BIOANALYTICAL LC-MS OF OLIGONUCLEOTIDES

by

BABAK BASIRI

(Under the Direction of Michael G. Bartlett)

ABSTRACT

The number of small oligonucleotides being used to improve human health is increasing rapidly. There are currently four FDA-approved therapeutic oligonucleotides and small endogenous RNA molecules have come to the forefront of both basic and translational research. For example, there have been enormous efforts made to investigate correlations between disease state and changes in the expression of various microRNAs. Improved methods to interrogate oligonucleotides will enhance our ability to use these powerful molecules in therapeutics and diagnostics. To date, such advances have been impeded by significant deficiencies in our ability to track and accurately quantify these macromolecules.

During the past decades, ion-pair reversed phase liquid chromatography combined with negative ion ESI-MS has become the method of choice for direct oligonucleotide analysis. LC-MS of various oligonucleotides has been accomplished using a mobile phase consisting of triethylamine (TEA) and hexafluoroisopropanol (HFIP). However, combinations of alternative alkylamines with HFIP were never carefully examined until studies from our laboratory showed that a mobile phase consisting of diisopropylethylamine (DIEA) and HFIP significantly increased the MS signal intensity for phosphorothioate DNA oligonucleotides when compared to the standard TEA/HFIP mobile phase. Early studies revealed that the choice of optimal ion-

pairing agents should be made on a case-by-case basis as it is strongly influenced by the physicochemical properties of the oligonucleotides and the mobile phase. This represented a major hurdle for researchers as they needed to rigorously investigate and optimize the mobile-phase composition prior to each study. To solve this issue, we developed a computational algorithm that can automate the process of ion-pair selection by introducing ion-pair and oligonucleotide parameters into a statistical regression model which will predict the MS signal intensity based on these inputs. Moreover, we demonstrated that HFIP can be substituted with other fluorinated alcohols and they would successfully act as the counter ions for the alkylamine ion pairing agents. Hexafluoromethylisopropanol (HFMIP), particularly showed superior performance compared to HFIP when utilized with more hydrophobic ion-pairs. All such improvements in the sensitivity of LC-MS methods, finally allowed for the detection and quantitation of miR-451 in plasma.

INDEX WORDS: Bioanalyis, Oligonucleotides, Quantitative mass spectrometry, LC-MS, microRNAs, miR-451, Ion pairing reagents, Alkylamines, Partial least squares (PLS), Bootstrap forest, Fluorinated alcohols, Ion mobility mass spectrometry, Charge state distribution, 1,1,1,3,3,3-Hexafluoro-2-methyl-2-propanol (HFMIP), Secondary structure, Nonafluoro-tert-butyl alcohol (NFTB), biotinylated capture, Streptavidin magnetic beads

BIOANALYTICAL LC-MS OF OLIGONUCLEOTIDES

by

BABAK BASIRI

B.S., Shiraz University, Iran, 2007

M.S., University of Delaware, 2012

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2017

BIOANALYTICAL LC-MS OF OLIGONUCLEOTIDES

by

BABAK BASIRI

Major Professor:	Michael G. Bartlett
Committee:	Shelley B. Hooks Mandi M. Murph Ron Orlando Lance Wells

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
August 2017

DEDICATION

To Mom,

For always being there for me.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Michael Bartlett for being a wonderful advisor. He was always there when I needed help, and his help was not just limited to educational support. He was also willing to offer his assistance with personal and career-related issues. I am very grateful that he accepted me into his lab. Hopefully, I have proven worthy of that trust. My committee members have always been a source of valuable insight into my project. Without their vision, my dissertation would have never reached its current depth.

Dr. Cary McGinnis walked me through all the steps of designing and performing a bioanalytical procedure with extraordinary patience and diligence. Dr. Pei Li taught me how to work with a mass spectrometer and Dr. Darren Gullick volunteered his help at all circumstances. I would also like to thank all other Bartlett lab members with whom I have had the opportunity to work throughout these years. In addition, I should thank all the faculty and staff at the University of Georgia College of Pharmacy.

None of the experiments described here could have been concluded without the continuous financial support of the UGA Graduate School, National Institutes of Health (NIH) and ProQR therapeutics and I shall remain grateful to them.

There are individuals who have been involved in my every success and I can never thank them enough. These include my parents, friends, and particularly my teachers. Specifically, I want to thank a very special teacher, Mr. Farshid Pahlavan, who uncovered my aptitude for life sciences for the first time. I am extremely thankful for having such influential people in my life and I do wish them success in all their endeavors.

Last but not least, I am eternally grateful to my lovely wife, Shirin. Because of her love and support, I had a very enjoyable time working toward completing my degree. Attending graduate school would have never been that easy if it wasn't for her.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The therapeutic potential of oligonucleotides has been explored for almost two decades. Fomivirsen (Vitravene) was registered in 1998 as the first oligonucleotide drug. It was used for treatment of cytomegalovirus-induced retinitis in immunocompromised patients with AIDS. In 2004 Pegatinib (Macugen), was introduced for the treatment of neovascular age-related macular degeneration (AMD) (Kole, Krainer et al. 2012), and finally in 2013 Mipomersen (Kynamro) became the first systemically-administered oligonucleotide drug for treatment of homozygous familial hypercholesterolemia (Gelsinger, Steinhagen-Thiessen et al. 2012, McGowan, Tardif et al. 2012, Parhofer 2012).

The most recent addition to the FDA-approved oligonucleotide drugs is Nusinersen marketed under the name Spinraza. It is the first drug approved for the treatment of spinal muscular atrophy, a rare neuromuscular disorder. Spinraza is administered directly to the central nervous system via intrathecal injection (Haché, Swoboda et al. 2016). Moreover, the diagnostic capability of oligonucleotides as biomarkers is just being understood as a result of the discovery of microRNAs. Today, miRNAs are the center of attention as biomarkers for various pathological conditions from cancer to heart disease (Gupta, Bang et al. 2010, Kosaka, Iguchi et al. 2010, Widera, Gupta et al. 2011, Lorenzen and Thum 2012).

Most therapeutic oligonucleotides exert their gene regulatory actions through one of the two major pathways. As shown in figure 1.1, antisense oligonucleotides hybridize to a complementary sequence on their target mRNA and guide its cleavage by RNase H (Wittrup and

Lieberman 2015), while siRNAs silence gene expression through their interaction with the RNA-induced silencing complex or RISC (Elbashir, Harborth et al. 2001, Elbashir, Lendeckel et al. 2001, Flynt and Lai 2008). The endogenous microRNAs also utilize a RISC-dependent pathway for gene regulation (Du and Zamore 2005). The similarities and differences of siRNA and miRNA mechanisms of action are depicted in figure 1.2.

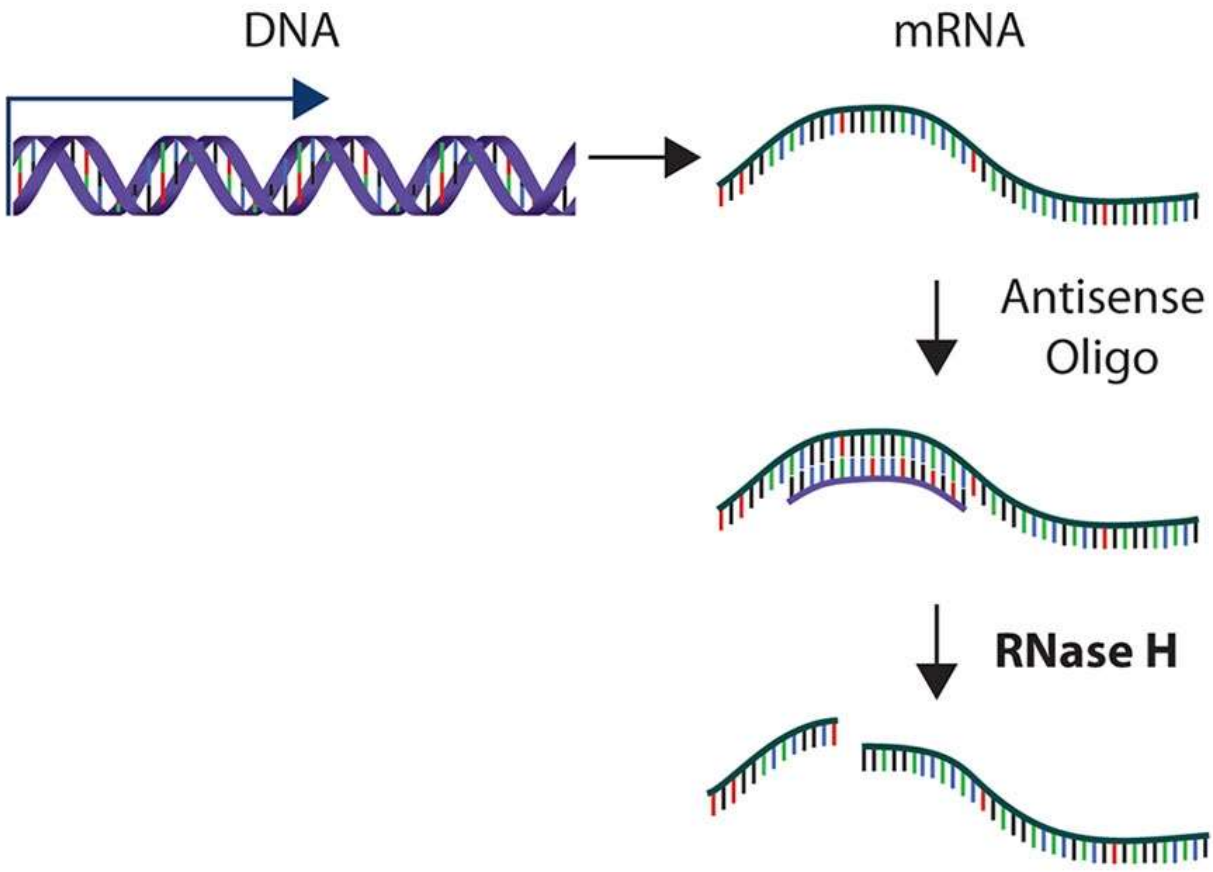


Figure 1.1- RNase H – mediated mRNA cleavage is the primary mechanism of action for most of the antisense oligonucleotides.

One of the major limitations for therapeutic application of oligonucleotides is caused by their very short half-lives due to the omnipresence of endo- and exo-nucleases in biological fluids (Kole, Krainer et al. 2012, McGinnis, Chen et al. 2012). Therefore, therapeutic oligonucleotides bear a plethora of chemical modifications in their structures in order to make them more resistant to degradation by endogenous nucleases. Different classes of such modifications, as well as their effect on the efficacy of therapeutic oligos and other practical limitations will be further discussed in chapter 2.

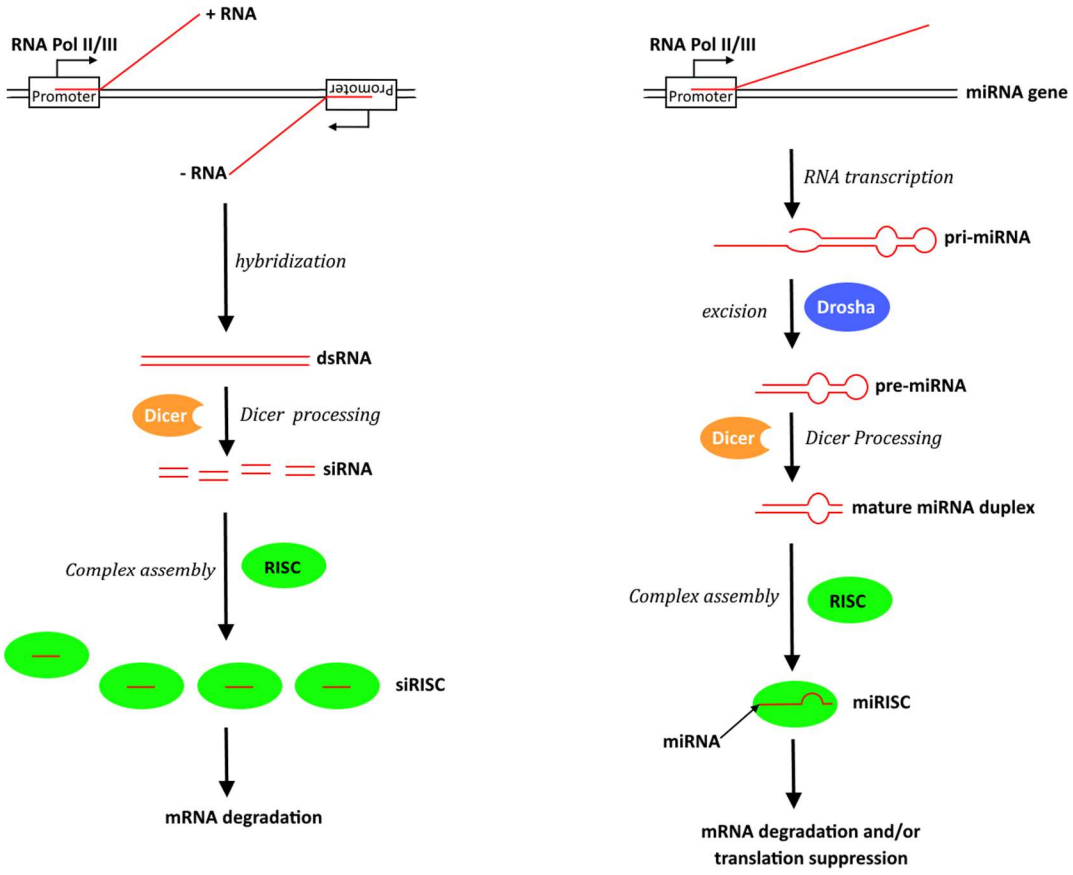


Figure 1.2- Biogenesis pathways and mechanisms of action for siRNAs and miRNAs.

Currently, at least for clinical studies, ELISA and qPCR are the methods of choice for the analysis of oligonucleotides. However, without time-consuming and complicated adjustments to the protocol, these methods are not capable of distinguishing between the full-length and truncated versions of oligonucleotides. It is even more difficult for these techniques to recognize the previously-mentioned modifications of therapeutic oligonucleotides. These limitations make such methods less suitable for quality control and metabolic studies. The PCR-based techniques also demonstrate specific disadvantages for biomarker discovery due to problems with specificity, normalization and biased performance of different PCR techniques (Wang, Meng et al. 2013). In contrast, because of its intrinsic property of measuring molecular masses of the analyzed compounds, mass spectrometry does not suffer from such detrimental effects and it can even reveal many details which are not obtainable by other methods.

Any bioanalytical procedure should start with a suitable and effective sample preparation method. There are five basic approaches for the isolation of oligonucleotides from biological samples. The first method is a classic liquid-liquid extraction using phenol-chloroform (Griffey, Greig et al. 1997, Murphy, Brown-Augsburger et al. 2005, Beverly, Hartsough et al. 2006). After adding phenol/ chloroform to the sample, the mixture is simply vortexed and centrifuged. The aqueous layer is next transferred to a new tube and washed with isopropyl ether to remove other endogenous compounds. The aqueous phase is mixed with glycogen and ethanol before storage at -80 °C for precipitation. The supernatant will be removed and the precipitated RNA will be air-dried in a fume hood. The final sample is then reconstituted in the initial chromatographic mobile phase prior to injection into the LC-MS system. A schematic of this process is presented in figure 1.3.

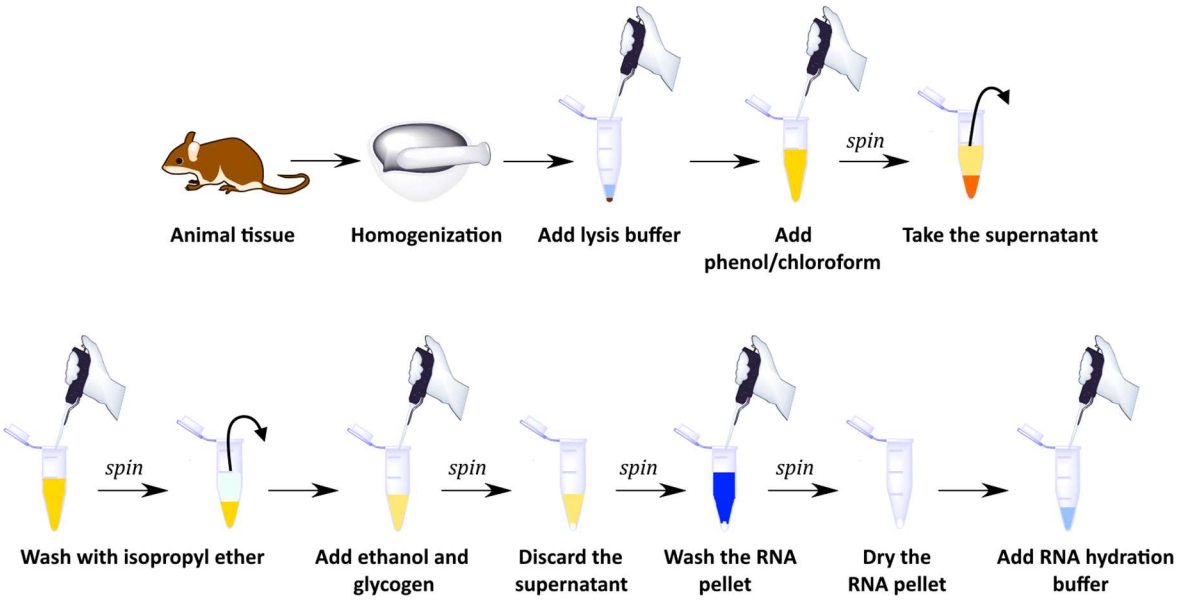


Figure 1.3- Schematic representation of phenol/chloroform LLE

The second approach involves using proteinase K to enzymatically remove the proteins from samples leaving the oligonucleotides behind (Bourque and Cohen 1993, Chen, Qian et al. 1997, Raynaud, Orr et al. 1997, Bellon, Maloney et al. 2000, Shimizu, Jinno et al. 2012). In this

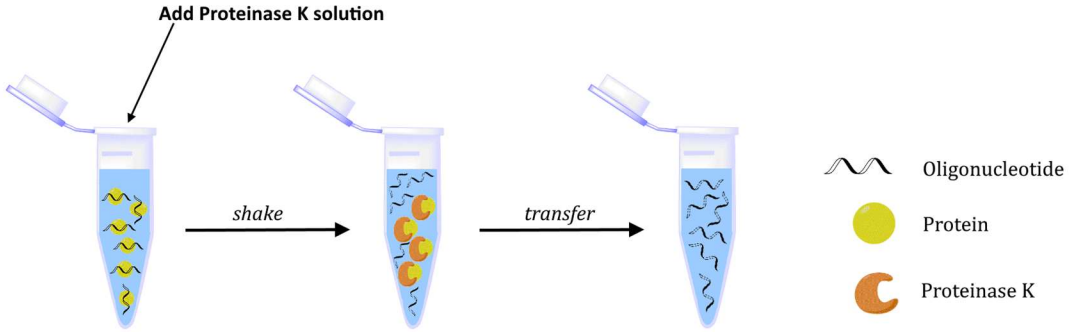


Figure 1.4- Proteinase K digestion for oligonucleotide extraction

method, the biological sample is buffered, proteinase K is added and the sample is digested for several hours while shaking. After digestion, the sample will be centrifuged and the supernatant taken for LC-MS analysis (McGinnis, Cummings et al. 2013). A schematic of this process is shown in Figure 1.4.

The third approach involves the use of magnetic ion-exchange beads (Ye and Beverly 2011). In this case, diluted plasma sample is mixed with the beads. The magnet is then applied to separate beads allowing removal of the supernatant. The beads are washed and the oligonucleotide is eluted from the beads by using a high concentration solution of ammonium chloride. Figure 1.5 shows a schematic of this extraction process.

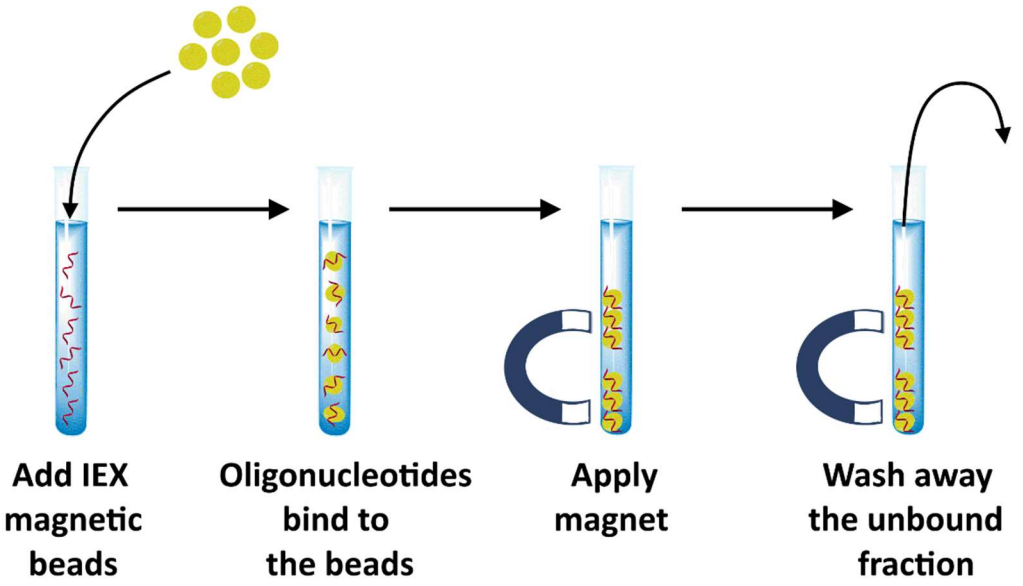


Figure 1.5- Schematic representation of the ion-exchange beads for oligonucleotide sample preparation.

The fourth approach involves the use of solid-phase extraction (SPE) for the isolation of RNA (Dai, Wei et al. 2005, Johnson, Guo et al. 2005). As shown in figure 1.6, the SPE cartridges are conditioned using methanol and the equilibration buffer. Plasma samples are then mixed with a lysis buffer and loaded onto the column. Proteins and other interfering compounds are removed using the wash buffer and then the analytes are collected using the elution buffer. The collected solutions are evaporated to near dryness under vacuum and reconstituted in deionized water prior to LC-MS analysis (Chen and Bartlett 2012).

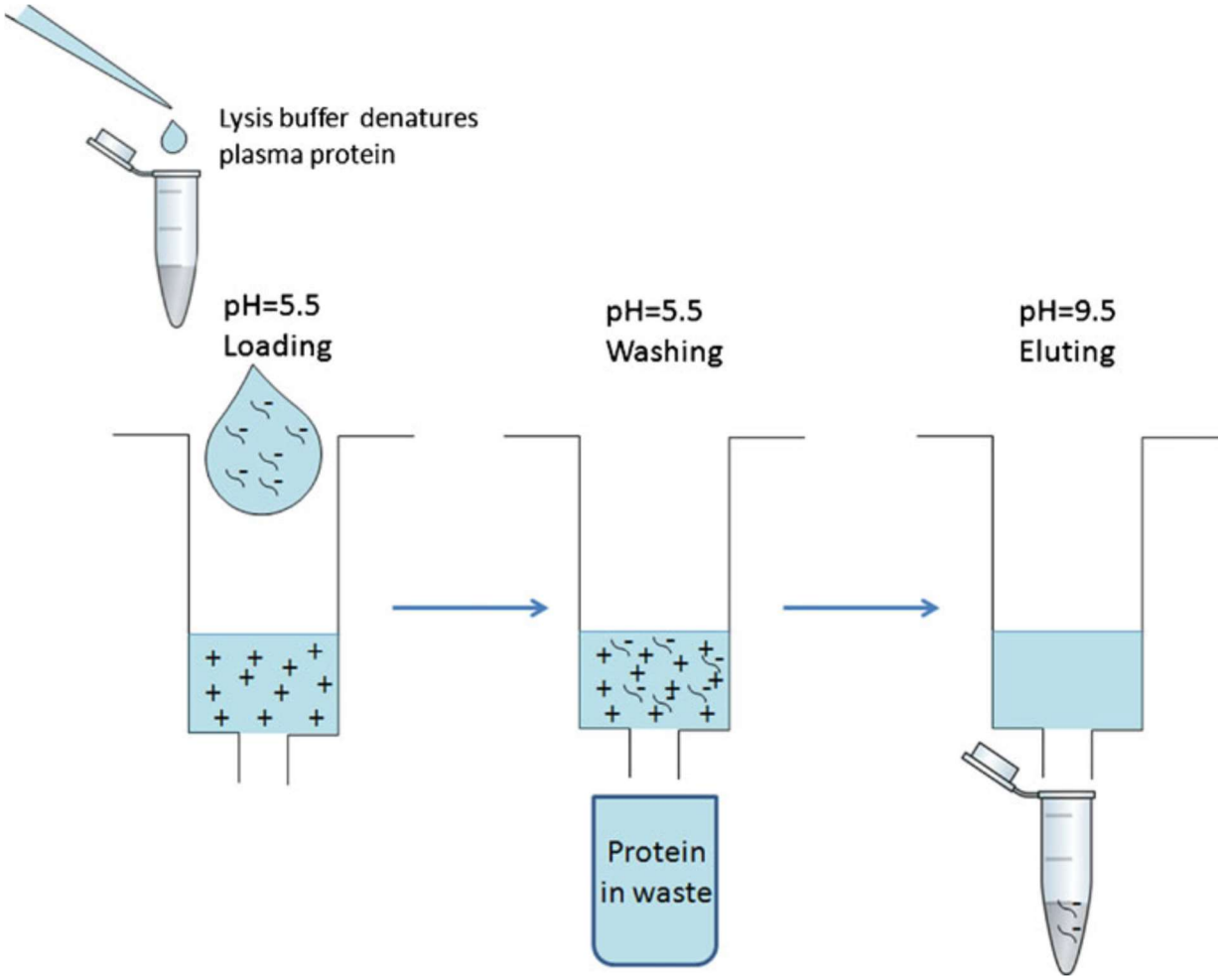


Figure 1.6- SPE sample preparation procedure [From (Chen and Bartlett 2012)].

The final approach involves combining magnetic beads with the immuno-capture of RISC-loaded RNAs using Argonaut 2 (Ago2) monoclonal antibodies (Zarovni, Corrado et al. 2015). In this approach shown in figure 1.7, protein G magnetic beads are used to capture either anti-mouse Ago2 (for mouse or rat samples) or anti-human Ago2 (for monkey or human samples).

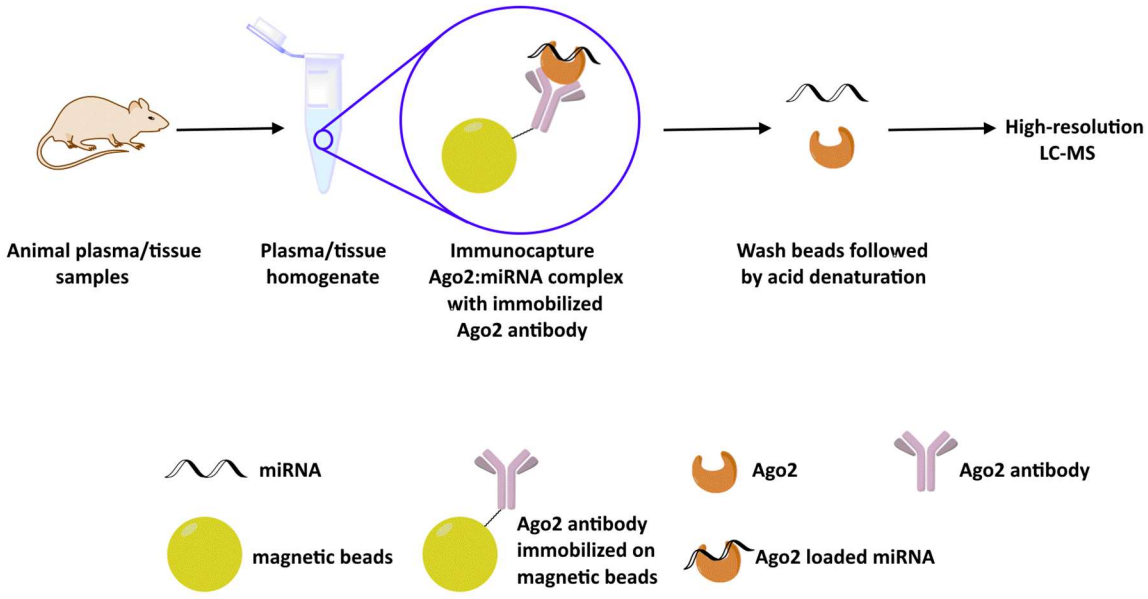


Figure 1.7- Immunocapture magnetic beads

All these previously-developed sample preparation methods are discussed in more detail in chapter 2, including their actual use in bioanalytical studies, advantages and limitations. In addition to these techniques, we have also developed a novel method for oligonucleotide sample preparation based on biotinylated capture strands. This method will be introduced in chapter 5.

Over the past two decades, ion-pair reversed phase liquid chromatography combined with ESI-MS has achieved lower detection limits and developed as the chromatographic method of choice for oligonucleotide analysis (van Dongen and Niessen 2011, McGinnis, Chen et al. 2012). Because of the highly charged hydrophilic backbone of oligonucleotides, they cannot be retained on hydrophobic columns without the aid of an ion-pairing (IP) agent. Several alkylamines can be used as ion-pair agents to improve the chromatographic separation of oligonucleotides (Sharma, Glick et al. 2012, McGinnis, Grubb et al. 2013, Gong and McCullagh 2014), although TEA is the most widely utilized ion-pairing reagent for LC-MS of oligonucleotides (van Dongen and Niessen 2011, McGinnis, Chen et al. 2012, Basiri and Bartlett 2014). In addition to improved chromatography, the evaporation of IP agents from the surface of the electrospray droplet, forms a concentration gradient from the surface to the interior of the droplet which would assist the oligonucleotides to approach the droplet surface along the IP concentration gradient (Chen, Mason et al. 2013). Alkylamines can also reduce the MS charge state distribution and cation adduction by binding to the oligonucleotides in the electrospray droplet and later dissociating from them in the gas phase (Muddiman, Cheng et al. 1996). However, the use of IP agents may also cause dramatic ion suppression in the ESI source and adversely affect the sensitivity of bioanalytical methods. Therefore, particular care must be taken when choosing an IP agent for a specific application. An extensive review of the prior use of different IP agents for analyzing various oligonucleotides as well as the benefits and challenges of each method is presented in Chapter 2.

One of the most important recent developments in the LC-MS analysis of small oligonucleotides was finding that the composition of these molecules plays a far more significant role in the electrospray desorption process than previously believed. This means that it will not

be possible to develop a set of generic LC-MS conditions for the determination of small oligonucleotides, as is done with tryptic digest analysis of small peptides. Having to optimize mobile phase solvents and additives for every different oligonucleotide will add substantial burden to method development time and hinder progress in the field. Chapter 3 is dedicated to explaining efforts toward elucidating the effect of physicochemical parameters of ion-pairing reagents on the signal intensity of oligonucleotides and determining the most significant ion-pair and oligonucleotide properties that can improve the sensitivity of LC-MS methods for oligonucleotide analysis. Successful identification of the factors that most influence the MS signal intensity of oligonucleotides, allowed for the development of an algorithm that can predict which ion-pairs would generate the highest MS signal intensity for a particular oligonucleotide composition. This predictive formula is also presented in Chapter 3.

In spite of the fact that alkylamines other than TEA have been occasionally used for LC-MS analysis of oligonucleotides, HFIP has been an invariant component of such mobile phase mixtures. In Chapter 4, evidence is presented in support of the hypothesis that different alkylamines should be paired with different fluorinated alcohols and that HFIP is not necessarily the best choice for every situation. It is also shown that some fluorinated alcohols can promote the preservation of oligonucleotide secondary structures in the gas phase.

Finally, as a result of these basic investigations into the nature of the ESI process for oligonucleotides, a practical biological application involving bioanalysis of miR-451 was developed as explained in Chapter 5. In order to develop this method, our accumulated knowledge of sample preparation was needed to design a novel extraction protocol. In addition, our predictive algorithm introduced in Chapter 3 was utilized to select an ion-pair for this application. After selection of the ion pair, our findings regarding the role of fluorinated alcohols

(Chapter 4) suggested the use of 1,1,1,3,3,3-Hexafluoro-2-methyl-2-propanol (HFMIP) instead of HFIP. These modifications resulted in a cleaner sample, better chromatographic retention and stronger MS responses. It was only because of these improvements that the direct detection of a microRNA from plasma, where its concentration is very low, was possible. The methods, algorithms and other selection criteria that are introduced in the following chapters are very robust and can be easily transferred to other laboratories to study other endogenous oligonucleotides. Therefore, the findings of this project can pave the way for further implementation of mass spectrometry in the fields of oligonucleotide metabolism and biomarker discovery. Considering the very high precision and accuracy of LC-MS methods compared to molecular biology techniques, fewer samples should be required to demonstrate a significant statistical difference when mass spectrometry is utilized for biomarker validation. This will considerably expedite the introduction of new oligonucleotide biomarkers.

CHAPTER 2

LC-MS OF OLIGONUCLEOTIDES: APPLICATIONS IN BIOMEDICAL RESEARCH[1]

[1] Babak Basiri and Michael G. Bartlett. 2014. *Bioanalysis*. 6(11): 1525-1542
Reprinted here with permission of the publisher.

Abstract: Recent findings have elucidated numerous novel biological functions for oligonucleotides. Current standard methods for the study of oligonucleotides (i.e. hybridization and PCR) are not fully equipped to deal with the experimental needs arising from these new discoveries. More importantly, as the intracellular capacity of oligonucleotides is being harnessed for biomedical applications, alternative bioanalytical techniques become indispensable in order to comply with ever-increasing regulatory requirements. Due to its ability to detect oligonucleotides independent of their sequence, LC-MS is emerging as the analytical method of choice for oligonucleotides. In this review, the current applications of LC-MS in the analysis of oligonucleotides, with an emphasis on RNA therapeutics and biomarkers, will be examined. In addition, the theoretical framework of oligonucleotide electrospray ionization is carefully inspected with the purpose of identifying the contributing factors to MS signal intensity.

Key Terms

Morpholino oligomers: A class of nucleic acid analogs where the pentose sugar rings in the backbone are substituted by morpholine rings that are linked through phosphorodiamidate groups.

Electrospray ionization (ESI): A soft ionization technique involving the dispersion of an analyte solution into a fine aerosol.

Ion evaporation model (IEM): This is one of the two major theories used to explain the production of gas phase ions in ESI. The IEM suggests that as the droplet radius approaches the Rayleigh limit, the field strength at the surface of the droplet is large enough to assist the field desorption of solvated ions.

Henry's law: One of the gas laws stating that the solubility of a gas in a liquid is directly proportional to the partial pressure of the gas above the liquid.

Charge state reduction: Generating fewer different multiply-charged *m/z* species when a molecule is analyzed by ESI mass spectrometry.

Wrong-way-round ionization: The observation of protonated or deprotonated ions during ESI ionization when such ions are not expected to exist in appreciable concentration considering the pH of the solution.

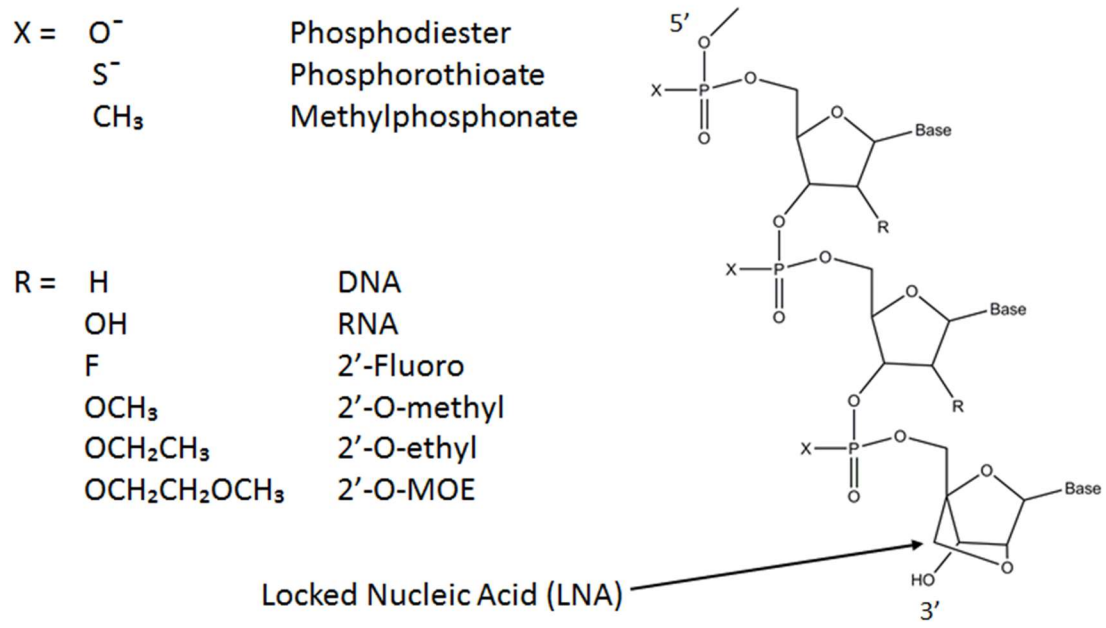
The 2013 approval of Mipomersen (Kynamro® [CA, USA]) by the United States Food and Drug Administration, brought oligonucleotides to the forefront of biomedical and pharmaceutical research once again after a period of increasing trepidation. Although oligonucleotides have been under intense study for pharmaceutical applications during the past three decades (Winkler 2013), the outcome of these efforts was not very promising. Before the approval of Mipomersen, there were only two oligo-based drugs available on the market and both of them were for local application in ocular diseases (Lin, Li et al. 2007). Fomivirsen (Vitravene) was registered in 1998 as the first oligonucleotide drug. It is used for treatment of cytomegalovirus-induced retinitis in immunocompromised patients with AIDS. Next came the aptamer, Pegatinib (Macugen), in 2004 for the treatment of neovascular age-related macular degeneration (AMD) (Kole, Krainer et al. 2012). Due to their distinctive structural properties, i.e. high molecular weight, hydrophilicity and multiple anionic charges, oligo-based therapeutics do not follow Lipinski's rules (Lipinski 2000) and suffer from poor drug-like properties. They generally do not show acceptable pharmacokinetic profiles (Dirin and Winkler 2013), cannot penetrate biological membranes (Koller, Vincent et al. 2011) and sometimes show off-target

effects (Stessl, Noe et al. 2012). These challenges along with the lack of a systemically-administered oligonucleotide therapeutic, until the beginning of 2013, raised serious doubts about the viability of oligonucleotide drugs (Krieg 2011). Mipomersen resolved many of these issues as a systemically delivered antisense oligonucleotide inhibitor of apolipoprotein B-100 synthesis in the liver for treatment of homozygous familial hypercholesterolemia (Gelsinger, Steinhagen-Thiessen et al. 2012, McGowan, Tardif et al. 2012, Parhofer 2012). Although the site of action for mipomersen, i.e. liver, is not a specifically hard target for drug delivery and even considering the FDA requirement for an enhanced pharmacovigilance program and a long-term registry of patients to determine the long-term risks of hepatotoxicity (Winkler 2013), the approval of mipomersen was more than a simple proof of concept that oligonucleotide drugs can be utilized in situations beyond the eye. Currently, several oligonucleotides are in different phases of development and clinical trials and hopefully some of them will be added to the list of approved oligo-based therapeutics in the next few years. Several recent reviews contain relatively complete listings of these investigational oligonucleotides (McGinnis, Chen et al. 2012, Winkler 2013).

Beside their hydrophilicity, one of the major challenges with oligonucleotide drug delivery is the universal presence of nucleases in biological fluids resulting in rapid hydrolysis and half-lives of approximately a few minutes *in vivo* (Kole, Krainer et al. 2012). Introducing chemical modifications to the backbone or sugar residues of oligonucleotides in order to promote their metabolic stability is one way to overcome this problem. The phosphate groups in the backbone could be replaced by phosphorothioate groups. Substitutions of the 2’ hydroxyl group of ribose including 2’-O-methyl, 2’-O-ethyl, 2’-O-methoxyethyl and 2’-fluorine have also been shown to protect oligonucleotides against nucleases. Locked nucleic acids (LNAs) are another

class of modified oligonucleotides with a covalent bond between the 2' oxygen and the 4' carbon (van Dongen and Niessen 2011). We can see some of these modifications in the structure of mipomersen (Figure 2.1).

a)



b) (3'→5')(P-thio)(G-C-C-U-C-dA-dG-dT-dC-dT-dG-dC-dT-dT-dC-G-C-A-C-C) Na_{19}

Modified residues:

A : 2'-O-(2-methoxyethyl)adenosine

C : 2'-O-(2-methoxyethyl)-5-methylcytidine

G : 2'-O-(2-methoxyethyl)guanosine

U : 2'-O-(2-methoxyethyl)-5-methyluridine

dC : 2'-deoxy-5-methylcytidine

Figure 2.1- Oligonucleotide modifications

a) Chemical modifications designed to improve the stability of oligonucleotides
b) Structural formula of "mipomersen sodium". As shown, it is a RNA oligonucleotide with several deoxyribonucleotides along its chain and with all backbone phosphates replaced with phosphorothioate groups. Also notice the presence of several 2'-O-MOE modifications. (FROM N07/79 STATEMENT ON A NONPROPRIETARY NAME ADOPTED BY THE USAN COUNCIL)

The therapeutic efficacy of oligonucleotides is based on the intracellular biochemical pathways that they trigger upon base-pairing with their molecular targets: Antisense (AS) and RNA interference (RNAi) oligonucleotides downregulate gene expression by inducing enzyme dependent degradation of targeted mRNA; steric-blocking oligonucleotides restrict the access of cellular machinery to pre- or mature mRNA without degrading it (Kole, Krainer et al. 2012). Antisense oligonucleotides recognize a specific sequence on the mRNA and after hybridization with mRNA, guide the nuclear enzyme RNase H to degrade that mRNA (Tafech, Bassett et al. 2006). Currently, a typical AS oligonucleotide drug is about 20 nucleotides long with phosphorothioate bonds (for example refer to the structure of mipomersen in figure 1). A central 10 nucleotide sequence is left with very few modifications (as required for interaction with RNase H) to make a gap between the five nucleotides at each end which have been heavily modified to improve drug stability (the origin of the term "gapmer"). Although a stretch of deoxynucleosides in the center of the gapmer is essential for activation of RNase H, the gapmer

sequence can tolerate extensive chemical modifications before obscuring RNase H activity (Kole, Krainer et al. 2012, Winkler 2013). RNAi was first discovered in the nematode worm *C. elegans* (Fire, Xu et al. 1998) where delivery of long, double-stranded RNAs (dsRNA) silenced gene expression. After entering the cell, these long dsRNAs were broken into 21-22 mer dsRNAs called small interfering RNAs (siRNA). siRNAs would then interact with the multiprotein RNA-induced silencing complex (RISC) leading to disposal of the sense strand and binding of the complex to its target mRNA through the guidance of the antisense strand (hence called the "guide strand"). If the siRNA was fully complementary to its target, argonaute 2, one of the RISC components, cleaves the mRNA 10-11 nucleotides from the 5' end of the guide strand (de Fougerolles, Vornlocher et al. 2007). Although mammalian cells lack the ability to cleave long dsRNA into siRNA, synthetic siRNAs can enter the RISC and degrade target mRNAs (Elbashir, Harborth et al. 2001). In contrast to antisense oligonucleotides, only a few of these classical chemical modifications can be tolerated in the structure of siRNA before eradicating its interaction with RISC. In the antisense strand, one or two internal nucleotides can bear 2'-O-methyl modifications and a phosphorothioate linkage can be inserted at the 3' end. The sense strand can contain more 2'-O-methyl nucleotide substitutions. However, recent findings point toward a new class of siRNA-optimized 2'-O modifications different from those mentioned previously. 2'-O-benzyl and 2'-O-methyl-4-pyridine are tolerated at multiple positions on the guide strand of siRNA sequences *in vivo* and even increase its potency (Butora, Kenski et al. 2011, Kenski, Butora et al. 2012). Nevertheless, the effective administration of siRNAs will most likely rely on their incorporation into delivery-enhancing bioconjugates (Kole, Krainer et al. 2012, Winkler 2013).

