Development and application of Differential Mobility- Mass Spectrometry as a rapid analytical platform

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A dissertation submitted to

The Faculty of
the College of Science of
Northeastern University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

April 11, 2014

Dissertation directed by

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Professor of Chemistry and Chemical Biology
Acknowledgements

First, I would like to thank Dr. Vouros for giving me the opportunity to be a part of his research group and guiding me throughout my stay here at Northeastern. Dr. Steve Coy, with his wealth of information and a passion for research, has constantly helped me with my projects. Next, on the list is Dr. James Glick for his unusual way of motivating graduate students and for his unrelenting support for lab members. I would like to thank all the group members of the Vouros Lab, past and present, for their help in the lab and delightful conversations over the years. I would also like to thank all the staff members at Northeastern who have helped me navigate graduate school.

I am blessed to have unconditional love from my parents, Kumar and Prava Kafle, who have always supported my endeavors and have been a source of inspiration to pursue higher education. With the support and laughter from my family members: Smriti, Ashish, Shristi, Samikshya, even the challenging times have passed seamlessly and their unwavering faith in me has helped me tremendously through graduate school. I would also like to thank all my relatives, friends from Nepal, Fairleigh Dickinson & Northeastern and all the teachers who have supported me over all these years.
Abstract of Dissertation

One of the key application areas in the field of mass spectrometry is the analysis of drugs, metabolites, endogenous/ exogenous compounds in matrices of varying complexities. Advances in liquid chromatography including sample clean-up methods (offline/ online) have greatly facilitated the analysis of these compounds, even bringing down the analysis time to a few minutes. However, method development for targeting analytes in complex matrices is often times challenging and sometime requires extensive sample clean-up steps prior to lengthy chromatography times which can affect the throughput of the analytical method. Another challenge that could arise in the chromatographic method development process is the presence of interferences (isomeric or isobaric) during the mass analysis. Hence, an analytical method eliminating or requiring minimal sample preparation steps while maintaining selectivity and providing good throughput for these analyses can be of great importance. Use of DMS has been mostly in conjunction with liquid chromatography; however, in recent years DMS is being increasingly applied to different kinds of matrices as a stand-alone technology for ion separation/ filtration prior to mass analysis. Recent advances in application of the DMS-MS platform to biologically relevant matrices, including contributions to understanding and usage of gas phase interactions is presented in this thesis.
Table of Contents

Acknowledgements ii
Abstract of Dissertation iii
Table of Contents iv
List of Figures viii
List of Tables xiv
List of Abbreviations and Symbols xv
Introduction 1

Chapter 1: Introduction to Differential Mobility Spectrometry (DMS) and rapid analysis using DMS-MS 5
1. 1 Introduction .......................................................................................................................... 6
1. 2 Three main types of ion mobility spectrometry: ................................................................. 7
1. 3 Differential Mobility Spectrometry (DMS) ........................................................................ 8
1. 4 Analyzer geometry ............................................................................................................... 9
1. 5 The alpha function ............................................................................................................ 10
1. 6 Chemical Modifiers in DMS ............................................................................................. 10
1. 7 History of DMS development ............................................................................................ 12
1. 8 Commercial DMS based instruments ............................................................................... 13
1. 9 Development of DMS in Vouros Lab ............................................................................... 16
1. 10 Recent interesting applications of DMS ......................................................................... 19
1. 11 Green Chemistry ............................................................................................................ 27
1. 12 Rapid analysis eliminating sample clean-up steps using nanoESI-DMS-MS ................. 28
1. 13 Results and Discussion ................................................................................................. 31
1. 14 Rapid Identification of β-carboline Hallucinogens by Pressure Assisted Extraction (PCT) and DMS-MS ................................................................. 32
1. 15 Quantitation of an immunosuppressant in transport media by DMS-MS/MS ................ 38
1. 16 Conclusion ........................................................................................................ 41

Chapter 2: Improving the Ion Trap’s qualitative and quantitative performance using Differential Mobility (DMS) as a prefilter  43

2. 1 Introduction ......................................................................................................... 44
2. 2 Ion Trap: Working Principles ................................................................................. 46
2. 3 Space Charges .................................................................................................... 47
2. 4 Automatic Gain Control (AGC) function ............................................................. 48
2. 5 Use of DMS as a prefilter .................................................................................. 49
2. 6 Experimental ....................................................................................................... 51
2. 7 Qualitative Comparison: DMS-on and DMS-uninstalled ...................................... 52
2. 8 Analysis of a dilute sample with DMS ................................................................. 55
2. 9 Analysis of a concentrated sample with DMS ..................................................... 56
2. 10 Quantitative comparison: DMS-on and DMS-uninstalled ...................................... 58
2. 11 DMS-transparent and DMS-on mode ............................................................... 61
2. 12 Removal of matrix ion interferences with DMS ................................................. 62
2. 13 Quantitation in MS mode .................................................................................. 67
2. 14 Quantitation in MS/MS mode .......................................................................... 69
2. 15 Conclusions ...................................................................................................... 71
Chapter 3: Understanding gas phase modifier interactions in rapid analysis by Differential Mobility-Tandem Mass Spectrometry

3. 1 Introduction
3. 2 Experimental Section
3. 3 Instrumentation
3. 4 Effect of Modifier on CoV of dG-ABP
3. 5 Effect of Modifier on CoV of dG-PhIP
3. 6 Clustering with gas phase modifiers
3. 7 Use of modifiers in separation
3. 8 Effect of separation voltages and modifiers on signal intensities in MRM mode
3. 9 Thermochemical kinetics of modifier-assisted DMS
3. 10 Conclusions

Chapter 4: Rapid detection and quantitation of DNA adducts dG-ABP and dG-PhIP using Differential Mobility-Mass Spectrometry

4. 1 Introduction
4. 2 Experimental
4. 3 Modification of calf-thymus DNA with 4-nitrobiphenyl
4. 4 Cell culture and dosing of MCL-5 cells
4. 5 DNA quantification, enzymatic digestion and protein precipitation
4. 6 Instrumentation
4. 7 Results and Discussion
4. 8 Detection of dG-ABP in calf thymus DNA using DMS-Ion Trap MS
4. 9 Quantitation of dG-ABP in Calf Thymus DNA by DMS-triple quadrupole MS
4. 10 Calibration curve of dG-ABP ........................................................................................................... 126
4. 11 Quantitation of dG-ABP in Modified CT-DNA: Comparison of DMS-MS/MS and LC-MS/MS Platforms ........................................................................................................................................... 129
4. 12 Quantitation of dG-PhIP in MRM mode using DMS-MS ................................................................. 131
4. 13 Calibration curve of dG-PhIP ........................................................................................................... 133
4. 14 Detection of dG-PhIP from procarcinogen PhIP dosing study on MCL5 cells ......................... 134
4. 15 CONCLUSIONS .......................................................................................................................... 136

Chapter 5: Conclusions and Future Directions ................................. 138

5. 1 Conclusions ........................................................................................................................................ 139
5. 2 Application in Radiation Biodosimetry: ......................................................................................... 144
5. 3 Proposed improvement in DMS software ....................................................................................... 149

Epilogue .................................................................................................................................................. 152

References .............................................................................................................................................. 155
List of Figures

Figure 1 Schematic of DMS ion filter operation ................................................................. 9

Figure 2 Graphical depiction of the clustering-declustering in DMS. During the low field the modifier molecules cluster with the analyte ion and during the high field the modifier ions decluster. This process is repeated as the analyte ions transmit through the DMS plates causing changes to the “effective cross sectional area” leading to better ion separation. ......................... 12

Figure 3 Different Commercial Instruments based on DMS .............................................. 14

Figure 4 Impact of ion mobility/ differential ion mobility in the field demonstrated through PubMed search (Years 2001-2013) as performed on March 11th, 2014. Note: Publications mentioning differential ion mobility (DMS) and field asymmetric ion mobility (FAIMS) were separately searched and presented as a sum and the final numbers are subset of the total publications mentioning Ion Mobility. ................................................................. 16

Figure 5 Left: DMS on the AB Sciex 3000 triple quadrupole mass spectrometer and Right: DMS on the Thermo Finnigan ion trap mass spectrometer ................................................................. 18

Figure 6 Separation of isobaric species using DMS (A) Isopropanol was used as the modifier at concentrations of a) 0%, b) 1.6%, c) 3.1%, and d) 6.2%. (B) Structures of analyzed compounds 1) oxfendazole, 2) clonazepam, 3) flusilazole, 4) bromazepam, 5) chlorprothixene, 6) pamaquin. ......................................................................................................................... 20

Figure 7: Separation of amine nitrogen protonated and carboxylic acid oxygen protonated 4-aminobenzoic acid by planar DMS ......................................................................................................................... 21

Figure 8 Analysis of Propranolol, 4-Hydroxypropranolol aromatic O-glucuronide (PRO-O-ARG) and 4-Hydroxypropranolol aliphatic O-glucuronide (PRO-O-ALG) from a dosed liver tissue extract by DMS-MS (A) without Modifier (B) with acetonitrile as the modifier.(ii) Structures of Propranolol, 4-Hydroxypropranolol aromatic O-glucuronide (PRO-O-ARG) and 4-Hydroxypropranolol aliphatic O-glucuronide (PRO-O-ALG). ................................................................. 23

Figure 9 Separation of isomeric dipeptides: glycine-alanine (GA) and alanine-glycine (AG), glycine-serine (GS) and serine-glycine (SG) with different modifiers ................................................................. 24

Figure 10 Separation of peptide mixture obtained from a tryptic digest of BSA performed with two component modifier mixture (2.0% methanol and 0.13% hexanol). Top panel was obtained
at SV 4000 volts without arcing suppressant and 0.05 % chloroform was added as the arcing suppressant in the bottom panel obtained at SV 4250 volts. .................................................. 25

Figure 11 DMS separation for three different charge states (2+, 3+, and 4+) of Syntide 2, measured using a mixture of He/N₂ as transport gas (% He is labeled). The w (full width at half maximum (FWHM)) can be used to compare peak shapes. .................................................. 26

Figure 12 Workflow for targeting analytes of interest (qualitative analysis of harmine & harmaline and quantitative analysis of cyclosporin A) using nanoESI-DMS-MS platform. It is worth noting that sample clean-up steps were eliminated for relatively complex matrices and samples were simply prepared for analysis by dilution in appropriate mobile phase .................... 31

Figure 13 Effect of modifiers on the separation of harmine and harmaline at a constant SV of 1500 volts. A) No modifier B) Isopropanol C) Acetone D) Ethyl acetate. Only acetone and ethyl acetate provide CoV shifts away from the zero value and the latter affords separation.............. 34

Figure 14 Mass spectrum showing separation and rapid qualitative analysis of harmine and harmaline using DMS-MS. A) DMS transparent B) DMS-on at fixed CoV of -8.0 volts showing selective introduction of harmine C) DMS-on at fixed CoV of -10.0 volts showing selective introduction of harmaline. Identities were further confirmed by MS-MS data. ......................... 36

Figure 15 Comparison of (A) Gas Chromatography (GC) and (B) Liquid Chromatography (LC) traces for the analysis of harmine and harmaline. GC work was performed under supervision of Dr. Adam Hall at Boston University and LC work was performed under supervision of Dr. James Glick at Northeastern University .......................................................... 37

Figure 16 Mass spectral reduction of background noise level and improvement of signal intensity by the use of DMS for Cyclosporin A and the labeled internal standard in transport media ...... 40

Figure 17 Calibration curve of Cyclosporin A in transport media. The error bars represent standard deviations of triplicate analyses and the inset shows a fit to the lowest part of the data. ................................................................................................................ 41

Figure 18 Graphical representation of DMS filtration when used with ion-trap mass spectrometer. Top: When there is no DMS filtration, the trap is filled with variety of ions including unwanted ions generated during the electrospray process. Bottom: With DMS filtration in line, the ion trap can be selectively filled with the desired ion population................................. 45
Figure 19 Depiction of regions of stability in the quadrupole ion trap[62]. Ions are stable in both z and r direction as long as their Mathieu parameters $a_z$ and $q_z$ fall within the stability zone. .......................... 46

Figure 20 Depiction of analytical cycle of ion trap mass spectrometer[64] ........................................ 48

Figure 21 Utility of DMS in cleaning up matrix ion during analysis of BE in urine. (a) mass spectra generated from blank with DMS-uninstalled (b) mass spectra of a 0.5 ng/ul BE sample with DMS-on ........................................................................................................ 53

Figure 22 Analysis of a dilute sample (0.1 ng/ul BE) with DMS filtration and comparison with DMS uninstalled spectra when the tip position was optimized for maximum ion signal intensity in the m/z 270-300 range. ........................................................................................................................................ 55

Figure 23 (i) Analysis of a concentrated sample (5.0 ng/ul BE) with DMS filtration and comparison with DMS uninstalled spectra when the tip position was optimized for maximum ion signal intensity in the m/z 270-300 range (ii) quantitative comparison of the signal gains/ losses by DMS filtration at two different fill times (a) AGC feature turned on (b) AGC feature turned off and trap set to a fill time of 500 ms.................................................................................................................................................. 57

Figure 24 Calibration curve of BE extracted from urine in MS mode using the DMS-MS platform. The error bars represent standard deviations of triplicate analyses ........................................ 59

Figure 25 (a) Calibration curve of BE extracted from urine in MS/MS mode using the DMS filtration. The error bars represent standard deviations of triplicate analyses. (b) Calibration curve of BE extracted from urine in MS/MS mode without the DMS filtration ................................................. 61

Figure 26 Analysis of BE in urine by DMS-MS. (a) Full scan mass spectrum (m/z 150 – m/z 500) of urine blank solution in the DMS-transparent mode; (b) Full scan mass spectrum (m/z 150 – m/z 500) of previous solution with DMS-on set at CoV = -16V showing removal of all matrix ions including potential interference at m/z 288.6; (c) Analysis of BE in urine (0.1ng/µL) with DMS set at CoV = -16V showing the ratio of 1:10 analyte : internal standard. MS scan range (m/z 182 – m/z 296). .......................................................................................................................... 63

Figure 27 Separation of m/z 290 and m/z 291 ions of BE demonstrating loss of resolution with increasing trap fill time due to space charge effects ................................................................. 65

Figure 28 Comparison of product ion signal intensities of BE in DMS-on mode across a range of concentrations (0.1 ng/ul- 25 ng/ul) under two different conditions: AGC-on and AGC-off 500 ms............................................................................................................................... 66
Figure 29 Calibration curves for quantitation of benzoylecgonine in urine by MS mode. The error bars represent the standard deviation of triplicate analyses. (ii,iv) were obtained with no DMS filtration (DMS-transparent), and (iii) AGC-off. (iv) AGC-on. (i,ii) were obtained using DMS filtration: (i) AGC-off (ii) AGC-on.

Figure 30 Calibration curves for quantitation of benzoylecgonine in urine by MS/MS (m/z 290 → m/z 168). The error bars represent the standard deviation of triplicate analyses. (a,c) were obtained with no DMS filtration (DMS-transparent), and (a) AGC-on. (c) AGC-off. (b,d) were obtained using DMS filtration: (b) AGC-on, (d) AGC-off. Results are much better with DMS filtration. In addition, the use of an extended fill time allows the calibration curve to extend an extra factor of 10 lower in BE concentration.

Figure 31 Structures of DNA adducts in this study.

Figure 32 Effect on compensation voltage of varying concentrations of modifiers (i) Ethyl acetate and (ii) Isopropanol on dG-ABP CoV shifts.

Figure 33 Comparison of peak shapes of dG-ABP with and without modifier. Improvements in peak shape by the introduction of modifiers can be realized by comparing the FWHM (full width at half maximum) at a constant SV of 3500 volts.

Figure 34 Effect of varying concentrations of modifiers (i) Isopropanol, (ii) Ethyl acetate.

