analysis of two mixtures of oligonucleotides linked through PO and PS linkages. As a matter of fact, the developed methods could be used in quality control to detect the presence of short- and longmers in the production of a specific phosphorothioate oligonucleotide. HILIC-UV seems an appropriate technique for the analysis of PS ranging from 5 to 22 units. CZE-UV showed a more limited resolving power but a complete separation of PS ranging from 5 to 10 units could be performed under the optimal conditions.

DTIMS-QTOF detection also demonstrated its potential for quality control purposes. This detection mode presented the advantages of giving the exact mass of the oligonucleotides and also of providing information about the conformational space of the ions. As expected, the method applied without any *a priori* about the oligonucleotide sequence was less accurate than that applied for sequence confirmation. But both of them bring an added value to the data provided by the determination of the exact mass of the studied oligonucleotides. Both HILIC and CZE-DTIMS-QTOF showed very good results with an accurate confirmation of the number of nucleotides for 15 out of 20 PS oligonucleotides in CZE and for all 20 PS oligonucleotides in HILIC.

1. **Acknowledgments**

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1. References

[1] C.A. Stein, D. Castanotto, FDA-Approved Oligonucleotide Therapies in 2017, Mol. Ther. 25 (2017) 1069–1075. doi:10.1016/j.ymthe.2017.03.023.

[2] C.I.E. Smith, R. Zain, Therapeutic Oligonucleotides : State of the Art, Annu. Rev. Pharmacol. Toxicol. 59 (2019) 605–630. doi:11146/annurev-pharmtox-010818-021050.

[3] H.G. Bohr, I. Shim, C. Stein, H. Ørum, H.F. Hansen, T. Koch, Electronic Structures of LNA Phosphorothioate Oligonucleotides, Mol. Ther. - Nucleic Acids. 8 (2017) 428–441. doi:10.1016/j.omtn.2017.05.011.

[4] W.J. Stec, B. Karwowski, M. Boczkowska, P. Guga, M. Koziołkiewicz, M. Sochacki, M.W. Wieczorek, J. Błaszczyk, Deoxyribonucleoside 3’-O-(2-thio- and 2-oxo-’spiro’-4,4-pentamethylene- 1,3,2-oxathiaphospholane)s: Monomers for stereocontrolled synthesis of oligo(deoxyribonucleoside phosphorothioate)s and chimeric PS/PO oligonucleotides, J. Am. Chem. Soc. 120 (1998) 7156–7167. doi:10.1021/ja973801j.

[5] F. Eckstein, Phosphorothioate Analogues of Nucleotides, Acc. Chem. Res. 12 (1979) 204–210. doi:10.1021/ar50138a003.

[6] V.K. Sharma, J. Glick, P. Vouros, Reversed-phase ion-pair liquid chromatography electrospray ionization tandem mass spectrometry for separation, sequencing and mapping of sites of base modification of isomeric oligonucleotide adducts using monolithic column, J. Chromatogr. A. 1245 (2012) 65–74. doi:10.1016/j.chroma.2012.05.003.

[7] R.N. Easter, K.K. Kröning, J.A. Caruso, P.A. Limbach, Separation and identification of oligonucleotides by hydrophilic interaction liquid chromatography (HILIC) - Inductively coupled plasma mass spectrometry (ICPMS), Analyst. 135 (2010) 2560–2565. doi:10.1039/c0an00399a.

[8] B. Chen, M.G. Bartlett, Determination of therapeutic oligonucleotides using capillary gel electrophoresis, Biomed. Chromatogr. 26 (2012) 409–418. doi:10.1002/bmc.1696.

[9] A. V. Willems, D.L. Deforce, C.H. Van Peteghem, J.F. Van Bocxlaer, Development of a quality control method for the characterization of oligonucleotides by capillary zone electrophoresis-electrospray ionization-quadrupole time of flight-mass spectrometry, Electrophoresis. 26 (2005) 1412–1423. doi:10.1002/elps.200406201.