Unlike antisense and siRNA oligonucleotides, steric-blocking oligonucleotides do not need to exploit cellular enzymes for their activity. They directly interact with specific sequences on pre- or mature mRNA and based on their target sequence, hinder the access of different cellular components (splicing machinery, ribosomes, protein factors, etc.) to mRNA without degrading it. Because this class of oligonucleotides does not rely on interaction with cellular enzymes, they tolerate the widest range of chemical modifications including locked nucleic acids, peptide nucleic acids and phosphorodiamidate morpholino oligomers (PMOs) (Kole, Krainer et al. 2012). This approach appears to be specifically promising for the treatment of Duchenne muscular dystrophy (DMD) (Matsuo, Masumura et al. 1991). Two splice-switching oligonucleotides have shown encouraging clinical results in patients with DMD: Drisapersen (PRO051) is an oligonucleotide with a full phosphorothioate backbone and 2'-O-methylation on all of its nucleosides (Goemans, Tulinius et al. 2011) and Eteplirsen is a phosphorodiamidate morpholino oligomer (Cirak, Arechavala-Gomeza et al. 2011).

Considering the current status of oligo-based therapeutics, it can be concluded that they offer a viable choice for drug development and although there are still significant challenges that need to be more fully resolved (e.g. poor systemic bioavailability, off-target effects, immunogenicity, etc.), it is not unreasonable to predict the arrival of new oligonucleotide drugs to the market in coming years. The potential emergence of these drugs calls for bioanalytical methods that are more suitable for their determination. Currently, at least for clinical studies, ELISA and qPCR are the methods of choice for the analysis of oligonucleotides. However, without time-consuming and complicated adjustments to the protocol, these methods are not capable of distinguishing between the full-length and truncated or unmodified and modified versions of oligonucleotides making them risky, especially for quality control or metabolic studies.

In addition to their therapeutic potential, oligonucleotides can also be used for diagnostic purposes as biomarkers (Gupta, Bang et al. 2010, Kosaka, Iguchi et al. 2010, Widera, Gupta et al. 2011, Lorenzen and Thum 2012). Again, the PCR-based methods demonstrate some disadvantages for biomarker identification/quantification as a result of problems with specificity, normalization and biased performance of different PCR techniques (Wang, Meng et al. 2013). In contrast, due to its intrinsic property of measuring molecular masses of the analyzed compounds, mass spectrometry does not suffer from such detrimental effects and it can even reveal many details which are not obtainable by other methods. However, the structural properties of nucleic acids (mainly their polyanionic charged state) make it challenging to develop efficient liquid chromatography combined with mass spectrometry (LC-MS) methods for their effective determination. The accumulated body of work to overcome this limitation will be discussed in the following sections.

A. General approaches to LC-MS of oligonucleotides

1- Sample preparation

Like any other analytical method, successful analysis of oligonucleotides begins with an efficient sample preparation step which would deplete the biological matrix (blood, plasma, urine, tissue) of interfering compounds and concentrate the analyte (oligonucleotide). Among different components of biological matrices, oligonucleotides seem to be highly associated with proteins (Yu, Geary et al. 2004, van Dongen and Niessen 2011, McGinnis, Cummings et al. 2013). Therefore, a suitable extraction method should be able to dissociate oligonucleotides from the bound proteins. Although protein precipitation (PP) with ammonium acetate or methanol would theoretically be the simplest approach, the strong protein binding of oligonucleotides

causes significant losses of the analyte during sample preparation using PP making this approach impractical (Deng, Chen et al. 2010, van Dongen and Niessen 2011). Solid-phase extraction (SPE) has also been used by several groups mostly using Oasis HLB cartridges (Dai, Wei et al. 2005, Johnson, Guo et al. 2005) and has usually suffered from low analyte recoveries because of nonspecific irreversible oligonucleotide binding to the SPE cartridges. This nonspecific binding is a problem not only with cartridges but also with other containers and glassware. It has been shown that silanizing sample containers, volumetric bottles, autosampler vials and other tubes which come in contact with oligonucleotides can significantly reduce the nonspecific binding (Zhang, Lin et al. 2007, Deng, Chen et al. 2010). More recently, a simpler approach to resolve this problem has been proposed: adding an internal standard at high concentration. This way, the internal standard will competitively bind to the exposed sites on the surface of containers and act as a sacrificial oligonucleotide preserving the real analytes in the solution (Chen and Bartlett 2012, McGinnis, Cummings et al. 2013). By utilizing the IS as the sacrificial oligonucleotide and adding ammonium acetate to the cartridge equilibration buffer, recoveries as high as 80% were observed for the first time using a one-step anion exchange SPE on a Clarity OTX cartridge (Chen and Bartlett 2012).

Liquid-liquid extraction (LLE) with phenol/chloroform is another widely-used extraction method for oligonucleotides (Griffey, Greig et al. 1997, Murphy, Brown-Augsburger et al. 2005, Beverly, Hartsough et al. 2006). Although this method usually results in high recoveries, it can be very labor intensive and time consuming rendering it ineffective for high-throughput studies. LLE has also been used in combination with SPE which has proven to be highly effective (Zhang, Lin et al. 2007, Deng, Chen et al. 2010). In a recent review (van Dongen and Niessen 2011), van Dongen and Niessen have summarized all the extraction techniques which have been

used in studies performed until the end of 2010 in a neatly-organized table that can be referred to for more detail. More recently, using a combination of phenol-chloroform LLE and ethanol precipitation, Chen and Bartlett managed to achieve recoveries of more than 75% while analyzing a phosphorothioate DNA oligonucleotide (Chen and Bartlett 2013).

Digestion of bound proteins with proteinase K is another technique that generally results in high recoveries. However, proteinase K digestion usually needs to be supplemented with additional purification steps (Bourque and Cohen 1993, Chen, Qian et al. 1997, Raynaud, Orr et al. 1997, Bellon, Maloney et al. 2000, Shimizu, Jinno et al. 2012) which makes it laborious and low-throughput. A break-through in using proteinase K for oligonucleotide sample extraction is the recent report by McGinnis et al. of a one-step sample prepation technique (McGinnis, Cummings et al. 2013). Samples were incubated for only 3 hours in a lysis buffer containing proteinase K, EDTA (for RNase inhibition and also, as it will be discussed later, cation adduct suppression), guanidine hydrochloride, DTT (for disulfide bond reduction) and Triton X-100 at pH 9.0 and then directly transferred to HPLC vials for analysis. The reported recovery for this method exceeds 95% and considering its simple and relatively fast procedure, it can be easily adopted to high-throughput procedures.

Ye and Beverly adopted an innovative approach for oligonucleotide extraction using magnetic beads. They optimized a method for utilizing commercially available strong anion exchange magnetic beads for extraction of siRNA from human serum in 100-200 µl of LC-MS compatible buffer (Ye and Beverly 2011). The extraction protocol took less than 1 hour and its compatibility with high-throughput analysis was demonstrated by its employment in a 96-well plate format. The recovery of siRNA oligonucleotides was different based on their modifications

as well as the type of magnetic beads averaging around 80%. However, recoveries as high as 99% were achieved for some siRNA molecules.

2- Ion-pair reversed phase liquid chromatography (IP-RPLC)

Although other chromatographic separation methods including anion-exchange chromatography (Arora, Knapp et al. 2002, Devi, Beer et al. 2005, McGinnis, Cummings et al. 2013) or hydrophilic interaction chromatography [HILIC] (Easter, Kroning et al. 2010, Gong and McCullagh 2011, Easter, Barry et al. 2013) can be used with oligonucleotides and despite some arguments regarding their merits over RPLC (Cook and Thayer 2011), ion-pair reverse phase liquid chromatography combined with ESI-MS has achieved lower detection limits and developed as the chromatographic method of choice for oligonucleotide analysis. Considering the highly charged hydrophilic backbone of oligonucleotides, it is obvious that they cannot be retained on hydrophobic columns without the aid of an ion-pairing agent. Ion pairing (IP) agents are amphipathic molecules that possess a charged or ionic group on one end and a hydrophobic tail on the other (Bartha and Ståhlberg 1994). Through electrostatic interactions involving their charged moiety, they form pairs that effectively mask the charge of the analyte. As a result, charged analyte molecules appear as neutrals to the RPLC column and display highly improved retention (van Dongen and Niessen 2011). For oligonucleotides, the charged part is in the form of a cationic amine and several alkylammonium salts have been used as IP agents including: Triethylammonium acetate (TEAA) (Huber, Oefner et al. 1993), triethylammonium bicarbonate (TEAB) (Huber and Krajete 1999), hexylammonium acetate (HAA) (McCarthy, Gilar et al. 2009), diisopropylammonium acetate (DIPAA) (Bothner, Chatman et al. 1995), butyldimethylammonium bicarbonate (BDMAB) (Oberacher, Oefner et al. 2001, Oberacher,

Parson et al. 2001), *N,N*-dimethylcyclohexylammonium bicarbonate (CycHDMAB) (Sharma, Glick et al. 2012), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) [58], tripropylammonium acetate and tributylammonium acetate (van Dongen and Niessen 2011). In most cases the separation has been performed on C18 columns and as represented by the work of Nikcevic and colleagues (Nikcevic, Wyrzykiewicz et al. 2011), XBridge and Acquity BEH columns have also shown excellent performance for HPLC and UPLC purposes accordingly. The monolithic PS-DVB columns are another possibility for this application (Premstaller, Oberacher et al. 2000, Premstaller, Oberacher et al. 2001, Sharma, Glick et al. 2012) (Figure 2). Interestingly, even mathematical equations have been developed that can predict the behavior of single stranded oligonucleotides on reverse phase columns based on their sequence and ability to form secondary structures (Gilar, Fountain et al. 2002, Gilar and Neue 2007, Sturm, Quinten et al. 2007). The applicability of various ion pair systems and mobile phase combinations for use with LC-MS is the focal point of the next section.

3- LC/MS Coupling

Although MALDI has played an important role in oligonucleotide ionization during various investigations (Lecchi, Le et al. 1995, Kirpekar, Berkenkamp et al. 1999, Wu and McLuckey 2004), due to the polyanionic phosphate backbone of oligonucleotides, negative ion ESI has generally produced better results. However, ESI efficiency is highly dependent on the nature of the mobile phase used. In general, ESI is most efficient with mobile phases of low viscosity, low surface tension, high volatility and low ionic strength (Deng, Chen et al. 2010, Li and Cole 2010) while efficient IP-RPLC of oligonucleotides is achieved in highly aqueous solutions of low pH (van Dongen and Niessen 2011) with high surface tension and conductivity. So it has been quite

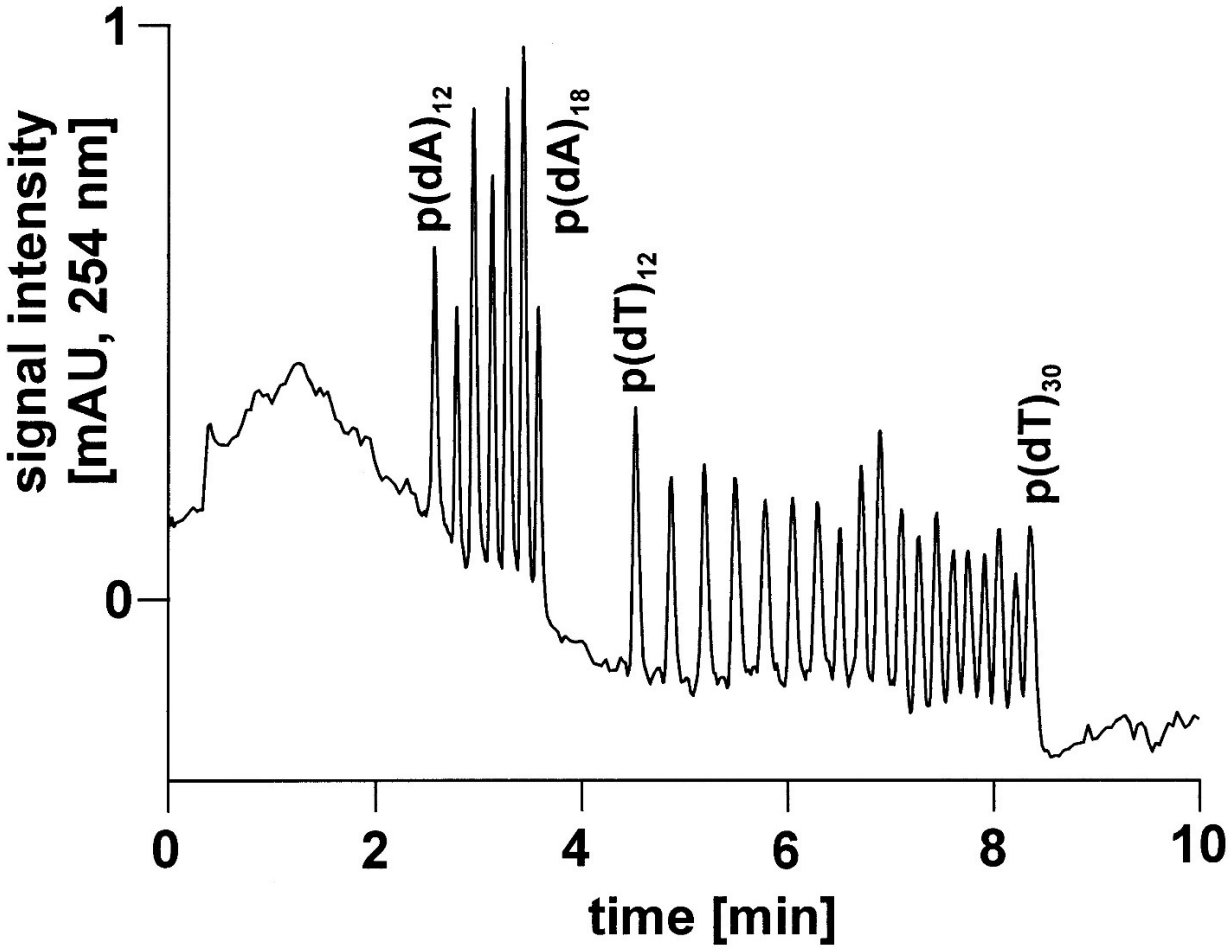


Figure 2.2- Separation of phosphorylated oligonucleotide ladders on a PS/DVB capillary column

Baseline separation of poly-dA and poly-dT oligonucleotides with only one nucleotide difference in length (12-18mer for poly-dA and 12-30mer for poly-dT) was achieved by using 100 mM TEAA as the ion-pairing agent. It is observed that the less hydrophobic poly-dA oligonucleotides elute before their hydrophobic poly-dT counterparts. (Reprinted with permission from (Huber and Krajete 1999). Copyright 1999 American Chemical Society).

challenging to find the right compromise in choosing a mobile phase that can support efficient online coupling of HPLC and ESI-MS. For example while using TEAA as the ion pairing agent for improved chromatographic separation (see Figure 2.2), dramatic ion suppression was noted in the electrospray ionization (Bleicher and Bayer 1994, Gilar, Fountain et al. 2002). However, replacing the acetate counter ion with a bicarbonate (i.e. using TEAB as an IP agent) improved the signal intensity up to seven times (Huber and Krajete 1999). The more hydrophobic ion-pair, BDMAB, by allowing an increase in the organic content of the mobile phase, raised the signal even higher (Oberacher, Oefner et al. 2001, Oberacher, Parson et al. 2001). Apffel et al. (Apffel, Chakel et al. 1997, Apffel, Chakel et al. 1997) used an ion pair system consisting of TEA and hexafluoroisopropanol (HFIP) in water/methanol [HFIP is very poorly miscible in acetonitrile]. This ion pair system allows for reasonable LC separation and reduces suppression in ESI. The presence of HFIP in the mobile phase makes TEA more hydrophobic and less soluble providing excellent chromatographic separation at much lower concentrations of TEA (van Dongen and Niessen 2011). McCarthy et al. compared the performance of the TEA/HFIP system to acetate salts of TEA, TBA, BDMA and HAA and showed that TEA/HFIP shows higher signal intensity as well as improved chromatographic resolution over all other investigated compounds (McCarthy, Gilar et al. 2009). This explains why the TEA/HFIP ion-pair system replaced almost all other mobile phase compositions soon after its introduction. It has been the principal mobile phase for LC-MS of oligonucleotides over the past 15 years without much effort to compare it to alternative combinations of alkylammonium ions with HFIP or to understand its mechanism of action (Chen, Mason et al. 2013). Originally, Apffell et al. proposed that HFIP is simply a dynamic liquid/gas phase pH adjuster that is used to lower the pH of the mobile phase and because of its low boiling point is rapidly depleted from the electrospray droplet without

competing with oligonucleotides for ionization. In contrast, acetic acid in TEAA has a higher boiling point than TEA so it would remain in the droplet and compete for ionization (Apffel, Chakel et al. 1997). It is worth mentioning here that adjusting the pH of the mobile phase is extremely important as it improves the chromatographic peak shape and also too low or too high pH values in the mobile phase can lead to depurination or deamination of oligonucleotides respectively (Nikcevic, Wyrzykiewicz et al. 2011). However, recent observations by Chen et al. assigned a much more sophisticated role to HFIP in this buffer system (Chen, Mason et al. 2013). They have demonstrated that by using trifluoroethanol (TFE) –another weak acid with a boiling point even lower than TEA- instead of HFIP, ESI signal intensity declined suggesting that HFIP is more than a pH adjuster with a low boiling point. Upon further evaluation of physicochemical properties of these compounds, they found a meaningful difference in the Henry's law constant ($k_{H,cc}(\frac{aq}{gas})$) between them: it equals to 0.96 for HFIP and 2.38 for TFE. In the ESI droplet, a compound with a $k_{H,cc} > 1$ would enrich in the solution while a compound with $k_{H,cc} < 1$ will deplete throughout droplet evaporation (McGinnis, Grubb et al. 2013). Therefore, TFE –especially at higher concentrations– would be enriched in the droplet and form a reservoir of intact molecules that could compete with oligonucleotides for ionization. In contrast, HFIP would be depleted fast enough not to suppress the deprotonation of oligonucleotides. It is through this "wrong way around ionization" mechanism that HFIP improves oligonucleotide ionization (Chen and Bartlett 2013, Chen, Mason et al. 2013). The general response of IP agents in modifying ESI charge state distribution and MS signal intensity seems to be governed by their $k_{H,cc}$ values, as well (Chen, Mason et al. 2013). Alkylamines with higher $k_{H,cc}$ values (larger than 1) reduce the charge state distribution by forming complexes with the oligonucleotide and dissociating from it in the gas phase and alkylamines with very low

$k_{H,cc}$ values facilitate ion emission from the droplet as explained by the ion evaporation model at earlier stages when it is not highly charged and reduce the charge state distribution this way. The evaporation of ion pairing agents from the surface of the electrospray droplet, forms a concentration gradient from the surface to the interior of the droplet which would assist the oligonucleotides to approach the droplet surface along the IP concentration gradient. The IP agents with lower $k_{H,cc}$ values are expected to generate steeper concentration gradients, hence stronger signals. This prediction is verified with DIEA and DMBA showing higher signal intensities than the widely used TEA due to their lower $k_{H,cc}$ values (Chen, Mason et al. 2013) (table 2.1).

Table 2.1- Physicochemical properties of some common alkylamines and HFIP

Compound name	MW (g/mol)	Boiling point (ºC)	pKa	Proton affinity (Kcal/mol)	Gas phase basicity (Kcal/mol)	Water solubility (g/L 25ºC)	Vapor pressure (Pa)	$k_{H,cc}(\frac{aq}{gas})$
Hexylamine	101.19	130.00	10.56	221.70	213.60	20.57	1.18 x 10^3	1.57
Diisopropylamine	101.19	83.90	11.05	232.30	224.30	75.31	1.06 x 10^4	0.42
Triethylamine	101.19	89.70	10.65	234.70	227.00	68.30	7.61 x 10^3	0.35
Dimethylbutylamine	101.19	75.72	10.02	231.60	224.20	41.80	6.19 x 10^3	0.27
Diisopropylethylamine	129.24	127.00	10.50	237.60	230.30	9.33	1.56 x 10^3	0.19
Tripropylamine	143.27	156.00	10.65	236.90	229.50	2.60	2.01 x 10^2	0.10
Tributylamine	185.35	216.50	10.89	238.60	231.30	0.08	12.5	0.08
HFIP	168.05	58.20	9.30	164.10	156.80	7.77	2.12 x 10^4	0.96

*Data taken from (Chen, Mason et al. 2013, McGinnis, Grubb et al. 2013)

After the original publication of the TEA/HFIP method by Apffel et al. (Apffel, Chakel et al. 1997, Apffel, Chakel et al. 1997) and optimizations of Gilar et al. (Gilar, Fountain et al. 2003),

HFIP has been generally used at the concentration of 400 mM along with 16.3 mM TEA which is the maximum concentration of TEA that is soluble in 400 mM HFIP (Cramer, Finn et al. 2011, Nikcevic, Wyrzykiewicz et al. 2011). However, according to the wrong way around ionization model, HFIP anions would accumulate in the droplet at increasing concentrations of HFIP and compete with the oligonucleotides for ionization. As a result, HFIP needs to be used at much lower concentrations. In the same manner, IP agents would not act more efficiently with increasing concentrations as they are amphipathic molecules and tend to accumulate on the surface of the droplet and interfere with desorption of the analyte into the gas phase. With these considerations, Chen et al. reported that the highest signal intensity would be generated by 10 mM DIEA and 50 mM HFIP in methanol instead of the traditional 400 mM HFIP/16 mM TEA (Chen, Mason et al. 2013). The role of organic solvents in the electrospray ionization of oligonucleotides is another subject which has been highly neglected as the TEA/HFIP buffer has always been used with methanol. However, it was concluded in a recent study that solvents with lower dielectric constants such as isopropanol or ethanol are more effective than methanol in increasing signal intensity because they do not disperse the HFIP anion from its proton or positively charged IP agent as efficiently (Chen and Bartlett 2013). Furthermore, McGinnis at al. found out that the choice of IP agent is highly dependent on the properties of the analyte and a one-size-fits-all approach is not applicable to this situation. Their results indicated that the hydrophobicity of the analyte should be considered when choosing ion pair systems: for more hydrophilic siRNA molecules, 10 mM DIPA with 25 mM HFIP is optimal while for highly hydrophobic phosphorothioate DNA oligonucleotides, 10 mM DIEA with 50 mM HFIP is the best choice (McGinnis, Grubb et al. 2013). In summary, lower detection limits are only obtainable by modifying the currently used TEA/HFIP buffer. HFIP needs to be used at much

lower concentrations along with alternative IP agents chosen by their $k_{H,cc}$ values. Methanol can be substituted by other alcohols with lower dielectric constants and mobile phases need to be optimized separately for differently modified oligonucleotides. Algorithms that take all these factors into account and calculate the composition of the optimal mobile phase for each application could be expected to emerge in the near future.

B. Implementation of LC-MS to oligonucleotide biomarker identification

1- Tandem mass spectrometry of oligonucleotides

As a major benefit when compared to other techniques, mass spectrometry can accommodate for the online sequencing of separated oligonucleotides. This superior capability facilitates the identification of those subtle changes such as substitutions/modifications at the single nucleotide level. Consequently, tandem mass spectrometry becomes instrumental in the discovery and identification of oligonucleotide biomarkers as well as natural intracellular nucleic acid metabolites. The gas-phase dissociation of polycharged oligonucleotides (through CID or alternative ion activation techniques) produces numerous types of fragment ions which can, in some cases, be used for construction of "mass ladders" for sequencing (Rozenski and McCloskey 1999). These fragment ions are usually named following the system proposed by McLuckey and colleagues (McLuckey, Van Berker et al. 1992, McLuckey and Habibi-Goudarzi 1993, Wu and McLuckey 2004) where cleavages at different locations along the phosphodiester backbone produces a/w, b/x, c/y and d/z ion series (Figure 2.3). In DNA, a-B (a-type ions that have lost their nucleobases) and w ion series are the most abundant and used for sequencing in forward and reverse directions (Wang, Wan et al. 1998), while in RNA the major ions belong to the c/y series (Tromp and Schürch 2005). Fortunately, these diagnostic ion series would still be formed

after backbone modifications (Bartlett, McCloskey et al. 1996), albeit in different proportions, and therefore they can be used for sequencing of modified oligonucleotides as well (See figure 2.6 for an example). In order to assist sequencing from mass spectrometric data, computer algorithms have been developed. The first computer algorithm for oligonucleotide sequence derivation from CID mass spectra was introduced in 1996 (Ni, Pomerantz et al. 1996) which eventually evolved to the simple oligonucleotide sequencer (SOS) (Rozenski and McCloskey 2002). These pioneer software were succeeded by several other computer programs (Oberacher, Wellenzohn et al. 2001, Oberacher, Mayr et al. 2004, Liao, Shen et al. 2009, Nakayama, Takahashi et al. 2011, Oberacher and Pitterl 2011, Sharma, Glick et al. 2012). A software package containing two algorithms, the oligonucleotide mass assembler (OMA) and the oligonucleotide peak analyzer (OPA) was introduced in 2012 (Nyakas, Blum et al. 2013). OMA/OPA is publicly available through the author's website and is capable of calculating all theoretically possible fragments of an input sequence and annotating them to an experimental spectrum. Several modifications are pre-defined in the program library and further modifications can be introduced into the library by users. More recently, Yang et al. reported another software for the MS analysis of oligonucleotides (Yang, Leopold et al. 2013). RNA mass calculator (RNA MC) offers great flexibility in the calculation of theoretical masses by allowing for a much larger number of modifications to be incorporated into an oligonucleotide sequence.

Ion mobility spectrometry (IMS) has also been shown to be extremely beneficial in assisting with oligonucleotide sequence determination. IMS is carried out by using an electric field to pull analyte ions through a gas-filled drift tube where ions with higher number of charges display a shorter transit time compared to their counterparts carrying fewer number of charges. IMS can be used to separate the ions arising from the gas phase dissociation of the oligonucleotides

according to their charge states. This approach provides a number of simplified mass spectra ideal for sequencing. Recently, Fisher et al. have reported the use of a negative ion nano-electrospray MS/MS with ion mobility spectrometry for sequencing of a 20-nucleotide RNA oligo (Fisher, Smith et al. 2013). They observed that while bases in the center of the sequence were unidentifiable after nESI-MS/MS because of a plethora of overlapping ions, adding an IMS step gave full sequence coverage. This improvement was a result of ion spectra arising only from product ions that carry the same charge. The spectra from the product ions with different numbers of charges on them was subsequently used for assignment confirmation. Taucher and Breuker exercised another technique for restricting the charge states of oligonucleotide ions. They showed that RNA ions of low precursor charge (~0.2 charges/nucleotide) can be electrosprayed from solutions of 25 mM piperidine/25 mM imidazole (Taucher and Breuker 2010). The sequence of a 61 nucleotide RNA was obtained with near-complete coverage by CID-FTICR MS of these low charge state ions.

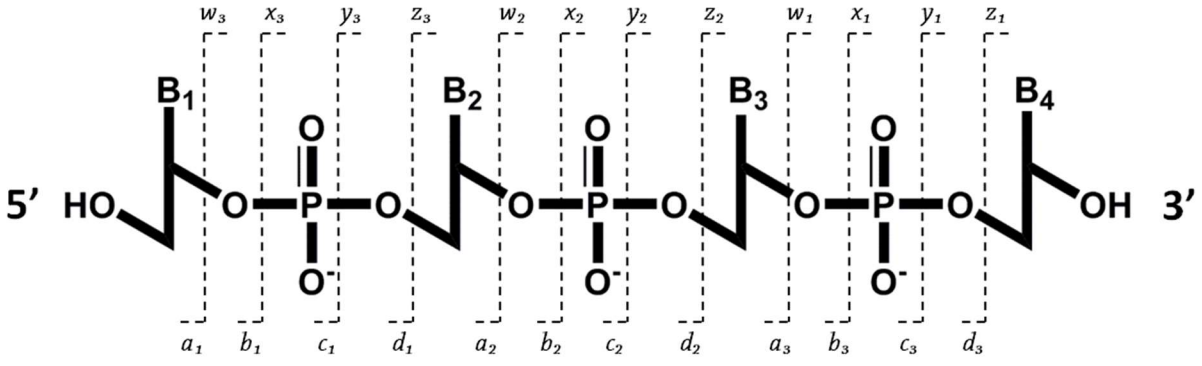


Figure 2. 3- Fragmentation pattern of oligonucleotides

The resulting fragment ions can be used for construction of mass ladders and oligonucleotide sequencing.

2- SNP genotyping

PCR-amplified fragments of intracellular nucleic acids can be subjected to MS/MS sequencing after an initial chromatographic separation for the purpose of identifying single nucleotide polymorphisms (SNPs) as potential biomarkers. In this case, it is clear that narrowing the charge state distribution would provide better detection limits by concentrating the ion current to fewer *m/z* channels. Moreover, it has been shown that precursor ions with higher charge states generate only a few nonspecific fragments at low *m/z* values while lower charge state precursor ions give high *m/z* sequence-specific fragments (Little, Thannhauser et al. 1995, Little, Aaserud et al. 1996, Deng, Chen et al. 2010). Cation adduction is another problem that needs to be considered: for polyanions such as oligonucleotides, the exchange of protons in the backbone for sodium or potassium ions causes further dispersion of the signal among multiple cation containing species which results in lower MS detection sensitivity and complicates the interpretation of observed peaks specifically for quantitative determination of allele frequencies (Smith, Loo et al. 1990, Oberacher, Parson et al. 2004). As discussed earlier, ion pairing agents with extremely low or high $k_{H,cc}$ values can reduce the charge state distribution. IP agents can also reduce cation adduction because they interact with the polyanionic backbone of the oligonucleotides and replace metal cations and later dissociate from the oligonucleotide backbone during the final stages of ionization. Proton transfer reactions are another possible way for charge state reduction of oligonucleotides. Protonated benzoquinoline (BQ) ions react with oligonucleotides predominantly by proton transfer and decrease their charge state (Wu and McLuckey 2003). Also, several factors have proven to affect adduct formation besides IP agents. Column temperatures higher than 50ºC are shown to be more efficient in exchange of adducted ions with TEA during the chromatographic separation. Elevated column temperature also has the

additional advantage of facilitating SNP detection by melting double-stranded DNA fragments as AT and GC base pairs have almost identical masses (Oberacher, Parson et al. 2004). Moreover, it has been shown that the desalting of single strands is easier than the duplex because single strands at higher temperatures do not form secondary structures (Beverly, Hartsough et al. 2005). However, if the purpose is to keep the double-stranded oligonucleotides intact, the temperature should be maintained at 10ºC below the melting point of the oligonucleotide (McCarthy, Gilar et al. 2009). The addition of chelating compounds such as EDTA to either the sample itself (Oberacher, Parson et al. 2004) or the mobile phase (Hail, Elliott et al. 2004) is another successful approach, although adding EDTA during sample preparation seems more compatible with electrospray ionization because the EDTA-cation complexes would be eluted in the void volume of LC and would not disturb the MS analysis. Even PCR mixtures containing detergents such as Tween-20 or Nonidet P-40 have been shown to interfere with MS detection and should be replaced with the solutions lacking any kind of detergent or PCR enhancer for the best mass spectrometric performance (Oberacher, Niederstätter et al. 2006).

Capillary ion-pair reversed phase HPLC combined with ESI-MS has been used for genotyping of polymorphic short tandem repeat (STR) loci from PCR amplified fragments of the human tyrosine hydroxylase (humTH01) gene (Oberacher, Parson et al. 2001). Figure 2.4 shows the identification of two closely-related STR alleles within this gene. Allele 9.3 is a common variant of allele 10 that has a deoxyadenosine deletion. As demonstrated, these two alleles with only a single nucleotide difference were readily identified based on their masses. Sequencing of amplified fragments of two distinct polymorphic sequence-tagged sites (STSs) with the lengths of 76 and 114 bp was successfully achieved by performing HPLC-ESI-MS/MS on their PCR amplicons representing 2 SNPs in the 76 bp and 3 SNPs in the 114 bp sequences. Furthermore,

the resolving power of HPLC-ESI-MS (3-6 Da in a total mass of 31,000 Da) proved to be sufficient to discriminate between different haplotypes just based on their molecular weight. This high mass accuracy was attributed to multiple representations of the molecular mass in the form of different charge states (Oberacher, Oefner et al. 2002). In another study, nearly the complete sequence of a 61 bp PCR-amplified fragment of Y-chromosomal locus M9 was reconstructed by a ladder of incompletely elongated primers that was obtained unintentionally (Oberacher, Niederstätter et al. 2006). However, these truncated PCR amplicons do not seem to be generated every time and more efficient enzymatic strategies have been introduced to provide a ladder of single stranded DNA fragments for sequence identification by ESI-MS. These methods include the application of exonuclease III and lambda exonuclease or a combination of restriction enzymes (Null, Benson et al. 2003). Optimized suppression of cation adducts during ion-pair HPLC/ESI-MS by increasing the column temperature and adding EDTA as a chelating agent has made the quantitative genotyping of SNPs amplified from the tetraploid genome of the potato possible (Oberacher, Parson et al. 2004).

3- Metabolomics of RNA

Unlike molecular biology assays, mass spectrometry provides a direct observation of an intrinsic property of oligonucleotides: their mass. The specificity provided by this measurement allows for the straightforward quantification and identification of post-transcriptional modifications which is not available by other methods. Consequently, mass spectrometry is clearly the superior method for the study of RNA metabolomics. Because tRNA is the most highly modified type of RNA (Limbach, Crain et al. 1994, Agris 2004), we will devote this section to the study of LC-MS applications in identification and analysis of tRNA modifications.

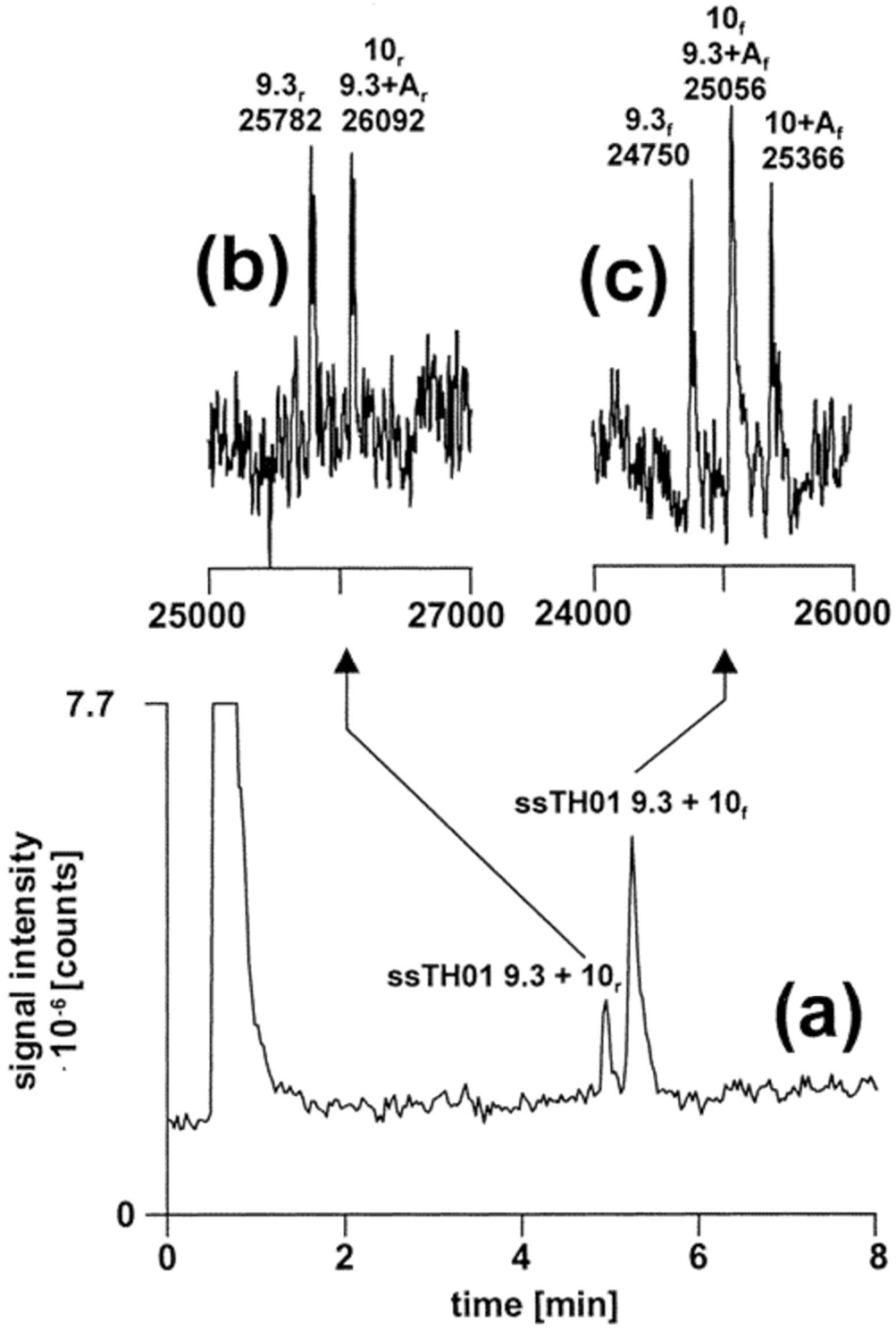


Figure 2.4- Determination of heterozygous alleles

Mixture of single stranded PCR-amplified fragments (f for forward and r for reverse strands respectively) from human tyrosine hydroxylase (TH01) alleles 9.3 and 10 were: a)

chromatographically separated and b,c) further identified by MS based on their theoretical masses (Reprinted with permission from (Oberacher, Parson et al. 2001). Copyright 2001 American Chemical Society).

Similar to the approach used for genotyping DNA polymorphisms, tRNA can be characterized through the selective digestion of intact molecules with endonucleases and sequencing of oligonucleotide fragments through the use of tandem mass spectrometry (without the need for the initial PCR amplification). These RNase mapping approaches have been proven successful in identification of post-transcriptionally modified RNAs (Kowalak, Pomerantz et al. 1993, Kowalak, Bruenger et al. 2000, McCloskey, Graham et al. 2001, Meng and Limbach 2004). A more efficient approach for tRNA separation and identification from the extremely complex cellular mixtures was introduced by Hossain and Limbach when they discovered that enzymatic digestion of individual tRNA species by a ribonuclease (e.g. RNase T1 or U2) can generate digestion products unique to that particular tRNA (Hossain and Limbach 2007). This set of unique or "signature" digestion products can be utilized to detect individual tRNAs in a total cellular tRNA pool. In an experiment, the entire tRNA families of *E. coli* could be identified via the unique *m/z* values of their signature enzymatic digestion products (SDPs) and further confirmation of SDP identities by obtaining their sequence information through tandem mass spectrometry (Wetzel and Limbach 2012). If tRNA samples from two different experimental conditions are digested in two parallel aqueous solutions of $H_2{}^{16}O$ or $H_2{}^{18}O$, a single ^{16}O or ^{18}O atom will be incorporated into each digestion product (remember that RNA digestion is a hydrolysis reaction). By combining these labeled digestion products, isotope pairs with a 2 Da

difference will emerge on the final mass spectrum and relative quantities of individual tRNAs can be calculated from the measured experimental ratios of $^{16}O/^{18}O$-labeled SDPs (Castleberry and Limbach 2010). Alternatively, a reference tRNA library (e.g. the fully sequenced tRNA pool of *E. coli*) can be labeled with one oxygen isotope during enzymatic digestion while an unknown library (e.g. cellular tRNA mixture from another bacterial species) is labeled with the other isotope of oxygen. After combining the two digests, digestion products that share the same post-transcriptional modification will appear as doublets separated by 2 Da. Modification differences, however, will generate singlets that could be further investigated (as shown in figure 2.5) to identify the exact nature of those alternative modifications (Li and Limbach 2012). This method, which is referred to as the comparative analysis of ribonucleic acid digests (CARD) (Li and Limbach 2013), provides a relatively fast and straightforward procedure for determination of post-transcriptional modifications given that a reference sample for the comparison can be acquired. In contrast to these "bottom-up" approaches, the "top-down" approach does not utilize any enzymatic digestion and the mixture is directly subjected to negative electrospray ionization followed by time-of-flight mass analysis. Here, individual tRNA components can be distinguished by their theoretical molecular masses or alternatively, precursor ions of interest can be selected to use their CID fragmentation pattern for sequence determination. The multiply charged fragment ions would be further charge-reduced via proton transfer ion/ion reactions to simplify the interpretation of the product ion mass spectrum. This approach was shown to be capable of distinguishing different tRNA molecules in a mixture provided by a commercial supplier and further demonstrating that the mass of $tRNA^{Phe}$ was not consistent with the nominal value for this tRNA; rather, the mass was consistent with $tRNA^{Phe}$ bearing an incomplete 3' terminus (Huang, Liu et al. 2010).