Figure 35 Separation of a mixture of dC, dA, dG and dG-ABP (A) in the presence of ethyl acetate modifier at a fixed concentration done on the DMS- ion trap (B) in the presence of isopropanol modifier at a fixed concentration done on the DMS- ion trap.

Figure 36 Separation of dG and dG-ABP (i) without modifier and using IPA modifier at two different modifier concentration done on DMS-triple quadrupole (ii) 0.60% and (iii) 1.10%.

Figure 37 Effect of separation voltage and modifier isopropanol (IPA) concentration on the MS/MS signal intensity of the DNA adduct dG-PhIP (A) at 0.6% (B) 1.1% (C) 2.2% (D) Trends of AB SCIEX DMS API 3000 signal intensities at separation voltages of 3500, 4000, 4500 volts with increasing modifier percentages.

Figure 38 Effect of modifiers on the product ion intensity at a fixed separation voltage of 3500 volts: (A) Modifiers Isopropanol (IPA) and 1-butanol on the DNA adduct dG-PhIP & (B) Modifiers Isopropanol (IPA) and Ethyl acetate (EtoAc) on the DNA adduct dG-ABP.
Figure 39 Using ab-initio thermochemical values, the mean number of bound neutrals is shown as a function of DMS field and transport gas temperature at 1 atm. These results for R-alpha-methylhistamine, which has a polar core similar to dG, with 1.5% isopropanol show that bulk gas temperature is an essential controlling parameter in modifier DMS selectivity. 107

Figure 40 Comparison of the workflow for the analysis of DNA-adducts by LC-MS and DMS-MS 120

Figure 41 Mass spectra of 100 pg dG-ABP in 2 ug calf thymus DNA under two different conditions: (A) DMS-transparent, No modifier (B) DMS-on, ethyl acetate modifier 123

Figure 42A) Normalized extracted ion profile showing CoV shifts for dG-ABP with and without ethyl acetate modifier at SV values of 0 and 3500 volts and (B) MRM transitions monitored for dG-ABP and internal standard 125

Figure 43 Calibration curve of dG-ABP in calf-thymus DNA. The error bars represent standard deviations of triplicate analysis (Inset shows linearity across lowest three points in the calibration curve) 126

Figure 44 Removal of interference using DMS. The signal from blank matrix CT (calf-thymus) DNA was removed in DMS on mode to produce a linear calibration curve using ethyl acetate as the modifier (A) Signal from blank calf thymus DNA in the DMS transparent (B) Signal from blank calf thymus DNA with DMS on (C) Signal from 5.8 femtomoles of the analyte in calf thymus DNA with DMS on 128

Figure 45 A) Extracted ion chromatogram showing CoV shifts for dG-PhIP with and without Isopropanol modifier at SV values of 0 and 3500 volts and (B) MRM transitions monitored for dG-PhIP and Internal standard 132

Figure 46 Calibration curve of dG-PhIP in calf-thymus DNA. The error bars represent standard deviations of triplicate analysis 133

Figure 47 Variable dosing study of procarcinogen PhIP on MCL5 cells over a 24 hour period. The error bars represent the standard deviation of triplicate measurements. No adducts were detected in the no dose control 135

Figure 48 Separation of three component biomarkers by DMS in the presence of ethyl acetate as modifier 145
Figure 49 Corresponding mass spectra for the total ion chromatogram and extracted ion chromotograms for three component biomarker mixture ................................. 146

Figure 50 Analysis of acylcarnitines in urine after SPE using DMS-MS. (A) The three acylcarnitines analyzed: acetylcarnitine, octanoylcarnitine and decanoylcarnitine were not separated under DMS transparent conditions (B) With application of the Separation Voltage (SV), the three analytes moved away from a CoV value of zero and were separated. (C) Mass spectra showing targeted analysis of acetylcarnitine (1ng/ul). Top: Mass spectrum is dominated by components from the matrix. Bottom: DMS allows selective introduction of acetylcarnitine (m/z→ 204) into the mass spectrometer and the mass analysis was performed in 30 seconds .......................... 148

Figure 51 Software interface on the Analyst software (version 1.5) as used on the AB Sciex 3000 DMS-triple quadrupole mass spectrometer used for the research. The DMS parameters are integrated in the Analyst software. Only one CoV value (circled) can be used for normal mode of operation and CoV scanning can be done by specifying two CoV points using the Edit Ramp feature in the software ........................................................................................................................................ 150

Figure 52 Expert Software interface as used on the Sionex DMS-Thermo Finnigan classic ion-trap used for the research. DMS is controlled by the separate software “Expert”. There are two fields for CoV values (circled) which are generally used for scanning mode ......................... 151
List of Tables

Table 1: Comparison of time scales for separation of harmine and harmaline by conventionally used GC, LC and emerging DMS technology. With analysis time of 1 minute or less, the DMS can be successfully applied for analytical applications ................................................................. 37

Table 2 Evaluation of quality control points for dG-ABP calibration curve in the DMS on mode .................................................................................................................................................. 129

Table 3 Quantitation of dG-ABP in Calf Thymus DNA (# Adducts in $10^7$ Normal Nucleosides) by DMS-MS and LC/MS ............................................................................................................................................. 131

Table 4: Evaluation of quality control points for dG-PhIP quantitation ......................................................... 134
### List of symbols and abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABP</td>
<td>Aminobiphenyl</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>AGC</td>
<td>Automatic gain control</td>
</tr>
<tr>
<td>BE</td>
<td>Benzoylecgonine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDS</td>
<td>Chemring Detection Systems</td>
</tr>
<tr>
<td>CE</td>
<td>Collision energy</td>
</tr>
<tr>
<td>CID</td>
<td>Collision-induced dissociation</td>
</tr>
<tr>
<td>CoV</td>
<td>Compensation Voltage</td>
</tr>
<tr>
<td>ct-DNA</td>
<td>calf thymus deoxyribonucleic acid</td>
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<tr>
<td>CWA</td>
<td>Chemical warfare agents</td>
</tr>
<tr>
<td>dA</td>
<td>deoxyadenosine</td>
</tr>
<tr>
<td>dC</td>
<td>deoxycytosine</td>
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<tr>
<td>dG-ABP</td>
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</tr>
<tr>
<td>dG-PhIP</td>
<td>N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-</td>
</tr>
<tr>
<td>DMS</td>
<td>Differential Mobility Spectrometry</td>
</tr>
<tr>
<td>DMS-MS</td>
<td>Differential Mobility- Mass Spectrometry</td>
</tr>
<tr>
<td>DMS-MS/MS</td>
<td>Differential Mobility- Tandem Mass Spectrometry</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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DNase I  deoxyribonuclease 1

ds  double strand

**DTIMS**  Drift Tube Ion Mobility Spectrometry

**ESI**  Electrospray ionisation

**EtoAc**  Ethyl acetate

**FA**  Formic acid

**FAIMS**  Field Asymmetric Ion Mobility Spectrometry

**FWHM**  Full width at half maximum

**GC**  Gas chromatography

**GC-MS**  Gas chromatography- mass spectrometry

**HBSS**  Hanks' balanced salt solution

**HCl**  Hydrochloric acid

**HEPES**  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

**IM**  Ion mobility

**IM-MS**  Ion mobility- mass spectrometry

**IPA**  Isopropanol

**JASMS**  Journal of American Society of Mass Spectrometry

**kV**  kilovolts

**LC**  Liquid chromatography

**LC-MS**  Liquid chromatography- mass spectrometry

**LOD**  limit of detection

**LOQ**  limit of quantitation

**m/z**  mass to charge
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>MCL5</td>
<td>metabolically competent human lymphoblast cells</td>
</tr>
<tr>
<td>mg/ml</td>
<td>milligrams per milliliter</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>nanoESI/ nESI</td>
<td>Nanoelectrospray ionisation</td>
</tr>
<tr>
<td>NASA</td>
<td>The National Aeronautics and Space Administration</td>
</tr>
<tr>
<td>ng/ul</td>
<td>nanograms per microliter</td>
</tr>
<tr>
<td>nl/min</td>
<td>nanoliters per minute</td>
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<tr>
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<td>pressure cycling technology</td>
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<tr>
<td>Q1</td>
<td>First Quadrupole</td>
</tr>
<tr>
<td>Q2</td>
<td>Second Quadrupole</td>
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<tr>
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<td>Solid Phase Extraction</td>
</tr>
<tr>
<td>SRM</td>
<td>Selected reaction monitoring</td>
</tr>
<tr>
<td>SV</td>
<td>Separation Voltage</td>
</tr>
<tr>
<td>TWIMS</td>
<td>Travelling Wave Ion Mobility Spectrometry</td>
</tr>
<tr>
<td>ug/ml</td>
<td>micrograms per milliliter</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-Violet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile organic compounds</td>
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Introduction

One of the biggest challenges in the field of analytical chemistry is the preparation of samples prior to analysis. A platform eliminating or requiring minimal sample preparation steps and capable of selective, rapid analysis is of interest to the analytical community. Differential Mobility is a rapidly evolving gas phase separations/filtration technique that can be performed in milliseconds regime. DMS has emerged as a powerful tool and in combination with a mass spectrometer, the DMS-MS platform can be utilized for rapid analytical purposes. Traditionally, elaborate sample preparation/clean-up steps are required before analyses in complex matrices, often resulting in loss of precious samples, followed by lengthy separation time. In order to address some of the concerns regarding sample preparation/clean-up steps and to speed up the overall analysis time, DMS has been hyphenated to a Thermo-Finnigan Ion-Trap and AB Sciex Triple Quadrupole Mass Spectrometers in the Vouros Lab at Northeastern University and has been a subject of considerable study for a number of years now. The goal of the thesis is to establish DMS-MS as a rapid stand-alone analytical platform and to contribute to the development and application of the same.

Chapter 1 of this thesis focuses on the fundamentals of planar DMS and a brief history of the relevant applications is also presented. The instrument design and currently accepted working principle of the DMS with modifiers is explained. Early on in the usage of DMS, it was realized that addition of polar compounds in the drift gas as modifiers produced a drastic effect on selectivity and sensitivity, so careful consideration has been given to the usage of modifiers with a more detailed investigation to follow up on the modifier interactions in Chapter 3. With hyphenation to a mass spectrometer, the DMS-MS platform can be exploited to perform rapid analyses without the need for lengthy sample preparation/chromatography steps for selected
applications. In order to demonstrate the usefulness and rapidity of this method, two distinct applications are presented in Chapter 1: qualitative analysis of two structurally similar β-carboline compounds: harmine & harmaline from *Peganum harmala* seed extracts and quantitative analysis of cyclosporin A in transport media (HBSS buffer supplemented with HEPES and glucose), both of which have been performed using DMS-MS eliminating sample clean-up steps.

Hyphenation of the DMS to the MS allows selective filtration of ion species going into the mass spectrometer for analysis. The results are more evident in mass spectrometers which depend on ion accumulation such as the Thermo-Finnigan Ion Trap when compared to the AB Sciex Triple Quadrupole, both of which were used over the course of this research. The original idea was proposed by Dr. Paul Vouros and Dr. Erkinjon Nazarov and this idea was previously explored in the Vouros lab. The work presented in Chapter 2 strengthens the original idea of selective filling of the trap using DMS to extend the qualitative and quantitative abilities of the trap which is demonstrated through the analysis of a drug metabolite in a biological fluid (urine). During the course of the work, it was realized that due to instrumental design of the DMS used in the study, ion losses were common. In addition to DMS-on and DMS-transparent data, DMS-uninstalled data will be presented as well as a benchmark for comparison, although during normal operation uninstalling DMS is not required as the DMS can be operated in the transparent mode. Setting the automatic gain control feature on the ion trap manually to a longer time afforded the instrument to be selectively filled with ions of interest and presented significant benefits with the usage of DMS.

The importance of modifiers in DMS-MS has already been established in the previous two chapters. Although cluster-decluster model has been widely accepted as the working model, the
exact nature of gas phase modifier interactions remain elusive. In the application examples presented earlier, the amount of analytes analyzed have been in the high picograms to nanograms range and have not required a stringent method development phase particularly with respect to modifier usage. In Chapter 3, a systematic study involving the use and optimization of gas phase modifiers in quantitative differential mobility-mass spectrometry (DMS-MS) analysis is presented using biomarkers of DNA damage as an important reference point for analysis in complex matrices. A brief introduction to DNA adducts is presented with details to follow in the next chapter. The target analyte amount is in the low picograms range and the optimizations of modifier usage along with DMS operational parameters have been deemed essential for effective selectivity and sensitivity. Particular attention has been paid to compensation voltage (CoV) shifts, peak shapes and product ion signal intensities while optimizing the DMS-MS conditions. Commonly used polar protic and polar aprotic modifiers have been screened for use against two deoxyguanosine adducts of DNA: N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-ABP) and N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (dG-PhIP). Based on these observations and thermochemical kinetics results, new insights and theories are developed into the complexity of DMS-modifier responses.

Chapter 4 is the continuation of the work presented in chapter 3 and the developed parameters are applied to the analysis of biomarkers of DNA damage. A brief background and history on the analysis of DNA adducts is presented. The potential of DMS over the widely used LC-MS method for adduct analysis is elucidated. DMS-MS platform has been utilized for the analysis of DNA adducts, specifically two carcinogenic deoxyguanosine adducts of: 4-aminobiphenyl (4-ABP) and 2-amino-1-methyl-6-phenylimidazo[4,5]pyridine (PhIP) have been studied as model analytes. After the DMS parameters were set, each sample was analyzed in just 30 seconds after
a simple protein precipitation step. Sample requirement has been reduced to two micrograms of DNA and detection limits of approximately 1 modification in $10^6$ nucleosides have been achieved using this method. An important quantitative comparison with liquid chromatography-mass spectrometry for DNA adducts analysis is presented. To demonstrate the applicability of the DMS-MS platform in biological systems, detection of DNA adduct dG-PhIP from a dosing study with PhIP is also presented.

Chapter 5 is the summary of the work presented in this thesis. Having established DMS-MS as a rapid and robust analytical platform, future directions and applications are proposed. A proposal is being developed between Northeastern University, Georgetown University and Draper Labs which incorporates exploration of DMS-MS for rapid targeted analysis for biomarkers of radiation damage and some preliminary data is presented. Current software controlling the DMS limits the number of simultaneous analysis to two analytes in the CoV stepping mode, so changes to the current version of DMS software to further accommodate simultaneous analysis of multiple analytes is proposed. The potential to reduce/eliminate sample clean-up/preparation steps, lower overall cost of analysis and the rapid analysis time demonstrated in this thesis through several analytes in complex matrices such as seed extracts, media, urine and DNA digest certainly calls for further applications of DMS-MS to the benefit of the analytical community.
Chapter 1: Introduction to Differential Mobility Spectrometry (DMS) and rapid analysis using DMS-MS
1. 1 Introduction

Much of today’s analytical research involves elaborate characterization and quantitation of molecules in matrices of varying complexities. Widely used liquid chromatography- mass spectrometry has established itself as the premier method for the analysis of complex samples. Advances in liquid chromatography have helped address many critical challenges prior to mass analysis. However, sample preparation steps and separation time still remain a bottleneck prior to mass analysis. In the field of analytical chemistry, it is common to apply a complementary method or explore/use alternate methods for detection, structural elucidation and quantitation of molecules especially if they afford advantages such as rapid analysis and reduced sample preparation steps. With the rising demand for rapid analytical methods capable of handling complex samples, there is a need for continuous advancement of analytical sciences to address these issues. A technology capable of rapid analysis, offering high-throughput, high selectivity and requiring minimal sample preparation has been identified as an important need. Ion mobility-mass spectrometry (IM-MS) has shown significant promise in gas-phase separations of complex samples.