[10] P. Holdšvendová, J. Suchánková, M. Bunček, V. Bačkovská, P. Coufal, Hydroxymethyl methacrylate-based monolithic columns designed for separation of oligonucleotides in hydrophilic-interaction capillary liquid chromatography, J. Biochem. Biophys. Methods. 70 (2007) 23–29. doi:10.1016/j.jbbm.2006.11.003.

[11] L. Qin, F. Lynen, G. Xu, J. Wang, P. Sandra, H. Li, Q. Li, Comprehensive hydrophilic interaction and ion-pair reversed-phase liquid chromatography for analysis of di- to deca-oligonucleotides, J. Chromatogr. A. 1255 (2012) 237–243. doi:10.1016/j.chroma.2011.11.062.

[12] L. Gong, J.S.O. McCullagh, Analysis of oligonucleotides by hydrophilic interaction liquid chromatography coupled to negative ion electrospray ionization mass spectrometry, J. Chromatogr. A. 1218 (2011) 5480–5486. doi:10.1016/j.chroma.2011.06.044.

[13] P.A. Lobue, M. Jora, B. Addepalli, P.A. Limbach, Oligonucleotide analysis by hydrophilic interaction liquid chromatography-mass spectrometry in the absence of ion-pair reagents, J. Chromatogr. A. 1595 (2019) 39–48. doi:10.1016/j.chroma.2019.02.016.

[14] S. Studzińska, F. Łobodziński, B. Buszewski, Application of hydrophilic interaction liquid chromatography coupled with mass spectrometry in the analysis of phosphorothioate oligonucleotides in serum, J. Chromatogr. B. 1040 (2017) 282–288. doi:10.1016/j.jchromb.2016.11.001.

[15] C. Gelfi, M. Perego, S. Morelli, A. Nicolin, P.G. Righetti, Analysis of Antisense Oligonucleotides by Capillary Electrophoresis, Gel-Slab Electrophoresis, and HPLC: A Comparison, Antisense Nucleic Acid Drug Dev. 6 (1996) 47–53. doi:10.1089/oli.1.1996.6.47.

[16] Q. Li, R. Chen, Y. Sun, Y. Hu, Separation of phosphodiester oligodeoxynucleotides and phosphorothioate antisense oligodeoxynucleotides by capillary zone electrophoresis at low pH, Chin J Chrimatogr. 25 (2007) 101–106. doi:10.1016/S1872-2059(07)60004-2.

[17] Y.L. Feng, H. Lian, J. Zhu, Application of pressure assisted electrokinetic injection technique in the measurements of DNA oligonucleotides and their adducts using capillary electrophoresis-mass spectrometry, J. Chromatogr. A. 1148 (2007) 244–249. doi:10.1016/j.chroma.2007.02.071.

[18] V. D’Atri, T. Causon, O. Hernandez-Alba, A. Mutabazi, J.L. Veuthey, S. Cianferani, D. Guillarme, Adding a new separation dimension to MS and LC–MS: What is the utility of ion mobility spectrometry?, J. Sep. Sci. 41 (2018) 20–67. doi:10.1002/jssc.201700919.

[19] C.S. Hoaglund, Y. Liu, A.D. Ellington, M. Pagel, D.E. Clemmer, Gas-phase DNA: Oligothymidine ion conformers, J. Am. Chem. Soc. 119 (1997) 9051–9052. doi:10.1021/ja970652w.

[20] A. Arcella, J. Dreyer, E. Ippoliti, I. Ivani, G. Portella, V. Gabelica, P. Carloni, M. Orozco, Structure and dynamics of oligonucleotides in the gas phase, Angew. Chemie Int. Ed. 54 (2015) 467–471. doi:10.1002/anie.201406910.