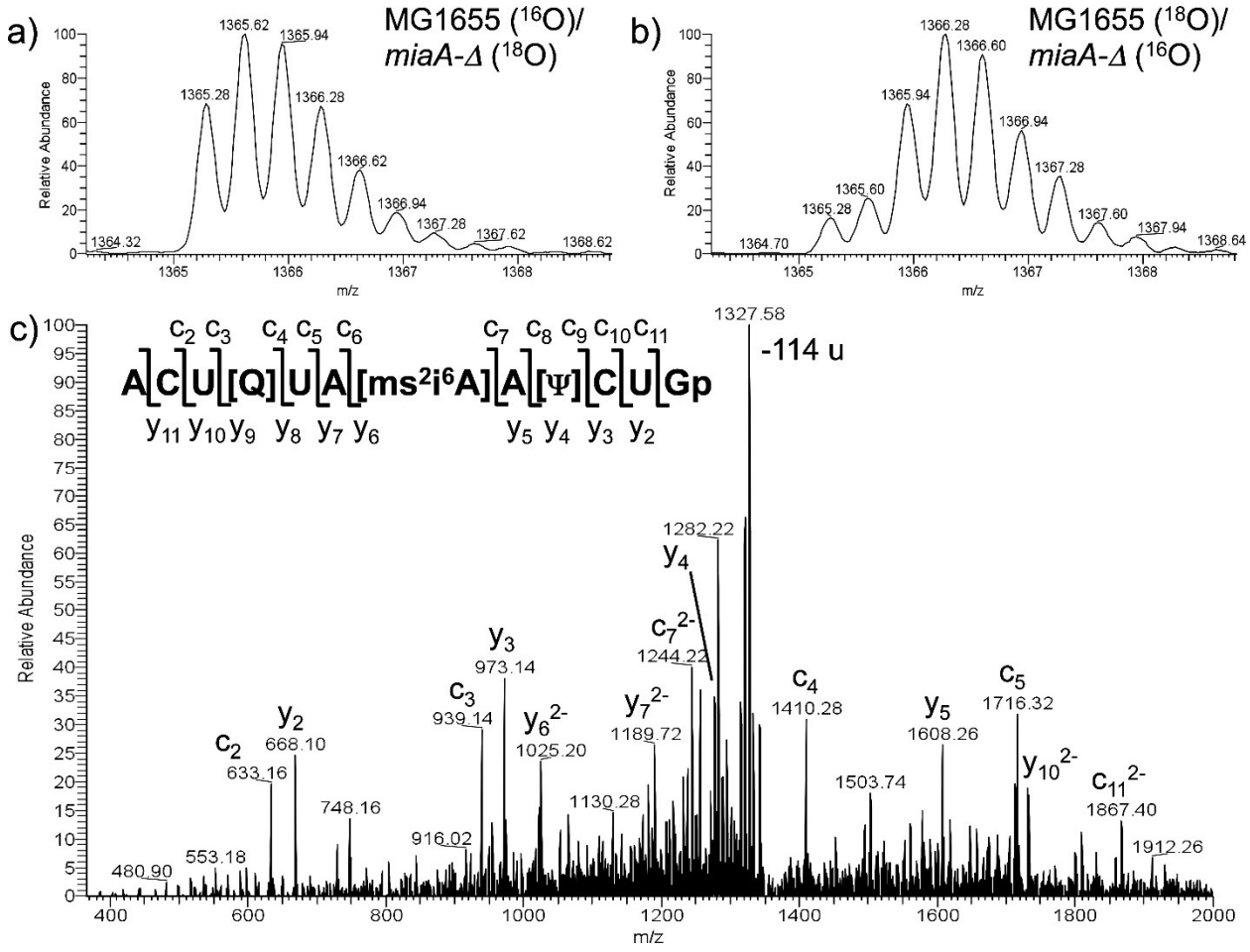


Figure 2.5- Comparative analysis of ribonucleic acid digests from wild-type (MG1655) and mutant miaA-Δ *E.coli* strains (Reprinted with permission from (Li and Limbach 2012). Copyright 2012 American Chemical Society).

a) Singlet ACU[Q]UA[ms^{2}i^{6}A]A[Ψ]CUGp (*m*/*z* 1365.2, 3$^-$ charge) arising from *E. coli* MG1655 after labeling miaA-Δ mutant with ^{18}O.

b) The same singlet after labeling *E. coli* MG1655 with ^{18}O. The +0.66 increase in the *m*/*z* isotopic envelope, corresponding to 2 Da at the −3 charge state, confirms the singlet arises from *E. coli* MG1655.

c) Collision-induced dissociation mass spectrum of the singlet ACU[Q]UA[ms^{2}i^{6}A]A[Ψ]CUGp from *E. coli* MG1655. The major peak corresponds

to a loss of 114 Da from the molecular ion. This loss is consistent with loss of the 2-methylthio-6-isopentenyl group during CID.

C. LC-MS and the study of therapeutics

1- Quality control and stability of oligonucleotide drugs

A suitable analytical method for use in pharmaceutical industry should be able to provide modification positions and aberrant chemical damage in addition to sequence information. The use of MS as an analytical tool for quality control and impurity identification during manufacturing of oligonucleotides is a common practice. Therapeutic oligonucleotides are produced via a stepwise synthetic process. Despite yields as high as 99.5% per step, a simple 21-mer oligonucleotide would have a maximum purity of 90% without further purification (McCarthy, Gilar et al. 2009). LC-MS has been critical to the identification of synthesis impurities such as failure sequences carrying 3'-terminal phosphate and phosphorothioate monoesters or incomplete backbone sulfurization and desulfurization products for phosphorothioate oligonucleotides (Ravikumar, Krishna Kumar et al. 2003, Capaldi, Gaus et al. 2004, Gaus, Olsen et al. 2005, Oberacher, Niederstätter et al. 2005, Kurata, Bradley et al. 2006, Nikcevic, Wyrzykiewicz et al. 2011). Ion pair RPLC has also been shown to be applicable to the separation and purification of siRNAs after synthesis (McCarthy, Gilar et al. 2009). For larger RNA molecules, such as rRNA, the use of macro-porous PS-DVB resin as the stationary phase along with a phosphate-containing mobile phase is thoroughly suited to preparative separations (Yamauchi, Taoka et al. 2013).

Understanding the exact location of oligonucleotide degradation is important for drug development as those sites can be chemically modified to improve drug stability. Identification of nuclease-derived fragments is often possible by measurements of their *m/z* values alone, as they usually have unique masses. In cases where the masses of different fragments are similar, identification can be achieved by tandem MS sequencing. Even oligonucleotides with multiple 2' fluoro and 2' O-methyl modifications have been determined by tandem mass spectrometry (Farand and Beverly 2008) and in one case an siRNA has been sequenced *de novo* using this approach (Farand and Gosselin 2009). More recently, after examining oligonucleotides with various types of 2'-position modifications (Figure 2.6), Gao et al. concluded that ion trap-CID is sufficient for characterizing 2'-chemically modified oligonucleotide sequences that do not contain DNA residues (Gao, Yang et al. 2013).

2- Metabolism of oligonucleotide drugs

Oligonucleotides are primarily degraded *in vivo* by nucleases that cleave the phosphodiester linkages at the ends of the strand (exonucleases) or in the middle of it (endonucleases) (McGinnis, Chen et al. 2012). As a result, identification of oligonucleotide metabolites is very similar to sequencing experiments with the aim of recognizing the truncated oligonucleotide strands. For sequencing, usually a high amount of oligonucleotide is available and the main focus is on simplifying the mass spectrum by reducing cation adduction and other reactions that may convolute the spectrum even at the expense of signal intensity and sample loss. Additionally, the distribution of analytes between different charge states can sometimes be helpful for sequence determination purposes as it provides multiple measurements for the mass of each fragment. In contrast, method development for the analysis of oligonucleotide drug metabolism only focuses

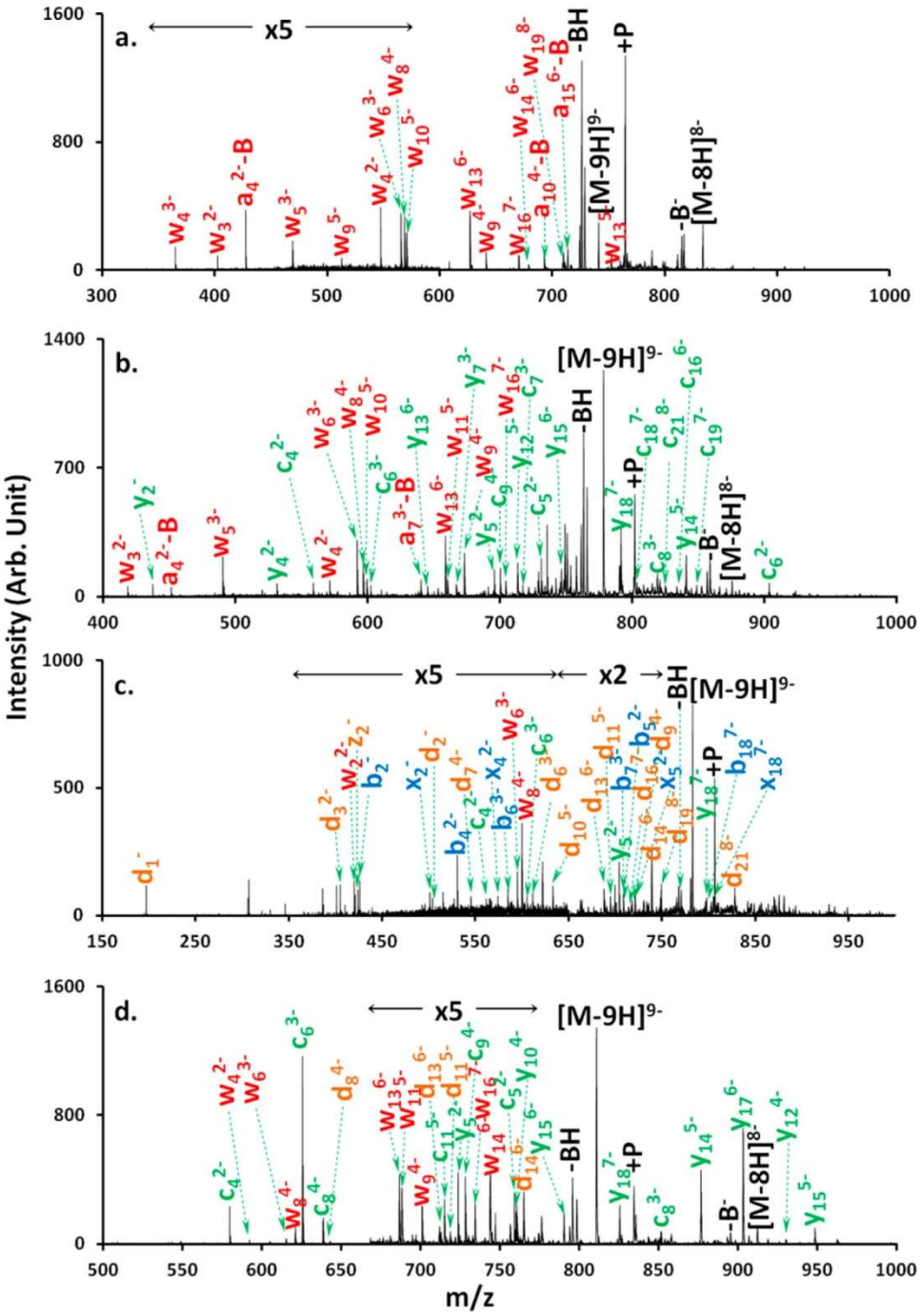


Figure 2.6- Product spectra from ion trap-CID of a 21-mer oligonucleotide with various 2' modifications

These spectra are obtained from CID of the common sequence 5′-CCAGGGCGAUGCCUUCCA UTT-3′ with a) 2'-H deoxyribose backbone, b) 2'-OH ribose backbone, c) 2'-F and d) 2'-OMe

ribose backbones. Notice the emergence of diagnostic a-B/w and c/y ion series in (a) and (b) respectively. The diagnostic c/y series are still produced after incorporation of the widely-used 2'-OMe modification. For a detailed discussion of fragment ion nomenclature see section B.1 and Figure 2.3. (Reprinted with permission from (Gao, Yang et al. 2013). Copyright 2013 American Chemical Society).

on lowering the detection limits because the analyte is present at very low concentrations and in this case, deconvolution of more complicated mass spectra is possible considering the *a priori* knowledge of the sequence. So the ideal LC-MS method for oligonucleotide drugs should preserve as much sample as possible and significantly reduce the charge state distribution. The excellent review by van Dongen and Niessen has extensively covered the previous applications of LC-MS for oligonucleotide drug bioanalysis in the literature (van Dongen and Niessen 2011) and the reader is encouraged to refer to that article for a complete list. However, a few examples will be provided here to demonstrate the capabilities of this analytical method.

In one study, ocular metabolites of a therapeutic siRNA targeting the VEGF receptor were extracted from ocular vitreous humor and retinal tissue using phenol/chloroform LLE and examined by LC-MS with TEA/HFIP. The siRNA was analyzed as an intact duplex as well as single strands after heating the column above the melting point. Metabolism products revealed a degradation pattern in which the end of the duplex with the weakest interstrand binding energy was most susceptible to degradation (Beverly, Hartsough et al. 2006). High resolution orbi-trap instruments have been used for identification of metabolites of a chemically modified siRNA in plasma and liver microsomes. The resulting siRNA metabolism profiles for liver microsomes

versus plasma highlighted the different nucleases present in each matrix (Zou, Tiller et al. 2008). Recently, quantification of a phosphorothioate oligonucleotide (PF-ODN) and its 5'N-1, 3'N-1, 5'N-2 and 5'N-3 metabolites with the LOQ of 4.0 ng/mL in rat plasma allowed for a preliminary pharmacokinetic study of this PF-ODN in rats (Deng, Chen et al. 2010). Chen and Bartlett observed similar PK parameters for a 24-mer phosphorothioate DNA and its truncated metabolites in rat plasma with a decreased LOQ of 2.5 ng/mL (Chen and Bartlett 2013). By using online SPE systems, two separate groups even managed to achieve sub-ng/mL detection limits for oligonucleotides: 0.5 ng/mL LOQ for an 18-mer phosphorothioate DNA oligonucleotide in human plasma (Wang, Yuan et al. 2009) and ~ 50 pg/mL for a 15-mer unmodified DNA oligomer (Hemsley, Ewles et al. 2012). Most recently, McGinnis et al. measured the amounts of a 21-mer RNA duplex with 2 deoxynucleotide overhangs at each 3' end in cell culture media and prostate cancer cells over a period of 36 hours and showed the cellular uptake of the siRNA along with its disappearance from the media. They were also able to identify and measure the concentrations of the metabolites arising from the truncations of the 5' ends of each strand by one or 2 nucleotides with a LOQ of 6.0 ng/mL, hence reassuring the capability of chromatographic methods for the study of siRNA duplexes (McGinnis, Cummings et al. 2013).

Conclusion

High performance liquid chromatography coupled with negative-ion electrospray MS provides a reliable method with unique capabilities for separation and identification of therapeutic oligonucleotides. It has been successfully utilized for quality control, purification, recognition, quantification, sequencing and metabolic profiling of oligonucleotides in

experimental settings and with decreasing LOQ levels, it becomes more applicable to clinical studies. As a result of recent findings, we have a much better understanding of the mechanism of electrospray ionization of oligonucleotides in the presence of ion-pairing agents and HFIP which makes systematic optimization of the mobile phase composition possible. LC-MS also plays an indispensable role for the study of RNA metabolomics because it is the only method that allows for the direct determination of RNA modifications. As previously discussed, innovative methods such as isotope labeling of signature enzymatic digestion products make it possible to perform metabolomic studies in a relatively fast and easy manner.

Future perspective

Although biochemical approaches such as PCR and ELISA are currently commonplace in pre-clinical and clinical studies of oligonucleotide therapeutics, their inherent disadvantages compared to LC-MS will eventually cause them to be replaced by mass spectrometric methods. Unlike other methods, mass spectrometry allows for quantification and identification of an oligonucleotide analyte, its impurities and its metabolites. MS techniques can also be used to sequence the oligonucleotide, often at the same time, offering an additional level of analysis. Moreover, double-stranded oligonucleotides can be analyzed intact which is more relevant to their physiological function. The major hindrance to the implementation of LC-MS methods to analysis of oligonucleotide drugs has been caused by their unsatisfactory detection limits. However, as recent studies are pushing this limit well below the 1.0 ng/mL level and with our current knowledge of the effect of ion pairing systems and organic solvents on signal intensity, it is predictable that LC-MS methods with adequate sensitivity will play a more important role in the bioanalysis of existing and emergent oligonucleotide biomarkers and drugs in the near future.

Executive summary:

LC/MS Coupling

- LC coupled with ESI-MS allows for the excellent separation and identification of oligonucleotides.
- For best analytical results, the mobile phase should consist of a suitable ion pairing agent along with HFIP dissolved in an alcohol.
- IP agents should be chosen based on their Henry's law constants in regard to the hydrophobicity of oligonucleotide analytes, and mobile phase solvents based on their dielectric constants.
- HFIP needs to be used at concentrations below 100 mM.

SNP Genotyping

- Several methods have been practiced for the reduction of the charge state distribution and cation adduction during oligonucleotide ESI. Utilizing these methods allows for the identification of oligonucleotides at lower concentrations.

Metabolism of Oligonucleotide Drugs

- LC-MS has currently reached sub-ng/mL detection limits and considering its successful application for preliminary PK studies in the past, it will soon be utilized during the PK studies of oligonucleotides in clinical trials.

CHAPTER 3

ASSESING THE INTERPLAY BETWEEN THE PHYSICOCHEMICAL PARAMETERS OF ION-PAIRING REAGENTS AND THE ANALYTE SEQUENCE ON THE ELECTROSPRAY DESORPTION PROCESS FOR OLIGONUCLEOTIDES[2]

[2] Babak Basiri, Mandi M. Murph and Michael G. Bartlett. Accepted by *Journal of the American Society for Mass Spectrometry*.

Reprinted here with permission of the publisher.

Abstract

Alkylamines are widely used as ion-pairing agents during LC-MS of oligonucleotides. In addition to a better chromatographic separation, they also assist with the desorption of oligonucleotide ions into the gas phase, cause charge state reduction and decrease cation adduction. However, the choice of such ion-pairing agents has considerable influence on the MS signal intensity of oligonucleotides as they can also cause significant ion suppression. Interestingly, optimal ion-pairing agents should be selected on a case by case basis as their choice is strongly influenced by the sequence of the oligonucleotide under investigation. Despite imposing major practical difficulties to analytical method development, such a highly variable system that responds very strongly to the nuances of the electrospray composition provides an excellent opportunity for a fundamental study of the electrospray ionization process. Our investigations using this system quantitatively revealed the major factors that influenced the ESI ionization efficiency of oligonucleotides. Parameters such as boiling point, proton affinity, partition coefficient, water solubility and Henry's law constants for the ion-pairing reagents and the hydrophobic thymine content of the oligonucleotides were found to be the most significant contributors. Identification of these parameters also allowed for the development of a statistical predictive algorithm that can assist with the choice of an optimum IP agent for each particular oligonucleotide sequence. We believe that research in the field of oligonucleotide bioanalysis will significantly benefit from this algorithm (included in supplementary material) as it advocates for the use of lesser-known but more suitable ion-pair alternatives to TEA for many oligonucleotide sequences.

Introduction

Mass spectrometry is one of the most widely used analytical techniques for biomedical applications (Woods and Darie 2014). This has only become possible as electrospray ionization (ESI) (Fenn 2003) and matrix-assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp 1988) provided the necessary means for generating gaseous ions from biological macromolecules (Tanaka, Waki et al. 1988, Fenn, Mann et al. 1989, Berkenkamp, Kirpekar et al. 1998). Electrospray ionization is particularly well suited for biochemical analysis due to its ease of coupling liquid chromatography with mass spectrometry, as well as its ability to preserve specific solution phase interactions between molecules into the gas phase (Heck and van den Heuvel 2004, Nilsson, Mann et al. 2010, Kitova, El-Hawiet et al. 2012, Liuni, Jeganathan et al. 2012, Percy, Rey et al. 2012). Nevertheless, despite the widespread expansion of applications involving ESI-MS, there is still significant debate on the mechanisms by which gaseous ions are formed through electrospray ionization (Nguyen and Fenn 2007, Hogan, Carroll et al. 2009).

According to the charged residue model (CRM) (Dole, Mack et al. 1968, Mack, Kralik et al. 1970) as the droplet size decreases by evaporation of solvent, it will reach the Rayleigh limit and break up into a number of smaller droplets. These second-generation droplets would continue to subdivide into still smaller droplets. A succession of such coulombic fissions would eventually lead to the formation of ultimate droplets so small that each of them would only contain one analyte molecule. Conversely, the ion evaporation model (IEM) argues that before a charged droplet becomes small enough to contain only one solute molecule, the charge density on its surface would become so high that the resulting electric field pushes one or more of those surface ions into the gas phase (Iribarne and Thomson 1976). The general agreement used to be that small molecules are primarily ionized via IEM, while the formation of large macromolecular

ions is better explained by CRM (Kebarle and Verkerk 2010). However, early nanospray experiments revealed that shorter chain-like proteins (such as insulin) can leave the ESI droplet when it is still quite large and contains more than one molecule of the analyte (Juraschek, Dülcks et al. 1999). The more recently proposed chain ejection model (CEM) applies to such conditions. Based on this model, unlike globular natively-folded proteins, unfolded proteins and disordered polymer chains immediately migrate to the surface when placed in a Rayleigh-charged nanodroplet in order to minimize contact between their exposed hydrophobic moieties and the aqueous microenvironment within the droplet interior. One chain terminus then gets expelled into the vapor phase, followed by stepwise sequential ejection of the remaining protein and separation from the droplet (Konermann, Ahadi et al. 2013, Yue, Vahidi et al. 2014). Despite its success in explaining the ionization of unfolded protein chains, the CEM is unlikely to apply to nucleic acid chains because their hydrophobic and hydrophilic moieties are homogenously distributed along the chain (Abi-Ghanem and Gabelica 2014).

An attempt for generating a predictive model for the MS signal intensity of small ions based on the IEM has been successful. Under this model (Tang and Kebarle 1991, Tang and Kebarle 1993), solvation energy and surface activity of the ions are the major determinants of their MS response. In contrast, the main focus of the theoretical models of CRM has been to predict the charge state of ionized proteins. Based on these models, the maximum charge of a protein in the gas phase does not depend on the analyte charge in solution. It is rather governed by the Rayleigh charge limit of the ultimate electrospray droplet that contains only one protein molecule. This value is only dependent on the size of a protein which can either be represented via the protein radius (Fernandez de la Mora 2000) or its surface area (Kaltashov and Mohimen

2005). However, no general theoretical framework for the prediction of ion intensity of large analytes which are produced by the CRM can be found.

The equilibrium partitioning model of Cech and Enke (Cech and Enke 2000) takes a distinct approach for predicting ESI ion intensities that could be applicable to both IEM and CRM. The basic postulation of this model is that because the excess charge resides on the surface of ESI droplets, only the analytes that can migrate to the surface will be charged and eventually detected by a mass spectrometer (Cech and Enke 2001). Therefore, the variability in the MS response of various compounds can be explained by their surface activity. Several parameters including nonpolar surface area, Gibbs free energy of transfer from nonpolar to polar solutions and reversed phase HPLC retention factors have been used as surrogates of surface activity in this model with varying degrees of success (Cech and Enke 2001).

There have also been empirical studies of the factors that influence ESI signal intensity of small organic compounds. Some parameters identified in various studies were: the degree of conjugation (Huffman, Poltash et al. 2012), charge delocalization (Kruve, Kaupmees et al. 2014), partition coefficient (Henriksen, Juhler et al. 2005, Chalcraft, Lee et al. 2009, Liigand, Kruve et al. 2014), gas phase basicity (Ehrmann, Henriksen et al. 2008), hydrogen to carbon (H/C) ratio (Nguyen, Nizkorodov et al. 2013) and molecular volume (Chalcraft, Lee et al. 2009). Nevertheless, in the particular case of oligonucleotides, very few investigations have been performed. It has been reported that solution pH (Bleicher and Bayer 1994), organic solvent percentage (Muddiman, Cheng et al. 1996) and analyte hydrophobicity (Null, Nepomuceno et al. 2003) can affect the signal intensity of oligonucleotides from pure solutions. But since LC-MS of oligonucleotides is almost always performed in the presence of alkylamine ion-pairing (IP) agents, the following studies that have included the use of IP agents are more relevant.

Gaus *et al.* (Gaus, Owens et al. 1997) compared the signal intensity for phosphorothioates when using seven different alkylamines as IP agents and observed the highest ion intensity with tripropylamine (TPA). Erb and Oberacher (Erb and Oberacher 2014) compared triethylamine (TEA) with dimethylcyclohexylamine (DMCHA) and concluded that TEA generates a stronger signal for a 27-mer DNA. Sharma *et al.* (Sharma, Glick et al. 2012) used six different alkylamines (including DMCHA, TPA and TEA) with a 17-mer DNA strand and observed the best MS sensitivity with dimethylbutylamine (DMBA). In an attempt to explain the effect of IP agents on oligonucleotide ion intensity, Chen *et al.* (Chen, Mason et al. 2013) carefully examined the signal intensity of a 24-mer DNA oligonucleotide in the presence of seven different IP agents and found a very strong relationship between the Henry's law constant of the alkylamines and MS signal intensity of the oligonucleotide. The IP agents with a lower Henry's law constant generated stronger MS signals for the oligonucleotide (Chen, Mason et al. 2013). Nevertheless, it was later demonstrated that the effect of IP agents on the signal intensity of oligonucleotides is very much sequence-dependent and cannot be solely explained by the Henry's law constant (McGinnis, Grubb et al. 2013, Gong 2015).

In this manuscript, we present a comprehensive study of the IP and oligonucleotide-related parameters that influence the ESI-MS signal intensity using 11 different DNA sequences and 15 different IP agents. Statistical analysis of this set of data reveals important details about the factors that impact ESI efficiency. It will be further discussed in the following sections that identification of these factors provides the necessary means for a more detailed mechanistic understanding of the ESI process. This understanding is particularly important for oligonucleotides – as well as many other macromolecules – because several explanations have been put forward regarding their interactions in the electrospray droplet (Muddiman, Cheng et al.

1996, Cech and Enke 2000, Null, Nepomuceno et al. 2003, Chen, Mason et al. 2013) and each explanation is successful for interpreting some experimental results, indicating that all of them are partially accurate. Therefore, a more quantitative account of such interactions is necessary for generating an integrative model of the oligonucleotide ESI process. Furthermore, as demonstrated in the results section, distinguishing the major parameters that affect the ESI signal intensity of oligonucleotides allows for the generation of an empirical formula for the prediction of oligonucleotide signal intensity in various mobile phase compositions. The proposed formula has extensive applications for LC-MS method development.

Materials and methods

Chemicals and reagents: The ion pairing agents N,N-dimethylbutylamine (DMBA), octylamine (OA), tripropylamine (TPA), N,N-dimethylhexylamine (DMHA), diisopropylamine (DIPA), N-methyldibutylamine (MDBA), propylamine (PA), triethylamine (TEA), hexylamine (HA), tributylamine (TBA), N,N-dimethylcyclohexylamine (DMCHA), N,N-diisopropylethylamine (DIEA), tetramethylethylenediamine (TMEDA), dibutylamine (DBA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), as well as LC-MS grade methanol and water were purchased from Sigma-Aldrich Inc. (St. Louis, MO). DNA Lobind microcentrifuge tubes were purchased from Eppendorf (Hauppauge, NY). The ssDNA strands with the following sequences were purchased from Eurogentec (Seraing, Belgium): AAAAAAAAAAAAAAAAAAAAAA, TTTTTTTTTTTTTTTTTTTTTT, CCCCCCCCCCCCCCCCCCCCCC, GTGTGTGTGTGTGTGTGTGTGT, ATTTCTTTGTTTATTTCTTTGTTT, ATTCTTGTTATTCTTGTTATTCTT, ATCTGTATCTGTATCTGTATCTGT, TCGTACTAGTGGTCCTAATCGTAC,

ATCGATCGATCGATCGATCGATCG, ACGACGACGTTTACGACGACGACG and CGGAGGAAACCTACGACGAGGAAA. The 24-mer TCGTGCTTTTGTTGTTTTCGCGTT was purchased from Integrated DNA Technologies (Coralville, IA).

Preparation of working solutions for direct infusion experiments: Pre-calculated volumes of various IP agents were added to aliquots of 20 μg/mL solutions of oligonucleotides in 50:50 methanol/water prepared fresh each day during the course of this investigation to the final concentration of 15 mM for all IP agents, except DBU. The final concentration of DBU was 2.5 mM. Importantly, all samples were prepared and analyzed in DNA Lobind tubes in order to eliminate any sample to sample variation due to differential non-specific losses to the tube walls. The pH of all of these oligonucleotide/ion-pair solutions fell in the very narrow range of 8.5 to 9.5 without adding any buffers.

Instrumental conditions: Samples were directly infused to a Waters (Milford, MA) Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer in the negative ion electrospray ionization mode via the instrument's built-in fluidics system. The TOF-MS tuning parameters were as follows: capillary voltage -2.0 kV, cone voltage 25 V, extraction cone voltage 2 V, source temperature 125 ˚C, desolvation temperature 450 ˚C, cone gas 0 L/h and desolvation gas (nitrogen) 1000 L/h. The infusion flow rate was set to 50 μL/min and the data were collected in continuum full-scan MS mode with a 1 s scan time over the mass range from 500–3000 m/z. All measurements were performed in triplicate and base peak signal intensities were measured by combining 50 scans.

Calculation of the normalized signal intensity of the oligonucleotide/ion-pair solutions: It is a well-known phenomenon that absolute ESI-MS signal intensities are arbitrary and quite variable from experiment to experiment. Therefore, the establishment of a reliable electrospray

ionization efficiency indicator that can be used to allow for comparisons among results obtained over several days of these experiments was an absolute necessity. It could be argued that the summation of all charge states detected for an oligonucleotide is necessary in order to represent its ionization efficiency. Nevertheless, since the charge state distribution of an oligonucleotide does not change dramatically with the use of different alkylamines, the ion count of the most intense charge state (the base peak) changes proportionally to the total current of all charge states. Therefore, for the sake of simplicity, we selected to use the MS signal intensity of the base analyte peak instead of the total ion current for calculating the ionization efficiency of oligonucleotides as described below. An inspection of figure 3.1 clearly indicates that calculations based on these two parameters (base peak intensity vs. sum of all charge states) does not generate any significant differences in the final results.

To deal with the highly varying base peak ESI-MS signal intensities for the same solution from day to day, we decided to use the oligonucleotide solutions in TMEDA as the reference. Therefore, the ionization efficiency of oligonucleotides in different solutions was reported as their normalized signal intensity which was calculated as the ratio of their base peak intensity in any particular solution to the MS signal intensity of the base peak for the same oligonucleotide in the TMEDA solution:

$$Normalized\ signal\ Intensity\ of\ oligo\ against\ IP_x = \frac{ESI-MS\ signal\ Intensity\ of Oligo\ in\ IP_x\ solution}{ESI-MS\ sign \quad Intensity\ of\ oligo\ in\ TMEDA\ solution}$$

The logic behind this method and other merits of this approach are further discussed in the "Results" section.

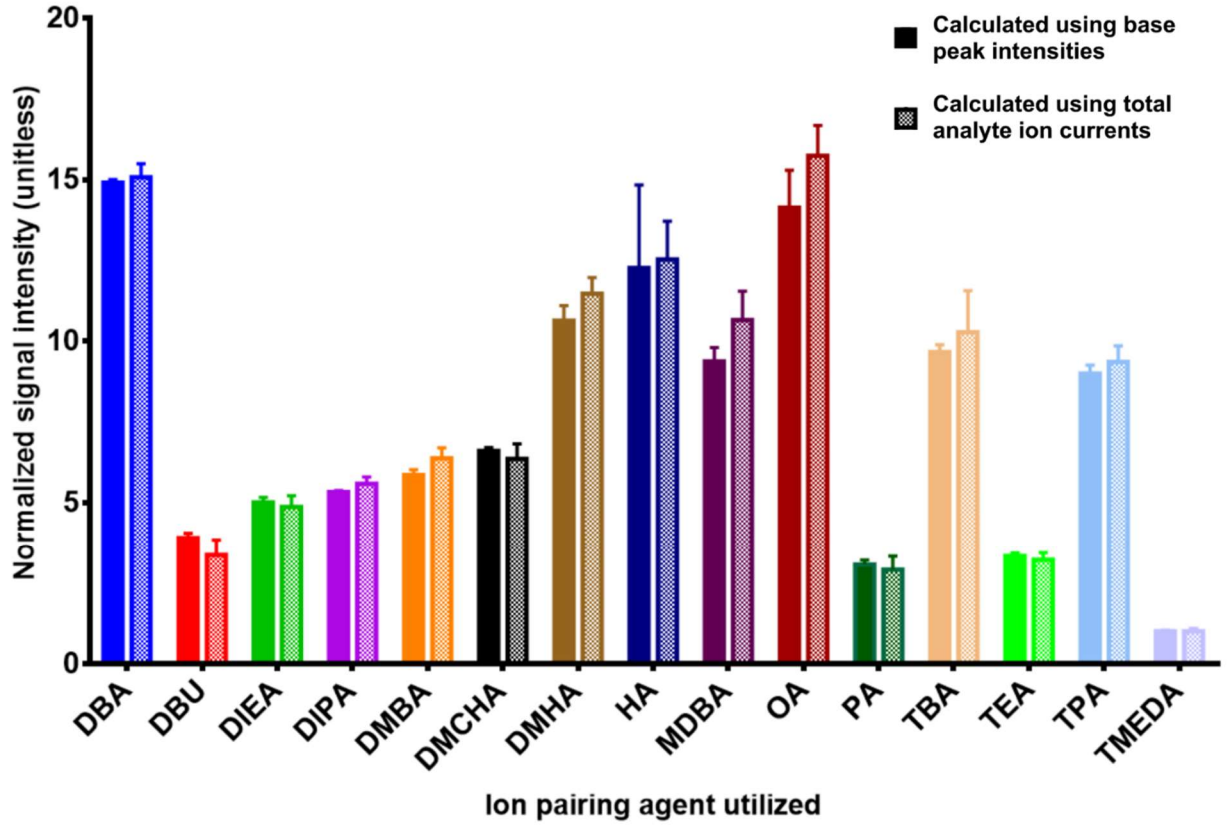


Figure 3.1- The normalized signal intensity of T_{24} in the presence of various IP agents calculated using either the oligonucleotide base peak signal intensities (solid bars) or the total ion counts of all oligonucleotide charge states (checkered bars)

Databases and computer software: The MS operation and data acquisition were performed using Waters (Milford, MA) MassLynx 4.1. Statistical analyses and graphs were created using GraphPad Software (La Jolla, CA) Prism 6 and SAS JMP Pro 12 (Cary, NC). Henry's law constants were computed using HENRYWIN module of the US Environmental Protection Agency's EPI suite v4.11 (Washington, DC). Proton affinity and gas-phase basicity values were obtained from the NIST database (http://webbook.nist.gov/chemistry/). The remaining

physicochemical parameters were obtained from the CAS REGISTRY of the American Chemical Society (https://scifinder.cas.org/).

Prediction Formula: The signal intensity prediction formula is as follows. We have also supplemented a Microsoft Excel sheet containing this formula with the online version of this manuscript for the convenience of our readers. Please note that Content A, T, C and G need to be entered as **decimals**.

$$\textit{Predicted Normalized Signal Intensity} = -0.00656\ \textit{MW} - 5.43532\ \textit{Density} + 0.02322\ \textit{Boiling Point} + 1.61079\ \textit{pKa} - 0.12832\ \textit{Proton Affinity} - 0.14625\ \textit{Gas Phase Basicity} + 0.23521\ \textit{Partition Coefficient} + 0.00005\ \textit{Water Solubility} - 0.00012\ \textit{Vapor Pressure} + 0.00340\ \textit{Henry's Law Constant} + 4.75149\ \textit{Content A} + 7.00368\ \textit{Content T} + 4.39043\ \textit{Content C} + 0.55245\ \textit{Content G} + 47.72180$$

Results and Discussion

In order to determine the extent to which different parameters influence the ESI ion intensity of oligonucleotides, we chose 11 DNA sequences of varying compositions and tested each one alongside 15 different ion-pairing agents with distinct physicochemical properties. Tables 3.1 and 3.2 list the oligonucleotide sequences and IP agents utilized for this study, respectively. Because previous studies in our laboratory (Chen, Mason et al. 2013, McGinnis, Grubb et al. 2013) had demonstrated that alkylamine IP agents produce the highest oligonucleotide MS signal intensity when used at concentrations around 15 mM, we used all alkylamine IP agents at this concentration. The only exception was DBU which was used at 2.5 mM in order to maximize its signal intensity as suggested by Sharma *et al.* (Sharma, Glick et al. 2012) and confirmed through our own observations. Furthermore, an oligonucleotide

concentration of 20 µg/mL was chosen because we were able to acquire robust mass spectra at this concentration which made data interpretation easier. We should also mention that the performance of ion-pairing reagents was not dependent on the concentration of the oligonucleotides; i.e., the ion-pairing reagent that generated the strongest MS signal intensity at higher oligonucleotide concentrations, also had the best performance at lower oligonucleotide concentrations. Therefore, the concentration of the studied oligonucleotides was kept constant throughout this investigation.

Table 3.1- Sequences of 11 different DNA strands that were used for this study.

Identifier	**Sequence (5'→3')**
100% A	AAAAAAAAAAAAAAAAAAAAAA
100% T	TTTTTTTTTTTTTTTTTTTTT
100% C	CCCCCCCCCCCCCCCCCCCCC
50% G	GTGTGTGTGTGTGTGTGTGTGT
75% T	ATTTCTTTGTTTATTTCTTTGTTT
66% T	ATTCTTGTTATTCTTGTTATTCTT
50% T	ATCTGTATCTGTATCTGTATCTGT
33% T	TCGTACTAGTGGTCCTAATCGTAC
25% T	ATCGATCGATCGATCGATCGATCG
13% T	ACGACGACGTTTACGACGACGACG
4% T	CGGAGGAAACCTACGACGAGGAAA

Table 3.2- Physicochemical parameters of 15 alkylamine ion-pairs of this study

IP agent	MW (g/mol)	Density (g/mL)	Boiling point (˚C)	pKa	Proton affinity (Kcal/mol)	Gas phase basicity (Kcal/mol)	Partition coefficient ($logP_{oct/wat}$)	Water solubility (g/L at pH=10)	Vapor pressure (Pa)	Henry's law constant (H^{cp}, $\frac{mol}{m^3\ Pa}$)
DIPA	101.19	0.72	83.9	11.05	232.3	224.3	1.364	235	10600	0.103
DIEA	129.24	0.74	127	10.5	237.6	230.3	2.354	87	1560	0.065
DMBA	101.19	0.72	95.9	10.02	231.6	224.2	1.647	51	6190	0.115
DMCHA	127.23	0.85	162	10.49	235.1	227.7	2.091	71	335	0.420
DMHA	129.24	0.74	147.1	9.99	231.5	223.5	2.666	13	771	0.065
HA	101.19	0.77	130	10.56	221.7	213.6	2.075	66	1180	0.368
MDBA	143.27	0.75	164.6	10.5	231.32	223.39	3.175	5.7	277	0.049
OA	129.24	0.78	179.6	10.65	221.86	213.77	3.094	17	129	0.012
PA	59.11	0.72	46.9	10.53	219.4	211.3	0.547	544	41500	0.667
TMEDA	116.2	0.78	121	8.97	242.07	232	-0.037	688	2000	409.836
TBA	185.35	0.78	216.5	10.89	238.6	231.3	4.704	0.67	12.5	0.062
TEA	101.19	0.73	89.7	10.65	234.7	227	1.647	155	7610	0.066
TPA	143.27	0.76	156	10.65	236.9	229.5	3.175	6.2	355	0.026
DBU	152.24	1.04	97	13.28	250.45	242.43	1.132	283	0.715	5.128
DBA	129.24	0.77	159	11.03	231.48	223.54	2.695	74	280	0.111

DIPA: diisopropylamine

DIEA: N,N-diisopropylethylamine

DMBA: N,N-dimethylbutylamine

DMCHA: N,N-dimethylcyclohexylamine

DMHA: N,N-dimethylhexylamine

HA: hexylamine

MDBA: N-methyldibutylamine

OA: octylamine

PA: propylamine

TMEDA: tetramethylethylenediamine

TBA: tributylamine

TEA: triethylamine

TPA: tripropylamine

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene

DBA: dibutylamine

Figure 3.2 shows the representative data acquired from our experiment with two different DNA strands. It is clear that in the absence of IP agents the oligonucleotide MS signal was weaker than when IP agents were being utilized. This observation might initially seem contradictive considering that IP agents are highly surface active and it is generally accepted that surface active additives cause ion suppression (Annesley 2003, Cech and Enke 2010). However, the model suggested by Chen *et al.* (Chen, Mason et al. 2013) can perfectly explain this phenomenon. Based on their model, the interaction between oligonucleotides and highly surface active IP agents helps oligonucleotides to reach the surface of the electrospray droplet. This effect is further amplified due to the alkylamine concentration gradient that exists from the center to the surface of the electrospray droplet because of the continuous ion-pair evaporation from the surface. Nevertheless, this explanation is only true when the ion-pair concentrations are not high enough to hinder oligo access to the surface of the droplet. That is why the concentrations of the ion-pairing reagents need to be chosen very carefully. The concentration of 15 mM (or 2.5 mM for DBU) completely satisfies this need.

Based on their model, Chen *et al.* also suggested that IP agents with a lower Henry's law constant would evaporate faster and create a more steep concentration gradient from the droplet center to the surface leading to stronger oligonucleotide MS signal intensity (Chen, Mason et al. 2013). Examining Figure 3.2 reveals that this scheme can only explain the extreme cases. For example, OA has the smallest Henry's law constant and it is often among the top performing IP agents while TMEDA with a very large Henry's law constant often performs poorly. Nevertheless, while HA has a much larger Henry's law constant than MDBA, it has performed better in both cases. Yet, perhaps the most interesting observation was the different performance patterns of IP agents based on the oligonocleotide sequence. These observations indicated the

need for a comprehensive set of experiments to understand the nuances that can influence the ESI ion intensity of oligonucleotides.