Coupling of IM with MS has been in existence since the 1960s, however, because of its ability to provide separations on the basis of ion structure and mass to charge ratio (m/z), the usage of this technology in recent years has boomed [1]. Today, commercial instruments that combine IM with MS are available from a wide number of vendors and a significant number of laboratories deploy these instruments for regular usage. In ion mobility, ions are generated and introduced into the mobility cell under the influence of an electric field. The generic equation that describes the mobility of ions given of a velocity, v in electric field E is:

\[ v = \frac{KE}{-} \]  
---Equation 1
Where, K is the ion mobility co-efficient and is also referred to as ion’s mobility factor.

1. 2 Three main types of ion mobility spectrometry:

I. Drift Tube Ion Mobility Spectrometry (DTIMS)

II. Travelling Wave Ion Mobility Spectrometry (TWIMS)

III. Differential Mobility Spectrometry (DMS)- Also referred to as Field Asymmetric Ion Mobility Spectrometry (FAIMS)

Drift tube is the most commonly used form of ion mobility spectrometry. It is a pulsed technique where ions generated from a source are introduced into the drift tube with a constant electric field. Based on ion cross section and the number of collisions with carrier buffer gas, the mobility of the ions through the tube can be determined and ions can be identified based on the time they spent in the drift tube.

Travelling wave is also a pulsed technique, and ions pass through a series of concentric ring electrodes in presence of a buffer gas [2, 3]. Rather than using a uniform electric field, the electric fields applied alternate between positive and zero. Ions accelerate during the application of high positive voltage and become stationary during when no voltage is applied and in the process the travelling wave allows separation on ions based on their cross sectional area.
1. 3 Differential Mobility Spectrometry (DMS)

Differential mobility spectrometer (DMS) is also referred to as high-field asymmetric waveform ion mobility spectrometry (FAIMS). DMS separates/filters ion population by passing them through a gap between two electrodes where an asymmetric field is applied tangential to the ion motion. The asymmetric waveform creates net ion motion which is dependent on the applied fields and this causes the ions to separate as they get carried by the transport gas through the gap of the electrodes.

The alternating RF voltage applied between two planar parallel electrodes is referred to as separation voltage (SV). In the asymmetric waveform, ions experience a short period of high field followed by a longer period of low field which causes a net ion displacement. In addition to the SV, a DC voltage also called the compensation voltage (CoV) is applied to transmit selected ions of interest through the electric field without being neutralized by the electrodes [4]. Application of SV and CoV allows both positively and negatively charged ions through the DMS cell and the ion transmission is optimized using a combination of SV and CoV values [5].

DMS can be operated in the following modes by scanning or fixing SV and CoV: 1) a fixed SV and CoV can be selected which results in continuous filtration of selected ion species 2) and CoV is scanned at a fixed SV which produces a linear DMS spectrum (often referred to as ionogram) 3) both SV and CoV are scanned which produces a full differential scan[4]. DMS can be used as a stand-alone detector or used as a pre-filter when coupled to other analytical instruments.
Figure 1: Schematic of DMS ion filter operation

1.4 Analyzer geometry

Two different types of differential mobility ion filters are commonly used: the cylindrical and the planar electrodes. Usually the name FAIMS (field-asymmetric waveform ion mobility spectrometry) is associated with cylindrical configuration and DMS (Differential Mobility Spectrometry) is used with the planar configuration; however, the names are used interchangeably. Both the cylindrical and planar configurations are continuous beam devices and utilize high frequency asymmetric waveforms to separate ions based on the difference between their mobilities in high field and low field. Schneider et al[4] have pointed out that FAIMS provides the potential advantage of ion focusing at atmospheric pressure because of RF fields and analyzer curvature. Whereas, DMS provides faster ion transit times and offers a transparent mode of operation which allows all ions to be transmitted without discrimination when SV is
turned off and CoV set to zero. Both configurations can simultaneously transmit both positive and negative ions and separate the ions based on their mobility differences.

1. 5 The alpha function
The α (alpha) function is the fundamental property of ion-neutral interactions which is directly related to conditions for DMS ion transmission. The field dependent part of the ion mobility is called the α function and it is given by the equation below:

$$\alpha(E) = \frac{K(E) - K(0)}{K(0)}$$

Where α(E) is the α function, K(E) is the high field mobility, and K(0) is the low-field mobility. α is a dimensionless normalized difference between the high-field and low-field mobility constants which gives the field dependence of ion mobility under different field strengths and transport gas conditions [5].

1. 6 Chemical Modifiers in DMS
During the initial years of DMS technology at Northeastern (brief history presented later), it was realized that addition of small polar molecules to the transport gas dramatically aids DMS separations. Since then, a number of studies have demonstrated and it has been well established that addition of modifiers, mainly small organic polar protic molecules to the transport gas, enhances DMS separations[6-8]. Small alcohols have proven effective as modifiers and in a recent publication, Hall et al. have reported on the use of ethyl acetate as a modifier in forensic applications[9]. In the presence of modifiers, the differential mobility separations are best described by the cluster-decluster model[10]. As mentioned, alternating RF voltages, known as the Separation Voltage (SV) are applied across the planar DMS electrodes. There are two types
of fields applied in differential mobility filter: high field and low field. In the cluster-decluster model, it is believed that during the low field portion of the asymmetric wave, analyte ions form clusters with the neutral gas modifiers and during the high field portion, the ion-neutral complex dissociates changing the effective cross sectional area for ion transmission. This change in the “effective cross sectional area” causes differences in ion transmission leading to improved separations. The compensation voltage (CoV) is applied to transmit the analyte of interest.

Over the course of this research work done at Northeastern, different modifiers such as isopropanol, ethyl acetate, 1-butanol were used for shifting the desired analyte(s) of interest to a different compensation voltage value resulting in separations of ion species and/or shifting the analyte away from the noise. Results on modifier usage are presented throughout this thesis with a special consideration to optimization of modifier usage presented in Chapter 3.
1.7 History of DMS development

The history of DMS presented in this section and the list of commercial DMS instruments presented in the following section was compiled by Dr. Erkinjon Nazarov. With his permission, the same section with modifications has been incorporated in this thesis and it presents a historical perspective on the development and application of DMS.

Functional prototypes of DMS sensors were first built in the mid-1980’s. The development process diverged with the efforts of two independently operated teams; Gorshkov’s team at the Institute of Thermo Physics of the Siberian Academy of Science built cylindrical geometry
devices that later came to be referred to as Field Asymmetric Waveform Ion Mobility Spectrometry, and a second team located in Siberia and Tashkent worked on planar designs of DMS sensors. The first public presentations of a planar DMS system were in 1987 in the USSR at 7th All-Union Conference on Low Temperature Plasma Physics, Tashkent, Uzbek Republic, and in 1991 outside of the USSR at the 8th Int. Colloquium on Plasma Process, Antibes, France.

In the mid to late 1990s, DMS/FAIMS technology started to migrate to North America; the cylindrical design to Mine Safety Appliances and the planar design to the laboratory of Prof. Gary Eiceman’s research group at New Mexico State University. The Charles Stark Draper Laboratory funded and provided technical support to build the first micro-machined planar DMS sensor with university research and development funding strategies. After demonstration of the analytical potential of DMS systems, additional efforts were focused on commercialization of this technique. A more detailed description of the DMS development is being included in an upcoming review from Nazarov et al. and prior shorter versions have been published [11, 12].

1. 8 Commercial DMS based instruments
The first commercial stand-alone DMS analyzer produced by Sionex Corporation called the SVAC unit released in 2005[13]. The SVAC unit was meant for testing and analyzing the DMS technology for several applications. Next stand-alone DMS analyzer was the Chemring Detection Systems (CDS) Juno™ handheld analyzer, which was developed in General Dynamics Corp. in 2004[14]. This was a light weight device and designed for use in detection and identification traces of chemical warfare agents (CWAs) in the field. The Varian CP-4900 analyzer was used with Micro-GC for chemical and petro-chemical processing [15, 16]. The ThermoFisher™ EGIS Defender was designed to operate with for fast detection of trace explosives[17].
Figure 3 Different Commercial Instruments based on DMS (Image compiled by Dr. Erkinjon Nazarov and reproduced with his permission)
Another launch of the DMS system was the Sionex microAnalyzer which was developed in 2007 for continuous, sensitive and precise monitoring of traces of individual volatile organic compounds (VOCs) and was targeted for many applications, including breath analysis, environmental monitoring, petrochemical and pharmaceutical analysis. Based on the same design principles, the Charles Stark Draper Laboratory built a modified version of the microAnalyzer in 2010 for NASA for continuous air quality monitoring in the International Space Station. This system has been successfully operating in the International Space Station[18, 19].

More sophisticated DMS systems such as the ones employed for the research presented in this thesis are used as prefilters in front of mass spectrometers. AB Sciex commercialized a planar DMS system in 2011 under the trade name SelexION™ Technology[20]. This is the most popular form of planar DMS technology out in the field today and is being increasingly used by researchers to solve challenging problems in analytical chemistry. Recently, Owlstone commercialized a chip based version of their micromachined DMS sensor which can be hyphenated to different mass spectrometers under the trade name UltraFAIMS[21].
To demonstrate the increasing impact of DMS in the field, a PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) search was performed on March 11th, 2014 and the results are presented in the graph below.

Figure 4 Impact of ion mobility/ differential ion mobility in the field demonstrated through PubMed search (Years 2001-2013) as performed on March 11th, 2014. Note: Publications mentioning differential ion mobility (DMS) and field asymmetric ion mobility (FAIMS) were separately searched and presented as a sum and the final numbers are subset of the total publications mentioning Ion Mobility.

1.9 Development of DMS in Vouros Lab

In 2005, the DMS was introduced in Vouros Lab at Northeastern University in collaboration with Sionex Corporation. Hyphenation of the planar DMS with mass spectrometer was done in order to evaluate its performance, develop fundamental considerations, study the feasibility, and exploit the platform for biologically relevant applications. Dr. Erkinjon Nazarov and Dr. Stephen
L. Coy have made significant contributions to the development of the DMS-MS platform here at Northeastern. Over the years, several graduate students have worked on the same in the Vouros Lab and contributed to practical applications and fundamental considerations.

Dr. Daren Levin was the first graduate student in the Vouros Lab to work on the DMS-MS platform. His research work on DMS-MS resulted in three significant publications. The DMS-MS platform was used to separate and quantitate a mixture of peptides[22]. Advantages of the DMS such as improvements to mass spectral signal-to-noise, orthogonal/complementary ion separation to mass spectrometry, enhanced ion and complexation structural analysis were realized[22]. In the next report from Levin et al., use of modifiers to enhance DMS analysis for separation of oligosaccharides was demonstrated[23]. A combination of tetrachloroethane/methanol solvent solution was successfully applied to reduce ESI oligosaccharide aggregate ion formation while enhancing the molecular ion signal[23]. In the third publication from Levin et al., gas phase interactions between five piperidine analogs as model analytes and three polar alcohols as modifiers were studied and the interactions were characterized based on strong and weak attractions between the analyte ions and gas phase modifiers[7]. Arguably the most important contribution from Dr. Levin’s work was the introduction of modifiers to aid DMS analysis and emphasis on understanding the resulting gas phase interactions to use the same advantageously for analytical applications.

Dr. Terrence Black was the next graduate student to work on the DMS platform and as a part of his research work here, he applied the DMS platform to study biomarkers of DNA damage, DNA adducts. He successfully demonstrated separation of a modified DNA nucleoside from unmodified nucleoside which setup the stage for further studies on DNA adducts.
Next graduate student, Dr. Adam Hall, applied the DMS platform for forensic analysis. Advantages of the DMS platform were demonstrated for rapid analysis of forensic drugs. A DMS-MS method for analyzing cocaine and cocaine cutting agents was published in the Journal of Forensic Sciences [9]. In another publication, Hall et al. applied the DMS-MS platform for rapid isolation and quantitation of drugs and drug metabolites[24].

In the early years of collaboration between Dr. Paul Vouros and Dr. Erkinjon Nazarov, they had conceived the original idea that DMS can be used to extend the dynamic range of mass analyzers that depend of ion accumulation and the idea was subsequently reduced to practice and was published in Journal of American Society of Mass Spectrometry in 2013[25]. Dr. Adam Hall during his research in the Vouros Lab conducted initial experiments to extend the range of ion-trap mass spectrometer using selective filtration by DMS and the same idea was explored over the course of my work which is presented in Chapter 2 of this thesis.

![Image](image1.jpg)  ![Image](image2.jpg)

**Figure 5 Left:** DMS on the AB Sciex 3000 triple quadrupole mass spectrometer and **Right:** DMS on the Thermo Finnigan ion trap mass spectrometer
1. Recent interesting applications of DMS

Early applications of DMS involved gas chromatography (GC) coupled to the DMS device [26]. DMS permitted detection of compounds not well resolved by GC and Sacks et al. used DMS coupled to a silicon microfabricated GC column to separate volatile organic compounds (VOC) [27]. DMS has been used as a GC detector to analyze chlorocarbons, ketones, alcohols, aldehydes, esters, ethers, pheromones [28] [29] [30]. Veasey and Thomas interfaced GC to DMS for fast quantification of nineteen organic species with mass fluxes ranging from 10 to 250 pg [31]. On the basis of the detection of a volatile organic compound mixture above bacterial culture, Shnayderman et al. used DMS and bioinformatics to identify the species of bacteria [32]. DMS as a portable device has been used to detect explosives and chemical warfare agent. The separation of ions from explosives by DMS using vapor modified drift gas has been performed by Eiceman et al [33].

A good review of DMS/FAIMS application was presented by Kolakowski and Mester in Analyst in the year 2007[34]. With the hyphenation to mass spectrometer, DMS applications have broadened in recent years and it has been increasingly applied for numerous applications such as separation of isobaric species, separation of isomeric species, and reduction of noise etc., prior to mass analysis. In an effort to highlight the development of planar DMS in the field of analytical chemistry, especially as a pre-filter for mass spectrometer, some relevant and interesting examples are presented. Furthermore, the examples provide a good indication of where the DMS stands in the field today and the nature of applications it is being used for.
Schneider et al. used the DMS-MS platform to separate six isobaric species (m/z→316) which is presented in Figure 6[35]. The six component mixture comprised of Oxendazole with molecular weight 315.06776, Flusilazole with molecular weight 315.10033, Pamaquin with molecular weight 315.23105, Clonazepam with molecular weight 315.715, Bromazepam with molecular weight 316.20, Chlorprothixene with molecular weight 315.861. To separate the molecular ions in the mass spectrum would require a mass spectrometer with a high resolution. Mass spectrometers alone with low resolution (such as the ones used for the study in this thesis) would not be able to resolve them. However, using DMS as the prefilter before mass analysis...
allowed separation of the six isobaric species. Increasing the concentration of the modifier afforded bigger shifts and better separations.

![Diagram of 4-aminobenzoic acid and its protonation sites](image)

**Figure 7:** Separation of amine nitrogen protonated and carboxylic acid oxygen protonated 4-aminobenzoic acid by planar DMS. Reprinted with permission from [36]

Although it is well known that the proton sits at the most basic site of the molecule during electrospray, it is hard to elucidate the exact site of protonation. In a recent publication, Campbell et al. have reported separation based on the protonation sites of 4-aminobenzoic acid molecule. Two other isomers of the aminobenzoic molecule: 2-aminobenzoic acid and 3-aminobenzoic acid did not produce multiple peaks. However, the analyte 4-aminobenzoic acid produced two different CoV peaks and it was concluded that the two distinct peaks were a result of protonation on the amine nitrogen (N-protonated) or the carboxylic acid oxygen (O-protonated)[36]. The presence of two protonation sites was further confirmed by MS/MS and hydrogen-deuterium exchange experiments.
In another application, two isomeric glucuronide metabolites of Propranolol were separated by DMS from a liver tissue dosing experiment[37]. Separation of isomeric glucuronide metabolites after dosing experiments represents applicability of the DMS to biological systems.