[21] S. Ickert, J. Hofmann, J. Riedel, S. Beck, K. Pagel, M.W. Linscheid, Charge-induced geometrical reorganization of DNA oligonucleotides studied by tandem mass spectrometry and ion mobility, Eur. J. Mass Spectrom. 24 (2018) 225–230. doi:10.1177/1469066717746896.

[22] B. Buszewski, S. Noga, Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique, Anal. Bioanal. Chem. 402 (2012) 231–247. doi:10.1007/s00216-011-5308-5.

[23] N.P. Dinh, T. Jonsson, K. Irgum, Probing the interaction mode in hydrophilic interaction chromatography, J. Chromatogr. A. 1218 (2011) 5880–5891. doi:10.1016/j.chroma.2011.06.037.

[24] Y. Kawachi, T. Ikegami, H. Takubo, Y. Ikegami, M. Miyamoto, N. Tanaka, Chromatographic characterization of hydrophilic interaction liquid chromatography stationary phases: Hydrophilicity, charge effects, structural selectivity, and separation efficiency, J. Chromatogr. A. 1218 (2011) 5903–5919. doi:10.1016/j.chroma.2011.06.048.

[25] P. Thaplyal, P.C. Bevilacqua, Experimental approaches for measuring pKa’s in RNA and DNA, Methods Enzymol. 549 (2014) 189–219. doi:10.1016/B978-0-12-801122-5.00009-X.

**Legend to figures**

**Figure 1**

Chromatograms of dT mix (A) and PS mix (B) by HILIC-UV obtained on Luna® HILIC (dihydroxypropane, 3 µm particle size), ZIC-cHILIC® (phosphorylcholine, 3 µm particle size) and BEH amide® columns (amide, 1.7 and 3.5 µm particle size) (cf. Supplemental Table 1). Figure 1A represents dT 5, 10, 15, 20, 30 and 50 compounds. Figure 1B represents PS compounds from PS 5 to PS 24. Additional information on those compounds can be found in Table 1. The peak width at half-height (W) of dT 5 and PS 5 were measured. Additional information about the mobile phase composition and the gradient for each column can be found in paragraph 2.5.1.

**Figure 2**

Electropherograms showing the effect of the electrolyte solution pH on the separation of dT mix (A) and PS mix (B). Figure 2A represents compounds dT 5, 10, 15, 20, 30 and 50. Figure 2B represents PS compounds from PS 5 to PS 10. Additional information on those compounds can be found in Table 1. Comparison of 300 mM ammonium acetate electrolyte solution adjusted to pH 6.0, 8.0 and 10.0. The number of plates for dT 5 and PS 5 was calculated. See paragraph 2.5.2. for additional information about CZE conditions.

**Figure 3**

Collision cross-section (CCS) values for the dT 5, 10, 15, 20 and 30 according to their charge, acquired in HILIC-DTIMS-QTOF (A) and CZE-DTIMS-QTOF (B). 3 replicates were performed and RSD values were plotted on the graph. Figure (C) represents CCS values for charge states 3, 4 and 5 plotted against the number of deoxythymidylic acids. The coefficients of determination were 0.9984 and 0.9992 for Z=3, 0.9789 and 0.9708 for Z=4 and 0.9603 and 0.9688 for Z=5, in HILIC-DTIMS-QTOF and CZE-DTIMS-QTOF, respectively. See paragraph 2.5.3 for additional information.

**Figure 4**

Accuracy on the number of nucleotides obtained by HILIC-DTIMS-QTOF and CZE-DTIMS-QTOF with the estimation (method 1) or the confirmation (method 2) method. The accuracy is represented as the percentage of the measured number of nucleotides versus the number of nucleotides. For the method 1, the number of nucleotides was calculated by reporting the measured CCS values of the ions of the charge indicated in the table in an established calibration curve. The calibration curve was drawn in the calibration range dT 5 to 15, dT 10 to 20 or dT 15 to 30. For the method 2, a correction factor was applied. See paragraph 2.5.3. for further information.