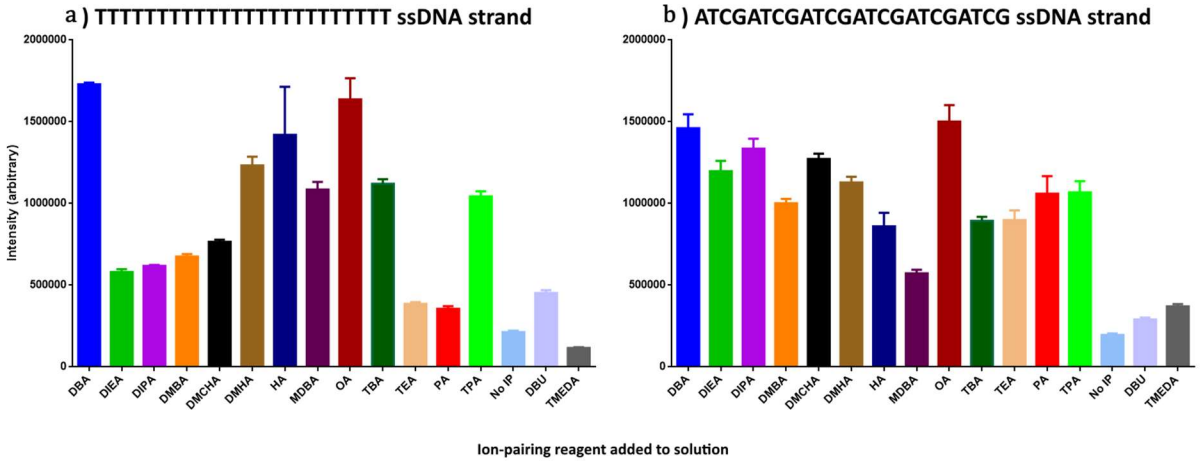


Figure 3.2- MS signal intensity of the base peak in the presence of various alkylamine IP agents for T_{24} (a) and $[ATCG]_6$ (b).

It is very well known that the absolute ESI-MS signal intensities are quite variable for an experiment performed at different occasions, even at similar experimental conditions. Therefore, a major prerequisite for performing this investigation was the establishment of a reliable ESI-MS intensity indicator that can be used to allow for comparisons among results obtained over several days of these experiments. To this end, we monitored the signal intensity of oligonucleotide solutions in the presence of various alkylamine ion-pairing agents over the course of a few days. Our results indicated that by choosing one of the oligo/IP solutions as a reference and reporting the ratio of MS signal intensity for other mixtures of the same oligonucleotide to the reference solution, a much more dependable measure of the MS signal intensity could be generated with

minimal variability between the experiments. This approach was consistent with the efforts of Leito and colleagues in generating an electrospray ionization efficiency scale (Leito, Herodes et al. 2008, Oss, Kruve et al. 2010, Liigand, Kruve et al. 2015). Since oligonucleotide solutions usually had the lowest signal intensities in TMEDA solutions, the oligonucleotide/TMEDA solutions were chosen as references and all other intensities were reported as their ratios to these reference solutions:

$$Normalized\ signal\ Intensity\ of\ oligo\ against\ IP_x = \frac{ESI-MS\ signal\ Intensity\ of Oligo\ in\ IP_x\ solution}{ESI-MS\ signal\ Intensity\ of\ oligo\ in\ TMEDA\ solution}$$

Table 3.3 shows the absolute and relative intensities of similar oligonucleotide solutions that were prepared and measured on two different days. It is seen that while the difference in absolute intensity from one day to another can easily exceed 30%, normalized intensities were not more than 15% different. Note that while only a representative number of measurements are shown here, these trends are consistent across much larger data sets.

Having a standardized response (normalized intensity) as well as variables related to the ion pair (physicochemical properties of Table 3.2) were important initial considerations toward modeling the ESI process for oligonucleotides. However, in order to proceed with statistical analysis, we still needed to construct a set of descriptors for the oligonucleotides. Studies have shown that the oligonucleotide hydrophobicity has the strongest correlation with its electrospray ionization efficiency (Null, Nepomuceno et al. 2003, McGinnis, Grubb et al. 2013). Furthermore, it has been demonstrated that the hydrophobicity of an oligonucleotide can be calculated solely from the percentages of its nucleobases with very good accuracy (Gilar, Fountain et al. 2002, Null, Nepomuceno et al. 2003). Therefore, we decided to use nucleotide proportions (%A, %T, %C, %G) of each oligonucleotide as the additional variables of our statistical model.

Table 3.3- Comparison of the absolute MS signal intensity vs. normalized intensity for oligonucleotide solutions infused on two different days

Sample Number	Absolute signal intensity			Normalized signal intensity		
	Day 1	**Day 2**	**% Difference**	**Day 1**	**Day 2**	**% Difference**
1	484920	636663	27	3.21	3.43	6
2	495899	621802	23	3.28	3.35	2
3	502573	701064	33	3.32	3.77	12
4	1.245×10^6	1.459×10^6	16	8.23	7.86	5
5	1.826×10^6	1.976×10^6	8	12.08	10.65	12

A partitioning (or decision tree-based) method was used for our analysis. Partitioning is a way to describe the relationship between a response and set of factors without a mathematical model (Cook and Goldman 1984). The goal is to divide the data into groups, which differ maximally with respect to MS response. Partitioning is an iterative process, the visualization of which resembles a tree – hence the term "decision tree". The bootstrap forest (or random forest) (Ho 1995) averages the results of many trees. For each of these trees, only a random sample of the observations is considered; then for each split, only a random subset of the candidate variables is considered. In this way, it is highly probable that all of the variables useful in predicting the response will eventually be chosen as splitting variables. Figure 3.3 shows the output of the bootstrap forest for our data. Not surprisingly, some familiar liquid-gas parameters such as boiling point and vapor pressure have been revealed to substantially contribute in MS

response determination. The Henry's law constant was also shown to have a considerable contribution.

Term	Number of Splits	SS	Portion
Boiling Point	849	106.523736	0.1540
Content T	1002	86.2546011	0.1247
Partition Coefficient	828	77.5333886	0.1121
Water Solubility	804	71.5574283	0.1035
Henry's Law Constant	796	61.5813594	0.0890
Gas phase basicity	715	49.0845156	0.0710
Proton affinity	794	48.9921181	0.0708
Vapor Pressure	643	37.6680386	0.0545
pKa	695	30.5009319	0.0441
Density	669	29.9094128	0.0432
Content A	602	29.1038845	0.0421
Content C	567	24.9289149	0.0360
MW	622	21.7318819	0.0314
Content G	514	16.2452799	0.0235

Figure 3.3- Contribution of various parameters to the ESI-MS signal intensity of oligonucleotides

The significant role of partition coefficient and water solubility of IP agents can be easily understood in light of the previously mentioned model of Chen *et al.* (Chen, Mason et al. 2013). Based on their model, the surface activity of alkylamine IP agents plays a vital role in the increased ion intensity of oligonucleotides. Water solubility and partition coefficient are both very closely related to surface activity. The effect of gas-phase basicity and proton affinity is

most probably brought forward during the dissociation process of oligonucleotides from ion-pairs in the gas phase and a hypothetical mechanism suggested by Muddiman et al. (Muddiman, Cheng et al. 1996) regarding the role of piperidine and imidazole in charge state reduction of oligonucleotides can help to explain this effect. It is reasonable to assume that alkylamines can hydrogen bond to the phosphate backbone of oligonucleotides. Through this hydrogen bonding, they can displace cations which results in reduced cation adduction and subsequently increases signal intensity. But for this to happen, the proton that originally resided on the alkylamine in solution should be transferred to the phosphate backbone in the gas phase so the hydrogen bound ion-pair can be released from the oligo. Therefore, it is expected for the gas-phase proton affinity of the top-performing IP agents to be lower than the oligonucleotide phosphodiester backbone. The proton affinity of the phosphodiester group is estimated at 315 Kcal/mol (Lum and Grabowski 1992), while all utilized alkylamines have proton affinities in the range of 220-240 Kcal/mol which is well below the estimated value for the phosphodiester backbone. One important observation in support of this proposal was the excellent capability of OA in suppressing cation adduction. In comparison, DBA with very closely related structure and physicochemical properties to OA that often resulted in similar effects on MS signal intensity of oligonucleotides, was not very effective in reducing cation adduction. Examination of table 3.2 reveals that the proton affinity of OA is about 10 Kcal/mol lower than DBA.

As expected, the composition of oligonucleotides also plays a major role in determining the ESI response in the form of a very large contribution for %T in the sequence of the oligo. A more significant contribution from thymine when compared to other nucleotides was not unexpected. It has been shown that T is the most hydrophobic DNA base (T>A>G>C) (Huber, Oefner et al. 1992, Huber, Oefner et al. 1993, Gilar, Fountain et al. 2002) and since

hydrophobicity significantly affects signal intensity, it is reasonable for T content to be more important than the other nucleobases. The major significance of hydrophobicity for electrospray ionization efficiency of our analytes is also in complete agreement with the previously mentioned equilibrium partitioning model (Cech and Enke 2000, Cech and Enke 2001).

Creating a predictive model with these data is also very desirable from a practical standpoint. Based on these experiments, it is clear that different DNA sequences generate the highest MS signal intensity with different alkylamine IP agents. Therefore, all different combinations of oligo/IP would need to be investigated to find the optimum ion-pair every time prior to the development of a LC-MS method. This would create additional burden on the analysis and hinder method development. Therefore, a predictive algorithm that can rapidly assist with the selection of optimal IP agents without the need to perform dozens of experiments would be extremely useful. We managed to generate a partial least squares (PLS) regression

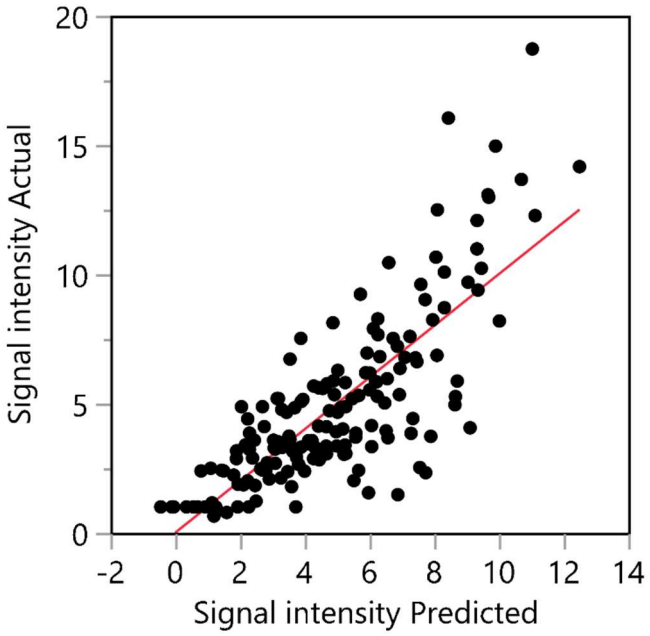


Figure 3.4- A PLS regression model was generated that can accurately predict the normalized MS signal intensity of any DNA sequence in the presence of any ion-pairing reagent

model capable of predicting intensity values that are very well aligned with our experimental observations (Figure 3.4).

To further confirm the prediction power of the resulting PLS model, we used a DNA strand with the sequence 5'-TCG TGC TTT TGT TGT TTT CGC GTT-3' which had not been examined in the previous experiments. The sequence was run through the algorithm and the expected normalized signal intensities calculated. Then the experimental values were obtained. As shown in Figure 3.5 the prediction of our model fit the observed data, well. The only exception was HA where prediction and observation showed opposite trends. We believe this is due to a complete lack of adenines in this particular sequence. Figure 3.6 shows the experimental MS signal intensities for two sequences that are both composed of 50% T. However, the remaining 50% is equally distributed among all other nucleotides for 3.6a in contrast to 3.6b which only has T and G nucleotides in its sequence. While the trend in 6a is more similar to the predictions of Figure 3.5, 6b more closely resembles the experimental results. Considering the particularly superb performance of HA with poly-A sequences (not shown), it seems that the presence of adenines is necessary for maximum HA performance. But neither the sequence in Figure 3.5, nor the sequence in Figure 3.6b contained any adenines. Therefore, HA was not among the best IP agents for any of them. However, HA performed well with the DNA sequence shown in 3.6a even though its T content was not very different from the other two. We believe

that this is because it contained a greater number of adenines. This very specific synergy between the adenine content and HA is too complicated for our model to accurately extract and therefore it has overestimated HA performance for the sequence in Figure 3.5. To further demonstrate this point, we repeated this experiment with a different sequence that contained adenine bases. As expected, the prediction pattern for HA and other IP agents was in line with the observations of

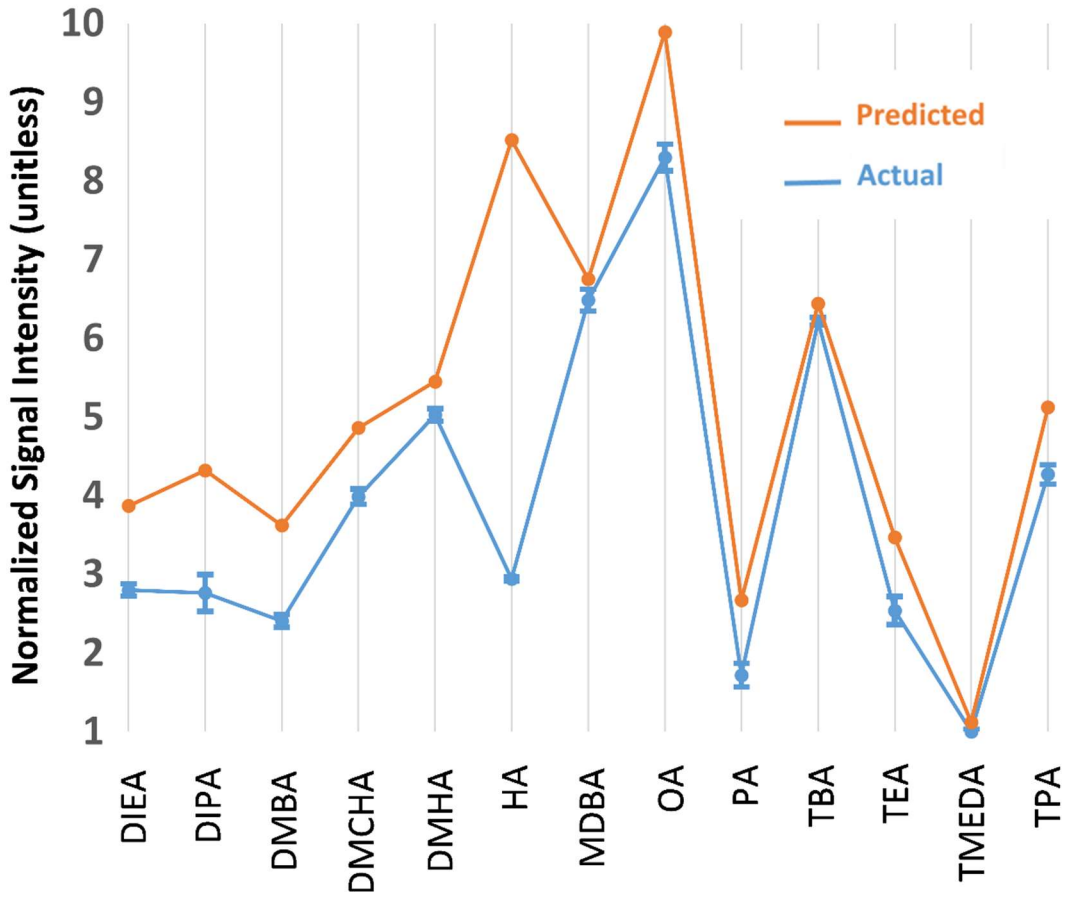


Figure 3.5- Experimental and predicted normalized signal intensities for the sequence 5'-TCG TGC TTT TGT TGT TTT CGC GTT-3' in the presence of various ion pairing reagents.

this experiment (Figure 3.7). Therefore, our model seems to predict the HA performance inaccurately for the sequences that are completely void of adenines. Nevertheless, this should not be a problem for most common sequences that contain all four nucleotides. Even for sequences like the one in Figure 3.5, our model accurately determined the response for all of the other ion-pairs.

Another important observation concerns TEA. It is the most widely used ion-pair for LC-MS analysis of oligonucleotides (McGinnis, Chen et al. 2012). However, when comparing the performance of TEA to other IP agents in Figures 3.2a, 3.5 and 3.6a the results indicate that using TEA with any of these sequences would cause a substantial decrease in the sensitivity of the resulting LC-MS method. Therefore, it is evident that the use of alternative alkylamine IP agents is necessary for improving the sensitivity of LC-MS methods for oligonucleotides. Of course, other considerations should also be taken into account when using IP agents for oligonucleotide analysis. One such important consideration is the ion suppression caused by these alkylamines when switching from negative to positive ESI. To our experience, all of the IP agents mentioned in this manuscript can cause such effect. The way we have found around this issue is to dedicate separate LC channels to positive and negative ESI buffers. This way, we have managed to keep the contamination at a minimum level and successfully switch between positive and negative ESI. We would also like to add that from the point of cost and ease of use, there are no dramatic differences among the alkylamines we have used for this study. Therefore, switching from TEA to an alternative ion-pair would not add any undue burden to the bioanalytical methods. In contrast, it provides an easy way for increasing the sensitivity of oligonucleotide quantitation by LC-MS which bears very important outcomes. Above all, it is only via increased

sensitivity that mass spectrometry can satisfy the many emerging needs of oligonucleotide researchers.

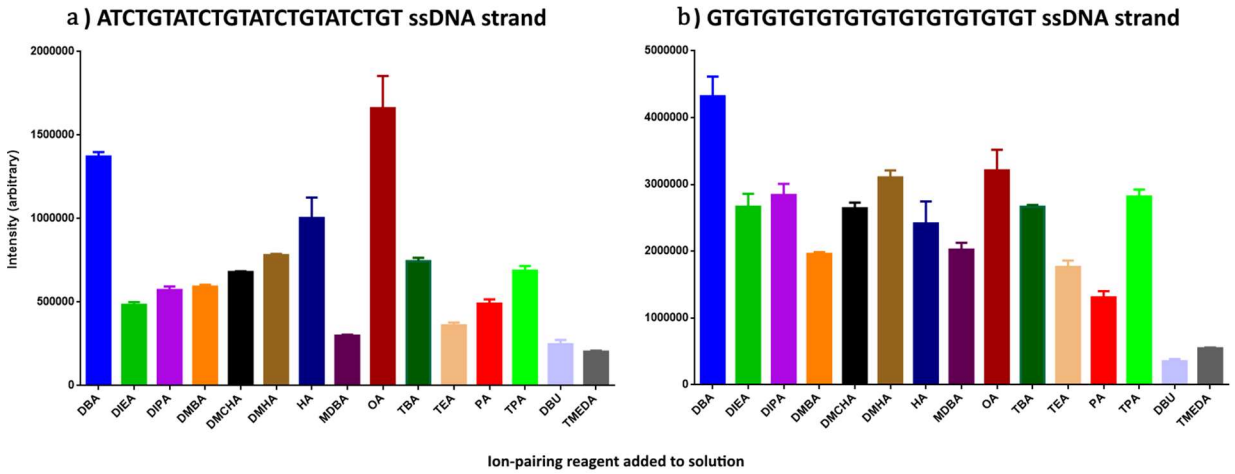


Figure 3.6- MS signal intensity of the base oligonucleotide peak in the presence of various alkylamine IP agents for $[ATCTGT]_4$ (a) and $[GT]_{12}$ (b).

It can be observed that HA has a much better performance alongside the DNA sequence examined in panel "a" compared to the sequence in panel "b".

Conclusion

We have performed a comprehensive set of experiments to help us better understand the physicochemical properties of analytes and mobile phase additives that govern electrospray ionization efficiency. Many of the factors identified through this process have been previously suggested to influence the ESI process by different mechanisms outlined in various models. Therefore, our study provides a unifying platform for several proposed mechanisms in addition to a more quantitative perspective regarding the relative contribution of factors suggested by one

model when compared to others. This would make it possible to assess the relative importance of each proposed mechanism toward the overall ESI process and combine those events in the right order to give rise to a generalized model with diverse applications.

We have also generated a PLS regression model with very good predictive power that can help with choosing the optimum IP agent based on the oligonucleotide composition for

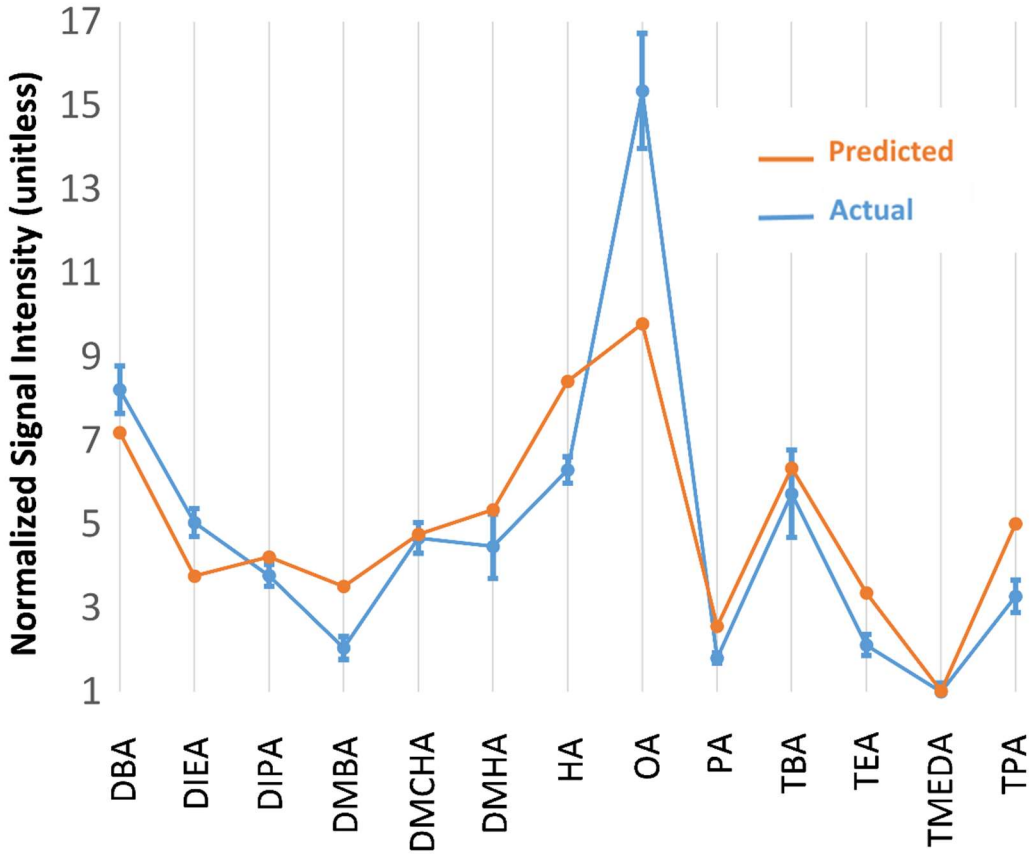


Figure 3.7- Experimental and predicted normalized signal intensities for miR-451 (5'- AAA CCG UUA CCA UUA CUG AGU U -3') in the presence of various ion pairing reagents. Note that in contrast to figure 3.5, both prediction and experiment follow similar patterns regarding HA performance.

performing LC-MS analysis. This is based on the fact that most of these alkylamine IP agents have been utilized previously in a few different investigations and all of them have shown acceptable chromatographic performance. Chen and Bartlett have used DIEA (Chen and Bartlett 2013). Gong has utilized DBA, DMBA, HA and TPA (Gong and McCullagh 2014, Gong 2015). Oberacher and colleagues have used DMCHA (Erb and Oberacher 2014). Mcginnis *et al.* have separated several modified and unmodified DNA and RNA strands using DIPA (McGinnis, Grubb et al. 2013) and Sharma *et al.* (Sharma, Glick et al. 2012) have reported using DBU among other IP agents. In all of mentioned studies, TEA has also been present as a reference point and the chromatographic performance of these alternative IP agents has been similar to or slightly better than TEA. More importantly, in two of these studies (Sharma, Glick et al. 2012, Gong and McCullagh 2014) several IP agents have been compared simultaneously and a close examination of the oligonucleotide retention factors further reveals that the difference in chromatographic performance between various alkylamines is minimal. Furthermore, the concentrations of IP agents in these studies have been in the general range of 10-15 mM which is totally in line with our experimental design. The only exceptions were DMBA and DBU which have been used at the concentrations of 5 and 2.5 mM, respectively.

The study performed by Gong and McCullagh (Gong and McCullagh 2014) is particularly important in supporting our proposition that the overall performance of oligonucleotide LC-MS methods is primarily governed by the ESI efficiency and the chromatographic performance of different IP agents is more or less the same. They have used mobile phases containing six different alkylamine IP agents including DBA, DIEA, DMBA, HA, TEA and TPA in order to separate different poly-T sequences (T10, T15, T25 and T40). The obtained resolutions for these oligonucleotides have been largely similar regardless of the choice

of ion-pair. Nevertheless, the overall method sensitivity has followed the same pattern as we have shown in Figure 3.2a with DBA>HA>TPA>DMBA>DIEA>TEA. This data clearly indicates that the alkylamine IP agents that generate the highest MS signal intensity for a particular oligonucleotide are usually the best choice for LC-MS method development as they will most probably have an acceptable chromatographic performance. Therefore, we can confidently suggest our PLS prediction algorithm as an efficient tool for ion-pair selection for LC-MS analysis of oligonucleotides although we have not fully studied the chromatographic performance of all IP agents discussed in this manuscript.

Acknowledgements

The experiments described in this manuscript were supported by the National Cancer Institute (CA176653).

CHAPTER 4

THE ROLE OF FLUORINATED ALCOHOLS AS MOBILE PHASE MODIFIERS FOR LC-MS ANALYSIS OF OLIGONUCLEOTIDES[3]

[3] Babak Basiri, Hilde van Hattum, William D. van Dongen, Mandi M. Murph and Michael G. Bartlett. 2017. *Journal of the American Society for Mass Spectrometry* 28(1): 190-199
Reprinted here with permission from the publisher.

Abstract

Hexafluoroisopropanol (HFIP) has been widely used as an acidic modifier for mobile phases for liquid chromatography-mass spectrometry (LC-MS) analysis of oligonucleotides ever since the first report of its use for this purpose. This is not surprising considering the exceptional performance of HFIP compared to carboxylic acids which cause significant MS signal suppression in electrospray ionization. However, we have found that other fluorinated alcohols can also be utilized for mobile phase preparation and the choice of optimal fluorinated alcohol is determined by the ion-pairing (IP) agent. While HFIP is a very good choice to be used alongside less hydrophobic IP agents, other fluorinated alcohols such as 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP) can significantly outperform HFIP when used with more hydrophobic IP agents. We also found that more acidic fluorinated alcohols assist with the transfer of oligonucleotides with secondary structure (e.g. folded strands and hairpins) into the gas phase.

Introduction

Since its invention, electrospray ionization (ESI) has evolved as a major technique for the analysis of biomolecules. It allows for seamless mass spectrometric (MS) study of large biomolecules, such as oligonucleotides, following their separation with liquid chromatography (LC) (Bartlett, Chen et al. 2013, Basiri and Bartlett 2014). However, the choice of optimal solvent systems has proven to be absolutely essential for the successful ionization of analytes by ESI. Positive ion ESI, particularly for peptides and proteins, has shifted toward the use of protonated solutions of acetonitrile/water or methanol/water. These protonated solvent clusters are created by reaction of the solvent with a weak acid, e.g. acetic or formic acid (Straub and Voyksner 1993, Marwah, Marwah et al. 2002, Cech and Enke 2010, Hua and Jenke 2012).

Analogous to this concept, studies of ESI-MS in the negative ion mode were initially performed by adding ammonium hydroxide to aqueous solutions of acidic analytes (Loo, Loo et al. 1992, Hoaglund, Liu et al. 1997, Kauppila, Talaty et al. 2008). However, negative ion ESI with basic solutions can be unfavorable as stable anions are not formed to any appreciable extent (Cech and Enke 2001). In fact, several studies have shown that ESI-MS in negative ion mode with volatile bases resulted in poorer detection limits and reduced stability of the electrospray plume (Kamel, Brown et al. 1999, Harvey 2005, Xia and Miller 2013). Although it might seem counterintuitive, weak acids are also very favorable modifiers for negative-ion ESI because they can create a very stable deprotonated anion pool which supports the negative charging process (Cech and Enke 2001, Wu, Gao et al. 2004).

Oligonucleotides are primarily ionized via negative-ion ESI. Considering that reversed phase LC retention of these molecules is only possible with the help of basic alkylamine ion-pairing agents, acidic modifiers play a dual role in this particular case: in addition to carrying excess negative charge, they also neutralize the solution pH. Various carboxylic acids such as acetate, bicarbonate and formate have been used for this purpose (Muddiman, Cheng et al. 1996, Huber and Krajete 1999, Oberacher, Parson et al. 2001) but all of them caused significant electrospray MS signal suppression. The introduction of hexafluoroisopropanol (HFIP) as an acid modifier by Apffel et al. (Apffel, Chakel et al. 1997, Apffel, Chakel et al. 1997) was a landmark achievement in the evolution of solvent systems for LC-MS of oligonucleotides. HFIP can adjust the solution pH and at the same time enhance MS signal intensity of oligonucleotides which explains why it has been used in every LC-MS study since its introduction. There have been several recent studies into alternative alkylamines as ion pairing reagents (Sharma, Glick et al. 2012, Chen and Bartlett 2013, McGinnis, Grubb et al. 2013, Erb and Oberacher 2014).

However, other fluorinated alcohols have never been thoroughly investigated as potential substitutes for HFIP. Cech and Enke (Cech and Enke 2001) briefly mentioned the use of trifluoroethanol (TFE) instead of HFIP for the analysis of oligonucleotides, but the outcome of their experiments was never discussed. To our knowledge, the only systematic comparison of TFE and HFIP for the ESI of oligonucleotides was reported by Chen et al. (Chen, Mason et al. 2013). They showed that while increasing concentrations of HFIP enhanced the oligonucleotide MS signal intensity, higher concentrations of TFE had the opposite effect and caused signal suppression. However, their study was confined to only one alternative alcohol and one oligonucleotide molecule (DNA) and it was performed in the absence of ion pairing agents. Here, we present a comprehensive study of the effect of utilizing different fluorinated alcohols as ESI solvent modifiers for several classes of oligonucleotides in the presence and absence of various ion pairing reagents in order to provide a more detailed guidance for optimizing the mobile phase composition for LC-MS analysis of oligonucleotides.

Materials and methods

Chemicals and reagents: The ion pairing agents N,N-dimethylbutylamine, octylamine, tripropylamine, N,N-dimethylhexylamine, diisopropylamine, N-methyldibutylamine, propylamine, triethylamine, hexylamine, tributylamine, N,N-dimethylcyclohexylamine, N,N-diisopropylethylamine, tetramethylethylenediamine, dibutylamine, methyldibutylamine and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), as well as bovine insulin and the fluorinated alcohols hexafluoroisopropanol, pentafluoropropanol, 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol, trifluoroethanol and nonafluoro tertiary butyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO). Nuclease-free water was acquired from Life Technologies (Carlsbad, CA) and LC-

MS grade methanol, acetonitrile, water and formic acid were purchased from Sigma-Aldrich, as well. The 24-mer ssDNA strand (5'-ATCGATCGATCGATCGATCGATCG-3') and two RNA strands with the sequences of miR-451 (AAACCGUUACCAUUACUGAGUU) and miR-30c (UGUAAACAUCCUACACUCUCAGC) were purchased from Eurogentec (Seraing, Belgium). The 33mer phosphorothioate with the sequence (UAUUCAAGUUACACUCAAGAAGGAAUA AUUUCU) and its 5'(n-1) truncation were provided by ProQR (Leiden, Netherlands) and were fully modified with 2'-O-methyl groups. DNA Lobind microcentrifuge tubes were purchased from Eppendorf (Hauppauge, NY).

Preparation of working solutions for direct infusion and LC-MS experiments: For direct infusion experiments, precise amounts of various IP agents and fluorinated alcohols were added to aliquots of 20 μg/mL solutions of each oligonucleotide in 50:50 methanol/water to the final concentration of 15 mM for the IP agent and 25 mM for the alcohol, except for NFTB which was used at the concentration of 2 mM. The samples used during LC-MS analysis were a mixture of the above-mentioned phosphorothioate and its n-1 truncation from the 5' side, both at 50 μg/mL and prepared in nuclease-free water. Solutions of intact insulin, insulin chain A and insulin chain B were prepared in 50:50 acetonitrile/water at the concentration of 20 μg/mL.

Instrumental conditions and software: Direct infusion experiments were made on a Waters (Milford, MA) Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer in the negative ion electrospray ionization mode via the instrument's built-in fluidics system. LC-MS experiments were performed using a Waters Acquity UPLC system coupled to the same mass spectrometer (Waters Synapt G2). The TOF-MS tuning parameters for all configurations were as follows: capillary voltage -2.0 kV, cone voltage 25 V, extraction cone voltage 2 V, source temperature 125 ˚C, desolvation temperature 450 ˚C, cone gas 0 L/h and desolvation gas

(nitrogen) 1000 L/h. The ion mobility experiments were performed at IMS gas (N_2) flow of 100 mL/min and trap gas (Ar) flow of 2 mL/min, while the source and helium cell gas flows were set to 0 mL/min. IMS wave velocity was 450 m/s and IMS wave height was 18 V. For direct infusion, a flow rate of 50 µL/min was utilized and the data were collected in continuum full-scan MS mode with a 1 s scan time over the mass range from 500–3000 m/z. All measurements were performed in triplicate and base peak signal intensities were measured by combining 50 scans.

UPLC separation was performed at 60˚C using a Waters Acquity UPLC OST C18 column (1.7 µm, 2.1 x 50 mm). Mobile phase A was 15 mM DMCHA and 25 mM HFIP (or HFMIP) in 10% methanol. Mobile phase B was 90% methanol. The flow rate was set at 1 mL/min and the injection volume at 15 µL. For DMCHA/HFIP mobile phase, a gradient from 20 to 25 percent B in 6 minutes was used during UPLC separation. In the case of DMCHA/HFMIP, the gradient was 12-17%, respectively.

Collision cross section (CCS) values were determined following the procedure of Ruotolo et al. (Ruotolo, Benesch et al. 2008). A calibration curve was created by plotting CCS (Ω) versus the charge-corrected drift time values ($t"_D$) for three calibrants: intact insulin with 3 and 4 negative charges (Ω = 654 Å²) (Fernandez-Lima, Blase et al. 2010), insulin chain A with two negative charges(Ω = 388 Å²) (Counterman, Valentine et al. 1998) and insulin chain B with two negative charges (Ω = 501 Å²) (Fernandez-Lima, Blase et al. 2010). The resulting calibration plot that showed very good linearity ($R^2 > 0.99$) was then used to convert drift time data for oligonucleotides to CCS measurements.

The UPLC and MS operation and data acquisition were performed using Waters MassLynx 4.1. IMS data were examined by Waters Driftscope v2.1. Statistical analyses and

graphs were made in Microsoft Office Excel 2016 or GraphPad Prism 6. All pKa, surface area and distribution coefficient (log *D*) values were determined using the calculator plugins of MarvinSketch 14.7.14, 2014 (ChemAxon, http://www.chemaxon.com). Henry's law constants were computed using HENRYWIN module of the US Environmental Protection Agency's EPI suite v4.11. Hairpin structure prediction was carried out with OligoAnalyzer 3.1.

Results and Discussion

Our initial study was to determine which of 14 different alkylamine IP agents including N,N-dimethylbutylamine (DMBA), octylamine (OA), tripropylamine (TPA), N,N-dimethylhexylamine (DMHA), diisopropylamine (DIPA), N-methyldibutylamine (MDBA), propylamine (PA), triethylamine (TEA), hexylamine (HA), tributylamine (TBA), N,N-dimethylcyclohexylamine (DMCHA), N,N-diisopropylethylamine (DIEA), tetramethylethylenediamine (TMEDA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) provided variable MS signal intensities for different classes of oligonucleotides. An interesting observation throughout these investigations (Figures 4.1 and 4.2) was the lower MS signal intensity of oligonucleotides in the absence of any ion pairing reagent compared to when IP reagents are present. There are two reasons for this phenomenon: IP agents can bring oligos to the surface of electrospray droplets with them as they move towards the surface. They also reduce cation adduction, resulting in an increase in the MS signal intensity of oligonuclaotides. Finally, four IP agents which provided variable, yet still high MS signal intensities for different types of oligonucleotides and possessed significantly different structural properties were chosen for the remaining studies. These IP agents were: DIEA, TEA, DMCHA and OA.

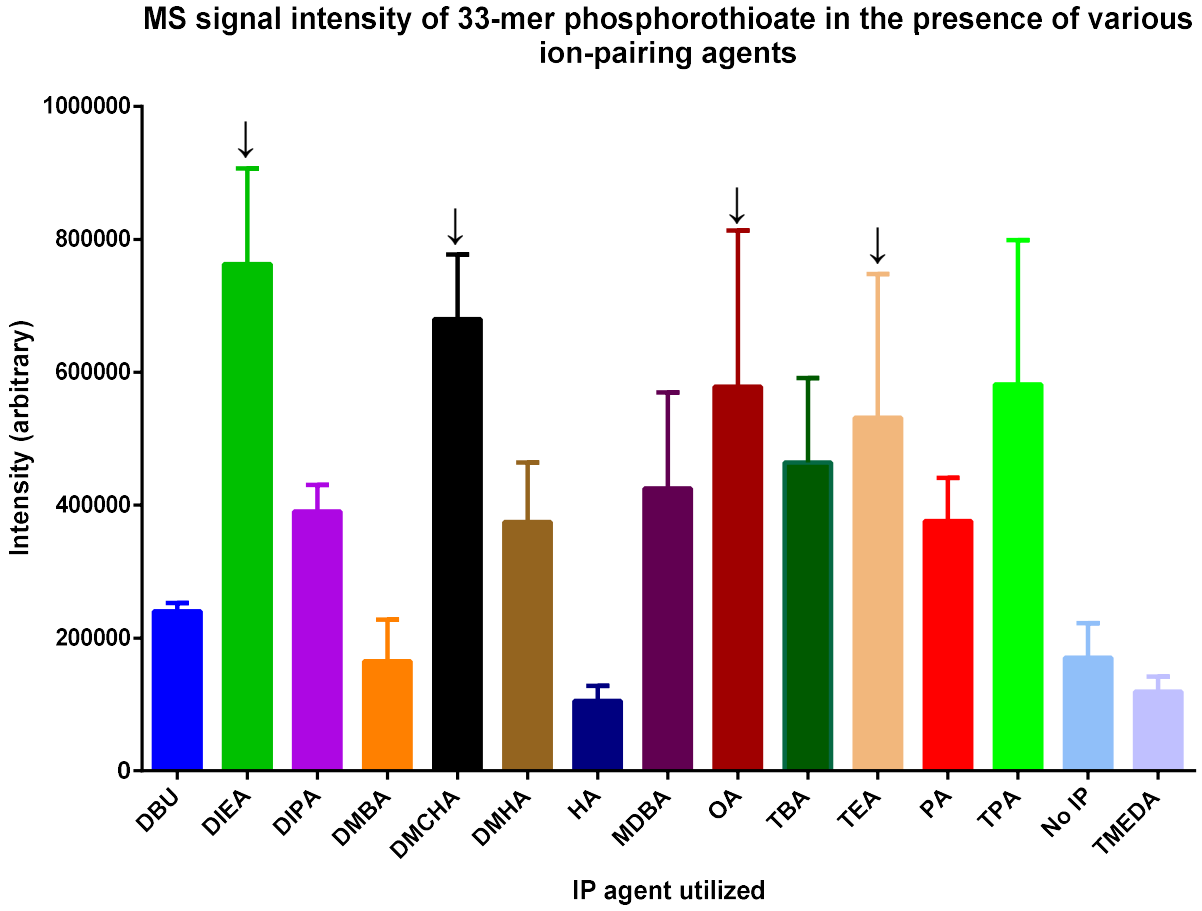
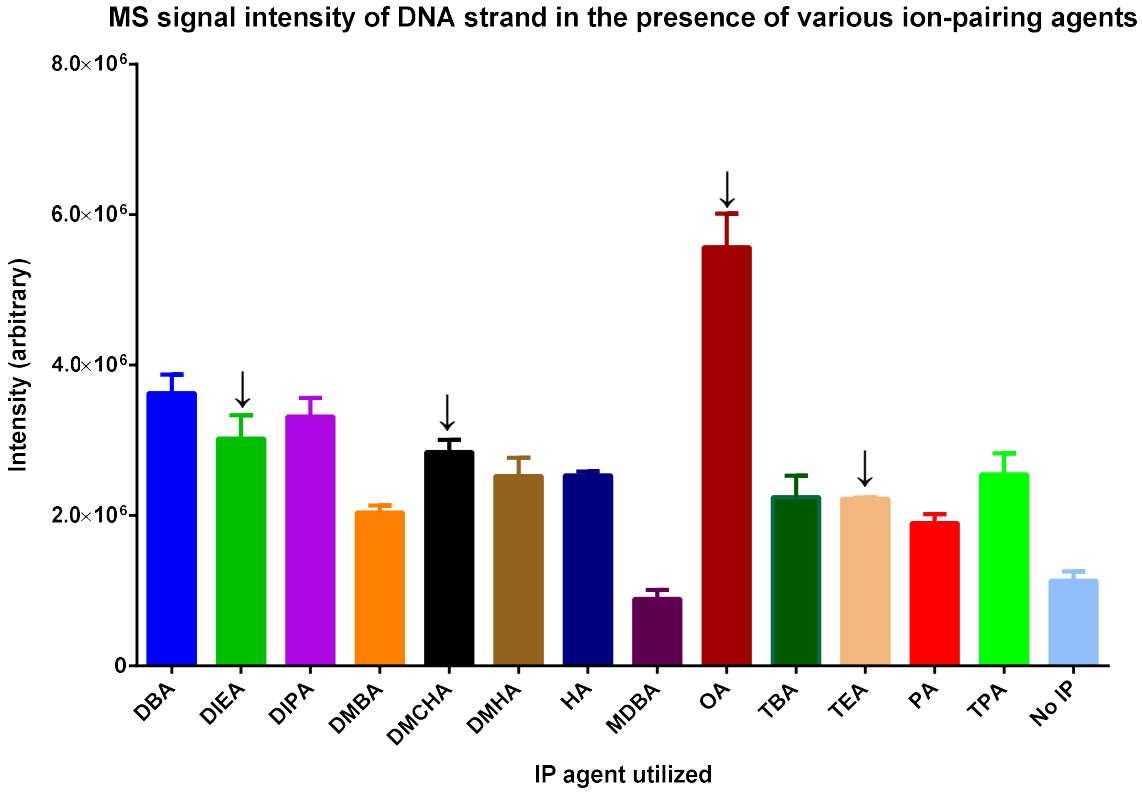
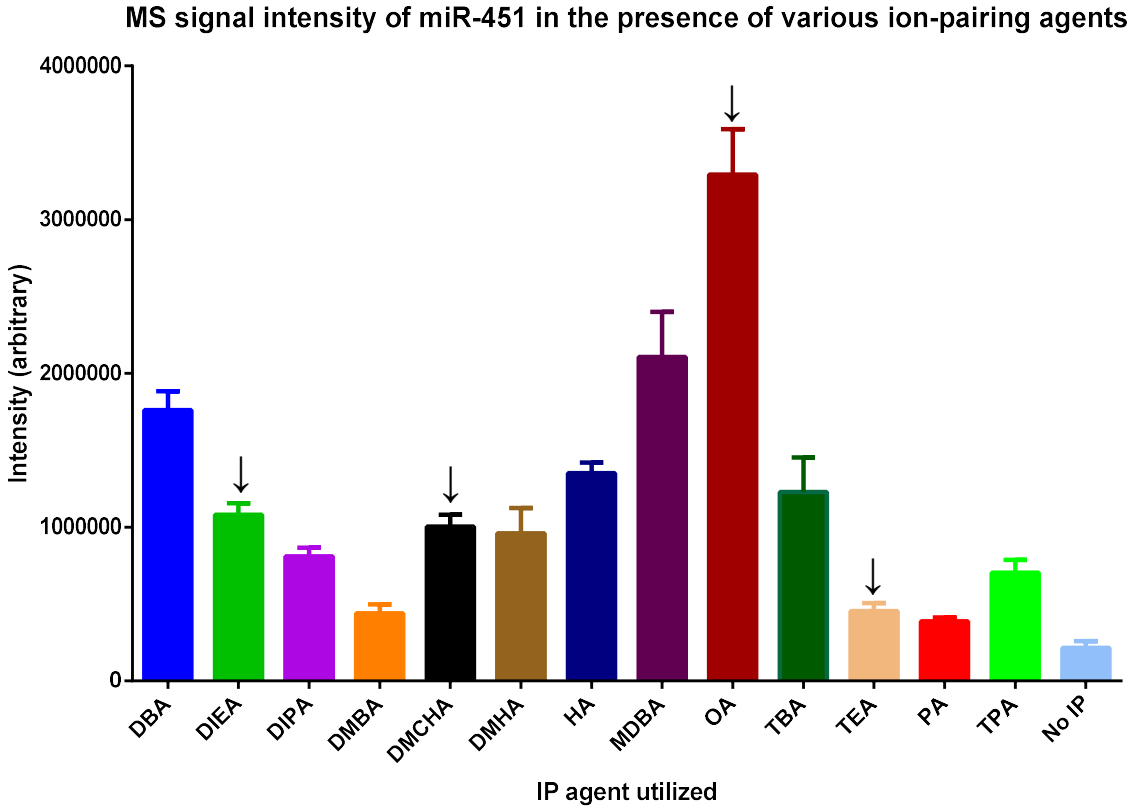


Figure 4.1- MS signal intensity of the phosphorothioate oligonucleotide in the presence of various ion pairing agents

The IP agents selected for this study have been marked with an arrow. All four of them generate strong and generally comparable ESI-MS signal intensities for this particular oligonucleotide.