Structures of Propranolol, 4-Hydroxypropranolol aromatic O-glucuronide (PRO-O-ARG) and 4-Hydroxypropranolol aliphatic O-glucuronide (PRO-O-ALG) are presented in Figure 8(ii). The mass transitions for MS/MS experiments are also given. When no modifier was added to the DMS transport gas, the two isomeric species 4-Hydroxypropranolol aromatic O-glucuronide (PRO-O-ARG) and 4-Hydroxypropranolol aliphatic O-glucuronide (PRO-O-ALG) could not be separated just by the application of separation voltage (Figure 8(i)(A)). When acetonitrile was added to the transport gas as a modifier, the two isomeric metabolites were well resolved as confirmed by the MS/MS peaks (Figure 8(i)(B)) [37].
Figure 8 Analysis of Propranolol, 4-Hydroxypropranolol aromatic O-glucuronide (PRO-O-ARG) and 4-Hydroxypropranolol aliphatic O-glucuronide (PRO-O-ALG) from a dosed liver tissue extract by DMS-MS (A) without Modifier (B) with acetonitrile as the modifier.

(ii) Structures of Propranolol, 4-Hydroxypropranolol aromatic O-glucuronide (PRO-O-ARG) and 4-Hydroxypropranolol aliphatic O-glucuronide (PRO-O-ALG).

Reprinted with permission from [37]
In the field of DMS-MS, there is continued interest in search for suitable modifiers and understanding of the modifier interactions. Blagojevic et al. have studied modifier and field effects using isomeric dipeptides as model system[38]. Two pairs of dipeptides [glycine-alanine (GA) and alanine-glycine (AG), glycine-serine (GS) and serine-glycine (SG)] and eight different modifiers (water, 2-propanol, 1,5-hexadiene, 2-chloropropane, chlorobenzene, dichloromethane, acetonitrile, and cyclohexane) were used in the study.

![Figure 9 Separation of isomeric dipeptides: glycine-alanine (GA) and alanine-glycine (AG), glycine-serine (GS) and serine-glycine (SG) with different modifiers. Reprinted with permission from [38]](image-url)
Separation and quantitation of peptides using a mixture of peptides was demonstrated by Levin et al. in 2006 [22]. Due to increasing interest of rapid peptide analysis, recently the DMS-MS is being applied to biologically relevant matrices for peptide analysis. In a 2014 JASMS paper by Blagojevic et al.[39], multicomponent modifier mixture along with a arcing suppressant has been applied to separate peptide mixture obtained after tryptic Bovine serum albumin (BSA) digestion(Figure 10).

Figure 10 Separation of peptide mixture obtained from a tryptic digest of BSA performed with two component modifier mixture (2.0% methanol and 0.13% hexanol). Top panel was obtained at SV 4000 volts without arcing suppressant and 0.05 % chloroform was added as the arcing suppressant in the bottom panel obtained at SV 4250 volts. Reprinted with permission from [39]
In the applications demonstrated above and used throughout this thesis, nitrogen has been used as the transport gas and modifiers were introduced at a few percent into the transport gas. Shvartsburg et al. have studied the role of transport gas on separations using mixture of helium (He) and nitrogen (N$_2$) at various composition [40]. In a 2014 JASMS publication[41], up to 100% helium has been used as the transport gas to enhance peak capacity.

Figure 11 DMS separation for three different charge states (2+, 3+, and 4+) of Syntide 2, measured using a mixture of He/N$_2$ as transport gas (% He is labeled). The w (full width at half maximum (FWHM)) can be used to compare peak shapes. Reprinted with permission from [41]
Three different charge states (2+, 3+ and 4+) of the peptide Syntide 2 (S2, PLARTLSVAGLPKK, 1508 Da) were separated by using a combination of He and N₂ mixture as the transport gas[41]. Use of high percentage of He gas (>70%) resulted in better separation of the 3+ and 4+ charge states of the peptide Syntide 2 as shown in Figure 11.

In recent years, the DMS-MS platform is being increasingly applied to biological matrices. Leading researchers in the field are applying this platform to challenging analytical applications as demonstrated by the examples presented above. More importantly, the applications presented highlight the continued interest in optimizing gas phase interactions for optimal DMS-MS performance.

1. 11 Green Chemistry
To reduce the impact on the environment, the field of analytical chemistry is looking for alternate greener methods. Not only there is an interest in treating the waste generated during the analysis, reduction of solvent waste during analytical processes has also been established as an important consideration in the Green Chemistry outlines presented by ACS (American Chemical Society)[42] [43]. Several studies have introduced the potential of DMS to conduct “greener” analysis [44, 45], however, the reduction of solvent waste with the DMS-MS platform is not often highlighted.

Throughout the work presented in this thesis, use of nanoflow for analysis has not only reduced the sample requirements, solvent requirement for analysis and the waste generated has been greatly reduced. Samples were reconstituted in ≤100 ul mobile phase per analysis point. Modifiers were introduced at the rate of 10-30 ul/min. Since the modifiers were converted into gas phase in the DMS cell and subsequently transmitted to the mass spectrometer, no physical
solvent waste was generated from the modifier usage. Use of electric fields in DMS as opposed to interaction between solvents and analytes used for isocratic or gradient run in LC eliminated the waste produced for separations. It is important to highlight that in the context of waste reduction or elimination, DMS-MS holds great potential for being preferred analytical “greener” method for analysis.

1.12 Rapid analysis eliminating sample clean-up steps using nanoESI-DMS-MS

The DMS-MS platform can be exploited to perform rapid analyses without the need for lengthy sample preparation/ chromatography steps for selected applications. In addition to its ion separation capabilities, DMS has also been demonstrated to be capable of reducing noise and enhancing sensitivity by allowing pre-filtration of ion species prior to mass analysis[25, 46]. Since DMS is used post-electrospray, DMS does not directly address matrix effects/ ion suppression issues and they still remain a concern. Flowing in the nanoflow regime not only reduces sample consumption, it greatly improves ionization efficiency hence mitigating the issue of matrix effects and ion suppression. Schmidt et al. have documented that at flow rates <20 nl/min, analyte suppression is practically absent[47]. A nanosplitter device was invented and used by Gangl et al. to reduce signal suppression effects[48]. The work presented here has been performed in the nanoflow (<400nl/min) regime comparable to the flowrates employed by Gangl et al., With an automated or highly efficient sample introduction/ electrospray setup, the flowrate can be further reduced to minimize matrix/ ion suppression effects and additional advantages can be taken of the DMS-MS platform.

In an effort to further demonstrate the qualitative and quantitative capabilities of DMS-MS and the advantages that it offers as a stand-alone platform, we present two distinct applications here: the first involves rapid separation and qualitative analysis of harmine/ harmaline from the seeds
of *Peganum harmala* and the second involves rapid quantitation of cyclosporin A in transport media eliminating the necessity for sample clean-up steps.

**Instrumentation**

*DMS-Ion Trap Mass Spectrometer:*

A planar DMS developed by Sionex Corporation with a filter gap 0.5mm high x 3.0mm wide x 10.0mm long was used for the work. The device was seated at the front end of the heated capillary of a Thermo-Finnigan, LCQ Classic mass spectrometer. Sionex Expert software was used to set the DMS parameters. The SV could be set at zero or set or scanned in the range from 500 V to 1500 V and the CoV could be set or scanned from -43 to +15 volts. Electrospray was performed using coated 10 µm PicoTip emitter from New Objective (Woburn, MA). Samples were introduced using a Harvard Apparatus syringe pump (Holliston, MA). The desolvation gas (ultra high purity nitrogen) was introduced at a flow rate of approximately 100 cc/min into the desolvation region at a temperature of 100°C. The vacuum drag of the mass spectrometer was measured to be 600 cc/min. External air flow of approximately 500 cc/min also merged in with the desolvation gas into the DMS with the bulk gas temperature at approximately 45°C. Modifiers were introduced into the desolvation region along with the nitrogen gas at a concentration of approximately at 1.5%. The electrospray was held at 2 kV throughout the analysis.

*DMS-Triple Quadrupole Mass Spectrometer:*

A prototype DMS- API 3000 Triple Quadrupole mass spectrometer (AB SCIEX, Concord, Ontario, Canada) which has the removable DMS filter online in front of the first quadrupole was
used for this study. The dimensions of the DMS were 1 mm x 10 mm x 15 mm. A modified version of the software Analyst version 1.5 which included parameters for SV and CoV was used. The SV could be varied from 0 to 5000 volts and the CoV could be varied from -100 to +100 volts. Electrospray was done using stainless steel 30 µm id emitter from Proxeon (Odense, Denmark). Samples were introduced at the rate of 400 nl/min using a Harvard Apparatus syringe pump. Electrospray was held constant at 3500 volts throughout the analysis. Isopronanol at 1.1% was introduced into the curtain gas (500 cc/min outflow at 40°C) using a second Harvard Apparatus syringe pump.

**Extraction of Harmine and Harmaline from the seeds of *Peganum harmala* using PCT**

Prior to evaluating the appropriate conditions for their rapid identification and analysis of the two harmala alkaloids, harmine and harmaline, must be first extracted from the seeds of *Peganum harmala*. In an effort to demonstrate a fast and efficient extraction method, harmine and harmaline were extracted from the seeds using pressure cycling technology (PCT) Barocycler NEP 2320 instrument from Pressure BioSciences Inc. The extraction method was adapted from a report by Kartal *et al.* which requires larger amount of starting sample and long macerations and liquid-liquid extraction time[49].

In order to evaluate the feasibility of PCT, a crushed single dried seed (average mass 3 mg) of *Peganum harmala* in 100 µL of methanol was run on the pressure cycler at 35,000 psi for 20 cycles with 20 seconds of high pressure and 20 seconds at ambient pressure for a total run time of approximately 20 minutes. The extracts were dissolved in 1 ml of 2% HCl and extracted twice with 2 ml of petroleum ether and four times with 2 ml of chloroform. The extraction proved to be a fast and efficient method. The PCT protocol resulted in an average extract yield of 0.3 mg, approximately 10% by weight.
1. 13 Results and Discussion

The projects reported here were initially scheduled to be performed by LC-MS. However, based on prior successful applications, the DMS-MS platform was applied in parallel to the LC-MS method to explore the feasibility of the DMS-MS approach. This work presents the potential of DMS-MS to be applied in matrices of varying complexities offering time and resource savings by reducing or eliminating sample clean-up steps and speeding up the overall analysis time demonstrated here through a qualitative application followed by another quantitative application.

The approaches taken for the two projects presented in this chapter is summarized in Figure 12.

**Figure 12:** Workflow for targeting analytes of interest (qualitative analysis of harmine & harmaline and quantitative analysis of cyclosporin A) using nanoESI-DMS-MS platform. It is worth noting that sample clean-up steps were eliminated for relatively complex matrices and samples were simply prepared for analysis by dilution in appropriate mobile phase.
1. 14 Rapid Identification of β-carboline Hallucinogens by Pressure Assisted Extraction (PCT) and DMS-MS

*Peganum harmala*, commonly known as African or Syrian rue, is a plant that contains several monoamine oxidase inhibiting alkaloids, including harmine and harmaline, which belong to a group of compounds with similar structures known as harmala alkaloids and β-carbolines. These alkaloids are hallucinogens, central nervous system stimulants, and serotonin antagonists. Consumption of these alkaloids may elicit these effects. Although the alkaloids found in the seeds of *Peganum harmala* are not yet regulated on the state or federal level, there have been numerous cases of its consumption in order to elicit the hallucinogenic properties. It is therefore imperative to develop robust methods for the identification and analysis of *Peganum harmala* alkaloids, in particular harmine and harmaline.

Currently several analytical methods with various sensitivity and selectivity have been utilized for the analysis of these two alkaloids. Such methods include HPLC in combination with UV[50], chemiluminescence[51], fluorometry[52], MS[53], and GC-MS[54]. Although GC offers many advantages, it often requires chemical derivatization to improve detection and separation of non-volatile, polar or thermally labile compounds, which results in increased sample preparation time and sometime results in unwanted modifications too. HPLC in combination with MS and fluorescence detection are the most often employed methods because of good selectivity and sensitivity[53, 54]. Despite their effectiveness, chromatographic separations remain time-consuming and there is an ongoing interest in higher throughput, and more efficient technologies for larger-scale analysis of drugs. With the aim of reducing sample preparation, analysis time and sample consumption, particularly for these two emerging drugs, the DMS-MS method has been applied for the same.
As previously mentioned, a key feature in DMS that improves ion selectivity and separation involves the use of transport-gas modifiers. The selection of an appropriate modifier for targeted analytes is a key component in DMS-based separations. In the present case, harmine and harmaline differ by one level of saturation in the pyridine ring fused to the indole ring of the β-carboline (structures presented in Figure 14). Commonly used modifiers: acetone, isopropanol, and ethyl acetate were evaluated in the separation of harmine and harmaline at a constant SV value of 1500 volts on the ion trap mass spectrometer. Without any modifier (Figure 13A), the peaks for both harmine and harmaline overlapped and no shift from a CoV value of zero was observed. Isopropanol, a widely used modifier in DMS-based separations, proved to be ineffective in shifting harmine and harmaline away from the CoV value of zero (Figure 13B). Next, acetone was introduced into the nitrogen gas as modifier and it was able to shift both harmine and harmaline to approximately -9 volts (Figure 13C), however, both peaks overlap for the most part resulting in ineffective separations. Lastly, ethyl acetate, which has been an effective modifier in prior studies [9, 55], proved to be effective in the separation of harmine and harmaline (Figure 13D). Although baseline separation was not obtained, the apex of the two peaks are well resolved which allows setting the DMS parameters in stepping mode for further analysis.
Figure 13: Effect of modifiers on the separation of harmine and harmaline at a constant SV of 1500 volts. A) No modifier B) Isopropanol C) Acetone D) Ethyl acetate. Only acetone and ethyl acetate provide CoV shifts away from the zero value and the latter affords separation.
Ethyl acetate was established as the modifier of choice for the analysis of harmine and harmaline. The PCT extract was diluted 1:100 in mobile phase (70% MeOH/30% H₂O/0.1% CH₂O₂ v/v/v) and infused at the rate of 400 nL/min. PCT extracts were analyzed in both DMS-transparent and DMS-On mode (Figure 14). There is no ion filtration in the DMS-transparent mode because the SV and CoV values are set to zero. Selective filtration can be achieved in the DMS-On mode by using an appropriate combination of SV and CoV values in presence of a suitable modifier. From Figure 14A, it can be seen in the DMS-transparent mode that there are residual matrix ions in addition to the harmine precursor ion (m/z 213) and harmaline precursor ion (m/z 215) in the mass spectrum. When the DMS was subsequently turned on with ethyl acetate as the modifier, harmine and harmaline could be selectively filtered and introduced into the ion trap mass spectrometer with a significant reduction in background noise (Figure 14). Although the two compounds by DMS-MS were not baseline resolved by DMS-MS (Figure 13D), selection of the apex of each individual peak resulted in a clean mass spectrum with only the target analyte signal. The identifications of harmine and harmaline were confirmed by MS-MS experiments relying on transitions from 213 to 198 for harmine and from 215 to 200 & 174 for harmaline respectively as previously reported [56, 57].
Figure 14: Mass spectrum showing separation and rapid qualitative analysis of harmine and harmaline using DMS-MS. A) DMS transparent B) DMS-on at fixed CoV of -8.0 volts showing selective introduction of harmine C) DMS-on at fixed CoV of -10.0 volts showing selective introduction of harmaline. Identities were further confirmed by MS-MS data.

Traces of LC and GC chromatograms are presented in Figure 15 for comparison. The GC work was done at Boston University under the supervision of Dr. Adam Hall and the LC work was done under the supervision of Dr. James Glick at Northeastern University. Per analysis point, the GC method required a total analysis time of 17 minutes and the LC required a total analysis time of 12 minutes; whereas the DMS required a total analysis time of 1 minute as presented in Table 1. With elimination of sample preparation steps and fast analysis time, the DMS-MS can be successfully applied to analysis of seed extracts.
Figure 15 Comparison of (A) Gas Chromatography (GC) and (B) Liquid Chromatography (LC) traces for the analysis of harmine and harmaline. GC work was performed under supervision of Dr. Adam Hall at Boston University and LC work was performed under supervision of Dr. James Glick at Northeastern University. Images reproduced with permission from Dr. Adam Hall and Dr. James Glick respectively.

<table>
<thead>
<tr>
<th>Method of Analysis</th>
<th>Time per sample point</th>
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<tbody>
<tr>
<td><strong>GC</strong></td>
<td>17 minutes</td>
</tr>
<tr>
<td><strong>LC</strong></td>
<td>12 minutes</td>
</tr>
<tr>
<td><strong>DMS</strong></td>
<td>1 minute</td>
</tr>
</tbody>
</table>

Table 1: Comparison of time scales for separation of harmine and harmaline by conventionally used GC, LC and emerging DMS technology. With analysis time of 1 minute or less, the DMS can be successfully applied for analytical applications.
1. 15 Quantitation of an immunosuppressant in transport media by DMS-MS/MS

Studying the permeability of different types of molecules is an important consideration in optimizing the drug delivery and evaluating the uptake of molecules by various cells. Caco-2 cells (human colon epithelial cancer cell line) are widely used as a model system for studying intestinal absorption of drugs and other compounds [58] and it has also been demonstrated that Caco-2 cells in transport media under culture conditions can be used to predict *in vivo* human absorption[59]. Hence, *in-vitro* studies of drug candidates in Caco-2 cells have become standard in early phases of drug development to help streamline the selection of drug candidates[60].

Analysis of permeability studies for drug candidates comes with significant analytical challenges. The compound usually has to be detected at a low concentration in a matrix of the transport media which is a mixture of salts like HBSS buffer supplemented with HEPES and glucose[59]. Furthermore, permeability is usually measured as a function of time which makes the throughput of the analytical technique important. Liquid Chromatography with Ultra-Violet detector (LC-UV) has been widely used in permeability studies; however the method largely depends on measurement of the absorbance of the analyte in the UV region. To overcome some of the detection issues using LC-UV, radioactive conjugates of the candidate molecules have been used in the permeability studies to enable detection [61] but radiolabelling adds additional costs and necessitates special safety procedures to work with during the synthesis, study and disposal. While LC-MS has established itself as the preferred detection method for the un-conjugated molecule in the complex buffer based system and has addressed the analysis requirements for these kinds of studies, a rapid method eliminating sample clean-up steps prior to analysis could prove highly beneficial.
Our focus has been to investigate the transport and permeability of Cyclosporin A, an immunosuppressive molecule, in an in-vitro model. Prior method development efforts in our laboratory using traditional LC-MS for the quantitation of cyclosporin A in media proved to be labor intensive. A SPE method or online clean-up was necessary prior to LC-MS analysis in order to produce a linear calibration curve. Using DMS, Schneider et al. have reported reduced contamination on the mass spectrometer’s interface with an optimized curtain and transport gas flow configuration after >2000 injections of undiluted HBSS (Hank’s Buffer salt solution) and reserpine samples. CoV reproducibility within ±0.2 volts was also demonstrated in the same study[46]. Hence, it was logical to try nESI-DMS-MS/MS platform for rapid quantitative analysis. With the goal of performing rapid quantitation of Cyclosporin A in the transport media without any sample clean-up steps, we have applied the differential mobility- mass spectrometry platform.

Prior to quantitation, the analyte and internal standard in transport media were analyzed in single quadrupole mode. Since, the parent ion of Cyclosporin A is at a higher mass, [M+NH₄]⁺ → 1220, than typical small molecules encountered during mass analysis, the spectrum was not dominated by matrix ions in the analyzed mass window (m/z 1200-1250). However, the background noise seemed to be high in the DMS-transparent mode. To determine if the DMS affords any benefit in this situation, the DMS-on mode was used. Not only does the background noise go down, the selective filtration by the use of DMS results in increased signal intensities as well (Figure 16).
For quantitation purposes, the transitions of the analyte, cyclosporin A 1220 $\rightarrow$ 1203 and internal standard (cyclosporin A-d4) 1224 $\rightarrow$ 1207 were monitored at unit resolution in the MRM mode. Isopropanol was used as the modifier of choice. The samples in media were diluted 1:20 in mobile phase [80:20 Methanol: Ammonium Acetate (40mM)] and infused at the rate of 400 nl/min. The ratios of the intensities of the products ions of the analyte and internal standard were recorded and a seven point calibration curve was constructed with each point analyzed in triplicate. Each sample was analyzed in thirty seconds producing excellent linearity over the range of 100 pg/ul- 10,000 pg/ul which is demonstrated in Figure 17. The data was processed using Microsoft Excel. A linear regression analysis was done in MS Excel through the seven points to get a $R^2$ value of 0.9994. To verify the linearity at the bottom of the calibration curve, a linear regression analysis was performed on the first three points (figure inset) which gave us a $R^2$ value of 0.9989, very much consistent with the whole calibration curve (Figure 17).
Figure 17: Calibration curve of Cyclosporin A in transport media. The error bars represent standard deviations of triplicate analyses and the inset shows a fit to the lowest part of the data.

1.16 Conclusion

In Chapter 1, an introduction of ion mobility and differential ion mobility is presented and the presently accepted theory of separation for differential ion mobility is described. A brief history on the development of DMS in the field and contributions to the development of DMS-MS in Vouros Lab at Northeastern University is included. Recent relevant examples of the DMS-MS are reviewed to compare where the application of this technology lies today and to emphasize the need to better understand modifier interactions in order to exploit the same for further analytical applications. Finally, a nanoESI-DMS-MS setup was employed for rapid qualitative analysis of
harmine & harmaline from *Peganum harmala* seed extracts and quantitative analysis of cyclosporin A in transport media. It has to be noted that sample clean-up steps were not necessitated and samples were simply analyzed by diluting in the appropriate mobile phase. The approach taken for analysis by using the CoV stepping mode (i.e. using the CoV apex value for analysis) to truly appreciate the rapidity of this method has been presented.
Chapter 2: Improving the Ion Trap’s qualitative and quantitative performance using Differential Mobility (DMS) as a prefilter
2. 1 Introduction

Ion traps are widely used mass spectrometers for MS, MS\(^2\) and MS\(^n\) experiments. The trap has a maximum storage capacity; overloading the trap affects performance leading to poor mass resolution, spectral quality and MS-MS capabilities as well. To mitigate the problems arising from overloading of the trap, an automatic gain control (AGC) is imposed which checks the ion population entering the trap and prevents overloading of the trap. When complex matrices are involved, the ion population going into the trap is heterogeneous including many unwanted ion components of the matrix and the AGC function cannot discriminate within the ion population, but simply controls the ion population going into the trap. In this scenario, a prefilter capable of selectively filling the trap with the desired ion population can prove highly beneficial. DMS has emerged as a powerful tool for ion filtration and in combination with an ion trap, it can increase the storage capacity of the trap by filtering out the unwanted matrix ions and filling the trap selectively with the analyze(s) of interest. Once the optimal parameters (Separation Voltage, Compensation Voltage, Modifier of Choice) have been identified, the DMS-MS platform can be exploited to perform rapid analysis without the need for lengthy chromatography steps.
Figure 18: Graphical representation of DMS filtration when used with ion-trap mass spectrometer. Top: When there is no DMS filtration, the trap is filled with variety of ions including unwanted ions generated during the electrospray process. Bottom: With DMS filtration in line, the ion trap can be selectively filled with the desired ion population.
2.2 Ion Trap: Working Principles

Developed by Nobel Prize winner, Wolfgang Paul, the trap consists of three hyperbolic electrodes: the ring electrode and the entrance and exit end-cap electrodes. RF voltages are applied to the ring electrode and end caps are held at ground potential. In an ion trap mass spectrometer, ions are contained within the trap by the application of appropriate RF voltages and are sequentially ejected based on mass selection. The Mathieu stability zone for quadrupole ion trap is presented in Figure 19. Mass spectra are generated by creating ion instability by mass selective ejection. Amplitude of the applied RF is increased so the ions move along the $q_z$ axis until they become unstable at boundary ($q_z = 0.908$). The ejected ions pass through a hole in the end cap electrode and reach a detector.

Figure 19: Depiction of regions of stability in the quadrupole ion trap[62]. Ions are stable in both $z$ and $r$ direction as long as their Mathieu parameters $a_z$ and $q_z$ fall within the stability zone.
2. 3 Space Charges

Ion traps have a limited storage capacity. The accumulation of high concentration of ions can have negative effects on resolution, sensitivity, mass accuracy and dynamic range because of development of space charges. The publication by Schwartz et al. on ion trap mass spectrometer is one of the most comprehensive articles on space charges [63]. They define the following three kinds of space charges in the article:

i) Activation Space Charge Limit—“the maximum number of ions which can be stored while maintaining the ability to activate ions with a specified fragmentation efficiency”

ii) Isolation Space Charge Limit—“the maximum number of ions which can be stored while maintaining the ability to isolate ions with a specified resolution and efficiency”

iii) Spectral Space Charge Limit—“the maximum number of ions which can be stored while maintaining the ability to obtain a mass spectrum of some specified resolution and mass accuracy”

Schwartz et al. further underscore that each space charge limit relates to a different ion capacity and the relative order of the different limits is:

Storage Limit> Activation Limit> Isolation Limit> Spectral Limit

The spectral limit is the most critical parameter in the trap to obtain a mass spectrum with desired resolution and mass accuracy. In practice, the spectral limit can be several orders of magnitude lower than the storage limit.
2. Automatic Gain Control (AGC) function

AGC is a patented technology that is specific to Thermo Scientific Ion Trap Instruments and is designed to optimize the number of ions inside the trap for mass accuracy and sensitivity. Major steps during the analytical cycle of the trap are demonstrated in Figure 20.

Figure 20: Depiction of analytical cycle of ion trap mass spectrometer[64]

The four main steps consist of:

1. AGC
2. Ion Injection
3. Isolation and activation of the parent ion within the trap
4. Scanning the ions out of the trap

While the rate determining step is the ion injection step, in normal operation mode, the AGC function precedes the ion injection and it determines the amount of time trap will remain open for the injection of ions. For ion population occurring at low concentration, the AGC sets a
longer injection time to allow accumulation of the target ion species and for abundant ions, it sets a shorter injection time to prevent saturation of the trap.

As mentioned above, the AGC function controls the number of ions inside the trap but does not discriminate within the ion population going to the trap. In the case of samples extracted from biological matrices, and when liquid chromatography is bypassed as in the case of DMS we are employing, the nature of ion population going into the trap is very heterogeneous and complex. In such a scenario, selective filtering of the ion species going into the trap using DMS could be highly beneficial and DMS-MS can be used as a rapid stand-alone platform for analytical purposes without the need for lengthy chromatography times.

2. 5 Use of DMS as a prefilter

Differential mobility spectrometry is an ambient ion separation/ filtration technique which is a post-electrospray continuous method. A stable electrospray is needed for the DMS to carry out its functions. When urine samples were analyzed right after dilution in mobile phase, a stable electrospray profile could not be achieved, and therefore the benzoylecgonine (BE) analyte samples were extracted from urine by SPE. BE is the primary metabolite of Cocaine, and its analysis from a biological fluid (urine) well represents typical challenges faced in many routine mass spectrometric applications. When the samples were introduced into the mass spectrometer for analysis, components from the matrix dominated the spectra when the DMS was uninstalled or was in the transparent mode. With DMS-on mode, the analyte of interest BE could be selectively introduced into the trap while removing the matrix ion components.

The DMS was evaluated in terms of its ability to deal with complex mixtures in order to improve the performance of an ion trap for qualitative and quantitative analysis. One of the benefits of using the planar configuration of DMS is that it allows operation of the MS in the DMS-
transparent mode, i.e. separation voltage and compensation voltage are not applied to the DMS. The DMS transparent mode affords a user the convenience to look at the total ion profile of the sample under investigation without having to remove the DMS setup. However, due to inevitable ion losses in the DMS filter gap and the DMS/MS interface, a reduction in mass spectral signal profile is expected. Under the DMS-MS setup we have employed, signal losses of as much as 85% were observed when the DMS was operated in the transparent mode. In an attempt to evaluate the true performance of the DMS – MS system and the efficacy of ion filtration by DMS prior to mass analysis, two DMS operational modes were evaluated first: (i) DMS-uninstalled mode- where the DMS setup is completely removed and the ions are directly sprayed into the heated capillary of the mass spectrometer and (ii) DMS-on mode- where the DMS is placed in front of the heated capillary of the mass spectrometer and analytes of interest are selectively introduced into the mass spectrometer by using a predetermined combination of separation voltage and compensation voltage along with a modifier of choice.

Using DMS as a prefilter, a series of experiments were done using the AGC-on feature and varying the trap fill times. Considerations were given to signal intensities and resolution. Without the DMS filter, the trap can be saturated by matrix ions building up space-charge effects which in turn impacts spectral quality and precursor ion isolation for further MS/MS analysis. In DMS-on mode, the prefiltering allowed the use of an extended fill time without any deleterious effect on the mass spectrum. For quantitative analysis, the use of DMS allowed lower detection level when compared to DMS-transparent mode and/or DMS-uninstalled mode and improved the linearity of the calibration curve over the whole concentration range analyzed as well. The quantitation method bypasses traditional chromatography and a calibration curve was prepared with DMS-on in under 3 hours.
2. 6 Experimental

A planar design of DMS developed by Sionex Corporation interfaced with Thermo-Finnigan LCQ classic is used. Alternating RF voltage (separation voltage, SV) is applied across the ion transport channel in the DMS which is perpendicular to the direction of the transport gas flow. Ions will leave the flight path and migrate toward the walls unless their trajectory is corrected by a counterbalancing voltage (compensation voltage, CoV). The samples were introduced into the mass spectrometer by infusion at the rate of 300 nl/min using a Harvard Apparatus syringe pump. The spray voltage was held constant throughout the analysis at 2 kV. Ethyl acetate was used as the modifier and was introduced into the DMS along with the nitrogen gas. The separation voltage was set at 1500 V and the compensation voltage was also fixed when the DMS was being used. Benzoylecgonine (BE), a metabolite of cocaine, was spiked and extracted from urine by using commercially available SPE cartridges.

Solid Phase Extraction (SPE) and calibration curve:

SPE was performed on 130mg Clean Screen Xcel I Column from UCT. The column was preconditioned with 2 ml of Methanol. Each sample was mixed with 1 ml of pH 6.0 phosphate buffer and loaded onto the column. The column was washed with 1 mL of 2% acetic acid/ 98% methanol. Finally the samples were eluted with 1 mL Methylene Chloride/ Iso-propanol/ Ammonium Hydroxide (78/20/2%). The samples were then dried down under vacuum for 1 hr and capped and stored at 4°C. For analysis, the samples were reconstituted in 200 ul mobile phase (70% Methanol: 30% Water: 0.1% FA)

The stock concentration of the analyte BE was 1 mg/ml and the concentration of the stock internal standard BE-d3 was 100 ug/ml. Eight different samples with final concentrations of BE at 25 ng/ul, 10 ng/ul, 7.5 ng/ul, 5ng/ul, 2.5 ng/ul, 1ng/ul, 0.5 ng/ul and 0.1 ng/ul were prepared in
1 ml of synthetic urine. Internal standard BE-d3 was spiked into each of the samples to get a final concentration of 0.5 ng/ul or 1 ng/ul.