Next, phosphorothioate oligonucleotide solutions containing these four IP reagents were mixed with five different fluorinated alcohols hexafluoroisopropanol (HFIP), pentafluoropropanol (PFP), 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP), trifluoroethanol (TFE) and nonafluoro tertiary butyl alcohol (NFTB) and the differential effect of


MS signal intensity of DNA strand in the presence of various ion-pairing agents
Intensity (arbitrary)
8.0×10⁶
6.0×10⁶
4.0×10⁶
2.0×10⁶
0
DBA
DIEA
DIPA
DMBA
DMCHA
DMHA
HA
MDBA
OA
TBA
TEA
PA
TPA
No IP
IP agent utilized


MS signal intensity of miR-451 in the presence of various ion-pairing agents
Intensity (arbitrary)
4000000
3000000
2000000
1000000
0
DBA
DIEA
DIPA
DMBA
DMCHA
DMHA
HA
MDBA
OA
TBA
TEA
PA
TPA
No IP
IP agent utilized

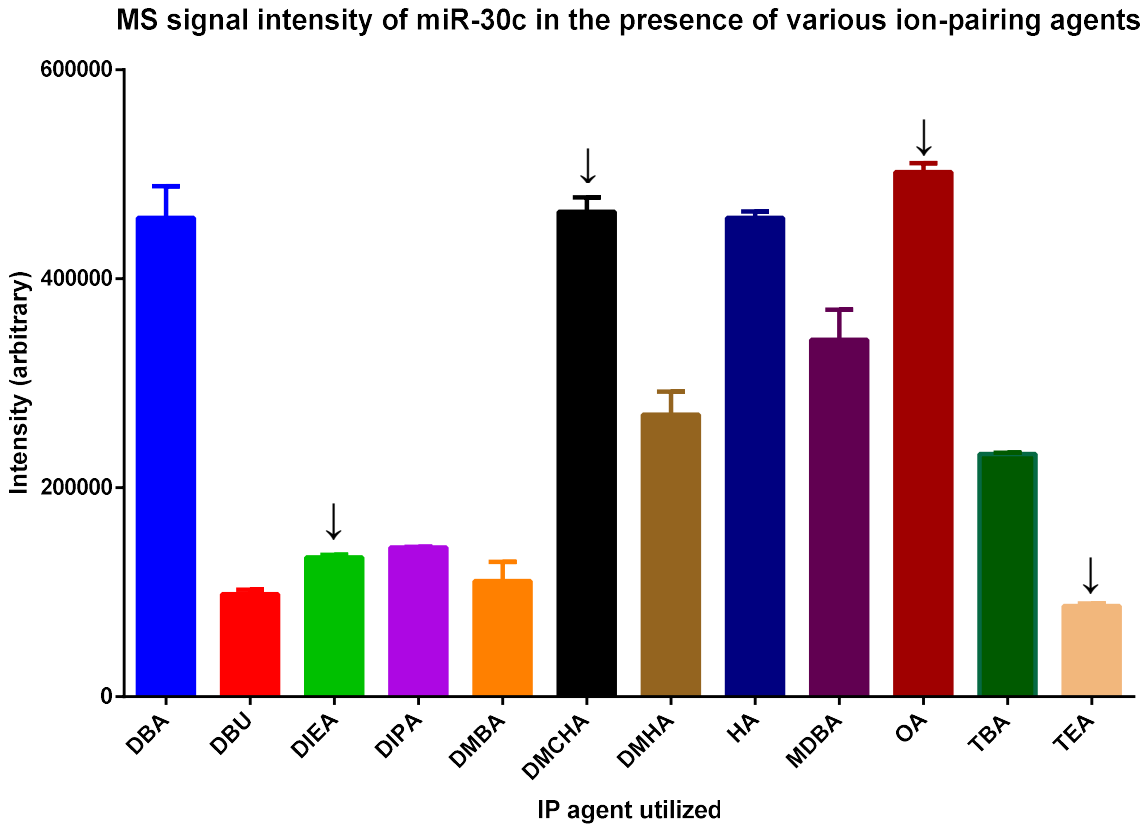


Figure 4.2- MS signal intensity of DNA and RNA oligonucleotides in the presence of various ion pairing reagents

By examining the panels in this figure and also comparing them to figure 4.1, it can be observed that the relative orders in which IP agents provide the highest MS signal intensities are different from one class of oligonucleotides to another. This is even true for the oligos that belong to the same class (miR-451 and miR-30c are both RNA strands). The IP agents selected for this study have been marked with an arrow. They provide MS signal intensities that are quite variable depending on the type of oligonucleotides.

these alcohols on the electrospray MS signal intensity was recorded. As shown in figure 4.3 while HFIP has a better performance in the presence of ion-pairs DIEA and TEA, it clearly is not the best choice for use with either DMCHA or OA. In fact, the mixture of DMCHA/HFMIP

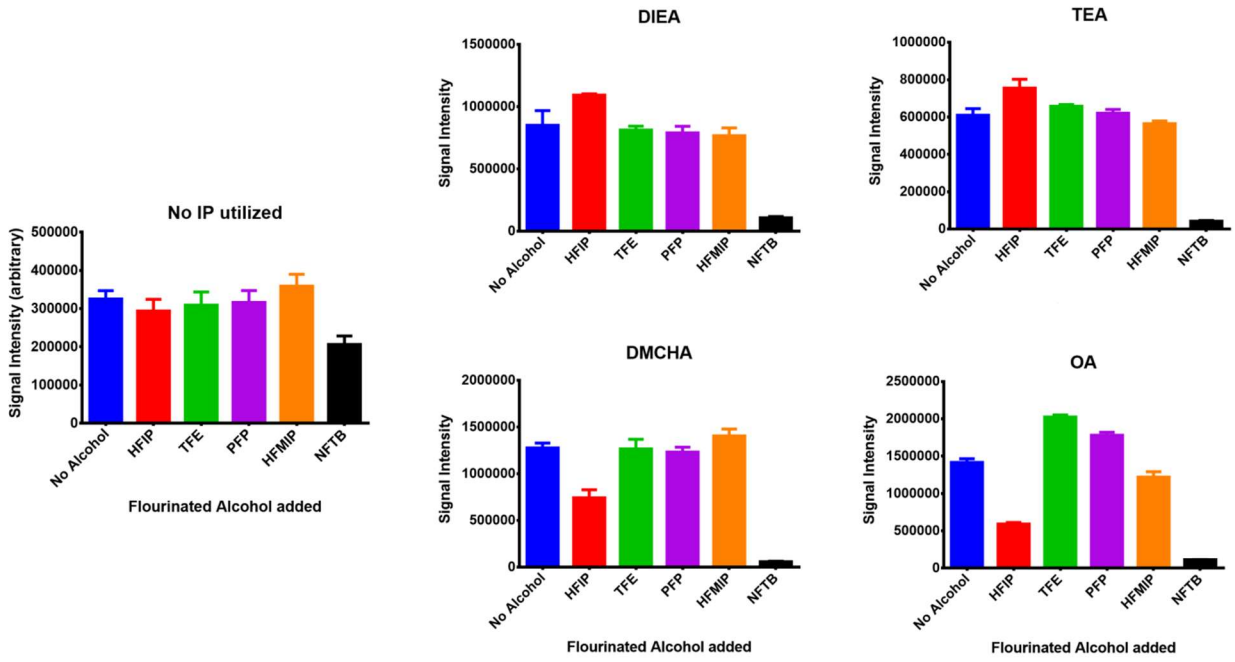


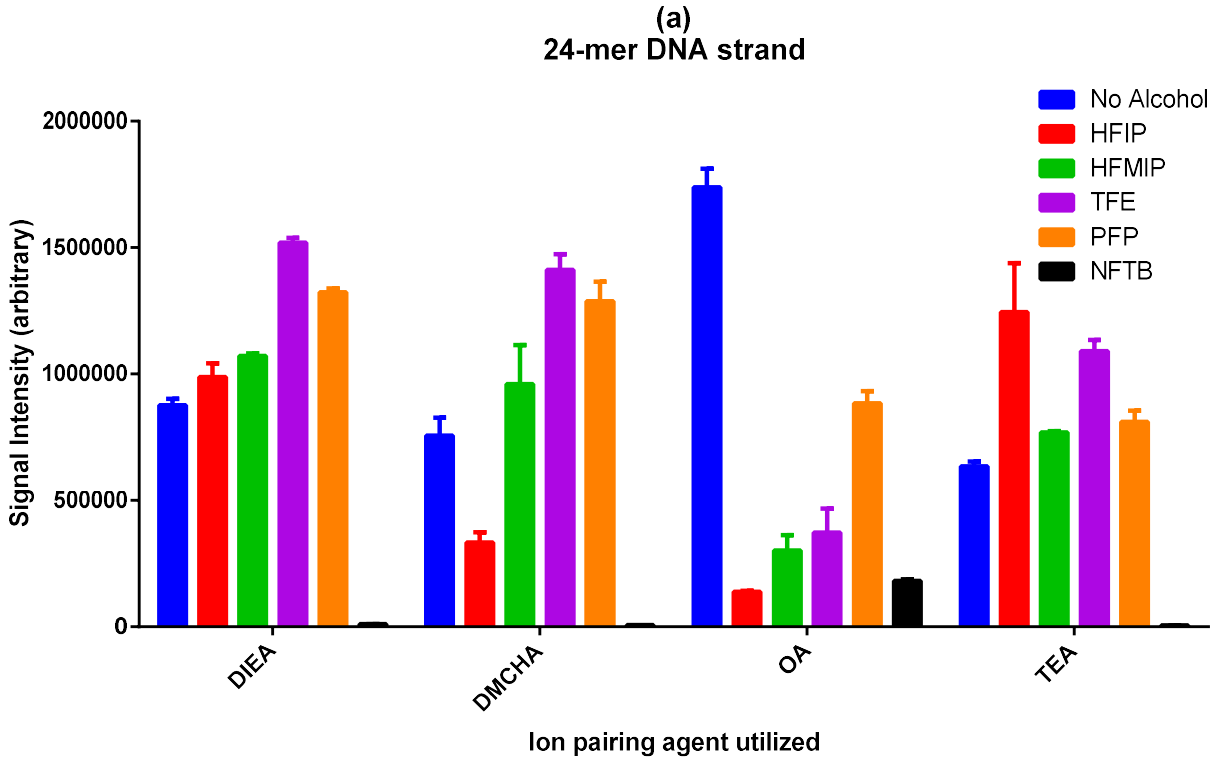
Figure 4.3- The effect of different fluorinated alcohols on MS signal intensity of a 33-mer phosphorothioate in the presence of four different alkylamine IP agents.

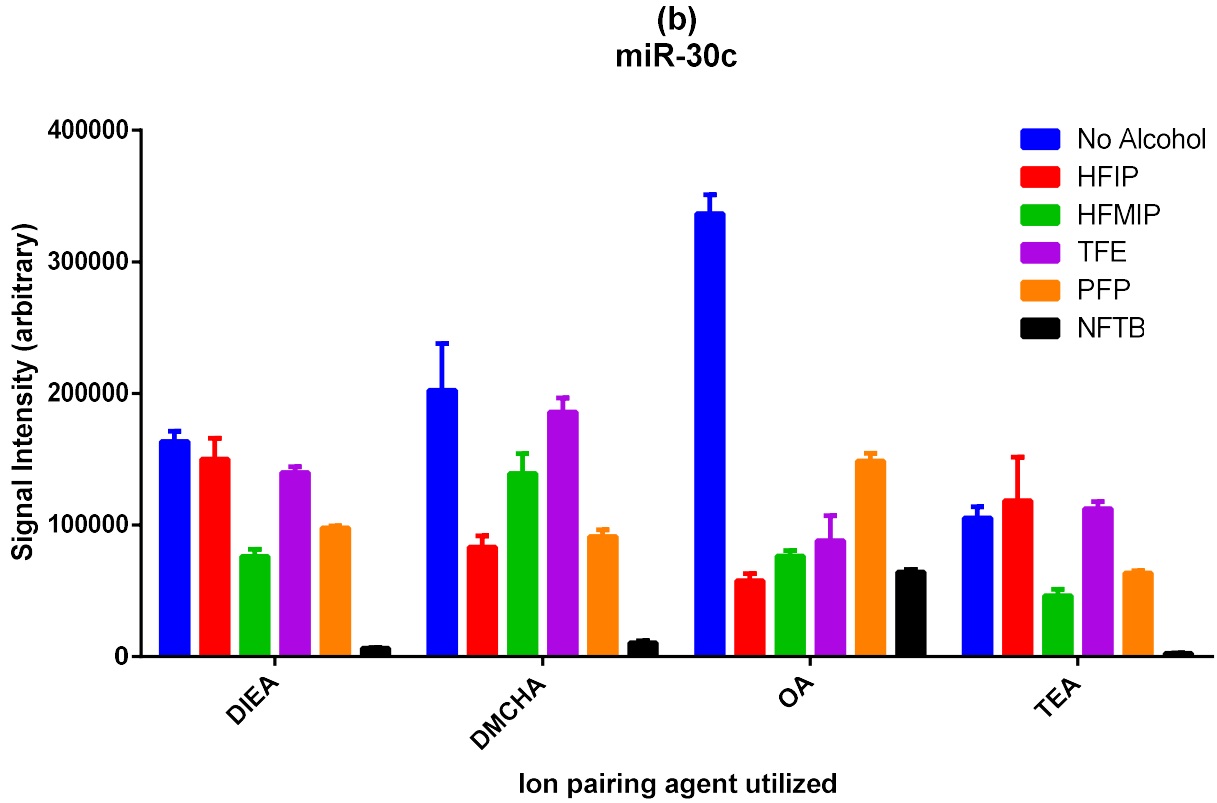
It can be observed that in the absence of IP agents, the addition of these alcohols does not have a significant effect on electrospray MS signal intensity. The only exception is NFTB that causes considerable MS signal suppression. It was exactly because of this suppression effect that NFTB was only used to a final concentration of 2 mM while other alcohols were used at a concentration of 25 mM. It is also clear that in the case of DMCHA and OA, other fluorinated alcohols (with the exception of NFTB) show a better performance than HFIP.

provided stronger signal intensity relative to DIEA/HFIP or TEA/HFIP. Therefore, using alternative fluorinated alcohols can help significantly in lowering the MS detection limit of this phosphorothioate.

It has been reported previously that different types of oligonucleotides (e.g. phosphorothioate vs. DNA or RNA) optimize with different IP agents and that the hydrophobicity of the oligonucleotide molecule has been suggested as an explanation for this behavior (McGinnis, Grubb et al. 2013). So it was reasonable to assume that the same principle may apply to fluorinated alcohols and different classes of oligonucleotides would show differential preference with respect to these alcohols. In order to elucidate this point, the same experiment was repeated with a DNA and two RNA strands. The results are presented in figure 4.4. While the behavior of the two RNA strands (with different sequences) were almost the same, some differences were observed among the three classes of oligonucleotides examined. For example, for phosphorothioate and RNA molecules, DIEA generated the highest MS signal intensity with HFIP while the combination of DIEA and TFE was the best for DNA. However, the overall pattern remained largely similar among the three classes of oligonucleotides. Namely, while HFIP showed good performance alongside DIEA and TEA, it performed poorly when utilized with DMCHA or OA.

Chen et al. (Chen, Mason et al. 2013) have previously reported superior performance with HFIP compared to TFE. Our results with DIEA and TEA were consistent with their observations. However, they never examined the IP agents DMCHA or OA for their study which explains why they did not observe the opposite effect reported here. They have also suggested that fluorinated alcohols with lower Henry's law constants generate stronger oligonucleotide MS signal intensities. Although this notion can be used to explain a few of our observations (for


(a)
24-mer DNA strand
No Alcohol
HFIP
HFMIP
TFE
PFP
NFTB
2000000
1500000
1000000
500000
0
Signal Intensity (arbitrary)
DIEA
DMCHA
OA
TEA
Ion pairing agent utilized


(b)
miR-30c
No Alcohol
HFIP
HFMIP
TFE
PFP
NFTB
400000
300000
200000
100000
0
Signal Intensity (arbitrary)
DIEA
DMCHA
OA
TEA
Ion pairing agent utilized

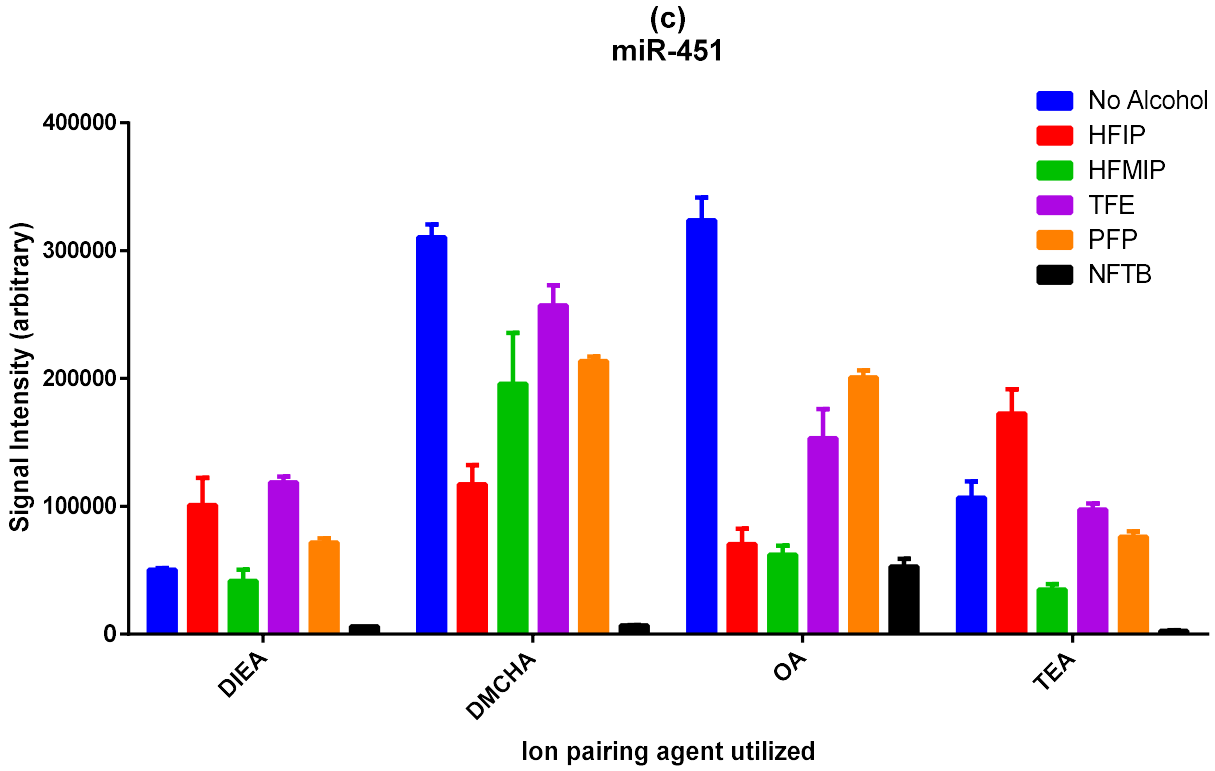


Figure 4.4- The effect of different fluorinated alcohols on MS signal intensity of DNA and RNA molecules in the presence of four different alkylamine IP agents

One DNA molecule (a) and two RNA molecules (b and c) were investigated in the presence of different fluorinated alcohols. While some differences can be observed, the overall response pattern of various classes of oligonucleotides to different combinations of IP agents and fluorinated alcohols remains very similar.

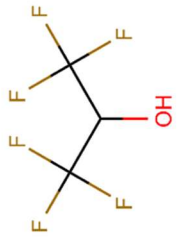
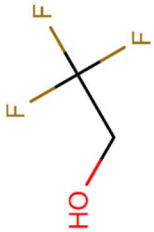
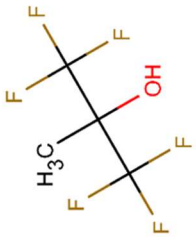
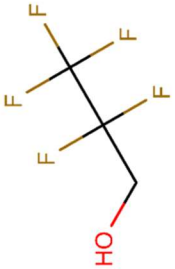
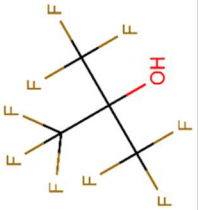
example, HFMIP has a lower Henry's law constant than HFIP and it produces stronger MS signal intensities when used with DMCHA), it does not coincide with the general pattern of our results. A brief examination of table 4.1 reveals that NFTB and TFE have the lowest and highest Henry's law constants among utilized fluorinated alcohols, respectively. Yet, NFTB causes

significant oligonucleotide ESI suppression, while TFE generates the highest signal intensity in several occasions (Figures 4.3 and 4.4). It appears that both the Henry's law constant and ion suppression each influence the signal in a way that the final results are from the combined effects of these two phenomena. With this reasoning, NFTB is not a very good modifier because despite a favorable Henry's law constant, it causes significant signal suppression.

We suggest here that due to their more hydrophobic characteristics, DMCHA and OA prefer more hydrophobic fluorinated alcohols over HFIP. It can be seen in table 4.1 that while HFIP and HFMIP have a similar polar surface area, the overall surface area of HFMIP is significantly larger than HFIP. These data indicate that HFMIP has a larger non-polar area and hence is more hydrophobic. Distribution coefficients of HFIP and HFMIP at pH = 9 (normal pH range of oligo/IP solutions) also imply the same fact. Based on log D values, HFIP is the least hydrophobic alcohol after NFTB. This explains why HFIP produced the strongest MS signal in the presence of less hydrophobic IP agents, TEA and DIEA. Poor performance of the most hydrophilic alcohol, NFTB, even in the presence of less hydrophobic IP agents should be attributed to its very low pKa. On the other hand, more hydrophobic alcohols such as HFMIP and PFP generate higher ESI responses when used alongside hydrophobic IP agents — i.e., DMCHA and OA. Such behavior is not unprecedented. In fact, it has been reported previously that during negative-ion ESI of selective androgen receptor modulators, TFE increased response of more hydrophobic compounds but decreased response of a more hydrophilic compound (Wu, Gao et al. 2004). The same pattern is seen with the oligonucleotides where TFE suppresses the signal in the presence of less hydrophobic IP reagents, while enhancing electrospray response in the presence of more hydrophobic compounds.

In general, the data from DMCHA and OA show that the presence of the fluorinated alcohols do not always contribute to improved electrospray detection for oligonucleotides. In the case of these two IP agents the electrospray response for both RNAs were reduced by the presence of the fluorinated alcohols while for DNA the fluorinated alcohols aided DMCHA but again reduced the response form OA. These results were different than the more classically used TEA or even the more recently published DIEA which had improved response with HFIP and several other fluorinated alcohols. These results show that in some cases the fluorinated alcohols may actually only be modifiers for the HPLC separation.

Table 4.1- Physicochemical properties of the utilized fluorinated alcohols

	HFIP	TFE	HFMIP	PFP	NFTB
Structure					
Boiling point (°C)	59	74	61	82	45
pKa	7.97	11.49	8.08	12.52	4.91
Henry's law constant, H^{cp} ($\frac{mol}{m^3\ Pa}$)	0.23	0.57	0.17	0.44	0.08
Polar surface area (Å²)	20.23	20.23	20.23	20.23	20.23
Molecular surface area (Å²)	165.94	120.38	201.06	164.26	216.70
log *D* (pH = 9)	0.39	0.44	0.77	1.14	–0.64

One conclusion from these studies was that NFTB appears to be a poor choice when MS signal enhancement is desired. However, we observed an unusual behavior particular to NFTB. When used with OA, it appears to preserve the secondary structure of the analyzed oligonucleotide. Figure 4.5 shows the shift toward higher m/z peaks when different types of oligonucleotides are sprayed from solutions containing OA and NFTB. It is a well-known phenomenon for proteins that the charge envelope at higher m/z (fewer charges) is representative of the folded protein, while the envelope at lower m/z represents the denatured and unfolded protein structures formed in acidic solutions (Chowdhury, Katta et al. 1990, Loo, Loo et al. 1991, Konermann and Douglas 1998, Dobo and Kaltashov 2001, Šamalikova, Matečko et al. 2004, Watt, Sheil et al. 2007). Among the oligonucleotides we used, RNA and phosphorothioates could form hairpins and ssDNA was capable of folding on itself and making a short double-helix (Figure 4.6). Moreover, stabilization of the secondary structure in OA/NFTB seemed to happen more readily when it was more favorable thermodynamically. Comparison of the top and bottom panels in figure 4.5 indicates that the hairpin structures are much more abundant in the mass spectrum of ssDNA compared to phosphorothioate, to the point that the hairpin is essentially the only major peak observed in the mass spectrum of ssDNA molecules sprayed from OA/NFTB solution. This observation is in complete agreement with predicted free energies of folding for these two oligonucleotides ($\Delta G = -9.30$ Kcal/mol for ssDNA vs. $\Delta G = -4.95$ Kcal/mol for the phosphorothioate). All of these evidence is suggesting that the same principle should govern the charge state distribution of the oligonucleotides that we examined. Nevertheless, this notion is not completely established for oligonucleotides as other parameters can also shift the charge state distribution. For example, Bartlett et al. (Bartlett, McCloskey et al. 1996) attributed the high number of charges observed on methylphosphonates to dielectric shielding by the backbone

methyl groups. Therefore, we decided to measure the collision cross sections of different charge states of the phosphorothioate sprayed from a NFTB/OA solution using ion-mobility spectrometry in order to show that the higher m/z envelope represents a much more compact geometry consistent with a higher-order structure. The CCS values for different charge states of the 33-mer phosphorothioate are presented in table 4.2. While the average CCS value for ions

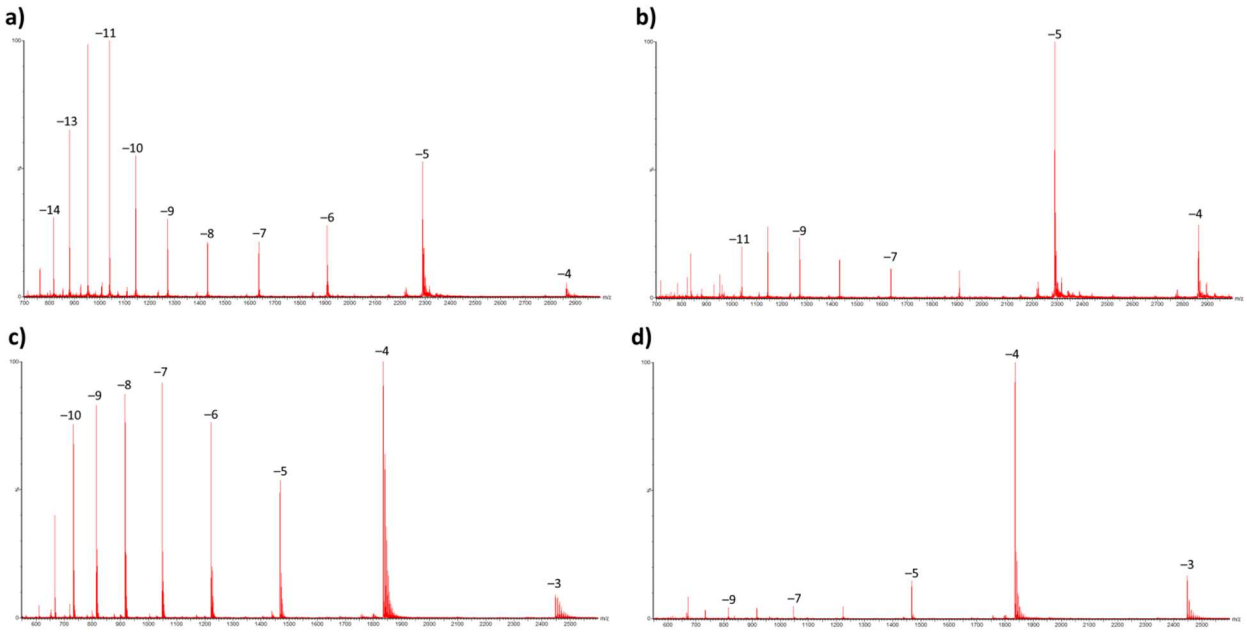


Figure 4.5- The effect of NFTB on stabilizing secondary oligonucleotide structures.

Top panels show the mass spectra of phosphorothioate infused in a solution of OA/HFIP (a) or OA/NFTB (b). Bottom panels show the mass spectra of the ssDNA strand in a solution of OA/HFIP (c) or OA/NFTB (d). Oligonucleotides sprayed from OA/NFTB solutions adopt a secondary structure with a lower charge state regardless of their type.

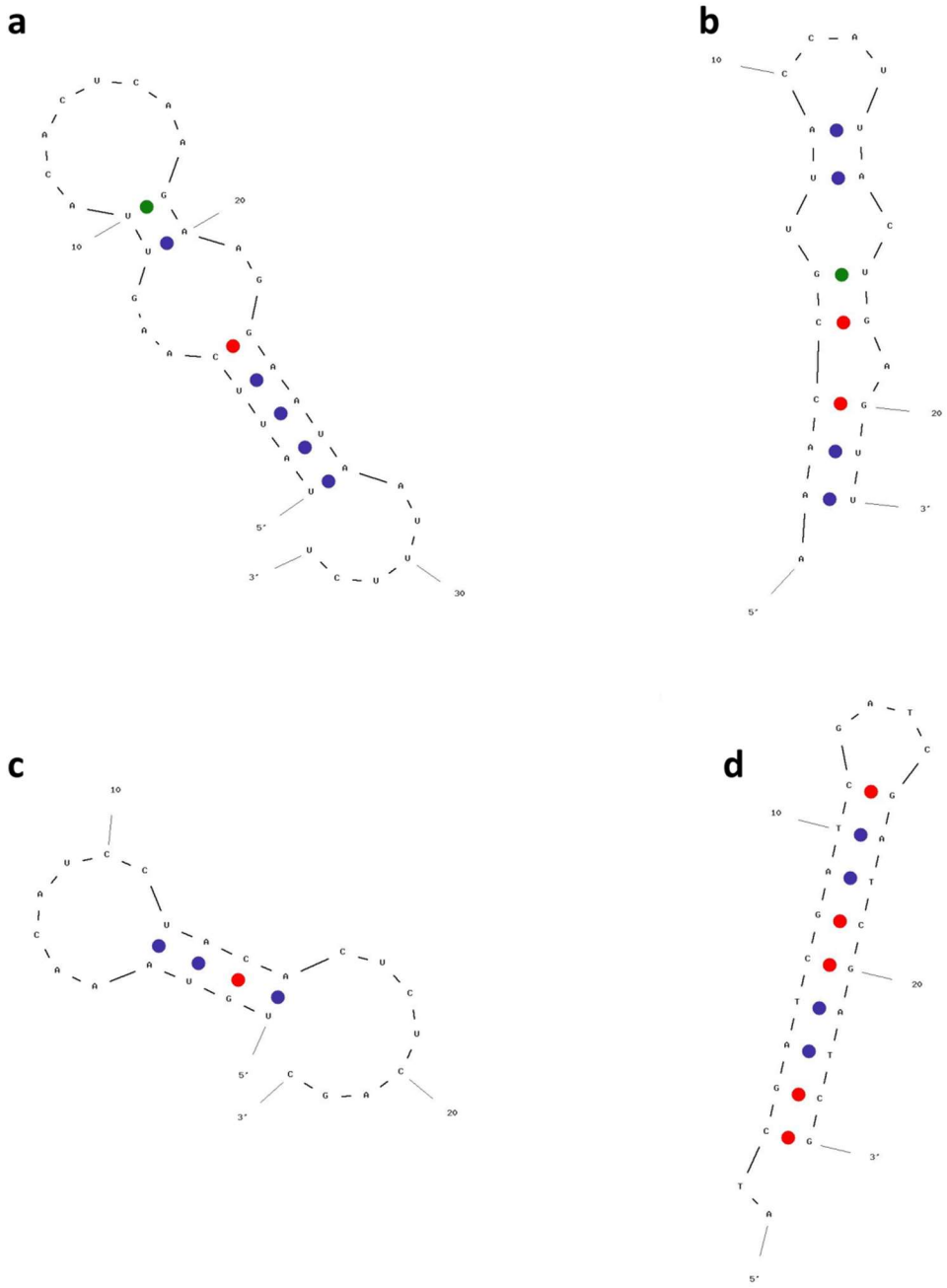


Figure 4.6- Predicted double-helical structures for the oligonucleotides used in this study.

(a) 33-mer phosphorothioate ΔG = –4.95 Kcal/mol, (b) miR-451 ΔG = –1.15 Kcal/mol, (c) miR-30c ΔG = –2.82 Kcal/mol, (d) 24-mer ssDNA ΔG = –9.30 Kcal/mol

carrying 8 to 12 negative charges is 1641 ± 166 Å², it declines rapidly to 984 ± 54 Å² for -5 and -6 ions.

Table 4.2- Collision cross section values for various charge states of the 33-mer phosphorothioate

m/z	Charge state	Ω (Å²)
954.76	–12	1814 ± 32
1041.65	–11	1763 ± 11
1145.92	–10	1673 ± 13
1273.35	–9	1557 ± 13
1432.65	–8	1400 ± 15
1637.45	–7	1228 ± 17
1910.53	–6	1022 ± 31
2292.84	–5	946 ± 11

It has been suggested previously that a transition from compact globular oligonucleotide conformers to more open elongated forms occurs at higher charge states because of an increase in coulombic repulsion (Hoaglund, Liu et al. 1997). However, the observed increase in collision cross section as a result of elongation was only a factor of 1.2 – 1.4. Our results indicate that the collision cross section at the highest charge states is almost 2X larger than the lowest charge state (Table 4.2). Furthermore, while the phosphorothioate conformer at 1400 Å² in -8 charge state very closely resembles an elongated form of a 36mer G-rich sequence with eight charges (Ω =

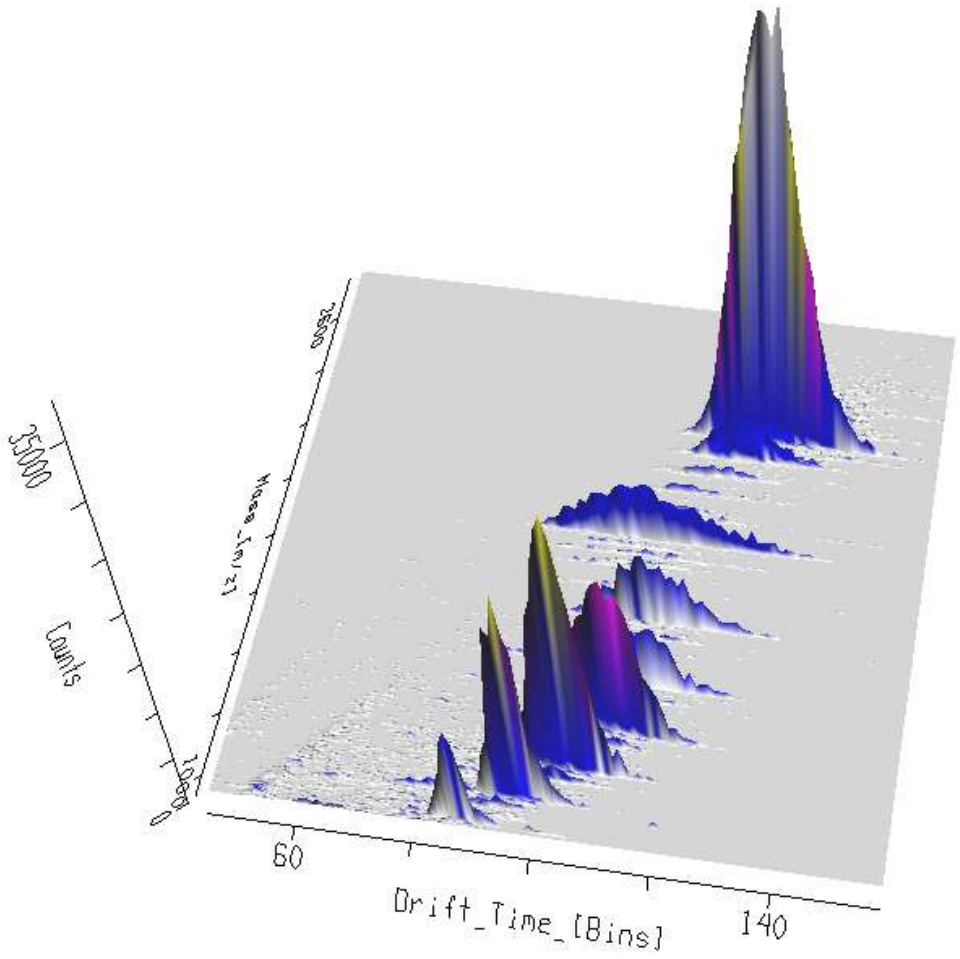


Figure 4.7- Drift time values for various ESI charge states of an oligonucleotide sprayed from a NFTB/OA solution.

Drift times for -5 to -12 charge states of the 33-mer phosphorothioate are shown here. It is clear that while the higher charge states at low m/z region (particularly the first four peaks) have fallen on a straight line indicative of the same drift time series, lower charge states have shifted to another series with shorter drift times (the last 2 peaks fall on a second line different from the line passing through higher charge states).

1416 Å²) (Baker, Bernstein et al. 2006), the collision cross section value of -5 ion (Ω = 946 ± 11 Å²) shows a very good correlation with the experimental cross section of the quadruplex geometry of the same G-rich sequence in -5 charge state (Ω = 989 Å²) (Baker, Bernstein et al. 2006) as well as a previously reported triple-helix with 36 nucleotides where Ω = 960 ± 30 Å² (Arcella, Portella et al. 2012). this strongly suggests that the oligo has adopted a secondary helical structure and not simply a compact globular form. It can also be observed in figure 4.7 that higher m/z peaks have clearly moved to a shorter drift time series. Through further experimentation, we realized that the same phenomenon can be reproduced with other hydrophobic linear alkylamines such as hexylamine (HA) and dibutylamine (DBA) when used with NFTB. However, just adding a single methyl side chain in methyldibutylamine (MDBA) is enough to eliminate this effect (Figure 4.8).

It is noticeable in figure 4.5 that the ssDNA already shows its secondary structure in an OA/HFIP solution, while this phenomenon was not observed with other IP agents — i.e., DIEA, TEA and DMCHA. This observation clearly indicated that the major driver of secondary structure stabilization is the IP agent. However, it is also noticeable that using NFTB alongside an appropriate IP reagent greatly increases the secondary structure formation. We believe this is due to the lower pKa of NFTB. Cech and Enke have mentioned previously that the oligonucleotide charge states achieved with the TFE solvent are significantly higher than those achieved with HFIP (Cech and Enke 2001) which is consistent with our observations. This could be explained based on the lower pKa of HFIP, which causes higher protonation of oligonucleotides and therefore a decreased charge state. In the same manner, since the pKa of NFTB is significantly lower than all other alcohols investigated here (Table 4.1), it drives the oligonucleotides to lower charge states and it is easier for these molecules with less charges to

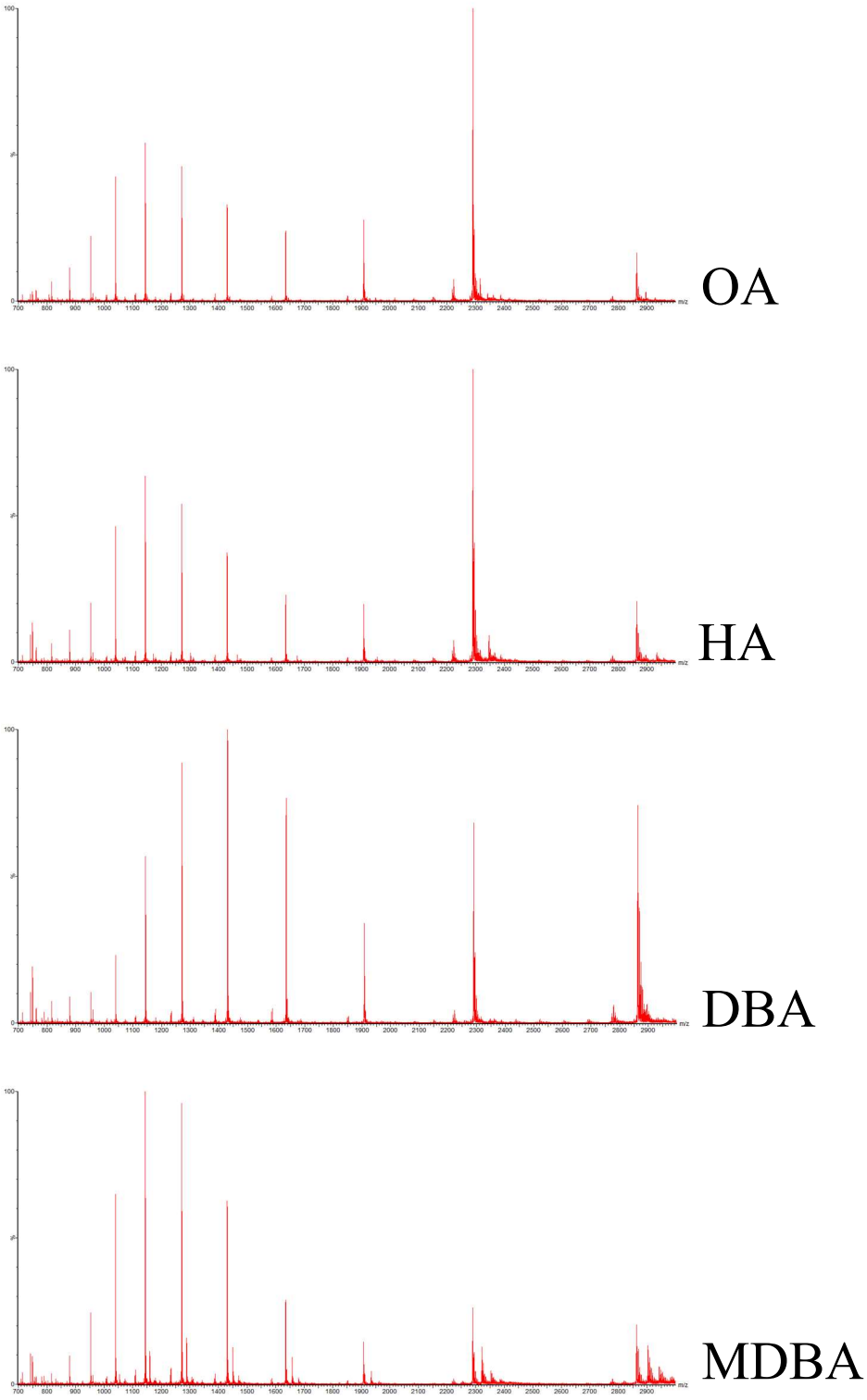


Figure 4.8- Only long, linear alkylamines promote the formation of secondary structures.

fold and form secondary structures. Consistent with this idea, adding formic acid to OA solutions generated similar results to NFTB addition (Figure 4.9). Although formic acid had to be used at much higher concentration than NFTB and it caused more ESI signal suppression and more cluster formation. Also, despite its lower pKa value, formic acid could not shift the charge state distribution as much as NFTB even at higher concentration. Of course, the poor performance of formic acid as an ESI modifier (compared to NFTB) was not unexpected considering its unfavorable properties, such as high Henry's law constant and boiling point.

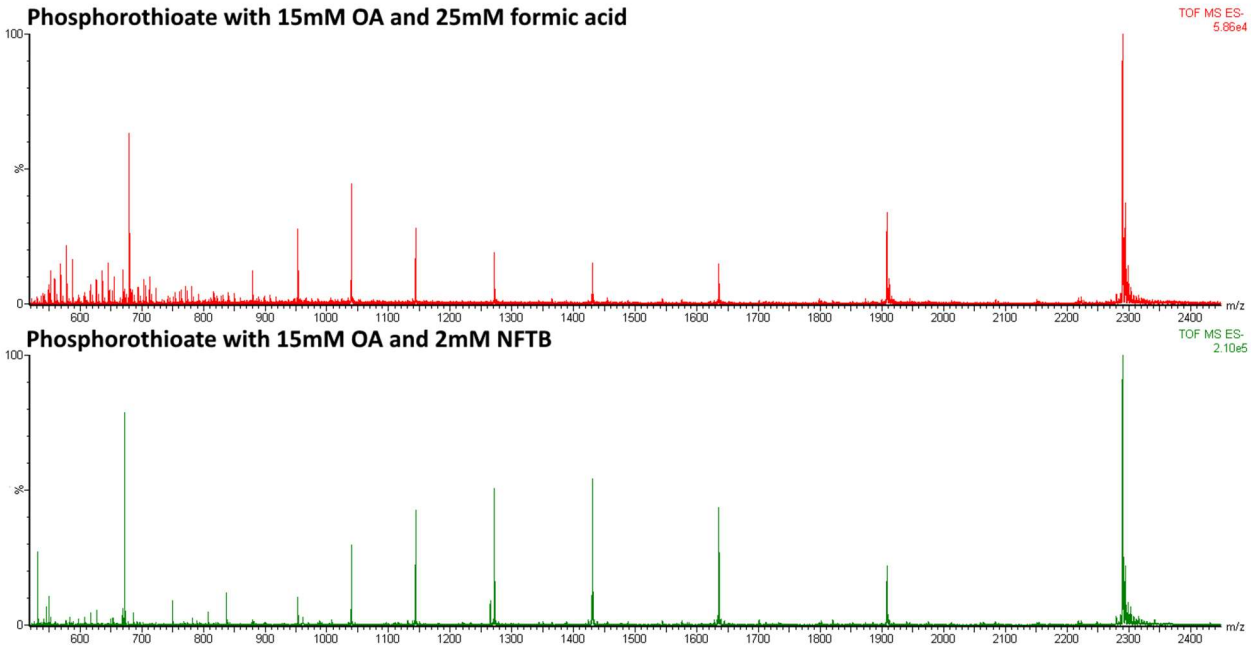


Figure 4.9- Oligonucleotide secondary structures are formed in OA/formic acid solutions.

Finally, in order to demonstrate the applicability of other fluorinated alcohols for LC-MS analysis, the full-length phosphorothioate and its n-1 truncation were separated using

DMCHA/HFIP and DMCHA/HFMIP mobile phases. As shown in figure 4-10, the DMCHA/HFMIP mobile phase can significantly enhance MS signal intensity without affecting the chromatographic separation.

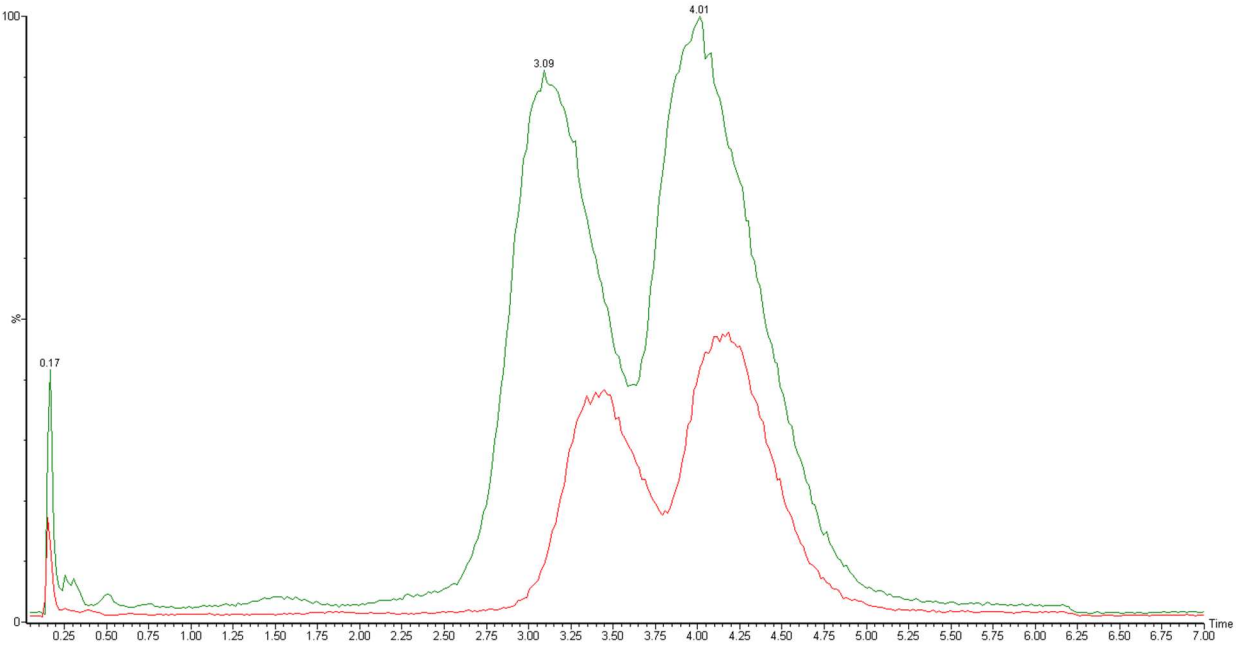


Figure 4.10- Comparison of HFMIP and HFIP as mobile phase modifiers.

Total ion chromatogram of the mixture of phosphorothioate and its n-1 truncation acquired using a DMCHA/HFIP (red) or DMCHA/HFMIP (green) mobile phase. HFMIP generates an enhanced MS signal, without affecting the chromatographic resolution.

Conclusion

The use of other fluorinated alcohols instead of HFIP for LC-MS analysis of oligonucleotides was investigated for the first time. The behavior of fluorinated alcohols was

similar to many reported studies on IP agents in that the maximum MS signal intensity was not always produced by HFIP. Two IP agents in particular, DMCHA and OA, could support much higher signal intensities when used with other fluorinated alcohols such as HFMIP and sometimes without the addition of any fluorinated alcohol. Our general guidelines for choosing IP/fluorinated alcohol based on the oligonucleotide type are summarized in table 4.3 below. These suggestions are made based on the fact that OA is less soluble in the aqueous mobile phase and requires higher organic content which makes it not applicable to all situations; particularly if we are analyzing a shorter oligonucleotide that cannot be retained with high organic content. We have also observed that TFE does not have good chromatographic properties. Therefore, its combinations with IP agents were omitted from the final list, as well.

Table 4.3- Optimized mobile phase conditions for the LC-MS analysis of various oligonucleotides

Oligonucleotide type	Optimum IP agent	Optimal fluorinated alcohol
ssDNA	TEA	HFIP
RNA	DBA	HFMIP
Phosphorothioate	DMCHA	HFMIP

Another interesting property of OA and other hydrophobic linear alkylamine IP agents was their ability to preserve the secondary structures of those oligonucleotides capable of forming such double-helical conformations. The shift in charge state distribution as well as very

sharp decrease in CCS values of the lower charge states of oligonucleotides confirmed that they have adopted secondary structures.

The change of MS charge state distribution as a result of folding/unfolding in various pH values is a well-known phenomenon for proteins. However, due to the very low pKa of the backbone phosphate groups, this approach is not applicable to oligonucleotides. Nevertheless, the ESI-MS structural study of oligonucleotides in their folded state compared to their unfolded structure is desirable for many purposes. One example of this is the study of G-quadruplexes. Previous reports have indicated that G-quadruplex structures are stable in the gas phase and they can be readily studied by mass spectrometry (Baker, Bernstein et al. 2006, Mazzitelli, Wang et al. 2007). But due to the role of cations in stabilizing the structure through coordination with the O6 atom of guanine, different quadruplex structures exist at different solution conditions. For example, antiparallel basket-type forms have been observed in Na^+ solutions (Wang and Patel 1993) in contrast to more parallel quadruplexes in K^+ solutions (Parkinson, Lee et al. 2002). Substitution of Na^+ and K^+ with NH_4^+ ions for electrospray mass spectrometry can result in less stable and more polymorphic quadruplex structures (Smargiasso, Rosu et al. 2008) that are not representative of physiological conformers. In order to deal with this issue, a recent study has suggested spraying G-quadruplexes from a trimethylammonium solution doped with KCl (Marchand and Gabelica 2014). The authors have reasoned that in contrast to NH_4^+ ions, triethylammonium ions cannot coordinate between G quartets due to their larger size. Therefore, only K^+ ions participate in the resulting G-quadruplexes making a more physiologically relevant structure that is also ESI compatible. The NFTB/OA solution may prove particularly useful in this case. Secondary oligonucleotide structures sprayed from this solution, show a significant amount of sodium and potassium adduction even though they were not added to the solutions

(Figure 4.5). This is not surprising since it is well-known that desalting the secondary structures is more difficult than single strands (Beverly, Hartsough et al. 2005) as cation adduction helps with salt bridge formation and the overall stability of the secondary structure. In the same manner, it could be expected that NFTB/OA solutions would substantially improve the formation of physiologically relevant Na^+ or K^+ coordinated G-quadruplexes by electrospray ionization.

Finally, by using a DMCHA/HFMIP mobile phase, we were able to enhance the MS signal intensity of a phosphorothioate molecule significantly more than it was possible with any combination of HFIP and IP agents. Moreover, HFMIP did not adversely affect the chromatography. These observations suggest that replacing HFIP with other fluorinated alcohols in some cases can significantly increase the sensitivity of LC-MS methods for oligonucleotide separation.

Acknowledgements

The experiments described in this manuscript were supported in part by grants from the National Cancer Institute (CA176653) and ProQR Therapeutics.

CHAPTER 5

DIRECT DETERMINATION OF MICRORNAS USING LC-MS: APPLICATION TO MIR-451[4]

[4] Babak Basiri, Shirin Hooshfar, Mandi M. Murph and Michael G. Bartlett. To be submitted to *Analytical Chemistry*

Abstract

In recent years, small endogenous RNAs have come to the forefront of both basic and translational research. For example, many studies have pointed to the potential role of microRNAs as disease biomarkers. However, precise quantitative methods for the analysis of microRNAs are still lacking. In this study, we report the first mass spectrometry-based quantitation of miR-451, a circulatory microRNA. Using a highly selective sample preparation method with an average recovery of 83.6% and a novel mobile phase chemistry, we were able to reach an LOQ of 0.5 ng/mL. Because of such high sensitivity, we could detect and quantify the endogenous miR-451 from both human and rat plasma. Considering the increased precision of LC-MS compared to other methods, these results usher in a new era of miRNA biomarker discovery and validation.

Introduction

There have been enormous efforts made to investigate correlations between disease state and changes in the expression of various microRNAs (Mitchell, Parkin et al. 2008, Widera, Gupta et al. 2011, Hauser, Wulfken et al. 2012, Lorenzen and Thum 2012, Jacob, Cooley et al. 2013, Kumar, Dezso et al. 2013, Mishra 2014, Prensner, Zhao et al. 2014, Schultz, Dehlendorff et al. 2014). Interestingly, many of these microRNAs are secreted from cells into extracellular fluids where they remain surprisingly stable (Gupta, Bang et al. 2010, Kosaka, Iguchi et al. 2010). This had led many to consider if bodily fluids such as plasma, urine and even saliva can provide similar diagnostic capabilities as more invasive biopsy procedures. Recent limited clinical studies have shown miRNAs are useful in predicting many diseases such as multiple

sclerosis (de Faria, Moore et al. 2012), Alzheimer's (Kumar, Dezso et al. 2013), cardiovascular dysfunction (Gupta, Bang et al. 2010), pulmonary hypertension (Wei, Henderson et al. 2013) and amyotrophic lateral sclerosis (Toivonen, Manzano et al. 2014). However, the greatest attention has been given to their potential role in the diagnosis and treatment of cancer (Mitchell, Parkin et al. 2008, Kosaka, Iguchi et al. 2010, Mishra 2014). For example, clinical trials involving patients with colorectal cancer revealed that elevated levels of miR-320e were strongly associated with poorer outcomes when treated with 5-fluorouracil (Perez-Carbonell, Sinicrope et al. 2015). A recent study also found that plasma levels of miR-199a-5p were significantly decreased in triple-negative breast cancer patients when compared to other types of breast cancer and normal patients, suggesting that this miRNA may be a specific biomarker for this type of breast cancer (Shin, Siu et al. 2015). Another study similarly found that miR-133a was significantly decreased in patients with non-small cell lung cancer (Lan, Zhang et al. 2015).

MiR-451 is a highly-conserved microRNA among all vertebrates (Yang, Maurin et al. 2010) and several studies have shown that it has the highest concentrations in serum and plasma in multiple species, including mice (Jacob, Cooley et al. 2013), rats (Gao, Liu et al. 2017), cattle (Spornraft, Kirchner et al. 2015) and humans (Wang, Zhu et al. 2010, Reid, Kirschner et al. 2011, Jones, Zabolotskaya et al. 2012, Wang, Yuan et al. 2012, Aushev, Zborovskaya et al. 2013, Williams, Ben-Dov et al. 2013). It acts as a positive regulator of erythroid maturation (Rasmussen, Simmini et al. 2010) and its antisense oligos have been speculated for the treatment of polycythemias (Zhan, Miller et al. 2007). It also plays a role in murine embryo implantation (Li, Jia et al. 2015) and numerous studies have established that miR-451 is widely dysregulated in human malignancies, including lung cancer (Bian, Pan et al. 2011, Wang, Wang et al. 2011), gastric cancer (Bandres, Bitarte et al. 2009), breast cancer (Kovalchuk, Filkowski et al. 2008,

Bergamaschi and Katzenellenbogen 2012), hepatocellular carcinoma (Shen, Siegel et al. 2016), glioma (Godlewski, Bronisz et al. 2010) and leukemia (Li, Sanda et al. 2011). More importantly, serum levels of miR-451 are altered in several types of cancer (Pan, Wang et al. 2013). Therefore, further investigation of the potential of circulating miR-451 as a noninvasive biomarker is essential. Nevertheless, such studies have been impeded by significant deficiencies in our ability to accurately quantify this macromolecule.

Currently, for both basic and clinical studies, methods based on hybridization such as ELISA, different variations of qPCR and micro-arrays are the methods of choice for the analysis of oligonucleotides. However, these methods demonstrate specific disadvantages for biomarker discovery due to problems with specificity, normalization and the biased performance of different PCR techniques (Gee, Buffa et al. 2011, Wang, Yuan et al. 2012, Wang, Meng et al. 2013). It has been reported previously that microarray-based miRNA detection methods cannot reliably distinguish between miRNAs that have one or only a few mismatches (Miska, Alvarez-Saavedra et al. 2004). And even though real-time reverse transcription polymerase chain reaction (qRT-PCR) is often considered as the ultimate standard for miRNA assays (Git, Dvinge et al. 2010), the use of miRNA qPCR panels from different commercial suppliers can provide inconsistent results. For example, serum miR-107 showed much higher levels when measured with Exiqon compared to Taqman qPCR kits as reported by Wang *et al.* (Wang, Yuan et al. 2012). More recently the use of cDNA libraries followed by next generation sequencing (Next-Gen) has been widely applied to the detection of microRNAs (Eminaga, Christodoulou et al. 2001, Lu, Meyers et al. 2007, Motameny, Wolters et al. 2010, Cobos Jiménez, Willemsen et al. 2014, Tam, de Borja et al. 2014). The quality of Next-Gen sequencing is completely dependent on the cDNA library and amplification has the potential to alter ratios of expressed miRNA

during library preparation. For example, Gao *et al.* observed that the expression of miR-486 was highly over-represented when they used miRNA-seq to characterize the miRNA profile of the whole blood in rats (Gao, Liu et al. 2017).

Compared to qPCR techniques, mass spectrometry can generate values that are more quantitative, easier to normalize and less prone to bias (Wang, Meng et al. 2013). The increased precision of LC-MS based methods in comparison to such alternatives, results in a significant reduction in the number of samples/patients needed to determine the statistical significance of a biomarker and so it is a much better method for validation and subsequent analysis of miRNA biomarkers. Moreover, endogenous nucleic acid molecules usually undergo chemical modifications that are of functional biological importance (Karijolich, Kantartzis et al. 2010). It has been reported that *Arabidopsis* miR-173 has a 2'-O-methylation on its 3' terminal ribose (Yu and Chen 2010). This modification has also been observed in *Drosophila* where mutations in the *hen1* gene, which is responsible for 2'-O-methylation of small RNAs, resulted in accelerated neurodegeneration and a shorter life span (Abe, Naqvi et al. 2014). Therefore, the reliable identification of microRNA modifications with high confidence is of great importance in elucidating both their biological function and kinetic profile. Despite their superior sensitivity and relative ease of use, hybridization-based methods have difficulty in detecting post-transcriptional modifications such as methylation as well as truncations. Therefore, MS-based techniques could play an indispensable role in the identification of modified miRNAs.

To our knowledge, there are only two direct MS analyses of an endogenous miRNA reported so far. One is of miR-122, a microRNA highly expressed in the liver, that was extracted from human hepatocytes and mouse livers. The acquired mass spectra revealed that one variant of miR-122 has a 3′-terminal adenosine that is introduced after processing by Dicer (Katoh,

Sakaguchi et al. 2009). Another study by Nakayama *et al.* (Nakayama, Yamauchi et al. 2015) identified 20 miRNAs from HeLa cells in an untargeted manner using LC-MS. Interestingly, several of them appear in multiple forms such as miR-23a which had variants missing the 3'-cytosine and a variant with an additional 3'-adenosine. Nevertheless, both studies have extracted microRNAs from tissues and cell samples where the concentration of miRNAs is relatively high and they have made no effort to quantitate the analyzed miRNAs. Here, we present a bioanalytical method for direct and accurate quantitation of miR-451 from plasma which can be easily adapted to the study of other miRNAs in plasma and other tissues.

Materials and methods

Chemicals and reagents: Tris, EDTA, NaCl, guanidine hydrochloride, Triton X-100, HCl, DTT, triethylamine (TEA), dibutylamine (DBA), hexafluoroisopropanol, 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol, lipase from *Candida rugosa* (catalog # L8525) and LC-MS grade methanol and water were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Proteinase K was purchased from Gold Biotechnology, Inc. (Olivette, MO). Nuclease-free water and pipette tips and RNaseZap solution were from Ambion (Thermo Fisher Scientific, Waltham, MA) and DNA Lobind microcentrifuge tubes were purchased from Eppendorf (Hauppauge, NY). Sprague Dawley male rat plasma, C57BL/6 mouse plasma and human plasma were obtained from Bioreclamation IVT (Westbury, NY). Hydrophilic streptavidin magnetic beads (part # S1421) were purchased from New England Biolabs (Ipswich, MA). Synthetic miR-451 (AAACCGUUACCAUUACUGAGUU) and its 5' n-1 truncation were purchased from Eurogentec (Seraing, Belgium) and the biotinylated capture strand (5'-Biotin-AACTCAGTAATGGTAAC) was from the Midland Certified Reagent Company (Midland, TX).

Preparation of working and stock solutions: 1 mg/mL stock solution of miR-451 was prepared in nuclease-free water and stored at -80˚C prior to use. The concentration of the stock solution for the biotinylated capture strand was 0.1 A260 units per µL (about 2.5 mg/mL) and was stored at -20˚C. Proteinase K (20 mg/mL) and lipase (15 mg/mL) solutions were made fresh in nuclease-free water before starting the experiment. The Proteinase K digestion buffer (McGinnis, Cummings et al. 2013) contained 60 mM Tris, 100 mM EDTA, 400 mM guanidine hydrochloride, and 0.1% Triton X-100 at pH 9 and was stored at 4 ˚C. 20 mM DTT was added to aliquots of this buffer before use. Working solutions of miR-451 were prepared at concentrations of 50, 5, 0.5 and 0.25 µg/mL by serial dilution of the stock solution with nuclease-free water. Importantly, all surfaces and equipment that came in contact with the samples were thoroughly wiped with RNaseZap prior to starting the analysis to avoid RNA degradation. Moreover, samples were prepared and analyzed in DNA Lobind tubes to minimize non-specific oligonucleotide losses to the tube walls.

Sample preparation: To 500 µL plasma, 100 µL of proteinase K digestion buffer, 70 µL of 20 mg/mL proteinase K and 70 µL of 15 mg/mL lipase from *Candida rugosa* were added. Samples were vortexed for 1 min, then heated at 55˚C with shaking (250 rpm) for 45 min.

In the meantime, the magnetic beads were prepared as follows:

Elution Buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] was pre-warmed in a 65°C bath and Low Salt Buffer [0.15 M NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA] was chilled in an ice bath.

2.0 A260 units (50 µg) of the biotinylated capture strand (5'-Biotin-AACTCAGTAATGGTAA C-3') was dissolved in 500 µL of Wash/Binding Buffer [0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA].

200 µL (1500 µg) of Hydrophilic Streptavidin Magnetic Beads were added into a clean RNase-free microcentrifuge tube. 200 µL of Wash/Binding Buffer was added and the beads vortexed to suspension. Supernatant was removed and discarded. 50 µL of biotinylated capture solution was added to the magnetic beads and vortexed to suspend the beads. They were incubated at room temperature for 10 minutes with occasional agitation. The magnet was applied and the supernatant removed and discarded. The beads were washed two times by adding 200 µL of Wash/Binding Buffer.

After 45 min, calibration curve samples were spiked with miR-451 and 750 µL of 2X Wash/Binding Buffer [1 M NaCl, 40 mM Tris-HCl (pH 7.5), 2 mM EDTA] was added to each plasma sample. The samples were then heated at 65°C for 5 minutes and quickly chilled on ice for 3 minutes.

The plasma samples from the previous step were added to prepared magnetic beads, vortexed to suspend the particles and incubated at room temperature for 20 minutes with occasional agitation. Next, the magnet was applied and the supernatant removed. 200 µL of Wash/Binding Buffer was added (2X). 200 µL of cold Low Salt Buffer was added, the magnet was applied and the supernatant discarded.

25 µL of prewarmed Elution Buffer was added followed by vortexing to suspend the beads, then incubated at 65°C bath for 2 minutes. The magnet was applied and the supernatant transferred to a clean auto-sampler vial. Elution was repeated with 25 µL of fresh Elution Buffer.

Instrumental conditions and software: LC-MS experiments were performed using a Waters Acquity UPLC system coupled to a Waters (Milford, MA) Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer. Tuning parameters were as follows: capillary voltage -2.0 kV, cone voltage 25 V, extraction cone voltage 2 V, source temperature 125 ˚C, desolvation

temperature 450 ˚C, cone gas 0 L/h and desolvation gas (nitrogen) 1000 L/h. The MS data acquisition was performed in negative-ion sensitivity mode with a 1 s scan time over the m/z range of 400-1400 with target enhancement at 578 m/z. The UPLC column flow-through was diverted to waste during the first two minutes to avoid entrance of salts to the mass spectrometer and the MS acquisition was performed from 2-8 minutes. The biotinylated capture strand (internal standard) was measured at 559.7 m/z and miR-451 at 577.9 m/z.

UPLC separation was performed at 65˚C using a Waters Acquity UPLC BEH C18 column (1.7 µm, 1.0 x 100 mm). Mobile phase A was 15 mM dibutylamine and 25 mM 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol in 5% methanol. Mobile phase B was 95% methanol. The flow rate was set to 0.15 mL/min and the injection volume was 20 µL. Autosampler temperature was maintained at 4˚C and the needle was cleaned with 600 µL of weak wash (10% methanol) and 200 µL of strong wash (70% methanol) after each injection. The gradient was as follows:

17% B from 0-2 min, 17-19% B from 2-3.5 min, 19-20% B from 3.5 to 5 min, 20-24% B from 5-6 min, 24-30% B from 6 to 7 min, 30% B for 7-8 min and 17% B from 8.01 to 12 min.

The UPLC and MS operation and data acquisition were performed using Waters MassLynx 4.1 (Milford, MA).

Results

Optimization of miR-451 extraction protocol: The overall scheme of our sample preparation strategy is summarized in figure 5.1. We decided to use a biotinylated capture strand with a complimentary sequence to miR-451 in conjunction with streptavidin magnetic beads to concentrate our target microRNA. Despite the seemingly straightforward nature of this sample extraction method, we had to overcome several challenges before successful implementation of

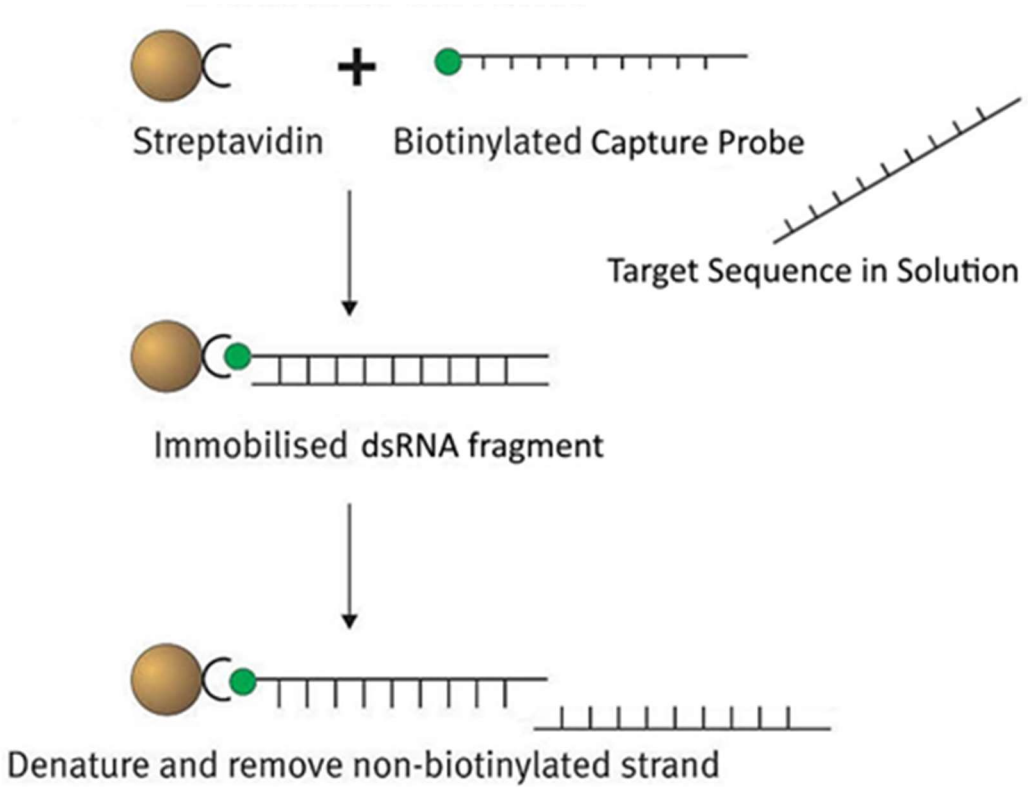


Figure 5.1- Schematic representation of the sample preparation approach developed for this study

this approach. In order to avoid difficulties arising from the fast degradation of spiked RNA in plasma, we performed our preliminary method optimization experiments using a ssDNA strand. Nevertheless, we were initially not able to even extract the spiked DNA strand from plasma. Since oligonucleotides have very high protein binding affinity (Yu, Geary et al. 2004, van Dongen and Niessen 2011, McGinnis, Cummings et al. 2013), we suspected that this problem could be caused because of DNA binding to plasma proteins and particularly albumin. McGinnis *et al.* have introduced a proteinase K digestion technique that can effectively lyse proteins in a

biological sample and release oligonucleotides into the solution (McGinnis, Cummings et al. 2013). Adding a proteinase K digestion step at the beginning of our sample preparation procedure resulted in successful ssDNA extraction from plasma using the biotinylated capture strand and streptavidin magnetic beads. For proteinase K treatment, we followed the same procedure as described by McGinnis *et al.* (McGinnis, Cummings et al. 2013) with two major alterations: first, we also included a lipase in our digestion mixture in addition to buffer and proteinase K. Second, we only incubated plasma samples for 45 minutes and then continued with the rest of our sample preparation procedure instead of using the 3-hour incubation from the original protocol. Our results indicated that this shortened incubation period did not influence oligonucleotide recovery when using streptavidin magnetic beads.

The next problem appeared when we started spiking plasma samples with RNA instead of ssDNA. Again, we could not recover any of the spiked RNA at the end of the sample preparation. This problem was caused by plasma RNases that degraded any spiked RNA in a matter of minutes. To overcome this issue, we had to spike calibration samples with miR-451 after the completion of the proteinase K digestion which successfully degraded the plasma RNases. Otherwise, this approach would not have succeeded for the quantitation of endogenous miR-451.

The final problem was with the adoption of an internal standard. Generally, an oligonucleotide with a different sequence from the analyte (usually a poly-T strand) is added to the samples and utilized as the internal standard. Nevertheless, since only target miRNA and its related sequences can hybridize with the capture strand, we cannot extract other oligonucleotides from plasma samples. Yet, the biotinylated capture strands are present in the final extract on top of miR-451. This happens because the streptavidin-biotin interaction is eliminated for the final

elution of miR-451 from the magnetic beads through denaturation of streptavidin. Therefore, biotinylated capture strands are also released into the final eluent. We investigated the possibility of using the capture strand as an internal standard and our analysis of a large number of injections revealed that the MS response from the capture strand is largely constant among different samples with a relative standard deviation below 10% (Figure 5.2).

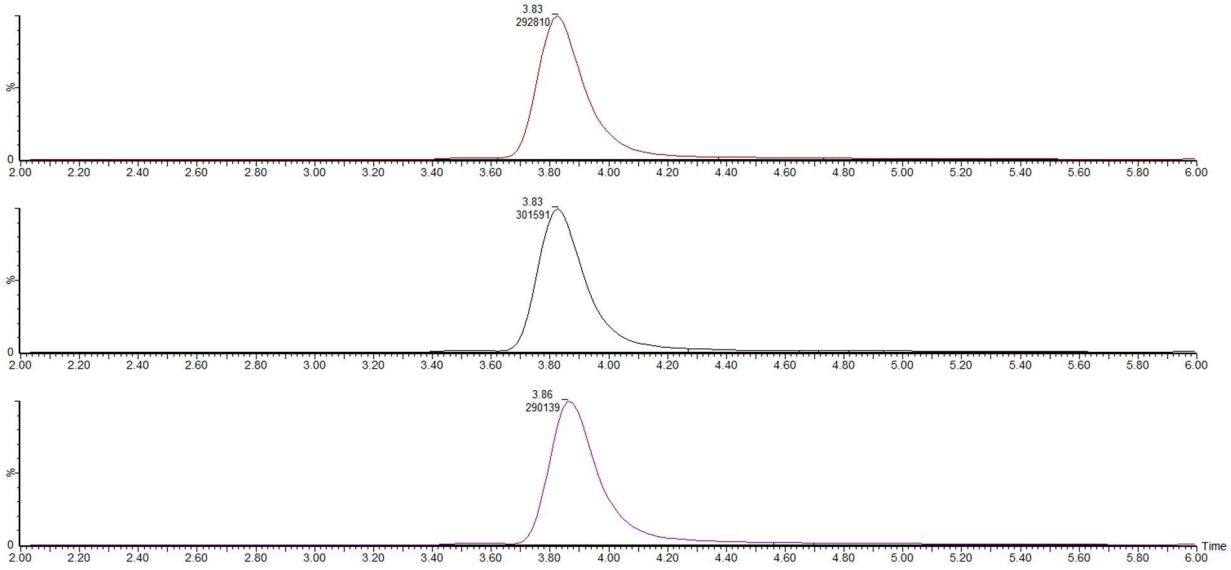


Figure 5.2- The MS peak at m/z = 559.7 for the biotinylated capture strand from 3 different samples. The peaks are annotated with retention times and area counts. Note that area counts have remained largely constant for the different samples.

Optimization of the liquid chromatography conditions for miR-451: Selecting the appropriate ion-pairing reagent among many different alkylamines that have been used as ion-

pairs is the single most important factor in the development of a sensitive LC-MS method for oligonucleotides. We have recently published an algorithm that helps with the selection of ion-pairs based on the composition of the analyzed oligonucleotide (Basiri, Murph et al. 2017). Based on the predictions of this algorithm which was further confirmed by experimental investigation, we concluded that miR-451 would generate the highest MS signal intensity in the presence of dibutylamine (DBA). Interestingly, using DBA not only increased the MS response, it also resulted in better chromatography. RNAs are hard to retain on a reversed-phase column and it is clear in figure 5.3 that miR-451 is hardly retained when triethylamine (TEA) is the ion-pair. Nevertheless, replacing TEA by DBA resulted in complete retention of RNAs on a C18

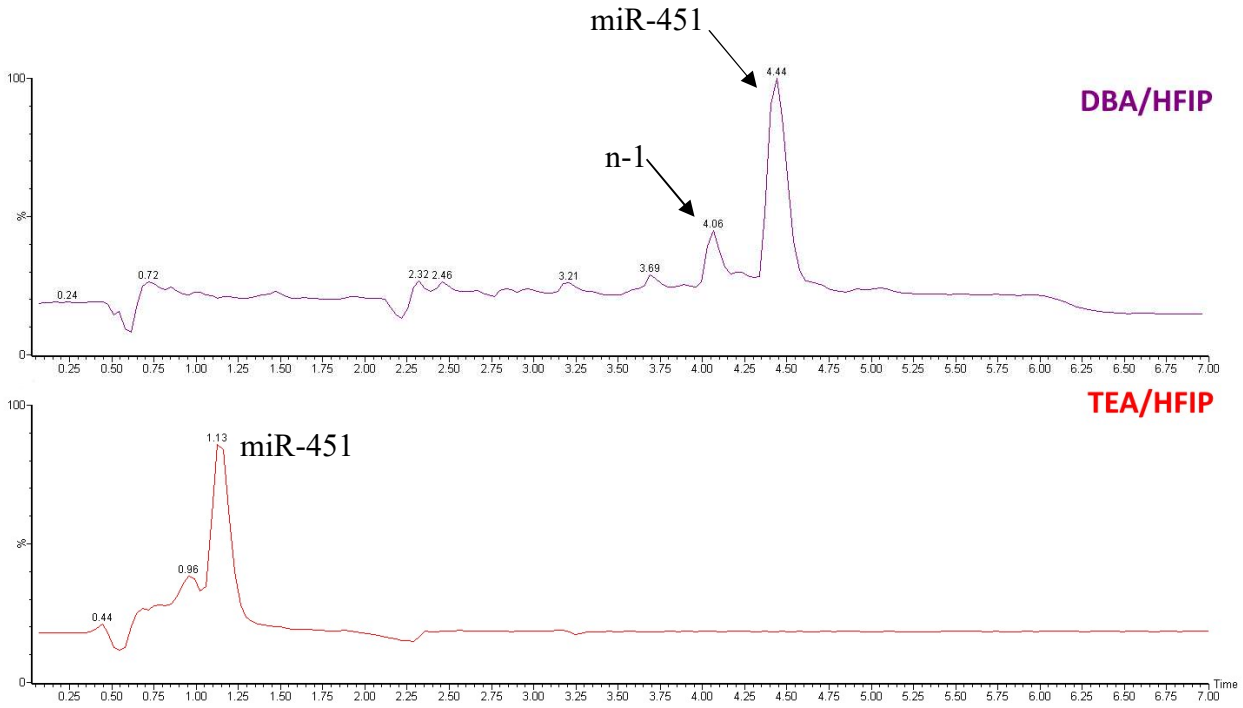


Figure 5.3- Chromatographic separation of miR-451 and its n-1 oligonucleotide using a DBA/HFIP mobile phase in comparison to the classical TEA/HFIP.

column and excellent separation of miR-451 and its n-1 truncation.

We have also reported previously that the use of 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP) with more hydrophobic ion-pairs results in better oligonucleotide MS response when compared to HFIP (Basiri, van Hattum et al. 2017). As shown in figure 5.4, this is the case for DBA. Using DBA with HFMIP instead of HFIP, results in a two-fold increase in the MS response of miR-451. Based on these observations, we chose the DBA/HFMIP mobile phase for our analysis. The final mobile phase (A) composition was 15 mM DBA and 25 mM HFMIP in 5% methanol. Considering the low concentration of microRNAs in plasma, it was only owing to the increased sensitivity of this mobile phase that we were able to detect the endogenous miR-

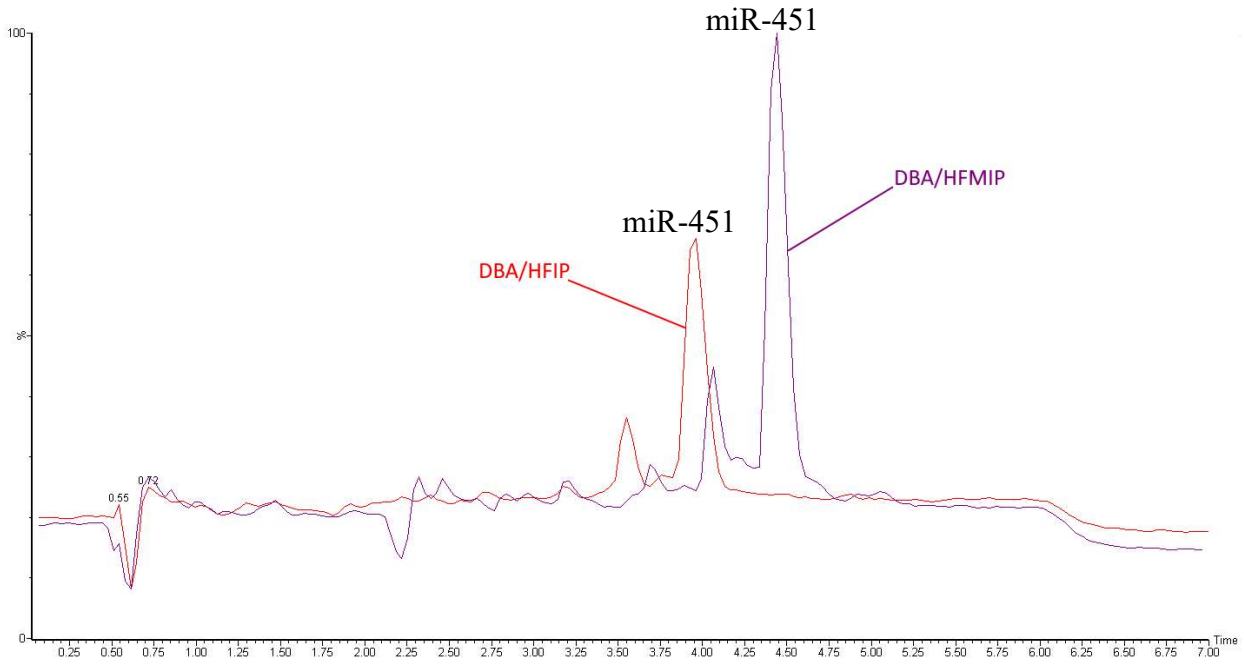


Figure 5.4- Replacing HFIP with HFMIP resulted in a significant increase in the MS signal intensity of miR-451 when DBA was used as the ion-pairing reagent.