RESULTS AND DISCUSSIONS:

2. 7 Qualitative Comparison: DMS-on and DMS-uninstalled
The capacity of DMS to prefilter ions species prior to mass analysis has been well established. In conjunction with that, we first evaluated the ability of DMS to clean up matrix ions from a biological matrix. Since the quantification of drug metabolites is a frequently encountered application in many DMPK studies, it was deemed of interest to consider the utility of the DMS to the analysis of Benzoylecgonine (BE), also known as 3-Benzoyloxy-8-methyl-8-azabicyclo octane-4-carboxylic acid, the primary metabolite of cocaine abuse in urine. BE at 0.5 ng/ul was spiked and extracted from urine according to the procedures mentioned above. The results are presented in Figure 21 below.
Figure 21 Utility of DMS in cleaning up matrix ion during analysis of BE in urine. (a) mass spectra generated from blank with DMS-uninstalled (b) mass spectra of a 0.5 ng/ul BE sample with DMS-on

As expected, the mass spectrum of blank matrix looks noisy with DMS-uninstalled [Figure 21 (a)]. When the separation voltage was set to 1500 volts and the compensation voltage was set to -16 volts with ethyl acetate introduced into the nitrogen transport gas as modifier, referred to as DMS-on mode, the ion filtration utility of DMS to remove matrix ions is evident by the clear mass spectrum as demonstrated by Figure 21(b). Under the same DMS on conditions, when 0.5 ng/ul BE sample is introduced, we can see that the DMS is able to selectively introduce the ion of interest into the mass spectrometer while filtering unwanted matrix ion components. On the operational side of the mass analyzer, space charge effects can limit the maximum number of ions that can be stored in the system so an automatic gain control (AGC) is imposed.
which provides for space charge minimization and optimal conditions for spectral acquisition.

The AGC-on settings used for the experiments on this instrument were: Full MS Target $\rightarrow 5\times10^7$ and SIM Target $\rightarrow 2\times10^7$ counts respectively. For acquiring the data in Figure 21, AGC-on feature on the mass spectrometer was utilized. It was expected that under DMS filtration, because of removal of matrix ions and selective introduction of the analyte of interest, the time the trap remains open under the AGC-on setting would be affected. Thus, the fill times with DMS- uninstalled and with DMS-on were noted during the experiment. It was observed that the trap remained open for 115 milliseconds for both conditions when the AGC was on. To evaluate whether DMS filtration improves the trap capacity for the storage of targeted ions of interest at higher trap fill times, further experiments were conducted by varying the fill times of the trap using the AGC-off feature. It was quickly realized that trap fill times in excess of 1000 milliseconds had an adverse effect on the resolution of M and M+1 ions of BE (Figure shown later). With resolution consideration, trap fill time of 500 milliseconds was set with AGC turned off for further evaluation of the utility of DMS. In an effort to quantify the ion signal gains/losses using DMS, data was acquired under four different conditions: (i) DMS-uninstalled AGC-on, (ii) DMS-on AGC-on, (iii) DMS-uninstalled AGC-off 500 msec fill time, and (iv) DMS-on AGC-off 500 msec fill time. The emitter tip position was adjusted for maximum signal intensity in the m/z 270- 300 range when the data was acquired.
2. 8 Analysis of a dilute sample with DMS

A 0.1 ng/ul BE was infused into the mass spectrometer and data was acquired under the four different conditions mentioned above. In the absence of DMS filtration in both AGC-on (Figure 22a) and AGC-off 500 (Figure 22c), very noisy spectra were obtained. When the DMS was turned on, the analyte of interest BE could be selectively introduced into the trap and detected. As expected, the signal intensity at the higher fill time AGC-off 500 is larger than with AGC-on.

![Figure 22: Analysis of a dilute sample (0.1 ng/ul BE) with DMS filtration and comparison with DMS uninstalled spectra when the tip position was optimized for maximum ion signal intensity in the m/z 270-300 range.](image)

A particular concern for quantifying the signal gains/losses using DMS was the presence of a prominent matrix ion at m/z 289 which can be seen in the Figure 22a. Due to indiscriminate introduction of matrix ions, we can clearly see from Figure 22a and c that the trap has extremely poor resolution. We suspect that the same matrix ion was contributing to the maximum peak intensity in that region. However, with the DMS on, the noise is removed and the
analyte BE can be detected even with just a few hundred counts signal intensity when the AGC is on (Figure 22b). Varying the trap fill time to a higher 500 msec increased the signal intensity by a factor of 50 to 3.93E+04 with the DMS on and both the M and M+1 peaks can be observed (Figure 22d).

2.9 Analysis of a concentrated sample with DMS
Under the same four conditions utilized, data was acquired for a 5 ng/ul BE sample. The trap exhibited poor resolution when the DMS was uninstalled under both AGC-on (Figure 23 (i)a) and AGC-off 500 msec fill time (Figure 23(i)c). However, this concentrated sample had a prominent signal and was assumed to be the primary contributor to the peak intensity in that region, dwarfing out the contributions by the matrix ions interferences which allowed us to do a direct comparison between the DMS on and DMS uninstalled signal intensities quantifying gains/losses. As anticipated, with DMS on in both AGC-on (Figure 23 (i)b) and AGC-off 500 (Figure 23 (i)d) modes, the analyte of interest BE was selectively introduced into the trap with acceptable resolution between the M and M+1 peaks. In the AGC-on mode (Figure 23 (ii)a), based on the highest peak intensities observed in that range, about 75% of the ions were lost in the DMS-on mode when compared to the DMS-uninstalled mode. However, when the trap was opened for an extended fill time of 500 msec, the selective DMS filtration resulted in a signal gain of about 20% when compared to the DMS-uninstalled mode (Figure 23 (ii)b).
Figure 23: (i) Analysis of a concentrated sample (5.0 ng/ul BE) with DMS filtration and comparison with DMS uninstalled spectra when the tip position was optimized for maximum ion signal intensity in the m/z 270-300 range (ii) quantitative comparison of the signal gains/losses by DMS filtration at two different fill times (a) AGC feature turned on (b) AGC feature turned off and trap set to a fill time of 500 ms

It has been proposed that there are four primary space charge limits associated with an ion trap, namely, storage space, activation space, isolation space and spectral space charge limits. The loss of resolution in the DMS uninstalled mode suggests that the system reached (or exceeded) its “storage space charge” limit. In the same mode, opening the trap for an extended period of time (500 msec) might have caused the trap to saturate with matrix ions and affected the signal intensity profiles as well. However, analyses in the DMS on mode have clearly demonstrated the utility of DMS through these two samples: dilute and concentrated for filtering uninstalled the
matrix effects and selectively transmitting the analyte of interest (BE) with acceptable resolution in AGC-on mode and even when the trap is opened for extended period of time of 500 msec. In terms of signal intensities, it has also been established from Figure 23(ii) that maximum benefit of the DMS utilization can be achieved by extending the fill time by switching off the AGC mode. For our analyte of interest, BE, 500 msec was used as the trap fill time in this DMS-MS platform with considerations to signal intensities and resolution.

2.10 Quantitative comparison: DMS-on and DMS-uninstalled
It is evident from the above results that differential mobility spectrometry in combination with an ion trap can increase the capacity of the trap by filtering uninstalled the matrix ions and filling the trap selectively with the analyte of interest. An important requirement in routine studies is quantitation, so we explored the possibilities of doing quantitative analysis in both MS mode and MS/MS mode using deuterated internal standard (BE-d3). For the quantitation in MS mode, precursor ions at m/z 290 and 293 were monitored for BE and BE-d3 respectively. For the quantitation in MS/MS mode, products ions at 168 and 171 were monitored for BE and BE-d3 respectively. For the MS/MS experiments, unit isolation width was selected and the normalized collision energy used for fragmentation was 25%.

The calibration curve obtained in the MS mode is presented in Figure 24. The DMS-uninstalled was deemed not suitable for quantitation purposes in MS mode because of poor resolution and high noise levels. In the DMS on mode, the SV was set at 1500 volts, CoV at -16 volts, ethyl acetate was used as the modifier of choice and the AGC feature was turned off and set at 500 milliseconds as previously established. The data was processed using Microsoft Excel. Y-axis represents the ratio of the precursor ion intensities of BE and BE-d3. X-axis represents the
concentration of BE in ng/ul (Figure 24). A linear regression analysis was done in MS Excel for the eight points used to get a $R^2$ value of 0.9991.

Figure 24: Calibration curve of BE extracted from urine in MS mode using the DMS-MS platform. The error bars represent standard deviations of triplicate analyses.

Next, we attempted quantitation in MS/MS mode with uninstalled DMS and DMS-on. This was deemed an important test for the DMS since it would test the ability of the trap to isolate an analyte of interest for fragmentation with and without the DMS filtration. The same eight points were analyzed under the same DMS and trap settings. The data was processed using Microsoft Excel. Y-axis represents the ratio of the product ion intensities of BE and BE-d3. X-axis
represents the concentration of BE in ng/ul (Figure 25). In the DMS on mode, a linear regression analysis was done in MS Excel for the eight points used to get a $R^2$ value of 0.9973. To verify the linearity at the bottom of the calibration curve, a linear regression analysis was performed on the first three points which gave us a $R^2$ value of 0.9994.

In the DMS uninstalled mode, where the trap had to isolate and fragment the analyte of interest (BE) from a noisy matrix environment, a poor linearity is present through the seven points with a $R^2$ value of 0.9437 (Figure 25b). Most notably, no appreciable product ion signal was seen for the lowest point, 0.1 ng/ul in the DMS uninstalled mode. The four points shown in the inset in Figure 25 demonstrate some linearity with a $R^2$ value of 0.9723. However, closer inspection of the ratios of the analyte to the internal standard (BE: BE-d3 product ions) in the DMS uninstalled mode revealed that the values are off from the expected theoretical ratios. Only when the DMS is turned on, did the observed BE: BE-d3 product ions ratios look reasonable. DMS aids in quantitative analysis by the trap through selective filtration and although there is a significant amount of ion losses in the DMS-on mode as compared to the DMS-uninstalled mode, manually setting the trap fill time allows the user to overcome ion loss issues.
Figure 25: (a) Calibration curve of BE extracted from urine in MS/MS mode using the DMS filtration. The error bars represent standard deviations of triplicate analyses. (b) Calibration curve of BE extracted from urine in MS/MS mode without the DMS filtration.

2. 11 DMS-transparent and DMS-on mode

In normal mode of ion trap operation, removal of the DMS filter is not required as the DMS can be placed in a transparent mode. Uninstalling and putting together the DMS after each run would be cumbersome and is not desirable. The DMS-transparent mode allows the user to look at the
total ion profile without having to physically remove the setup. The data presented hence forth in Chapter 2 compares the following two modes:

1) DMS-transparent: The CoV and SV values are set to zero and this allows the ion species to pass through the DMS filter into the mass spectrometer without any selectivity.

2) DMS-on mode: The CoV and SV are set to unique values which allow selective filtration of ion species into the mass spectrometer.

As mentioned earlier, the mass spectrometer can be operated in AGC-on mode, the normal operation of the trap and AGC-off mode, where the AGC-on mode can be overridden to manually fill the trap at a desirable timescale. In an effort to optimize the performance of the DMS with the ion-trap a series of experiments were performed in the DMS-transparent mode and DMS-on mode with the mass spectrometer set to AGC-on or AGC-off. The results are presented in the following sections.

2.12 Removal of matrix ion interferences with DMS

In an effort to compare the DMS-transparent mode with the DMS-on mode demonstrating the utility of the DMS, a blank urine sample was infused into the mass spectrometer as shown in Figure 26. As expected, in the DMS-transparent mode, the mass spectrum is dominated by undesirable matrix ions from the urine blank sample. Of particular concern is the presence of mass peak at m/z->288.6 which can interfere with the target analyte peak at m/z->290.
Figure 26 Analysis of BE in urine by DMS-MS. (a) Full scan mass spectrum (m/z 150 – m/z 500) of urine blank solution in the DMS-transparent mode; (b) Full scan mass spectrum (m/z 150 – m/z 500) of previous solution with DMS-on set at CoV = -16V showing removal of all matrix ions including potential interference at m/z 288.6; (c) Analysis of BE in urine (0.1ng/µL) with DMS set at CoV = -16V showing the ratio of 1:10 analyte : internal standard. MS scan range (m/z 182 – m/z 296).

In order to ascertain if DMS can remove the matrix ion components, especially the potential interference at m/z 288.6, the blank solution was infused with the DMS turned on. As expected, with DMS filtration on, all the matrix ion components were effectively removed in the m/z 150-500 range (Figure 26b). Next, a sample containing 0.1 ng/ul BE and 1 ng/ul BE-d3 were infused and the corresponding mass spectrum was recorded with DMS on. The DMS allowed selective
introduction of the analyte and the internal standard at the same combination of CoV and SV. The ratio between analyte to internal standard was maintained at 1:10 which suggested that DMS can be used for further quantitation purposes (Figure 26c).

After the selectivity of DMS was demonstrated, the next consideration was the degree to which the trap fill time could be extended in order to increase the population of the precursor ions of the BE metabolite when extracted from a biological matrix so that acceptable mass resolution is obtained. The easiest way to monitor the spectral quality was to track the ability of the ion trap to distinguish between the M and M+1 ions. In order to test the trap’s ability to maintain acceptable resolution, trap fill times were varied from the normal operating AGC-on mode and additional fill times of 200 ms, 500 ms, 1000 ms and 5000 ms were manually set and the corresponding massspectras recorded (Figure 27). While the improvement in ion accumulation was dramatic as given by the increase in signal intensity, a decrease in the resolving power of the trap also became apparent with overloading of the analyzer ion trap. As shown in Figure 27, deterioration of resolution began at fill times approaching 1,000 ms and at trap fill time of 5000 ms, the trap was unable to distinguish between the M and M+1 ions. As defined above, the trap has exceeded its spectral charge limit i.e. the ability to obtain mass spectrum with acceptable resolution and mass accuracy. This further makes it evident that the storage limit is significantly bigger than the spectral charge limit. Hence, setting the ion trap for a reasonable time to obtain spectra with quality resolution is very important.
Figure 27: Separation of m/z 290 and m/z 291 ions of BE demonstrating loss of resolution with increasing trap fill time due to space charge effects

More importantly, for the analysis of BE, base line resolution between these two peaks was observed at a trap fill time of 500 ms which is comparable to resolution observed in the AGC-on mode. It is interesting to point out that the increase in signal intensity was almost 25 fold when comparing the AGC-off 500 ms and AGC-on mode. In order to ascertain, if this phenomenon was unique for a fixed concentration of BE or if the gains in signal intensity was uniformly applicable to a wide range of concentrations and instrument settings, additional experiments were performed.
Generation of calibration curves in MS/MS mode is routinely done for quantitation purposes and product ion signals are monitored for the same. Without incorporation of internal standard, ion signal intensities were monitored across a range of concentrations between 0.1 ng/ul to 25 ng/ul in two different modes: AGC-on and AGC-off 500 ms (Figure 28). The transition 290→168 was monitored for the product ions and in both the cases, the DMS was turned on for data acquisition.

![DMS-on: product ion signal intensity as a function of concentration](image)

**Figure 28:** Comparison of product ion signal intensities of BE in DMS-on mode across a range of concentrations (0.1 ng/ul- 25 ng/ul) under two different conditions: AGC-on and AGC-off 500 ms.
The linearity of signal intensity across the concentration range analyzed was good under both the conditions as demonstrated in Figure 28. The slopes between the two conditions vary by a factor of approximately 29 which is very much in line with earlier observations. Hence, setting the AGC to the off mode and manually selecting a fill time of 500 ms afforded us the optimal mass resolution and sensitivity.

For quantitation purposes, a deuterated internal standard was used and the performance of the trap was evaluated in both MS and MS/MS mode. For comparison purposes the DMS was operated in transparent mode or DMS-on mode. Absolute signal values cannot be directly compared between AGC-on and AGC-off, but the LOD or LOQ values in the two conditions can be meaningfully compared. So, experiments were performed with AGC-on and AGC-off 500 ms.