451 as described later. To further demonstrate the robustness and sensitivity of the developed LC-MS method, we performed a limited validation study as discussed in the following sections.

Selectivity and specificity: Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. To ensure the selectivity of this method, rat plasma samples were spiked with miR-451 after proteinase K treatment and the MS traces of the extracted samples were investigated for any interferences. As demonstrated in figure 5.5, baseline separation from other interfering compounds in the biological matrix was achieved for miR-451 and the biotinylated oligonucleotide extracted from rat plasma.

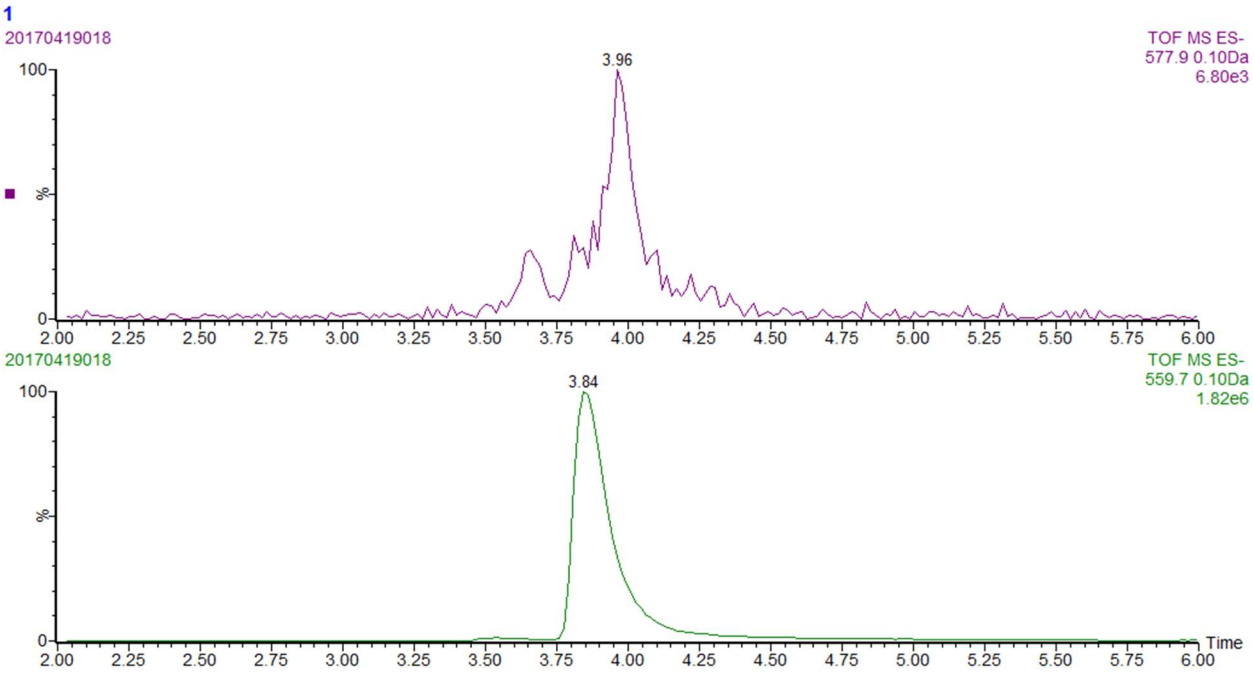


Figure 5.5- MS traces for miR-451 (top) and biotinylated capture strand (bottom) extracted from rat plasma samples spiked with 1.0 ng/mL of miR-451.

Symmetrical peak shape, good resolution and reasonable retention time for all compounds are indicative of a selective and specific UPLC separation.

Linearity and calibration curve: A calibration curve was generated by spiking proteinase K-digested rat plasma samples with 11 increasing concentrations miR-451. The spiked concentrations were 0.5, 1, 2, 4, 6, 10, 30, 50, 100, 200 and 400 ng/mL. The mentioned calibration curves also consisted of a non-spiked sample. The correlation coefficient of the resulting calibration curve was larger than 0.99 without any weighting (Table 5.1) indicating very good linearity over the examined range.

Table 5.1- Calibration curve parameters for LC-MS analysis of miR-451

Retention time	Slope	Intercept	Correlation coefficient
3.95 $\pm$ 0.3	0.977	8.085	0.993

Precision and accuracy: The limit of quantitation (LOQ) was established at 0.5 ng/mL by careful examination of the MS traces acquired during the preparation of the calibration curve and further verification of precision and accuracy criteria. The precision of the method indicates the degree of dispersion within a series of determinations on the same sample. The precision of the method was determined by analysis of four different concentrations: 0.5 ng/mL (LOQ), 1.0 ng/mL (low-QC), 20 ng/mL (mid-QC) and 200 ng/mL (high-QC) in five replicates on the same day. The data were evaluated as percent relative standard deviation (RSD%) for the

determinations. The accuracy of the assay method was evaluated by performing the analysis against a calibration curve and calculating the mean percent differences between the theoretical values and the measured values. As represented in table 2, all of the values did fall within the FDA specifications (<15% for QC and <20% for LOQ).

Table 5.2- Precision and accuracy of the biotinylated capture method for determination of miR-451

QC Point	Expected Concentration (ng/mL)	Measured Concentration (ng/mL)	Precision RSD %	Accuracy %
LOQ	0.5	0.6	19.5	20.0
LOW	1.0	1.1	14.2	10.0
MID	20	22.7	12.6	13.5
HIGH	200	174.2	7.6	12.9

Other considerations: The analyte recovery was evaluated by comparing the analytical results for the extracted samples at low, medium and high concentrations with spiked elution buffers. The observed values for recovery at low, medium and high concentrations were 79.8% $\pm$ 8.6%, 81.5% $\pm$ 5.8% and 89.4% $\pm$ 8.0%, respectively. MiR-451 working solutions and extracted samples were stable for more than 12 hours at 4˚C or on the refrigerated autosampler if kept in nuclease-free solvents and vials.

Application of the method: In order to demonstrate the applicability of this method for biomarker studies, non-spiked rat plasma samples were analyzed and their miR-451 content determined. Figure 5.6 is showing the MS traces for the biotinylated capture and miR-451 in blank rat plasma. The endogenous concentration of miR-451 in rat plasma was estimated between 0.3 to 0.7 ng/mL based on the extrapolation of our standard calibration curve and it remained stable for three freeze-thaw cycles at -80˚C.

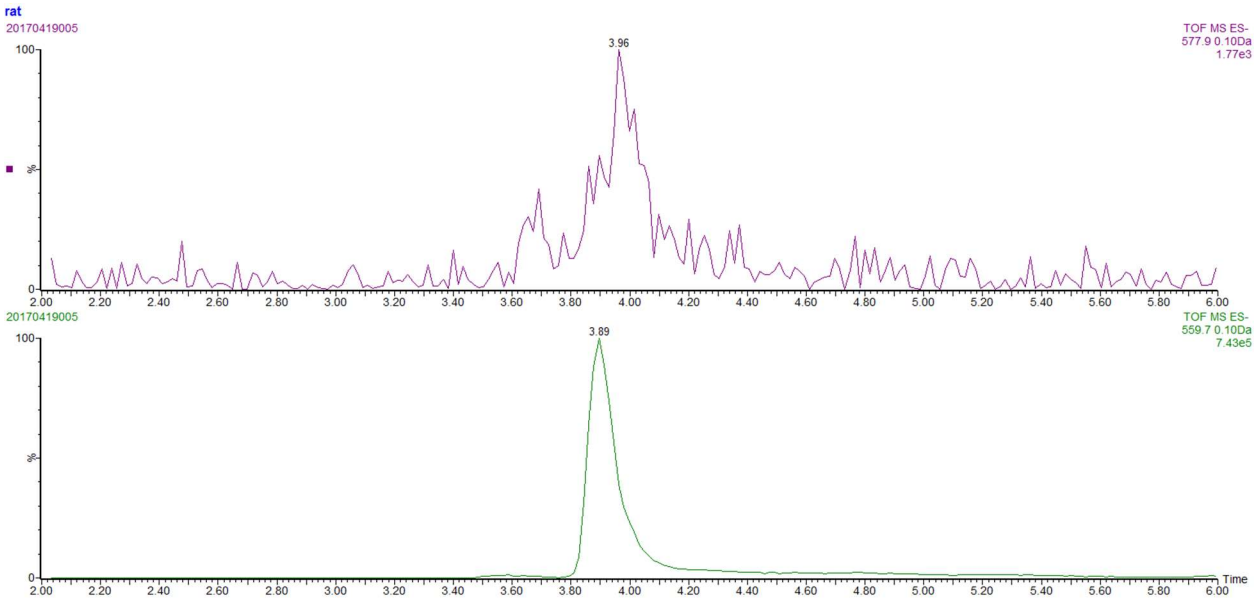


Figure 5.6- MS traces for miR-451 (top) and biotinylated capture strand (bottom) extracted from blank rat plasma. The endogenous miR-451 has a distinguishable peak at RT = 3.96 min.

We also analyzed mouse and human plasma samples. Interestingly, while miR-451 was present in human plasma, we were not able to detect it in mouse plasma (Figure 5.7). Based on

this observation, we can conclude that the concentration of miR-451 is higher in human and rat plasma compared to mice.

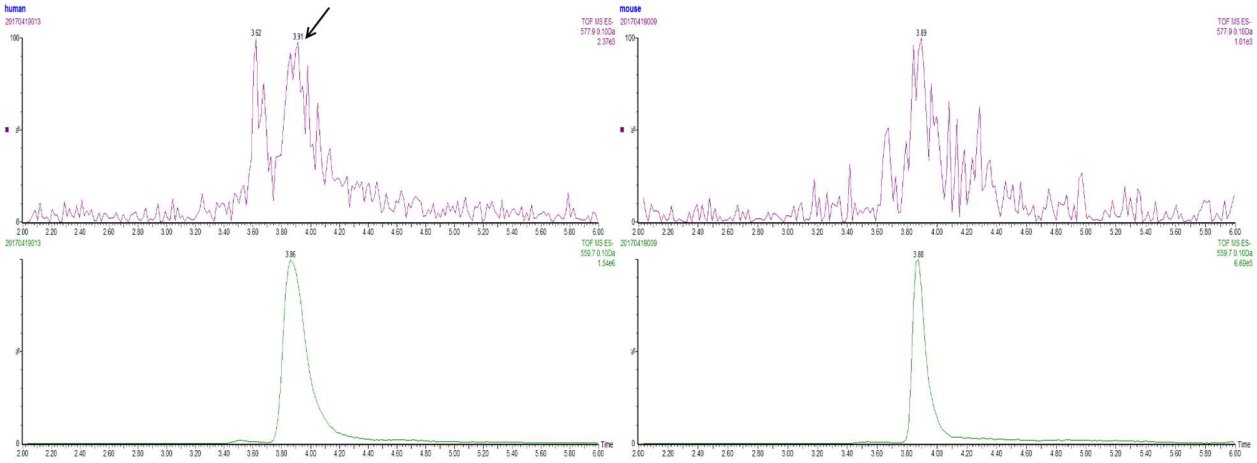


Figure 5.7- While miR-451 is present in human plasma (indicated by an arrow), it cannot be detected in mouse.

Discussion

In this report, we have presented a new sample preparation method and a new mobile phase chemistry for the characterization of endogenous small RNAs using LC-MS. We believe that our sample preparation method could be easily utilized for the study of any endogenous RNA following the determination of an appropriate capture sequence. The designated capture sequence would then play a second role as an internal standard just as we demonstrated here. Proteinase K digestion of samples at the beginning of this protocol is absolutely necessary for satisfactory results. In addition to RNases, proteinase K also digests albumin and other proteins

that bind to nucleic acids. Based on our experience with various extraction methods for oligonucleotides, we can say with confidence that almost any sample preparation method for oligos would benefit from adding a proteinase K digestion to the protocol. For RNAs, it is important to spike the samples with standard RNA solution only after the completion of proteinase K digestion as native RNases can degrade the spiked RNA in a few minutes. Interestingly, endogenous RNAs (such as circulatory miRNAs) are protected from the action of RNases as they are stably placed inside microvesicles (Hunter, Ismail et al. 2008) or exosomes (Valadi, Ekstrom et al. 2007, Chevillet, Kang et al. 2014, Hannafon, Carpenter et al. 2015, Hannafon, Trigoso et al. 2016).

The selection of NEB hydrophilic streptavidin magnetic beads for this protocol was based on the manufacturer specifications indicating low non-specific nucleic acid binding to these beads. Although we suspect other brands of streptavidin magnetic beads should generate satisfactory results, as well. Nevertheless, it is very important to closely study the ingredients of the final elution buffer for different magnetic beads and make sure that they do not contain any strong MS ion suppressors.

The introduced mobile phase in this manuscript should also be appropriate for many endogenous RNAs, although we encourage the readers to refer to our other publication (Basiri, Murph et al. 2017) and choose an ion-pair based on the nucleotide composition of their desired miRNA. Hopefully, the significant increase in sensitivity achieved as a result of using DBA/HFMIP instead of TEA/HFIP has convinced the readers of the important role of mobile phase chemistry in determining method sensitivity for oligonucleotides.

Conclusions

Here, we have reported the use of a novel mobile phase composition consisting of 15 mM DBA and 25 mM HFMIP for LC-MS analysis of microRNAs. We also developed an efficient and highly-specific sample preparation method based on the use of biotinylated capture strands and streptavidin magnetic beads. This sample preparation method is ideal for extracting and concentrating low-abundance endogenous oligonucleotides. The combination of this novel sample preparation technique with the DBA/HFMIP mobile phase resulted in a very low LOQ of 0.5 ng/mL which allowed for detection of miR-451 from non-spiked plasma. This report demonstrates the feasibility of using LC-MS as a tool for miRNA biomarker validation as we were able to distinguish a significant difference between the plasma miR-451 concentration in mice and rats. Although we didn't identify any modified forms of miR-451 in this study, we anticipate that other groups can identify modified forms for different miRNA molecules using the approaches that were presented here.

Acknowledgements

The experiments described in this manuscript were supported by the National Cancer Institute (CA176653).

CHAPTER 6

CONCLUSIONS

The results presented throughout this dissertation reveal some new aspects of the electrospray ionization of oligonucleotides. They also provide a general framework for future investigations into the interaction between various compounds within an electrospray droplet during the ionization process. This is particularly important for challenging LC separations that require complex mobile phase mixtures for acceptable chromatographic resolution. It will also help us to better understand the basic ESI mechanism by unraveling the factors that affect ionization efficiency.

Despite its many advantages over routine molecular biology techniques for identification and quantitation of oligonucleotides, LC-MS suffers from a major drawback: its sensitivity does not even come close to those methods. Therefore, any attempt at expanding the applications of LC-MS for oligonucleotide analysis should include lowering the MS detection limits for this class of molecules. This goal has been partly achieved via the advent of more sensitive mass spectrometers in recent years. Nevertheless, a major reason for poor MS sensitivity of oligonucleotides is the interference/ion suppression caused by other components of the sample or mobile phase. Therefore, it is necessary to understand and minimize such effects in order to harness the full potential of ever more sensitive instruments.

The ingredients of the mobile phase are by far the most important factors that influence the ionization efficiency of oligonucleotides. The results presented in Chapters 3 and 4 clearly indicate that by choosing an optimal IP/fluorinated alcohol combination for each particular

oligonucleotide instead of using a generic mobile phase combination for every analyte, the sensitivity of methods can be easily increased by an order of magnitude or more. The only problem with this approach is that it requires a complete survey of all alkylamine ion-pairs prior to each study. In order to resolve this problem, careful characterization of the factors that influence the MS signal intensity of oligonucleotides allowed for the development of a predictive statistical model to help with the choice of optimal IP agent based on the base composition of oligonucleotides. The choice of fluorinated alcohols seems to be more straightforward as less hydrophobic IP agents need to be paired with less hydrophobic alcohols such as HFIP, while more hydrophobic alkylamines are best paired with more hydrophobic alcohols such as HFMIP.

Mass spectrometry, particularly IMS, is a powerful tool for the study of spatial conformations of biomolecules. Nevertheless, the transfer of an intact secondary oligonucleotide structure from solution to the gas phase is quite challenging. During these studies, it was shown that one particular fluorinated alcohol, nonafluoro-tert-butyl alcohol, promoted the preservation of oligo hairpin structures in the gas phase when utilized with long, unbranched alkylamines, such as OA. This observation can have a significant impact on many investigations that involve the study of G-quadruplexes and other higher-order oligonucleotide structures.

MicroRNAs have been among the most studied biomolecules in recent years and with each new study more fascinating aspects of their regulatory role has been revealed. One of such interesting observations has been the discovery of multiple modified species for many miRNAs. Due to its unique capability in characterizing modifications, mass spectrometry is the perfect tool for further study of modified miRNAs. The previous chapter showed the results of a successful microRNA bioanalysis using a new sample preparation method and mobile phase chemistry. Extraction of microRNAs by a highly-specific capture strand resulted in a very clean background

and increased signal to noise ratio. In addition, unlike the traditional phenol/chloroform LLE, the presented sample preparation method can be implemented in high-throughput studies of oligonucleotides using LC-MS. Therefore, the developed sample preparation method is expected to facilitate further progress in the field.

The selected mobile phase for miR-451 characterization consisted of dibutylamine (DBA) and 1,1,1,3,3,3-Hexafluoro-2-methyl-2-propanol (HFMIP). In addition to increasing the MS signal intensity for miR-451, it also resulted in a much better chromatographic retention and resolution when compared to the classical TEA/HFIP mobile phase. Because retention of small RNAs on reversed-phase columns has proven to be challenging, DBA could be a viable choice as an ion-pair even for applications that do not include the use of a mass spectrometer.

Despite successful characterization of miR-451 throughout these studies, a lot is left to be done. We only identified and measured miR-451 in a pooled plasma sample of control animals as a proof-of-principle experiment to indicate the capability of LC-MS bioanalytical methods for the measurement of trace amounts of endogenous microRNAs in biological samples. Nevertheless, to fully establish the power of LC-MS for microRNA biomarker validation, we require several samples from control animals in addition to animals that are affected by a pathological condition, such as cancer, in order to demonstrate a statistically significant difference in miRNA levels between these two cohorts. A comparison between the results of such study and a classical PCR-based miRNA biomarker discovery is expected to better highlight the advantages of MS-based oligonucleotide biomarker validation.

Utilizing the methods presented here, the next major investigation should involve the characterization and quantitation of modified microRNA species. Unfortunately, due to very minute amounts of miR-451 in plasma, we were not able to detect any modified species.

Nevertheless, we know that some tissues contain very large amounts of particular miRNAs. For example, miR-122 has a very high concentration in hepatocytes. With minor adjustment of the sample preparation protocol presented in the previous chapter, it should be possible to extract those highly-expressed miRNAs from various tissues and characterize their modifications. A comprehensive study of the alteration of miRNA modifications in different stages of normal development and pathological progression is warranted to generate significant results.

Characterization of the modified oligonucleotide species is also a matter of extreme interest to the pharmaceutical industry and as it was revealed as a result of their experience with antibody-drug conjugates (ADCs), the enzyme-based bioanalytical methods have a very limited capability for this purpose. We predict that in the same manner that LC-MS found a pivotal role in the study of ADCs as a tool that complements ELISA, it will also become a much more important part of the routine pharmacokinetic studies of oligonucleotide drug candidates. This is particularly because more pharmaceutical companies are showing interest in oligonucleotides as a viable drug modality and with that more complex modifications are being made to the structure of therapeutic oligonucleotides to improve their drug-like characteristics. The oligonucleotides which are currently under investigation as potential drug candidates bear many modifications to their backbones as well as their nucleobases which makes them more resistant to nuclease activity, thus ensuring an acceptable half-life in the body. They may even be conjugated to different carbohydrate constructs to target them to specific tissues. Therefore, it is highly anticipated that some of the major metabolites of such therapeutic oligonucleotides arise from the dissociation of oligos from their conjugated sugars or involve the oxidation of their chemically-modified backbones. As such metabolites would be unrecognizable from the parent

drugs when using hybridization-based assays, the use of LC-MS is necessary for the metabolite identification of oligonucleotide drugs.

The results discussed in the former chapters indicate the potential of LC-MS for in-depth study of oligonucleotide therapeutics and biomarkers. Nevertheless, this potential can only be actualized through careful optimization of sample preparation and chromatography conditions. In effect, what has been presented in this writing can provide the necessary tools for other researchers who are seeking to perform such optimizations.

REFERENCES

Abe, M., A. Naqvi, G.-J. Hendriks, V. Feltzin, Y. Zhu, A. Grigoriev and N. M. Bonini (2014). "Impact of age-associated increase in 2′-O-methylation of miRNAs on aging and neurodegeneration in Drosophila." Genes & Development **28**(1): 44-57.

Abi-Ghanem, J. and V. Gabelica (2014). "Nucleic acid ion structures in the gas phase." Physical Chemistry Chemical Physics **16**(39): 21204-21218.

Agris, P. F. (2004). "Decoding the genome: a modified view." Nucleic Acids Research **32**(1): 223-238.

Annesley, T. M. (2003). "Ion Suppression in Mass Spectrometry." Clinical Chemistry **49**(7): 1041-1044.

Apffel, A., J. A. Chakel, S. Fischer, K. Lichtenwalter and W. S. Hancock (1997). "Analysis of Oligonucleotides by HPLC−Electrospray Ionization Mass Spectrometry." Analytical Chemistry **69**(7): 1320-1325.

Apffel, A., J. A. Chakel, S. Fischer, K. Lichtenwalter and W. S. Hancock (1997). "New procedure for the use of high-performance liquid chromatography–electrospray ionization mass spectrometry for the analysis of nucleotides and oligonucleotides." Journal of Chromatography A **777**(1): 3-21.

Arcella, A., G. Portella, M. L. Ruiz, R. Eritja, M. Vilaseca, V. Gabelica and M. Orozco (2012). "Structure of Triplex DNA in the Gas Phase." Journal of the American Chemical Society **134**(15): 6596-6606.

Arora, V., D. C. Knapp, M. T. Reddy, D. D. Weller and P. L. Iversen (2002). "Bioavailability and efficacy of antisense morpholino oligomers targeted to c-myc and cytochrome P-450 3A2 following oral administration in rats." Journal of Pharmaceutical Sciences **91**(4): 1009-1018.

Aushev, V. N., I. B. Zborovskaya, K. K. Laktionov, N. Girard, M.-P. Cros, Z. Herceg and V. Krutovskikh (2013). "Comparisons of microRNA Patterns in Plasma before and after Tumor Removal Reveal New Biomarkers of Lung Squamous Cell Carcinoma." PLOS ONE **8**(10): e78649.

Baker, E. S., S. L. Bernstein, V. Gabelica, E. De Pauw and M. T. Bowers (2006). "G-quadruplexes in telomeric repeats are conserved in a solvent-free environment." International Journal of Mass Spectrometry **253**(3): 225-237.

Bandres, E., N. Bitarte, F. Arias, J. Agorreta, P. Fortes, X. Agirre, R. Zarate, J. A. Diaz-Gonzalez, N. Ramirez, J. J. Sola, P. Jimenez, J. Rodriguez and J. Garcia-Foncillas (2009). "microRNA-451 Regulates Macrophage Migration Inhibitory Factor Production and Proliferation of Gastrointestinal Cancer Cells." Clinical Cancer Research **15**(7): 2281-2290.

Bartha, Á. and J. Ståhlberg (1994). "Electrostatic retention model of reversed-phase ion-pair chromatography." Journal of Chromatography A **668**(2): 255-284.

Bartlett, M. G., B. Chen and A. C. McGinnis (2013). LC-MS Bioanalysis of Oligonucleotides. Handbook of LC-MS Bioanalysis, John Wiley & Sons Inc.**:** 607-627.

Bartlett, M. G., J. A. McCloskey, S. Manalili and R. H. Griffey (1996). "The Effect of Backbone Charge on the Collision-Induced Dissociation of Oligonucleotides." Journal of Mass Spectrometry **31**(11): 1277-1283.

Basiri, B. and Michael G. Bartlett (2014). "LC–MS of oligonucleotides: applications in biomedical research." Bioanalysis **6**(11): 1525-1542.

Basiri, B., M. M. Murph and M. G. Bartlett (2017). "Assessing the Interplay between the Physicochemical Parameters of Ion-Pairing Reagents and the Analyte Sequence on the Electrospray Desorption Process for Oligonucleotides." J Am Soc Mass Spectrom.

Basiri, B., H. van Hattum, W. D. van Dongen, M. M. Murph and M. G. Bartlett (2017). "The Role of Fluorinated Alcohols as Mobile Phase Modifiers for LC-MS Analysis of Oligonucleotides." J Am Soc Mass Spectrom **28**(1): 190-199.

Bellon, L., L. Maloney, S. P. Zinnen, J. A. Sandberg and K. E. Johnson (2000). "Quantitative Determination of a Chemically Modified Hammerhead Ribozyme in Blood Plasma Using 96-Well Solid-Phase Extraction Coupled with High-Performance Liquid Chromatography or Capillary Gel Electrophoresis." Analytical Biochemistry **283**(2): 228-240.

Bergamaschi, A. and B. S. Katzenellenbogen (2012). "Tamoxifen downregulation of miR-451 increases 14-3-3[zeta] and promotes breast cancer cell survival and endocrine resistance." Oncogene **31**(1): 39-47.

Berkenkamp, S., F. Kirpekar and F. Hillenkamp (1998). "Infrared MALDI mass spectrometry of large nucleic acids." Science **281**(5374): 260-262.

Beverly, M., K. Hartsough and L. Machemer (2005). "Liquid chromatography/electrospray mass spectrometric analysis of metabolites from an inhibitory RNA duplex." Rapid Communications in Mass Spectrometry **19**(12): 1675-1682.

Beverly, M., K. Hartsough, L. Machemer, P. Pavco and J. Lockridge (2006). "Liquid chromatography electrospray ionization mass spectrometry analysis of the ocular metabolites from a short interfering RNA duplex." Journal of Chromatography B **835**(1–2): 62-70.

Bian, H.-B., X. Pan, J.-S. Yang, Z.-X. Wang and W. De (2011). "Upregulation of microRNA-451 increases cisplatin sensitivity of non-small cell lung cancer cell line (A549)." Journal of Experimental & Clinical Cancer Research : CR **30**(1): 20-20.

Bleicher, K. and E. Bayer (1994). "Analysis of oligonucleotides using coupled high performance liquid chromatography-electrospray mass spectrometry." Chromatographia **39**(7-8): 405-408.

Bleicher, K. and E. Bayer (1994). "Various factors influencing the signal intensity of oligonucleotides in electrospray mass spectrometry." Biological Mass Spectrometry **23**(6): 320-322.

Bothner, B., K. Chatman, M. Sarkisian and G. Siuzdak (1995). "Liquid chromatography mass spectrometry of antisense oligonucleotides." Bioorganic & Medicinal Chemistry Letters **5**(23): 2863-2868.

Bourque, A. J. and A. S. Cohen (1993). "Quantitative analysis of phosphorothioate oligonucleotides in biological fluids using fast anion-exchange chromatography." Journal of Chromatography B: Biomedical Sciences and Applications **617**(1): 43-49.

Butora, G., D. M. Kenski, A. J. Cooper, W. Fu, N. Qi, J. J. Li, W. M. Flanagan and I. W. Davies (2011). "Nucleoside Optimization for RNAi: A High-Throughput Platform." Journal of the American Chemical Society **133**(42): 16766-16769.

Capaldi, D. C., H. J. Gaus, R. L. Carty, M. N. Moore, B. J. Turney, S. D. Decottignies, J. V. McArdle, A. N. Scozzari, V. T. Ravikumar and A. H. Krotz (2004). "Formation of 4,4′-dimethoxytrityl-C-phosphonate oligonucleotides." Bioorganic & Medicinal Chemistry Letters **14**(18): 4683-4690.

Castleberry, C. M. and P. A. Limbach (2010). "Relative quantitation of transfer RNAs using liquid chromatography mass spectrometry and signature digestion products." Nucleic Acids Research **38**(16): e162.

Cech, N. B. and C. G. Enke (2000). "Relating Electrospray Ionization Response to Nonpolar Character of Small Peptides." Analytical Chemistry **72**(13): 2717-2723.

Cech, N. B. and C. G. Enke (2001). "Effect of Affinity for Droplet Surfaces on the Fraction of Analyte Molecules Charged during Electrospray Droplet Fission." Analytical Chemistry **73**(19): 4632-4639.

Cech, N. B. and C. G. Enke (2001). "Practical implications of some recent studies in electrospray ionization fundamentals." Mass Spectrometry Reviews **20**(6): 362-387.

Cech, N. B. and C. G. Enke (2010). Selectivity in Electrospray Ionization Mass Spectrometry. Electrospray and MALDI Mass Spectrometry. R. B. Cole. Hoboken, NJ, John Wiley & Sons, Inc.**:** 49-73.

Chalcraft, K. R., R. Lee, C. Mills and P. Britz-McKibbin (2009). "Virtual Quantification of Metabolites by Capillary Electrophoresis-Electrospray Ionization-Mass Spectrometry: Predicting Ionization Efficiency Without Chemical Standards." Analytical Chemistry **81**(7): 2506-2515.

Chen, B. and M. Bartlett (2012). "A One-Step Solid Phase Extraction Method for Bioanalysis of a Phosphorothioate Oligonucleotide and Its 3′ n-1 Metabolite from Rat Plasma by uHPLC–MS/MS." The AAPS Journal **14**(4): 772-780.

Chen, B. and M. G. Bartlett (2013). "Evaluation of mobile phase composition for enhancing sensitivity of targeted quantification of oligonucleotides using ultra-high performance liquid chromatography and mass spectrometry: Application to phosphorothioate deoxyribonucleic acid." Journal of Chromatography A **1288**(0): 73-81.

Chen, B., S. Mason and M. Bartlett (2013). "The Effect of Organic Modifiers on Electrospray Ionization Charge-State Distribution and Desorption Efficiency for Oligonucleotides." Journal of The American Society for Mass Spectrometry **24**(2): 257-264.

Chen, S.-H., M. Qian, J. M. Brennan and J. M. Gallo (1997). "Determination of antisense phosphorothioate oligonucleotides and catabolites in biological fluids and tissue extracts using anion-exchange high-performance liquid chromatography and capillary gel electrophoresis." Journal of Chromatography B: Biomedical Sciences and Applications **692**(1): 43-51.

Chevillet, J. R., Q. Kang, I. K. Ruf, H. A. Briggs, L. N. Vojtech, S. M. Hughes, H. H. Cheng, J. D. Arroyo, E. K. Meredith, E. N. Gallichotte, E. L. Pogosova-Agadjanyan, C. Morrissey, D. L. Stirewalt, F. Hladik, E. Y. Yu, C. S. Higano and M. Tewari (2014). "Quantitative and stoichiometric analysis of the microRNA content of exosomes." Proceedings of the National Academy of Sciences **111**(41): 14888-14893.

Cirak, S., V. Arechavala-Gomeza, M. Guglieri, L. Feng, S. Torelli, K. Anthony, S. Abbs, M. E. Garralda, J. Bourke, D. J. Wells, G. Dickson, M. J. A. Wood, S. D. Wilton, V. Straub, R. Kole, S. B. Shrewsbury, C. Sewry, J. E. Morgan, K. Bushby and F. Muntoni (2011). "Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study." The Lancet **378**(9791): 595-605.

Cobos Jiménez, V., A. M. Willemsen, E. J. Bradley, F. Baas, A. H. C. van Kampen and N. A. Kootstra (2014). "Next-generation sequencing of microRNAs in primary human polarized macrophages." Genomics Data **2**: 181-183.

Cook, E. F. and L. Goldman (1984). "Empiric comparison of multivariate analytic techniques: Advantages and disadvantages of recursive partitioning analysis." Journal of Chronic Diseases **37**(9): 721-731.

Cook, K. and J. Thayer (2011). "Advantages of ion-exchange chromatography for oligonucleotide analysis." Bioanalysis **3**(10): 1109-1120.

Counterman, A. E., S. J. Valentine, C. A. Srebalus, S. C. Henderson, C. S. Hoaglund and D. E. Clemmer (1998). "High-order structure and dissociation of gaseous peptide aggregates that are hidden in mass spectra." Journal of the American Society for Mass Spectrometry **9**(8): 743-759.

Cramer, H., K. J. Finn and E. Herzberg (2011). Purity Analysis and Impurities Determination by Reversed-Phase High-Performance Liquid Chromatography. Handbook of Analysis of Oligonucleotides and Related Products. J. V. Bonilla and G. S. Srivatsa, CRC Press**:** 1-46.

Dai, G., X. Wei, Z. Liu, S. Liu, G. Marcucci and K. K. Chan (2005). "Characterization and quantification of Bcl-2 antisense G3139 and metabolites in plasma and urine by ion-pair reversed phase HPLC coupled with electrospray ion-trap mass spectrometry." Journal of Chromatography B **825**(2): 201-213.

de Faria, O. J., C. S. Moore, T. E. Kennedy, J. P. Antel, A. Bar-Or and A. S. Dhaunchak (2012). "MicroRNA dysregulation in multiple sclerosis." Frontiers in Genetics **3**: 311.

de Fougerolles, A., H.-P. Vornlocher, J. Maraganore and J. Lieberman (2007). "Interfering with disease: a progress report on siRNA-based therapeutics." Nat Rev Drug Discov **6**(6): 443-453.

Deng, P., X. Chen, G. Zhang and D. Zhong (2010). "Bioanalysis of an oligonucleotide and its metabolites by liquid chromatography–tandem mass spectrometry." Journal of Pharmaceutical and Biomedical Analysis **52**(4): 571-579.

Devi, G. R., T. M. Beer, C. L. Corless, V. Arora, D. L. Weller and P. L. Iversen (2005). "In vivo Bioavailability and Pharmacokinetics of a c-MYC Antisense Phosphorodiamidate Morpholino Oligomer, AVI-4126, in Solid Tumors." Clinical Cancer Research **11**(10): 3930-3938.

Dirin, M. and J. Winkler (2013). "Influence of diverse chemical modifications on the ADME characteristics and toxicology of antisense oligonucleotides." Expert Opinion on Biological Therapy **13**(6): 875-888.

Dole, M., L. L. Mack, R. L. Hines, R. C. Mobley, L. D. Ferguson and M. B. Alice (1968). "Molecular Beams of Macroions." The Journal of Chemical Physics **49**(5): 2240-2249.

Du, T. and P. D. Zamore (2005). "microPrimer: the biogenesis and function of microRNA." Development **132**(21): 4645-4652.

Easter, R., C. Barry, J. Caruso and P. Limbach (2013). "Separation and identification of phosphorothioate oligonucleotides by HILIC-ESIMS." Analytical Methods **5**(11): 2657-2659.

Easter, R. N., K. K. Kroning, J. A. Caruso and P. A. Limbach (2010). "Separation and identification of oligonucleotides by hydrophilic interaction liquid chromatography (HILIC)-inductively coupled plasma mass spectrometry (ICPMS)." Analyst **135**(10): 2560-2565.

Ehrmann, B. M., T. Henriksen and N. B. Cech (2008). "Relative importance of basicity in the gas phase and in solution for determining selectivity in electrospray ionization mass spectrometry." J Am Soc Mass Spectrom **19**(5): 719-728.

Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl (2001). "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells." Nature **411**(6836): 494-498.

Elbashir, S. M., W. Lendeckel and T. Tuschl (2001). "RNA interference is mediated by 21- and 22-nucleotide RNAs." Genes & Development **15**(2): 188-200.

Eminaga, S., D. C. Christodoulou, F. Vigneault, G. M. Church and J. G. Seidman (2001). Quantification of microRNA Expression with Next-Generation Sequencing. Current Protocols in Molecular Biology, John Wiley & Sons, Inc.

Erb, R. and H. Oberacher (2014). "Comparison of mobile-phase systems commonly applied in liquid chromatography-mass spectrometry of nucleic acids." ELECTROPHORESIS **35**(9): 1226-1235.

Farand, J. and M. Beverly (2008). "Sequence Confirmation of Modified Oligonucleotides Using Chemical Degradation, Electrospray Ionization, Time-of-Flight, and Tandem Mass Spectrometry." Analytical Chemistry **80**(19): 7414-7421.

Farand, J. and F. Gosselin (2009). "De Novo Sequence Determination of Modified Oligonucleotides." Analytical Chemistry **81**(10): 3723-3730.

Fenn, J., M. Mann, C. Meng, S. Wong and C. Whitehouse (1989). "Electrospray ionization for mass spectrometry of large biomolecules." Science **246**(4926): 64-71.

Fenn, J. B. (2003). "Electrospray wings for molecular elephants (Nobel lecture)." Angew Chem Int Ed Engl **42**(33): 3871-3894.

Fernandez-Lima, F. A., R. C. Blase and D. H. Russell (2010). "A Study of Ion-Neutral Collision Cross Section Values for Low Charge States of Peptides, Proteins, and Peptide/Protein Complexes." International journal of mass spectrometry **298**(1-3): 111-118.

Fernandez de la Mora, J. (2000). "Electrospray ionization of large multiply charged species proceeds via Dole's charged residue mechanism." Analytica Chimica Acta **406**(1): 93-104.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-811.

Fisher, H. C., M. Smith and A. E. Ashcroft (2013). "De novo sequencing of short interfering ribonucleic acids facilitated by use of tandem mass spectrometry with ion mobility spectrometry." Rapid Communications in Mass Spectrometry **27**(20): 2247-2254.

Flynt, A. S. and E. C. Lai (2008). "Biological principles of microRNA-mediated regulation: shared themes amid diversity." Nat Rev Genet **9**(11): 831-842.

Gao, F., P. Liu, J. Narayanan, M. Yang, B. L. Fish, Y. Liu, M. Liang, E. R. Jacobs and M. Medhora (2017). "Changes in miRNA in the lung and whole blood after whole thorax irradiation in rats." Scientific Reports **7**: 44132.

Gao, Y., J. Yang, M. T. Cancilla, F. Meng and S. A. McLuckey (2013). "Top-Down Interrogation of Chemically Modified Oligonucleotides by Negative Electron Transfer and Collision Induced Dissociation." Analytical Chemistry **85**(9): 4713-4720.

Gaus, H., P. Olsen, K. V. Sooy, C. Rentel, B. Turney, K. L. Walker, J. V. McArdle and D. C. Capaldi (2005). "Trichloroacetaldehyde modified oligonucleotides." Bioorganic & Medicinal Chemistry Letters **15**(18): 4118-4124.

Gaus, H. J., S. R. Owens, M. Winniman, S. Cooper and L. L. Cummins (1997). "On-Line HPLC Electrospray Mass Spectrometry of Phosphorothioate Oligonucleotide Metabolites." Analytical Chemistry **69**(3): 313-319.

Gee, H. E., F. M. Buffa, C. Camps, A. Ramachandran, R. Leek, M. Taylor, M. Patil, H. Sheldon, G. Betts, J. Homer, C. West, J. Ragoussis and A. L. Harris (2011). "The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis." Br J Cancer **104**(7): 1168-1177.

Gelsinger, C., E. Steinhagen-Thiessen and U. Kassner (2012). "Therapeutic Potential of Mipomersen in the Management of Familial Hypercholesterolaemia." Drugs **72**(11): 1445-1455.

Gilar, M., K. J. Fountain, Y. Budman, J. L. Holyoke, H. Davoudi and J. C. Gebler (2003). "Characterization of therapeutic oligonucleotides using liquid chromatography with on-line mass spectrometry detection." Oligonucleotides **13**(4): 229-243.

Gilar, M., K. J. Fountain, Y. Budman, U. D. Neue, K. R. Yardley, P. D. Rainville, R. J. Russell Ii and J. C. Gebler (2002). "Ion-pair reversed-phase high-performance liquid chromatography analysis of oligonucleotides:: Retention prediction." Journal of Chromatography A **958**(1–2): 167-182.

Gilar, M. and U. D. Neue (2007). "Peak capacity in gradient reversed-phase liquid chromatography of biopolymers: Theoretical and practical implications for the separation of oligonucleotides." Journal of Chromatography A **1169**(1–2): 139-150.

Git, A., H. Dvinge, M. Salmon-Divon, M. Osborne, C. Kutter, J. Hadfield, P. Bertone and C. Caldas (2010). "Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression." RNA **16**(5): 991-1006.

Godlewski, J., A. Bronisz, M. O. Nowicki, E. A. Chiocca and S. Lawler (2010). "microRNA-451: A conditional switch controlling glioma cell proliferation and migration." Cell Cycle **9**(14): 2814-2820.

Goemans, N. M., M. Tulinius, J. T. van den Akker, B. E. Burm, P. F. Ekhart, N. Heuvelmans, T. Holling, A. A. Janson, G. J. Platenburg, J. A. Sipkens, J. M. A. Sitsen, A. Aartsma-Rus, G.-J. B. van Ommen, G. Buyse, N. Darin, J. J. Verschuuren, G. V. Campion, S. J. de Kimpe and J. C. van Deutekom (2011). "Systemic Administration of PRO051 in Duchenne's Muscular Dystrophy." New England Journal of Medicine **364**(16): 1513-1522.

Gong, L. (2015). "Comparing ion-pairing reagents and counter anions for ion-pair reversed-phase liquid chromatography/electrospray ionization mass spectrometry analysis of synthetic oligonucleotides." Rapid Communications in Mass Spectrometry **29**(24): 2402-2410.

Gong, L. and J. S. O. McCullagh (2011). "Analysis of oligonucleotides by hydrophilic interaction liquid chromatography coupled to negative ion electrospray ionization mass spectrometry." Journal of Chromatography A **1218**(32): 5480-5486.

Gong, L. and J. S. O. McCullagh (2014). "Comparing ion-pairing reagents and sample dissolution solvents for ion-pairing reversed-phase liquid chromatography/electrospray ionization mass spectrometry analysis of oligonucleotides." Rapid Communications in Mass Spectrometry **28**(4): 339-350.

Griffey, R. H., M. J. Greig, H. J. Gaus, K. Liu, D. Monteith, M. Winniman and L. L. Cummins (1997). "Characterization of Oligonucleotide MetabolismIn Vivovia Liquid Chromatography/Electrospray Tandem Mass Spectrometry with a Quadrupole Ion Trap Mass Spectrometer." Journal of Mass Spectrometry **32**(3): 305-313.

Gupta, S. K., C. Bang and T. Thum (2010). "Circulating MicroRNAs as Biomarkers and Potential Paracrine Mediators of Cardiovascular Disease." Circulation: Cardiovascular Genetics **3**(5): 484-488.

Haché, M., K. J. Swoboda, N. Sethna, A. Farrow-Gillespie, A. Khandji, S. Xia and K. M. Bishop (2016). "Intrathecal Injections in Children With Spinal Muscular Atrophy." Journal of Child Neurology **31**(7): 899-906.

Hail, M. E., B. Elliott and K. Anderson (2004). "High-throughput analysis of oligonucleotides using automated electrospray ionization mass spectrometry." American Biotechnology Laboratory **22**(1): 12-15.

Hannafon, B. N., K. J. Carpenter, W. L. Berry, R. Janknecht, W. C. Dooley and W. Q. Ding (2015). "Exosome-mediated microRNA signaling from breast cancer cells is altered by the anti-angiogenesis agent docosahexaenoic acid (DHA)." Mol Cancer. **14**.

Hannafon, B. N., Y. D. Trigoso, C. L. Calloway, Y. D. Zhao, D. H. Lum, A. L. Welm, Z. J. Zhao, K. E. Blick, W. C. Dooley and W. Q. Ding (2016). "Plasma exosome microRNAs are indicative of breast cancer." Breast Cancer Research **18**(1): 90.

Harvey, D. J. (2005). "Fragmentation of Negative Ions from Carbohydrates: Part 1. Use of Nitrate and Other Anionic Adducts for the Production of Negative Ion Electrospray Spectra from N-linked Carbohydrates." Journal of the American Society for Mass Spectrometry **16**(5): 622-630.

Hauser, S., L. M. Wulfken, S. Holdenrieder, R. Moritz, C.-H. Ohlmann, V. Jung, F. Becker, E. Herrmann, G. Walgenbach-Brünagel, A. von Ruecker, S. C. Müller and J. Ellinger (2012). "Analysis of serum microRNAs (miR-26a-2*, miR-191, miR-337-3p and miR-378) as potential biomarkers in renal cell carcinoma." Cancer Epidemiology **36**(4): 391-394.

Heck, A. J. R. and R. H. H. van den Heuvel (2004). "Investigation of intact protein complexes by mass spectrometry." Mass Spectrometry Reviews **23**(5): 368-389.

Hemsley, M., M. Ewles and L. Goodwin (2012). "Development of a bioanalytical method for quantification of a 15-mer oligonucleotide at sub-ng/ml concentrations using LC–MS/MS." Bioanalysis **4**(12): 1457-1469.

Henriksen, T., R. K. Juhler, B. Svensmark and N. B. Cech (2005). "The relative influences of acidity and polarity on responsiveness of small organic molecules to analysis with negative ion electrospray ionization mass spectrometry (ESI-MS)." J Am Soc Mass Spectrom **16**(4): 446-455.

Ho, T. K. (1995). "Random decision forests." Proceedings of 3rd International Conference on Document Analysis and Recognition **1**: 278-282 vol.271.

Hoaglund, C. S., Y. Liu, A. D. Ellington, M. Pagel and D. E. Clemmer (1997). "Gas-Phase DNA: Oligothymidine Ion Conformers." Journal of the American Chemical Society **119**(38): 9051-9052.

Hogan, C. J., J. A. Carroll, H. W. Rohrs, P. Biswas and M. L. Gross (2009). "Combined Charged Residue-Field Emission Model of Macromolecular Electrospray Ionization." Analytical Chemistry **81**(1): 369-377.

Hossain, M. and P. A. Limbach (2007). "Mass spectrometry-based detection of transfer RNAs by their signature endonuclease digestion products." RNA **13**(2): 295-303.

Hua, Y. and D. Jenke (2012). "Increasing the Sensitivity of an LC–MS Method for Screening Material Extracts for Organic Extractables via Mobile Phase Optimization." Journal of Chromatographic Science **50**(3): 213-227.

Huang, T.-Y., J. Liu and S. A. McLuckey (2010). "Top-Down Tandem Mass Spectrometry of tRNA Via Ion Trap Collision-Induced Dissociation." Journal of the American Society for Mass Spectrometry **21**(6): 890-898.

Huber, C. G. and A. Krajete (1999). "Analysis of Nucleic Acids by Capillary Ion-Pair Reversed-Phase HPLC Coupled to Negative-Ion Electrospray Ionization Mass Spectrometry." Analytical Chemistry **71**(17): 3730-3739.

Huber, C. G., P. J. Oefner and G. K. Bonn (1992). "High-performance liquid chromatographic separation of detritylated oligonucleotides on highly cross-linked poly-(styrene-divinylbenzene) particles." Journal of Chromatography A **599**(1): 113-118.

Huber, C. G., P. J. Oefner and G. K. Bonn (1993). "High-Resolution Liquid Chromatography of Oligonucleotides on Nonporous Alkylated Styrene-Divinylbenzene Copolymers." Analytical Biochemistry **212**(2): 351-358.

Huffman, B. A., M. L. Poltash and C. A. Hughey (2012). "Effect of Polar Protic and Polar Aprotic Solvents on Negative-Ion Electrospray Ionization and Chromatographic Separation of Small Acidic Molecules." Analytical Chemistry **84**(22): 9942-9950.

Hunter, M. P., N. Ismail, X. Zhang, B. D. Aguda, E. J. Lee, L. Yu, T. Xiao, J. Schafer, M.-L. T. Lee, T. D. Schmittgen, S. P. Nana-Sinkam, D. Jarjoura and C. B. Marsh (2008). "Detection of microRNA Expression in Human Peripheral Blood Microvesicles." PLoS ONE **3**(11): e3694.

Iribarne, J. V. and B. A. Thomson (1976). "On the evaporation of small ions from charged droplets." The Journal of Chemical Physics **64**(6): 2287-2294.

Jacob, N. K., J. V. Cooley, T. N. Yee, J. Jacob, H. Alder, P. Wickramasinghe, K. H. Maclean and A. Chakravarti (2013). "Identification of Sensitive Serum microRNA Biomarkers for Radiation Biodosimetry." PLoS ONE **8**(2): e57603.

Johnson, J. L., W. Guo, J. Zang, S. Khan, S. Bardin, A. Ahmad, J. X. Duggan and I. Ahmad (2005). "Quantification of raf antisense oligonucleotide (rafAON) in biological matrices by LC-MS/MS to support pharmacokinetics of a liposome-entrapped rafAON formulation." Biomedical Chromatography **19**(4): 272-278.

Jones, C. I., M. V. Zabolotskaya, A. J. King, H. J. S. Stewart, G. A. Horne, T. J. Chevassut and S. F. Newbury (2012). "Identification of circulating microRNAs as diagnostic biomarkers for use in multiple myeloma." Br J Cancer **107**(12): 1987-1996.

Juraschek, R., T. Dülcks and M. Karas (1999). "Nanoelectrospray—more than just a minimized-flow electrospray ionization source." Journal of the American Society for Mass Spectrometry **10**(4): 300-308.

Kaltashov, I. A. and A. Mohimen (2005). "Estimates of protein surface areas in solution by electrospray ionization mass spectrometry." Anal Chem **77**(16): 5370-5379.

Kamel, A. M., P. R. Brown and B. Munson (1999). "Effects of Mobile-Phase Additives, Solution pH, Ionization Constant, and Analyte Concentration on the Sensitivities and Electrospray Ionization Mass Spectra of Nucleoside Antiviral Agents." Analytical Chemistry **71**(24): 5481-5492.

Karas, M. and F. Hillenkamp (1988). "Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons." Analytical Chemistry **60**(20): 2299-2301.

Karijolich, J., A. Kantartzis and Y.-T. Yu (2010). RNA Modifications: A Mechanism that Modulates Gene Expression. RNA Therapeutics. M. Sioud, Humana Press. **629:** 1-19.

Katoh, T., Y. Sakaguchi, K. Miyauchi, T. Suzuki, S.-i. Kashiwabara, T. Baba and T. Suzuki (2009). "Selective stabilization of mammalian microRNAs by 3′ adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2." Genes & Development **23**(4): 433-438.

Kauppila, T. J., N. Talaty, A. U. Jackson, T. Kotiaho, R. Kostiainen and R. G. Cooks (2008). "Carbohydrate and steroid analysis by desorption electrospray ionization mass spectrometry." Chemical Communications(23): 2674-2676.

Kebarle, P. and U. H. Verkerk (2010). On the Mechanism of Electrospray Ionization Mass Spectrometry (ESIMS). Electrospray and MALDI Mass Spectrometry: Fundamentals, Instrumentation, Practicalities, and Biological Applications. R. B. Cole. Hoboken, NJ, John Wiley & Sons, Inc.**:** 1-48.

Kenski, D. M., G. Butora, A. T. Willingham, A. J. Cooper, W. Fu, N. Qi, F. Soriano, I. W. Davies and W. M. Flanagan (2012). "siRNA-optimized Modifications for Enhanced In Vivo Activity." Mol Ther Nucleic Acids **1**: e5.

Kirpekar, F., S. Berkenkamp and F. Hillenkamp (1999). "Detection of Double-Stranded DNA by IR- and UV-MALDI Mass Spectrometry." Analytical Chemistry **71**(13): 2334-2339.

Kitova, E. N., A. El-Hawiet, P. D. Schnier and J. S. Klassen (2012). "Reliable determinations of protein-ligand interactions by direct ESI-MS measurements. Are we there yet?" J Am Soc Mass Spectrom **23**(3): 431-441.

Kole, R., A. R. Krainer and S. Altman (2012). "RNA therapeutics: beyond RNA interference and antisense oligonucleotides." Nat Rev Drug Discov **11**(2): 125-140.

Koller, E., T. M. Vincent, A. Chappell, S. De, M. Manoharan and C. F. Bennett (2011). "Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes." Nucleic Acids Research **39**(11): 4795-4807.

Konermann, L., E. Ahadi, A. D. Rodriguez and S. Vahidi (2013). "Unraveling the Mechanism of Electrospray Ionization." Analytical Chemistry **85**(1): 2-9.

Kosaka, N., H. Iguchi and T. Ochiya (2010). "Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis." Cancer Science **101**(10): 2087-2092.

Kovalchuk, O., J. Filkowski, J. Meservy, Y. Ilnytskyy, V. P. Tryndyak, V. F. Chekhun and I. P. Pogribny (2008). "Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin." Molecular Cancer Therapeutics **7**(7): 2152-2159.

Kowalak, J. A., E. Bruenger, P. F. Crain and J. A. McCloskey (2000). "Identities and Phylogenetic Comparisons of Posttranscriptional Modifications in 16 S Ribosomal RNA from Haloferax volcanii." Journal of Biological Chemistry **275**(32): 24484-24489.

Kowalak, J. A., S. C. Pomerantz, P. F. Crain and J. A. McCloskey (1993). "A novel method for the determination of posttranscriptional modification in RNA by mass spectrometry." Nucleic Acids Research **21**(19): 4577-4585.

Krieg, A. M. (2011). "Is RNAi Dead?" Mol Ther **19**(6): 1001-1002.

Kruve, A., K. Kaupmees, J. Liigand and I. Leito (2014). "Negative Electrospray Ionization via Deprotonation: Predicting the Ionization Efficiency." Analytical Chemistry **86**(10): 4822-4830.

Kumar, P., Z. Dezso, C. MacKenzie, J. Oestreicher, S. Agoulnik, M. Byrne, F. Bernier, M. Yanagimachi, K. Aoshima and Y. Oda (2013). "Circulating miRNA Biomarkers for Alzheimer's Disease." PLoS ONE **8**(7): e69807.

Kurata, C., K. Bradley, H. Gaus, N. Luu, I. Cedillo, V. T. Ravikumar, K. V. Sooy, J. V. McArdle and D. C. Capaldi (2006). "Characterization of high molecular weight impurities in synthetic phosphorothioate oligonucleotides." Bioorganic & Medicinal Chemistry Letters **16**(3): 607-614.

Lan, D., X. Zhang, R. He, R. Tang, P. Li, Q. He and G. Chen (2015). "MiR-133a is downregulated in non-small cell lung cancer: a study of clinical significance." European Journal of Medical Research **20**(1): 50.

Lecchi, P., H. M. T. Le and L. K. Pannell (1995). "6-Aza-2-thiothymine: a matrix for MALDI spectra of oligonucleotides." Nucleic Acids Research **23**(7): 1276-1277.

Leito, I., K. Herodes, M. Huopolainen, K. Virro, A. Künnapas, A. Kruve and R. Tanner (2008). "Towards the electrospray ionization mass spectrometry ionization efficiency scale of organic compounds." Rapid Communications in Mass Spectrometry **22**(3): 379-384.

Li, S. and P. A. Limbach (2012). "Method for Comparative Analysis of Ribonucleic Acids Using Isotope Labeling and Mass Spectrometry." Analytical Chemistry **84**(20): 8607-8613.

Li, S. and P. A. Limbach (2013). "Mass spectrometry sequencing of transfer ribonucleic acids by the comparative analysis of RNA digests (CARD) approach." Analyst **138**(5): 1386-1394.

Li, X., T. Sanda, A. T. Look, C. D. Novina and H. von Boehmer (2011). "Repression of tumor suppressor miR-451 is essential for NOTCH1-induced oncogenesis in T-ALL." The Journal of Experimental Medicine **208**(4): 663-675.

Li, Y. and R. B. Cole (2010). Charge State Distributions in Electrospray and MALDI. Electrospray and MALDI Mass Spectrometry. R. B. Cole, John Wiley & Sons, Inc.**:** 491-534.

Li, Z., J. Jia, J. Gou, X. Zhao and T. Yi (2015). "MicroRNA-451 plays a role in murine embryo implantation through targeting Ankrd46, as implicated by a microarray-based analysis." Fertility and Sterility **103**(3): 834-844.e834.

Liao, Q., C. Shen and P. Vouros (2009). "GenoMass - a computer software for automated identification of oligonucleotide DNA adducts from LC-MS analysis of DNA digests." Journal of Mass Spectrometry **44**(4): 549-560.

Liigand, J., A. Kruve, I. Leito, M. Girod and R. Antoine (2014). "Effect of Mobile Phase on Electrospray Ionization Efficiency." Journal of The American Society for Mass Spectrometry **25**(11): 1853-1861.

Liigand, J., A. Kruve, P. Liigand, A. Laaniste, M. Girod, R. Antoine and I. Leito (2015). "Transferability of the Electrospray Ionization Efficiency Scale between Different Instruments." Journal of The American Society for Mass Spectrometry **26**(11): 1923-1930.

Limbach, P. A., P. F. Crain and J. A. McCloskey (1994). "Summary: the modified nucleosides of RNA." Nucleic Acids Research **22**(12): 2183-2196.

Lin, Z. J., W. Li and G. Dai (2007). "Application of LC–MS for quantitative analysis and metabolite identification of therapeutic oligonucleotides." Journal of Pharmaceutical and Biomedical Analysis **44**(2): 330-341.

Lipinski, C. A. (2000). "Drug-like properties and the causes of poor solubility and poor permeability." Journal of Pharmacological and Toxicological Methods **44**(1): 235-249.

Little, D. P., D. J. Aaserud, G. A. Valaskovic and F. W. McLafferty (1996). "Sequence Information from 42−108-mer DNAs (Complete for a 50-mer) by Tandem Mass Spectrometry." Journal of the American Chemical Society **118**(39): 9352-9359.

Little, D. P., T. Thannhauser and F. W. McLafferty (1995). "Verification of 50-to 100-mer DNA and RNA sequences with high-resolution mass spectrometry." Proceedings of the National Academy of Sciences **92**(6): 2318-2322.

Liuni, P., A. Jeganathan and D. J. Wilson (2012). "Conformer Selection and Intensified Dynamics During Catalytic Turnover in Chymotrypsin." Angewandte Chemie International Edition **51**(38): 9666-9669.

Loo, J. A., R. R. O. Loo, K. J. Light, C. G. Edmonds and R. D. Smith (1992). "Multiply charged negative ions by electrospray ionization of polypeptides and proteins." Analytical Chemistry **64**(1): 81-88.

Lorenzen, J. M. and T. Thum (2012). "Circulating and Urinary microRNAs in Kidney Disease." Clinical Journal of the American Society of Nephrology **7**(9): 1528-1533.

Lu, C., B. C. Meyers and P. J. Green (2007). "Construction of small RNA cDNA libraries for deep sequencing." Methods **43**(2): 110-117.

Lum, R. C. and J. J. Grabowski (1992). "Trimethyl phosphate: the intrinsic reactivity of carbon versus phosphorus sites with anionic nucleophiles." Journal of the American Chemical Society **114**(22): 8619-8627.

Mack, L. L., P. Kralik, A. Rheude and M. Dole (1970). "Molecular Beams of Macroions. II." The Journal of Chemical Physics **52**(10): 4977-4986.

Marchand, A. and V. Gabelica (2014). "Native Electrospray Mass Spectrometry of DNA G-Quadruplexes in Potassium Solution." Journal of the American Society for Mass Spectrometry **25**(7): 1146-1154.

Marwah, A., P. Marwah and H. Lardy (2002). "Analysis of ergosteroids: VIII: Enhancement of signal response of neutral steroidal compounds in liquid chromatographic–electrospray ionization mass spectrometric analysis by mobile phase additives." Journal of Chromatography A **964**(1–2): 137-151.

Matsuo, M., T. Masumura, H. Nishio, T. Nakajima, Y. Kitoh, T. Takumi, J. Koga and H. Nakamura (1991). "Exon skipping during splicing of dystrophin mRNA precursor due to an intraexon deletion in the dystrophin gene of Duchenne muscular dystrophy kobe." Journal of Clinical Investigation **87**(6): 2127.

Mazzitelli, C. L., J. Wang, S. I. Smith and J. S. Brodbelt (2007). "Gas-Phase Stability of G-quadruplex DNA Determined by Electrospray Ionization Tandem Mass Spectrometry and Molecular Dynamics Simulations." Journal of the American Society for Mass Spectrometry **18**(10): 1760-1773.

McCarthy, S. M., M. Gilar and J. Gebler (2009). "Reversed-phase ion-pair liquid chromatography analysis and purification of small interfering RNA." Analytical Biochemistry **390**(2): 181-188.

McCloskey, J. A., D. E. Graham, S. Zhou, P. F. Crain, M. Ibba, J. Konisky, D. Söll and G. J. Olsen (2001). "Post-transcriptional modification in archaeal tRNAs: identities and phylogenetic relations of nucleotides from mesophilic and hyperthermophilic Methanococcales." Nucleic Acids Research **29**(22): 4699-4706.

McGinnis, A. C., B. Chen and M. G. Bartlett (2012). "Chromatographic methods for the determination of therapeutic oligonucleotides." Journal of Chromatography B **883–884**(0): 76-94.

McGinnis, A. C., B. S. Cummings and M. G. Bartlett (2013). "Ion exchange liquid chromatography method for the direct determination of small ribonucleic acids." Analytica Chimica Acta **799**(0): 57-67.

McGinnis, A. C., E. C. Grubb and M. G. Bartlett (2013). "Systematic optimization of ion-pairing agents and hexafluoroisopropanol for enhanced electrospray ionization mass spectrometry of oligonucleotides." Rapid Communications in Mass Spectrometry **27**(23): 2655-2664.

McGowan, M. P., J.-C. Tardif, R. Ceska, L. J. Burgess, H. Soran, I. Gouni-Berthold, G. Wagener and S. Chasan-Taber (2012). "Randomized, placebo-controlled trial of mipomersen in patients with severe hypercholesterolemia receiving maximally tolerated lipid-lowering therapy." PloS one **7**(11): e49006.

McLuckey, S. A. and S. Habibi-Goudarzi (1993). "Decompositions of multiply charged oligonucleotide anions." Journal of the American Chemical Society **115**(25): 12085-12095.

McLuckey, S. A., G. J. Van Berker and G. L. Glish (1992). "Tandem mass spectrometry of small, multiply charged oligonucleotides." Journal of the American Society for Mass Spectrometry **3**(1): 60-70.

Meng, Z. and P. A. Limbach (2004). "RNase mapping of intact nucleic acids by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICRMS) and 18O labeling." International Journal of Mass Spectrometry **234**(1–3): 37-44.

Mishra, P. (2014). "MicroRNAs as promising biomarkers in cancer diagnostics." Biomarker Research **2**(1): 19.

Miska, E., E. Alvarez-Saavedra, M. Townsend, A. Yoshii, N. Sestan, P. Rakic, M. Constantine-Paton and H. R. Horvitz (2004). "Microarray analysis of microRNA expression in the developing mammalian brain." Genome Biology **5**(9): R68.

Mitchell, P. S., R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher, B. S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B. Martin and M. Tewari (2008). "Circulating microRNAs as stable blood-based markers for cancer detection." Proceedings of the National Academy of Sciences **105**(30): 10513-10518.

Motameny, S., S. Wolters, P. Nurnberg and B. Schumacher (2010). "Next Generation Sequencing of miRNAs - Strategies, Resources and Methods." Genes (Basel) **1**(1): 70-84.

Muddiman, D. C., X. Cheng, H. R. Udseth and R. D. Smith (1996). "Charge-state reduction with improved signal intensity of oligonucleotides in electrospray ionization mass spectrometry." Journal of the American Society for Mass Spectrometry **7**(8): 697-706.

Murphy, A. T., P. Brown-Augsburger, R. Z. Yu, R. S. Geary, S. Thibodeaux and B. L. Ackermann (2005). "Development of an ion-pair reverse-phase liquid chromatographic/tandem mass spectrometry method for the determination of an 18-mer phosphorothioate oligonucleotide in mouse liver tissue." European journal of mass spectrometry (Chichester, England) **11**(2): 209.

Nakayama, H., N. Takahashi and T. Isobe (2011). "Informatics for mass spectrometry-based RNA analysis." Mass Spectrometry Reviews **30**(6): 1000-1012.

Nakayama, H., Y. Yamauchi, M. Taoka and T. Isobe (2015). "Direct identification of human cellular microRNAs by nano-flow liquid chromatography–high-resolution tandem mass spectrometry and database searching." Analytical Chemistry.

Nguyen, S. and J. B. Fenn (2007). "Gas-phase ions of solute species from charged droplets of solutions." Proceedings of the National Academy of Sciences **104**(4): 1111-1117.

Nguyen, T. B., S. A. Nizkorodov, A. Laskin and J. Laskin (2013). "An approach toward quantification of organic compounds in complex environmental samples using high-resolution electrospray ionization mass spectrometry." Analytical Methods **5**(1): 72-80.

Ni, J., S. C. Pomerantz, J. Rozenski, Y. Zhang and J. A. McCloskey (1996). "Interpretation of Oligonucleotide Mass Spectra for Determination of Sequence Using Electrospray Ionization and Tandem Mass Spectrometry." Analytical Chemistry **68**(13): 1989-1999.

Nikcevic, I., T. K. Wyrzykiewicz and P. A. Limbach (2011). "Detecting low-level synthesis impurities in modified phosphorothioate oligonucleotides using liquid chromatography–high resolution mass spectrometry." International Journal of Mass Spectrometry **304**(2–3): 98-104.

Nilsson, T., M. Mann, R. Aebersold, J. R. Yates, A. Bairoch and J. J. M. Bergeron (2010). "Mass spectrometry in high-throughput proteomics: ready for the big time." Nat Meth **7**(9): 681-685.

Null, A. P., L. M. Benson and D. C. Muddiman (2003). "Enzymatic strategies for the characterization of nucleic acids by electrospray ionization mass spectrometry." Rapid Communications in Mass Spectrometry **17**(24): 2699-2706.

Null, A. P., A. I. Nepomuceno and D. C. Muddiman (2003). "Implications of Hydrophobicity and Free Energy of Solvation for Characterization of Nucleic Acids by Electrospray Ionization Mass Spectrometry." Analytical Chemistry **75**(6): 1331-1339.

Nyakas, A., L. Blum, S. Stucki, J.-L. Reymond and S. Schürch (2013). "OMA and OPA—Software-Supported Mass Spectra Analysis of Native and Modified Nucleic Acids." Journal of The American Society for Mass Spectrometry **24**(2): 249-256.

Oberacher, H., B. Mayr and C. Huber (2004). "Automated de novo sequencing of nucleic acids by liquid chromatography-tandem mass spectrometry." Journal of the American Society for Mass Spectrometry **15**(1): 32-42.

Oberacher, H., H. Niederstätter, B. Casetta and W. Parson (2006). "Some guidelines for the analysis of genomic DNA by PCR-LC-ESI-MS." Journal of the American Society for Mass Spectrometry **17**(2): 124-129.

Oberacher, H., H. Niederstätter and W. Parson (2005). "Characterization of synthetic nucleic acids by electrospray ionization quadrupole time-of-flight mass spectrometry." Journal of Mass Spectrometry **40**(7): 932-945.

Oberacher, H., P. J. Oefner, G. Hölzl, A. Premstaller, K. Davis and C. G. Huber (2002). "Re-sequencing of multiple single nucleotide polymorphisms by liquid chromatography–electrospray ionization mass spectrometry." Nucleic Acids Research **30**(14): e67.

Oberacher, H., P. J. Oefner, W. Parson and C. G. Huber (2001). "On-Line Liquid Chromatography Mass Spectrometry: A Useful Tool for the Detection of DNA Sequence Variation." Angewandte Chemie International Edition **40**(20): 3828-3830.

Oberacher, H., W. Parson, G. Hölzl, P. J. Oefner and C. G. Huber (2004). "Optimized suppression of adducts in polymerase chain reaction products for semi-quantitative SNP

genotyping by liquid chromatography-mass spectrometry." Journal of the American Society for Mass Spectrometry **15**(12): 1897-1906.

Oberacher, H., W. Parson, R. Mühlmann and C. G. Huber (2001). "Analysis of Polymerase Chain Reaction Products by On-Line Liquid Chromatography−Mass Spectrometry for Genotyping of Polymorphic Short Tandem Repeat Loci." Analytical Chemistry **73**(21): 5109-5115.

Oberacher, H. and F. Pitterl (2011). "Tandem mass spectrometric de novo sequencing of oligonucleotides using simulated annealing for stochastic optimization." International Journal of Mass Spectrometry **304**(2–3): 124-129.

Oberacher, H., B. Wellenzohn and C. G. Huber (2001). "Comparative Sequencing of Nucleic Acids by Liquid Chromatography−Tandem Mass Spectrometry." Analytical Chemistry **74**(1): 211-218.

Oss, M., A. Kruve, K. Herodes and I. Leito (2010). "Electrospray Ionization Efficiency Scale of Organic Compounds." Analytical Chemistry **82**(7): 2865-2872.

Pan, X., R. Wang and Z.-X. Wang (2013). "The Potential Role of miR-451 in Cancer Diagnosis, Prognosis, and Therapy." Molecular Cancer Therapeutics **12**(7): 1153-1162.

Parhofer, K. G. (2012). "Mipomersen: evidence-based review of its potential in the treatment of homozygous and severe heterozygous familial hypercholesterolemia." Core evidence **7**: 29.

Parkinson, G. N., M. P. H. Lee and S. Neidle (2002). "Crystal structure of parallel quadruplexes from human telomeric DNA." Nature **417**(6891): 876-880.

Percy, A. J., M. Rey, K. M. Burns and D. C. Schriemer (2012). "Probing protein interactions with hydrogen/deuterium exchange and mass spectrometry-a review." Anal Chim Acta **721**: 7-21.

Perez-Carbonell, L., F. A. Sinicrope, S. R. Alberts, A. L. Oberg, F. Balaguer, A. Castells, C. R. Boland and A. Goel (2015). "MiR-320e is a novel prognostic biomarker in colorectal cancer." Br J Cancer **113**(1): 83-90.

Premstaller, A., H. Oberacher and C. G. Huber (2000). "High-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry of Single- and Double-Stranded Nucleic Acids Using Monolithic Capillary Columns." Analytical Chemistry **72**(18): 4386-4393.

Premstaller, A., H. Oberacher, W. Walcher, A. M. Timperio, L. Zolla, J.-P. Chervet, N. Cavusoglu, A. van Dorsselaer and C. G. Huber (2001). "High-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry Using Monolithic Capillary Columns for Proteomic Studies." Analytical Chemistry **73**(11): 2390-2396.

Prensner, J. R., S. Zhao, N. Erho, M. Schipper, M. K. Iyer, S. M. Dhanasekaran, C. Magi-Galluzzi, R. Mehra, A. Sahu, J. Siddiqui, E. Davicioni, R. B. Den, A. P. Dicker, R. J. Karnes, J. T. Wei, E. A. Klein, R. B. Jenkins, A. M. Chinnaiyan and F. Y. Feng (2014). "RNA biomarkers associated with metastatic progression in prostate cancer: a multi-institutional high-throughput analysis of SChLAP1." The Lancet Oncology **15**(13): 1469-1480.

Rasmussen, K. D., S. Simmini, C. Abreu-Goodger, N. Bartonicek, M. Di Giacomo, D. Bilbao-Cortes, R. Horos, M. Von Lindern, A. J. Enright and D. O'Carroll (2010). "The miR-144/451 locus is required for erythroid homeostasis." J Exp Med **207**(7): 1351-1358.

Ravikumar, V. T., R. Krishna Kumar, D. C. Capaldi and D. L. Cole (2003). "Synthesis of high quality phosphorothioate oligonucleotides as antisense drugs. Use of I-linker in the elimination of 3′-terminal phosphorothioate monoesters." Nucleosides, Nucleotides and Nucleic Acids **22**(5-8): 1421-1425.

Raynaud, F. I., R. M. Orr, P. M. Goddard, H. A. Lacey, H. Lancashire, I. R. Judson, T. Beck, B. Bryan and F. E. Cotter (1997). "Pharmacokinetics of G3139, a Phosphorothioate Oligodeoxynucleotide Antisense to bcl-2, after Intravenous Administration or Continuous Subcutaneous Infusion to Mice." Journal of Pharmacology and Experimental Therapeutics **281**(1): 420-427.

Reid, G., M. B. Kirschner and N. van Zandwijk (2011). "Circulating microRNAs: Association with disease and potential use as biomarkers." Crit Rev Oncol Hematol **80**(2): 193-208.

Rozenski, J. and J. McCloskey (2002). "SOS: A simple interactive program for ab initio oligonucleotide sequencing by mass spectrometry." Journal of the American Society for Mass Spectrometry **13**(3): 200-203.

Rozenski, J. and J. A. McCloskey (1999). "Determination of Nearest Neighbors in Nucleic Acids by Mass Spectrometry." Analytical Chemistry **71**(7): 1454-1459.

Ruotolo, B. T., J. L. P. Benesch, A. M. Sandercock, S.-J. Hyung and C. V. Robinson (2008). "Ion mobility-mass spectrometry analysis of large protein complexes." Nat. Protocols **3**(7): 1139-1152.

Schultz, N. A., C. Dehlendorff, B. V. Jensen and et al. (2014). "MIcrorna biomarkers in whole blood for detection of pancreatic cancer." JAMA **311**(4): 392-404.

Sharma, V. K., J. Glick, Q. Liao, C. Shen and P. Vouros (2012). "GenoMass software: a tool based on electrospray ionization tandem mass spectrometry for characterization and sequencing of oligonucleotide adducts." Journal of Mass Spectrometry **47**(4): 490-501.

Sharma, V. K., J. Glick and P. Vouros (2012). "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." Journal of Chromatography A **1245**(0): 65-74.

Shen, J., A. B. Siegel, H. Remotti, Q. Wang and R. M. Santella (2016). "Identifying microRNA panels specifically associated with hepatocellular carcinoma and its different etiologies." Hepatoma Research; Vol 2, No 6 (2016).

Shimizu, H., F. Jinno, A. Morohashi, Y. Yamazaki, M. Yamada, T. Kondo and S. Asahi (2012). "Application of high-resolution ESI and MALDI mass spectrometry to metabolite profiling of small interfering RNA duplex." Journal of Mass Spectrometry **47**(8): 1015-1022.

Shin, V. Y., J. M. Siu, I. Cheuk, E. K. Ng and A. Kwong (2015). "Circulating cell-free miRNAs as biomarker for triple-negative breast cancer." Br J Cancer **112**(11): 1751-1759.

Smargiasso, N., F. Rosu, W. Hsia, P. Colson, E. S. Baker, M. T. Bowers, E. De Pauw and V. Gabelica (2008). "G-Quadruplex DNA Assemblies: Loop Length, Cation Identity, and Multimer Formation." Journal of the American Chemical Society **130**(31): 10208-10216.

Smith, R. D., J. A. Loo, C. G. Edmonds, C. J. Barinaga and H. R. Udseth (1990). "New developments in biochemical mass spectrometry: electrospray ionization." Analytical Chemistry **62**(9): 882-899.

Spornraft, M., B. Kirchner, M. W. Pfaffl and I. Riedmaier (2015). "Comparison of the miRNome and piRNome of bovine blood and plasma by small RNA sequencing." Biotechnology Letters **37**(6): 1165-1176.

Stessl, M., C. Noe and J. Winkler (2012). Off-Target Effects and Safety Aspects of Phosphorothioate Oligonucleotides. From Nucleic Acids Sequences to Molecular Medicine. V. A. Erdmann and J. Barciszewski, Springer Berlin Heidelberg**:** 67-83.

Straub, R. F. and R. D. Voyksner (1993). "Determination of penicillin G, ampicillin, amoxicillin, cloxacillin and cephapirin by high-performance liquid chromatography—electrospray mass spectrometry." Journal of Chromatography A **647**(1): 167-181.

Sturm, M., S. Quinten, C. G. Huber and O. Kohlbacher (2007). "A statistical learning approach to the modeling of chromatographic retention of oligonucleotides incorporating sequence and secondary structure data." Nucleic Acids Research **35**(12): 4195-4202.

Tafech, A., T. Bassett, D. Sparanese and C. H. Lee (2006). "Destroying RNA as a therapeutic approach." Current medicinal chemistry **13**(8): 863-881.

Tam, S., R. de Borja, M.-S. Tsao and J. D. McPherson (2014). "Robust global microRNA expression profiling using next-generation sequencing technologies." Lab Invest **94**(3): 350-358.

Tanaka, K., H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida and T. Matsuo (1988). "Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry." Rapid Communications in Mass Spectrometry **2**(8): 151-153.

Tang, L. and P. Kebarle (1991). "Effect of the conductivity of the electrosprayed solution on the electrospray current. Factors determining analyte sensitivity in electrospray mass spectrometry." Analytical Chemistry **63**(23): 2709-2715.

Tang, L. and P. Kebarle (1993). "Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution." Analytical Chemistry **65**(24): 3654-3668.

Taucher, M. and K. Breuker (2010). "Top-Down Mass Spectrometry for Sequencing of Larger (up to 61 nt) RNA by CAD and EDD." Journal of the American Society for Mass Spectrometry **21**(6): 918-929.

Toivonen, J. M., R. Manzano, S. Oliván, P. Zaragoza, A. García-Redondo and R. Osta (2014). "MicroRNA-206: A Potential Circulating Biomarker Candidate for Amyotrophic Lateral Sclerosis." PLoS ONE **9**(2): e89065.

Tromp, J. and S. Schürch (2005). "Gas-phase dissociation of oligoribonucleotides and their analogs studied by electrospray ionization tandem mass spectrometry." Journal of the American Society for Mass Spectrometry **16**(8): 1262-1268.

Valadi, H., K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee and J. O. Lotvall (2007). "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells." Nat Cell Biol **9**(6): 654-659.

van Dongen, W. D. and W. M. A. Niessen (2011). "Bioanalytical LC–MS of therapeutic oligonucleotides." Bioanalysis **3**(5): 541-564.

Wang, G.-K., J.-Q. Zhu, J.-T. Zhang, Q. Li, Y. Li, J. He, Y.-W. Qin and Q. Jing (2010). "Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans." European Heart Journal **31**(6): 659-666.

Wang, K., Y. Yuan, J.-H. Cho, S. McClarty, D. Baxter and D. J. Galas (2012). "Comparing the MicroRNA Spectrum between Serum and Plasma." PLoS ONE **7**(7): e41561.

Wang, L., M. Meng and S. Reuschel (2013). "Regulated bioanalysis of oligonucleotide therapeutics and biomarkers: qPCR versus chromatographic assays." Bioanalysis **5**(22): 2747-2751.

Wang, L., W. Yuan, Y. Zhao, J. Chen, G. vonArx, M. Meng and P. Bennett (2009). "Qantitation of TL0901 oligonucleotide in human plasma using LC-MS/MS." AAPS Annual Meeting and Exposition November 8 - 12, Los Angeles, CA.

Wang, R., Z. X. Wang, J. S. Yang, X. Pan, W. De and L. B. Chen (2011). "MicroRNA-451 functions as a tumor suppressor in human non-small cell lung cancer by targeting ras-related protein 14 (RAB14)." Oncogene **30**(23): 2644-2658.

Wang, Y. and D. J. Patel (1993). "Solution structure of the human telomeric repeat d[$AG_3(T_2AG_3)_3$] G-tetraplex." Structure **1**(4): 263-282.

Wang, Z., K. Wan, R. Ramanathan, J. Taylor and M. Gross (1998). "Structure and fragmentation mechanisms of isomeric T-rich oligodeoxynucleotides: A comparison of four tandem mass spectrometric methods." Journal of the American Society for Mass Spectrometry **9**(7): 683-691.

Wei, C., H. Henderson, C. Spradley, L. Li, I.-K. Kim, S. Kumar, N. Hong, A. C. Arroliga and S. Gupta (2013). "Circulating miRNAs as Potential Marker for Pulmonary Hypertension." PLOS ONE **8**(5): e64396.

Wetzel, C. and P. A. Limbach (2012). "Global identification of transfer RNAs by liquid chromatography–mass spectrometry (LC–MS)." Journal of Proteomics **75**(12): 3450-3464.

Widera, C., S. K. Gupta, J. M. Lorenzen, C. Bang, J. Bauersachs, K. Bethmann, T. Kempf, K. C. Wollert and T. Thum (2011). "Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome." Journal of Molecular and Cellular Cardiology **51**(5): 872-875.

Williams, Z., I. Z. Ben-Dov, R. Elias, A. Mihailovic, M. Brown, Z. Rosenwaks and T. Tuschl (2013). "Comprehensive profiling of circulating microRNA via small RNA sequencing of cDNA libraries reveals biomarker potential and limitations." Proc Natl Acad Sci U S A **110**(11): 4255-4260.

Winkler, J. (2013). "Oligonucleotide conjugates for therapeutic applications." Therapeutic Delivery **4**(7): 791-809.

Wittrup, A. and J. Lieberman (2015). "Knocking down disease: a progress report on siRNA therapeutics." Nat Rev Genet **16**(9): 543-552.

Woods, A. G. and C. C. Darie (2014). Advancements of Mass Spectrometry in Biomedical Research. Cham, Switzerland, Springer International Publishing.

Wu, J. and S. A. McLuckey (2003). "Ion/ion reactions of multiply charged nucleic acid anions: electron transfer, proton transfer, and ion attachment." International Journal of Mass Spectrometry **228**(2–3): 577-597.

Wu, J. and S. A. McLuckey (2004). "Gas-phase fragmentation of oligonucleotide ions." International Journal of Mass Spectrometry **237**(2–3): 197-241.

Wu, Z., W. Gao, M. A. Phelps, D. Wu, D. D. Miller and J. T. Dalton (2004). "Favorable Effects of Weak Acids on Negative-Ion Electrospray Ionization Mass Spectrometry." Analytical Chemistry **76**(3): 839-847.

Xia, Y.-Q. and J. D. Miller (2013). LC-MS Method Development Strategies for Enhancing Mass Spectrometric Detection. Handbook of LC-MS Bioanalysis, John Wiley & Sons Inc.**:** 391-401.

Yamauchi, Y., M. Taoka, Y. Nobe, K. Izumikawa, N. Takahashi, H. Nakayama and T. Isobe (2013). "Denaturing reversed phase liquid chromatographic separation of non-coding ribonucleic acids on macro-porous polystyrene-divinylbenzene resins." Journal of Chromatography A **1312**(0): 87-92.

Yang, J., P. Leopold, R. Helmy, C. Parish, B. Arvary, B. Mao and F. Meng (2013). "Design and Application of an Easy to Use Oligonucleotide Mass Calculation Program." Journal of The American Society for Mass Spectrometry **24**(8): 1315-1318.

Yang, J. S., T. Maurin, N. Robine, K. D. Rasmussen, K. L. Jeffrey, R. Chandwani, E. P. Papapetrou, M. Sadelain, D. O'Carroll and E. C. Lai (2010). "Conserved vertebrate mir-451

provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis." Proc Natl Acad Sci U S A **107**(34): 15163-15168.

Ye, G. and M. Beverly (2011). "The use of strong anion-exchange (SAX) magnetic particles for the extraction of therapeutic siRNA and their analysis by liquid chromatography/mass spectrometry." Rapid Communications in Mass Spectrometry **25**(21): 3207-3215.

Yu, B. and X. Chen (2010). Analysis of miRNA Modifications. Plant MicroRNAs. B. C. Meyers and P. J. Green, Humana Press. **592:** 137-148.

Yu, R. Z., R. S. Geary, D. K. Monteith, J. Matson, L. Truong, J. Fitchett and A. A. Levin (2004). "Tissue disposition of 2′-O-(2-methoxy) ethyl modified antisense oligonucleotides in monkeys." Journal of Pharmaceutical Sciences **93**(1): 48-59.

Yue, X., S. Vahidi and L. Konermann (2014). "Insights into the Mechanism of Protein Electrospray Ionization From Salt Adduction Measurements." Journal of The American Society for Mass Spectrometry **25**(8): 1322-1331.

Zarovni, N., A. Corrado, P. Guazzi, D. Zocco, E. Lari, G. Radano, J. Muhhina, C. Fondelli, J. Gavrilova and A. Chiesi (2015). "Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches." Methods.

Zhan, M., C. P. Miller, T. Papayannopoulou, G. Stamatoyannopoulos and C.-Z. Song (2007). "MicroRNA expression dynamics during murine and human erythroid differentiation." Experimental Hematology **35**(7): 1015-1025.

Zhang, G., J. Lin, K. Srinivasan, O. Kavetskaia and J. N. Duncan (2007). "Strategies for Bioanalysis of an Oligonucleotide Class Macromolecule from Rat Plasma Using Liquid Chromatography−Tandem Mass Spectrometry." Analytical Chemistry **79**(9): 3416-3424.

Zou, Y., P. Tiller, I. W. Chen, M. Beverly and J. Hochman (2008). "Metabolite identification of small interfering RNA duplex by high-resolution accurate mass spectrometry." Rapid Communications in Mass Spectrometry **22**(12): 1871-1881.