2. 13 Quantitation in MS mode
To demonstrate the linearity of DMS, quantitation of BE after extraction from urine was performed in MS mode. In MS mode, only the precursor ion signal is monitored making it vulnerable to interferences from matrix ion components. In the Figure 29 below, the data in panels ii and iv were obtained with no DMS filtration (DMS-transparent), and (iii) AGC-off. (iv) AGC-on. (i,ii) were obtained using DMS filtration: (i) AGC-off (ii) AGC-on. The linearity is much better with the DMS on as demonstrated by Figure 29(i) and (iii). The LOQ is 0.1 ng/ul with the use of DMS with trap set at AGC-off 500 ms whereas the LOQs is 0.5 ng/ul without DMS at the same trap fill time. Demonstration of selectivity of DMS through quantitation in MS mode in a biological matrix suggests that the same platform can be easily extended to less complex or comparable matrices without having to perform laborious method development for MS/MS mode if an easier/ quicker method is desired for quantitation.
Figure 29 Calibration curves for quantitation of benzoylecgonine in urine by MS mode. The error bars represent the standard deviation of triplicate analyses. (ii,iv) were obtained with no DMS filtration (DMS-transparent), and (iii) AGC-off. (iv) AGC-on. (i,ii) were obtained using DMS filtration: (i) AGC-off (ii) AGC-on.
2. 14 Quantitation in MS/MS mode

MS/MS mode provides additional structural information and is the preferred method for quantitation especially when the matrix is biological. To further determine the potential advantages that may be realized by selective DMS filtration, the quantitation of BE by MS/MS on the m/z 290 → m/z 168 transition was determined under DMS-on and DMS-transparent conditions. (Figure 30(a,b,c,d)). Panels (a,c) were obtained with no DMS filtration (DMS-transparent): (a) AGC-on, (c) AGC-off. Panels (b,d) were obtained using DMS filtration: (b) AGC-on, (d) AGC-off. Overall, calibration curve accuracies are much better with DMS filtration.

In reviewing the data of Figure 30, most striking is the benefit derived from DMS filtration. In addition, the use of an extended fill time along with DMS filtration allows the calibration curve to extend lower in BE concentration than any other configuration. Dramatic improvement is evident when the DMS-on results are compared to the DMS-transparent mode where there is indiscriminate introduction of all sample ions into the trap. In addition to the much better linearity of the calibration curve, the result of higher loading of precursor ions due to DMS filtration is also shown by the improved sensitivity at the lower BE concentrations where the lowest point in the calibration curve is 0.1 ng/µL, a level that was unobservable with DMS transparent. This demonstrates that the matrix suppression provided by DMS filtration is especially useful in extending the quantitation range of ion trap analyses by MS/MS. The scatter in triplicate results with AGC-on at the lowest achievable concentration of 1 ng/µL is very much lower with DMS-on than DMS-transparent [(Figure 30(a,c)].
Figure 30 Calibration curves for quantitation of benzoylecgonine in urine by MS/MS (m/z 290 → m/z 168). The error bars represent the standard deviation of triplicate analyses. (a,c) were obtained with no DMS filtration (DMS-transparent), and (a) AGC-on. (c) AGC-off. (b,d) were obtained using DMS filtration: (b) AGC-on, (d) AGC-off. Results are much better with DMS filtration. In addition, the use of an extended fill time allows the calibration curve to extend an extra factor of 10 lower in BE concentration.
2. 15 Conclusions

DMS can be used to filter/introduce desired ion population into the mass spectrometer. In the absence of DMS, indiscriminate introduction of matrix ions from biological samples often results in premature trap saturation with irrelevant ion populations that dominate the trap contents, and prevent MS/MS for minor ions at moderate or low concentrations. DMS with extended trap fill time allows optimal performance in a stand-alone DMS-Ion trap system as demonstrated in this chapter by selectively targeting benzoylcegonine in a biological matrix. While the improvement in sensitivity observed by incorporation of DMS filtration for the analysis of BE in urine tested here was approximately 10-fold, this number is likely to be higher for more complex matrices and lower for less complex ones. It is expected, however, that the advantages will be best realized in trace level analyses or for improving the limits of detection in an assay as observed here for the quantitation of BE. Moreover, the use of DMS filtration and by-passing the AGC control functions adds a level of flexibility for conducting quantitative analyses or other related MS/MS operations in ion trap systems.
Chapter 3: Understanding gas phase modifier interactions in rapid analysis by Differential Mobility-Tandem Mass Spectrometry
3.1 Introduction

Differential Mobility is a rapidly evolving new analytical technology which offers rapid gas phase ion separation/filtration prior to mass analysis, and the advantages of this platform such as improved signal to noise ratio, separation of closely related compounds, and removal of interferences have been well demonstrated[9, 35, 46, 65-67]. The mobility of charged ion species in an applied electric field forms the basis for ion separation in a DMS cell. Krylov and Nazarov [10] have studied three different models of ion-neutral interactions in the applied electric field: (a) rigid sphere scattering, (b) long-range ion–dipole attraction and (c) clustering. They concluded that ion-neutral clustering is the most relevant phenomenon to explain the dependence of ion mobility on field strength that is the source of DMS selectivity. The mobility of ions between the electrodes in a differential mobility cell has been well described in a paper by Schneider et al [66] and more extensively in monographs by Shvartsburg [68], Eiceman and Karpas [69], and in a discussion on the fundamentals of ion mobility by Mason and McDaniel [70]. We present here a brief discussion of these principles that provides a framework for understanding the role of modifiers in DMS separations as they particularly pertain to the analysis of selected model DNA adducts, biomarkers indicative of DNA damage from carcinogens.

Ion mobility as used in DMS is a high pressure phenomenon in which ions in an electric field quickly reach a limiting speed, described by a field-dependent ion mobility coefficient, \( K(E) \),

\[
\bar{v}_d(E) = K(E) \bar{E},
\]

\[
K(E) = K(0)(1 + \alpha(E)), \quad \text{Equation 2}
\]
where $v_d(E)$ is the drift velocity of the ion, $E$ the electric field strength, and $\alpha(E)$ contains the deviation from low-field behavior, and is known as the alpha parameter, or the differential mobility. $K(0)$ is the low-field mobility coefficient, with a dependence on the ion’s chemical identity, but also depending on pressure, temperature, and chemical environment. Low field mobility, $K(0)$, is the property measured in ion mobility spectrometry (IMS) under the same conditions. However, the ion separations used in differential mobility spectrometry (DMS) are determined solely by the differential mobility parameter, $\alpha(E)$, [71, 72] because of the DMS filter condition which determines the relationship between the DMS separation peak voltage, $SV$, and the DMS compensation voltage, $CoV$, for a particular DMS waveform shape, $f(t)$ [73]. Ion transmission in a planar DMS geometry requires that the ion remain along the axis of the DMS analytical region after each $f(t)$ waveform cycle of period $T_f$

$$\int_0^{T_f} dt f(t) E(t) \left(1 + \alpha(E(t))\right) = 0,$$

Equation 3

where $E(t) = SV \cdot f(t) + CoV$.

As a result of this filter condition, there is complete equivalence between the observed DMS compensation voltages ($CoV$) for a range of field amplitudes ($SV$) and the differential mobility, $\alpha(E)$ [66].

The dependence on chemical composition of the transport gas has been found to be the largest contributor to differential mobility. This is both a hindrance and an advantage. Ions of similar molecular weight and related structure can usually be separated with the assistance of a modifier and peak capacity is greatly increased, but the underlying cause is still obscure. While useful for improved resolution and reduced chemical noise, the modifier effect is the most difficult to predict; however, it is important to recognize that the mobility coefficient is directly related to
the effective size of the ion, whether it is a bare ion or a ion-neutral cluster (effective size is referred to as the cross-section for collisions, between the ionic species and molecules in the neutral transport gas).

Early on in the application of DMS technology, it was realized that the use of modifiers in the transport gas is an important step for analytical applications [8, 74]. Prior works from our laboratory and several others have demonstrated the advantages of introducing drift gas modifiers to aid in DMS separations [23, 44]. In a recent publication by Schneider et al., the effects of transport gas modifiers have been extensively documented with particular attention to resulting peak capacity [75] and other groups are active as well in areas like Green Chemistry [44, 45]. These results are part of a growing interest in the use of modifiers with the DMS-MS platform as it offers a promising alternative for rapid analytical applications as well as possible field applications such as those proposed for NASA applications [76, 77].

Our group has long been involved in the development of DMS technology for analytical applications [6, 23, 78] and in recent years, we have been particularly interested in the use of DMS-MS for quantitative analysis with reduced sample cleanup and minimal or zero LC time. There are a number of advantages to the use of DMS in rapid quantitative biomarker analysis, many previously discussed, as in Coy et al. [65], but one point is of special interest when DMS-MS quantitative analysis is compared to LC-MS: LC-MS as well as drift-time or travelling-wave IMS require integration of an ion current over an elution or drift time profile, but DMS-MS intensities for quantitation are obtained continuously by monitoring the CoV for peak ion transmission. The DMS analytical region is a continuous ion filter that transmits with minimum diffusion losses at characteristic (SV,CoV) values. Because the width of the peak in CoV units is inversely related to the low field ion mobility of the ion, integrating over the DMS peak width
would contaminate the observed data with an unknown scale factor, one which has a variable environmental dependence. Thus, DMS at the peak CoV value provides a continuous measurement of ion concentration, limited in response time only by the transit time of the ion through the DMS analytical region, and the agility of the electronics of the mass spectrometer.

Prior works from our laboratory have demonstrated that the judicious use of modifiers provided conditions for rapid quantitation of selected analytes in matrices of varying complexities [6, 55]. We have shown that, in addition to the rapid analysis time afforded by use of the DMS, sample preparation steps can be minimized or even eliminated by taking advantage of the unique post-electrospray selective ion filtration capabilities. As with any other analytical platform, proper method development is necessary to achieve optimal sensitivity and efficacy. In the work presented here, we demonstrate the process of stepwise evaluation of modifier effects from a method development perspective in order to establish the optimal conditions for quantitation of selected DNA damage biomarker analytes by differential mobility- mass spectrometry.

Commonly used polar protic and polar aprotic modifiers have been screened for use against two deoxyguanosine adducts of DNA: N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-ABP) and N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (dG-PhIP). N-(2-deoxyguanosine-8-yl)-4-ABP (dG-ABP), the deoxyguanosine adduct of the bladder carcinogen 4-aminobiphenyl (4-ABP) is a known carcinogen found in cigarette smoke, paints, food colors, hair dyes and fumes from heated oils and fuels[79-82]. The heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5]pyridine (PhIP) is found in grilled meats [83]. Particular attention was paid to compensation voltage (CoV) shifts, peak shapes and product ion signal intensities while optimizing the DMS-MS conditions. DNA adducts provide direct evidence of genetic exposure and damage in cells and monitoring their levels from a health perspective is
important. So-called bulky DNA adducts share common structural features in that, other than the carcinogen, they all contain a deoxyribose moiety along with the nucleobase, typically a guanine. In this regard, the compounds selected for this study provide excellent models for the purpose of evaluating the DMS conditions that might be appropriate for the DMS – MS analysis of this class of analytes. The two selected adducts have been related to lifestyles of people and we have used them as model analytes to explore and optimize the gas phase interactions in DMS and develop differential mobility- mass spectrometry as a rapid quantitative platform. The work presented in this chapter was recently accepted for publication in JASMS[84].

![Figure 31 Structures of DNA adducts in this study](image-url)
3. 2 Experimental Section

Chemicals and Standards:

Caution: 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5]pyridine and 4-Aminobiphenyl and its derivatives are carcinogenic and should be handled carefully.

Calf-thymus DNA, nuclease p1 from *Penicillium citrinum*, deoxyribonuclease 1 (DNase I) type 2 from bovine pancreas, alkaline phosphatase from *Escherichia coli* (type III), ethanol, dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Snake venom phosphodiesterase was purchased from USB Corporation (Cleveland, OH). $N$-(2-deoxyguanosine-8-yl)-4-ABP (dG-ABP) was purchased from Toronto Research Chemicals (Toronto, ON, Canada, D239600, CAS 84283-08-9, C$_{22}$H$_{22}$N$_6$O$_4$). $N$-(deoxyguanosine-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-β]pyridine (dG-PhIP) were purchased from Toronto Research Chemicals (Toronto, ON, Canada, D239630, CAS 142784-25-6, C$_{23}$H$_{23}$N$_9$O$_4$). Formic acid solution was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). HPLC grade water and methanol were purchased from Fisher Scientific (Fair Lawn, NJ).

DNA quantification, enzymatic digestion and protein precipitation:

DNA quantification was done using an Invitrogen Corporation (Carlsbad, CA) Quant-iTM double strand (ds) DNA BR Assay kit with a Qubit fluorometer. Aliquots containing 2 ug DNA (dissolved in 5mM Tris-Cl/ 10 mM ZnCl$_2$) were removed for digestion and analysis for each sample point.
Calf thymus DNA was hydrolyzed similarly to a method previously described[85]. Samples were incubated at 98 °C for 3–5 min and chilled in the freezer down to room temperature. 0.3 units of nuclease P1 (0.3 units μL⁻¹ solution of 5 mM Tris–Cl, pH 7.4) and 3.1 Kunits of DNase I (1 μg μL⁻¹ solution in 5 mM TRIS/10 mM MgCl₂, pH 7.4) were then added per μg of DNA and incubated in a water bath maintained at 37 °C. After 5 hours, 0.003 units of phosphodiesterase (100 ng μL⁻¹ in 5 mM TRIS/10 mM MgCl₂, pH 7.4), and 0.002 units of alkaline phosphatase per μg of DNA were added and the mixture was further incubated at 37 °C for 18 h.

Protein precipitation was done by adding five volumes of ice cold ethanol and centrifuging at 10000 rpm for 15 minutes. The samples were dried down and stored at -80°C until analysis.

3. 3 Instrumentation

The fundamental goals of the proposed approach to the optimization of modifiers in quantitative analysis by DMS-MS was tested using two different planar DMS systems, one attached to a 3-D ion trap (Thermo Finnigan LCQ Classic) (San Jose, CA) and the second interfaced to an AB SCIEX API 3000 triple quadrupole MS [AB SCIEX, Framingham, MA]. The DMS filters varied only in terms of their dimensions, and this comparison provided an assessment of the general effect and applicability of the DMS configuration to such analyses.

DMS-Ion Trap Mass Spectrometer:

A planar DMS developed by Sionex Corporation (Bedford, MA) with a filter gap 0.5mm high x 3.0mm wide x 10.0mm long which was positioned at the entrance of the heated capillary of a Thermo-Finnigan, LCQ Classic mass spectrometer was used for the work. Sionex Expert software was used to set the DMS parameters. The SV could be set at zero or scanned in the range from 500 V to 1500 V, and the CoV could be set or scanned from -43 to +15 volts.
Although the system in use here is no longer commercially available, the underlying electronics technology has been described, and successor commercial instrumentation (SelexION, AB SCIEX) has become commercially available.

Electrospray was performed using coated 10 µm PicoTip emitters from New Objective (Woburn, MA). The syringe was connected to the emitter tip using a 150 um (ID) capillary tubing and the ESI voltage was applied to the union at the liquid-liquid junction between the capillary and the tip. Samples were introduced at the rate of 300 nl/min using a Harvard Apparatus syringe pump (Holliston, MA). The desolvation gas (ultra high purity nitrogen) was introduced at a flow rate of approximately 100 cc/min into the desolvation region at a temperature of 100°C. The vacuum drag of the mass spectrometer was measured to be 500 cc/min. External air flow of approximately 400 cc/min also merged in with the desolvation gas into the DMS. The bulk gas temperature was estimated to be 45°C. Modifiers were introduced into the desolvation region along with the nitrogen gas. The electrospray emitter voltage was held at 2 kV throughout the analysis.

**DMS-Triple Quadrupole Mass Spectrometer:**

A prototype DMS- API 3000 Triple Quadrupole mass spectrometer (AB SCIEX, Concord, Ontario, Canada) which has an integrated DMS filter online in front of the vacuum orifice was used for this study. The dimensions of the DMS analytical region were 1 mm x 10 mm x 15 mm. A modified version of the software Analyst version 1.5 which included parameters for SV and CoV was used. The SV could be varied from 0 to 5000 volts and the CoV could be varied from -100 to +100 volts. Electrospray was performed using stainless steel 30 µm id emitter from
Proxeon (Thermo Fisher, West Palm Beach, FL). Samples were introduced at the rate of 400 nl/min using a Harvard Apparatus syringe pump. Electrospray was held constant at 3500 volts throughout the analysis. Modifiers were introduced into the curtain gas (nitrogen, 1.1 L/min, 600 cc/min vacuum drag 500 cc/min curtain gas outflow at 40°C) using a second Harvard Apparatus syringe pump.

RESULTS AND DISCUSSION

Effect of modifiers on CoV shifts

In an effort to understand and characterize the gas phase molecular interactions, varying percentages of modifiers were introduced into the transport gas and the CoV shifts were recorded with increasing separation voltage. Voltages corresponding to the apex of extracted ion chromatograms are reported here as CoV values. The goal of using the modifiers in this work is to shift the analyte of interest selectively to a CoV value free of interferences where DMS can be exploited to perform rapid quantitation in the presence of a complex matrix.
Figure 32: Effect on compensation voltage of varying concentrations of modifiers (i) Ethyl acetate and (ii) Isopropanol on dG-ABP CoV shifts
3. 4 Effect of Modifier on CoV of dG-ABP

The clustering effects of two gaseous modifiers of differing size and polarity, ethyl acetate (C₄H₈O₂, mw 88.11) and isopropanol (C₃H₈O, mw 60.10), on dG-ABP at varying concentrations were investigated by monitoring the CoV shift as a function of separation voltage (SV). As shown in Figure 32, in the absence of a modifier, the analyte ion does not exhibit any shifts in CoV at SV values up to 2000 volts. Even when the SV is increased beyond 2000 volts, the CoV starts shifting only slightly towards the negative voltage reaching a maximum of -2 volts at SV=4500 volts.

With ethyl acetate as a modifier, dG-ABP shifts rapidly to negative CoV values, as expected for charge-dipole interactions[10], reaching -5V at only 0.30% ethyl acetate. Interestingly, at higher ethyl acetate concentrations of 0.6%, 1.20% and 2.50%, identical CoV responses are observed, as indicated by the overlapping CoV curves. This strongly suggests that steric and thermodynamic effects limit the maximum number of coordinated ethyl acetate molecules to a value which is reached between 0.3% and 0.6% by volume, under the AB-SCIEX 3000 triple-quad conditions. It can also be noted that the CoV shifts for all SV values were intermediate between those of no modifier and higher modifier concentrations.

With isopropanol as a modifier, in comparison to ethyl acetate, the CoV value of the adduct shifted to even greater negative values with isopropanol than for ethyl acetate, and the effect was even more substantial with increasing concentrations (Figure 32). For example, going from no modifier to progressively higher modifier concentrations of 0.60% , 1.10% and 2.20%, the respective CoV values at SV=4,500V increased to -4.6, -10.4 and -14.2 V. For isopropanol the greatest CoV shift observed at 2.20% was -14.2 volts compared to ethyl acetate’s maximum shift
of -9 volts. The observation of a greater shift toward negative CoV values with isopropanol than with ethyl acetate, even though isopropanol is of lower molecular weight and of smaller geometric cross-section, can be interpreted in different ways. On one hand, it might indicate that the limiting coordination number with isopropanol is greater than with ethyl acetate, or, alternatively, thermochemical effects related to the free energy changes on cluster formation, and the related role of hydrogen bonding in cluster formation in the two systems may be important. This is discussed further below.

It has been reported that the modifier effect is sensitive to small variations in ion structure, so that even closely related structures may be separated (citrate/isocitrate, [65]; ephedrine / pseudoephedrine [46], and others [86]). Prior works in the field of DMS have established the importance of modifiers to not only enhance CoV shifts, i.e. selectivity but, using the analogy to chromatography, to improve peak capacity in the DMS analysis [66], as well as to suppress chemical noise. We thus next examined the peak shapes of the analyte of interest dG-ABP at different DMS conditions. Selected extracted ion chromatograms from the CoV scan are shown in Figure 33. For dG-ABP, the improvements in peak shape by the introduction of modifiers can be understood by comparing the FWHM (full width at half maximum) at a constant SV of 3500 volts in Figure 33 (no modifier: Figure 33B, Ethyl acetate modifier at 0.6%: Figure 33C and Isopropanol modifier at 0.6%: Figure 33E). The FWHM without modifier is ~4 volts compared to ~3 volts in presence of ethyl acetate and ~3 volts in presence of isopropanol, which highlights the role of DMS and gas phase clustering in the presence of modifiers on peak shape improvements during DMS analysis. It should also be pointed out that that the FWHM increased initially by 1.5 volts when the SV was set to 3500 volts (Figure 33B) as compared to zero SV (Figure 33A) which was subsequently improved by introducing modifiers. Much of this effect
may be due to DMS suppression of chemical noise due to ions of higher molecular weight, which are detected with a wider peak width due to lower mobility.

It is interesting to point out the effect of increasing modifier percentages on peak shape. Specifically, when ethyl acetate modifier is introduced at 2.5% (Figure 33D) which is roughly 4x the amount of the same modifier introduced at 0.6% (Figure 33C), not only do the CoV shifts overlap, but the peak shapes look very similar which supports our notion of “saturation effect” as discussed above. However, a 4-fold increase in the isopropanol concentration (Figure 33E and Figure 33F) also shows that the peak shape can begin to suffer with larger CoV shifts. It is reasonable to surmise that formation of larger clusters with increasing isopropanol concentration may have caused the transmission of analyte ion dG-ABP over a slightly larger CoV range as expected from the higher cluster molecular weight (DMS peak width ~ (1/K(E)). The broadening effect is seen to be greater near the baseline than at half-height, as would be expected for some fraction of larger clusters with broader peaks, that are in rapid equilibrium with single adducts. It is therefore deemed necessary to optimize the modifier percentage being introduced to avoid excessive peak broadening during DMS-MS analysis.
Figure 33: Comparison of peak shapes of dG-ABP with and without modifier. Improvements in peak shape by the introduction of modifiers can be realized by comparing the FWHM (full width at half maximum) at a constant SV of 3500 volts.
3. 5 Effect of Modifier on CoV of dG-PhIP

The principal DNA adduct of PhIP, a diet related carcinogen, is also formed by covalent bonding at the C-8 position of guanine. In that sense this adduct provides an interesting example for comparison with dG-ABP since the two compounds share the 2’-deoxyguanosine structure \((C_{10}H_{13}N_{5}O_{4}, \text{mw} = 267.2413)\) and differ since the PhIP moiety is larger, richer in heteroatoms, and more polar than ABP. In evaluating the modifier effects on CoV, ethyl acetate and isopropanol were tested as before, and 1-butanol \((C_{4}H_{10}O, \text{mw} = 74.1216, \mu=1.584 \text{ D (1.660 D expt.)} [87])\) was also screened for use in DMS-MS analysis of the adduct and the results are presented in Figure 34.

In the absence of a modifier, the dG-PhIP CoV values started shifting in the positive direction reaching +3 volts at SV value of 4500 volts. As discussed by Krylov, a positive CoV shift is associated with short-range collisions, while long-range, charge–dipole interactions result in negative CoV shifts. Upon the introduction of modifiers, the CoV values reversed direction to the negative side and this trend increased significantly as a function of modifier concentrations and SV. Ethyl acetate was the least effective in shifting the CoV to a negative direction while 1-butanol exhibited larger shifts than isopropanol, reaching a maximum peak value of -10 volts at 2.20%. It is conceivable that the stronger action of 1-butanol compared to isopropanol might be attributable to the larger geometric size of 1-butanol, to the end position of the -OH which increases the cluster profile, and to lower steric inhibition for the end-attached –OH by the alpha methyl group, especially since the dipole moments of the two alcohols are nearly identical and thus have the same long-range attractive interaction with an ion.
Figure 34: Effect of varying concentrations of modifiers (i) Isopropanol, (ii) Ethyl acetate, (iii) 1-butanol on dG-PhIP CoV shifts
Also especially interesting was the limited effect exhibited by ethyl acetate with a maximum peak value of -2.2 volts at 2.50%. This may be interpreted in two radically different ways: (1) difference in hydrogen-bonding donor-acceptor characteristics may cause ethyl acetate (acceptor) to cluster less strongly than the alcohols (donor), or (2) the much greater dipole moment of ethyl acetate and the greater polarity of the PhIP part of dG-PhIP may cause the clustering with dG-PhIP to be so strong that the complex remains fully saturated throughout the DMS waveform, quenching the differential mobility effect. Ab-initio calculations of Gibbs free energies of cluster formation do show significantly stronger binding with ethyl acetate than with isopropanol as expected from the charge-dipole interaction. The stronger effect of 1-butanol compared to isopropanol may be due to the larger geometric size of 1-butanol so that the cross-section increases more on binding 1-butanol. Steric inhibition of the methyl groups located alpha to the hydroxylic carbon might also play a role in determining the free energy change on complex formation since isopropanol is disadvantaged by the mid-chain position of the hydroxyl group.

3. 6 Clustering with gas phase modifiers

Ion-neutral clustering has been the subject of considerable study, although mechanisms are complex and not fully elucidated. A useful early review is that of Castleman and Keesee [88], but there are a number of more recent studies, including those from the Leone group[89], and others. A quantitative analysis of comparative modifier effects requires a detailed examination of conformations, and steric hindrance, in cluster energetics as well as computations of mobility [90] from predicted structures, nonetheless, the experimental data through its exceptional cases provides insight into the principles governing the DMS modifier effect. Based on the experimental observations made during the DMS analysis of the adducts of interest with
appropriate modifiers, the scheme for one of dynamic equilibrium between the ion and ion
clusters containing one or more modifier neutrals is presented. The kinetic analysis of ion-neutral
clustering can be based on the following scheme describing the chemical equilibria among
clustering polar modifier, C, protonated ion, MH\(^+\), and ion-neutral clusters of different sizes,
\((C_n)\cdot MH^+\) in the gas phase.

For protonated electrospray cations, this can be written as

\[
\begin{align*}
MH^+ + C & \overset{k_1}{\rightleftharpoons} (C)_1 \cdot MH^+ \\
(C)_1 \cdot MH^+ + C & \overset{k_2}{\rightleftharpoons} (C)_2 \cdot MH^+ 
\end{align*}
\]

Using an abbreviated notation, we can write the partition of the initial ion concentration into ion-
neutral clusters as follows.

\[
\begin{align*}
A_j & \equiv \left( (C)_j \cdot MH^+ \right) \\
C & \equiv [C] \\
A & \equiv [MH^+]_{\text{total}} \\
A_{\text{total}} & = A_0 + A_1 + A_2 + \ldots
\end{align*}
\]

Here we include a limit to the number of clustered modifiers, based on the number of modifier
molecules geometrically and electrically compatible with the ion, a modifier-shell-size limit.

There is also a kinetic limit that to the maximum change in cluster number between the low and
high field cases, which is related to the collision rate and the electric field frequency that can be
approximated in the same way. These two effects differ: only the shell model can lead to the
solvent saturation effect that is observed in dG-ABP with ethyl acetate, but not with isopropanol.
3. 7 Use of modifiers in separation

The ability of gaseous modifiers to control the compensation voltage of an analyte provides the means for enhancing separations during DMS analysis. Even more importantly, in a targeted analysis, control of the CoV shift can provide a means for isolating the analyte of interest into a transmission zone that is free of matrix interferences, thereby improving signal to noise ratio and sensitivity. In the studies of the biomarkers of DNA damage, the matrix is a DNA enzymatic digest comprised of normal deoxynucleosides and enzyme-related breakdown products. Ensuring the separation of the adduct from normal (unmodified) nucleosides is of particular importance since they have the common deoxynucleoside functional group.

As an initial test, a mixture of 1 ng of each deoxyguanosine (dG), deoxycytosine (dC), deoxyadenosine (dA) and 100 pg of the DNA adduct dG-ABP was prepared to investigate the role of modifiers on separation. Two modifiers: ethyl acetate and isopropanol were evaluated for separation of the dG-ABP adduct from the excess unmodified DNA bases. CoV scans at a fixed SV value were performed and the analytes of interest were extracted from the TIC. It should be noted that effective separations were not observed in the absence of a modifier.
Figure 35: Separation of a mixture of dC, dA, dG and dG-ABP (A) in the presence of ethyl acetate modifier at a fixed concentration done on the DMS- ion trap (B) in the presence of isopropanol modifier at a fixed concentration done on the DMS- ion trap.
From Figure 35A, we can see that the four component nucleoside mixture is separated well by the DMS using ethyl acetate as the modifier. As expected, dG-ABP being the bulkiest molecule in the mixture, moves the least towards the negative compensation voltage compared to other unmodified nucleosides because its mobility (~1/cross-section) will be less modified by cluster formation. dA appears as a double peak whose characteristics have not been investigated further. Ethyl acetate and isopropanol have been successfully applied as effective modifiers for other applications with DMS, so we decided to screen isopropanol for separation purposes as well with dG-ABP. Introduction of isopropanol as the modifier, provided the necessary conditions for separation of the normal nucleosides dA and dC but failed to resolve dG from dG-ABP as the latter two were transmitted at the same CoV value. It was therefore decided to ascertain if an increase in modifier concentration could be used in order to achieve desired separations. As can be seen from (Figure 36(i) and (ii)), dG and dG-ABP could not be separated without the modifier and when the modifier Isopropanol was introduced at a lower rate of 0.6% into the curtain gas. However, when the modifier was introduced at the rate of 1.1%, separation was achieved between dG and dG-ABP. The small dG signal at the dG-ABP CoV is interpreted as arising from fragmentation of dG-ABP to dG after the DMS and in the mass spectrometer transition to vacuum (Figure 36(iii)). Using the example of dG-ABP and unmodified dG, we have demonstrated here that the percentage of modifier being introduced can be used as an another tool in achieving or enhancing separations using a DMS based platform.
Figure 36: Separation of dG and dG-ABP (i) without modifier and using IPA modifier at two different modifier concentration done on DMS-triple quadrupole (ii) 0.60% and (iii) 1.10%
3. 8 Effect of separation voltages and modifiers on signal intensities in MRM mode

In the experiments discussed above, the focus was on the CoV shifts of dG-ABP and dG-PhIP. These studies established that the use of organic modifiers can have a dramatic effect on analytical performance. Given our goal of using the DMS-MS platform to perform rapid quantitation of these biomarkers, we examined next the influence of modifiers on signal intensity in order to integrate all of these variables into a comprehensive analytical protocol. The rapid scanning features of the DMS-MS platform for performing rapid quantitation can be best appreciated by using it in the CoV stepping mode and the signal intensities monitored in the MRM mode. Under those conditions, once the CoV apex for any given analyte has been identified, this voltage value can be set for subsequent quantitative analysis. Setting the CoV value allows the user to filter out matrix contaminants and selectively transmit analyte(s) of interest continuously into the mass spectrometer without physically adding time for the mass spectral acquisition. With the DMS-on, signal intensities in the MRM mode were thus monitored for a fixed concentration of each compound to optimize the best DMS conditions for selected analyte ion transmission.

As mentioned above, in light of the variations in CoV shifts as a function of modifier concentration, it was deemed useful to monitor the effect of modifier choice on the product ion signal intensity. Accordingly, samples containing 0.100 picomoles of dG-PhIP were analyzed and, initially, CoV scans were performed at different values of separation voltages and modifier percentages in order to identify the peak apex values. Once these values had been identified, product ion counts were monitored in the MRM mode at the respective operating values of separation voltage, compensation voltage and modifier percentage (only combinations of...
separation voltages and modifier percentages that exhibited shift away from the zero compensation voltage were investigated). It is shown that, with increasing separation voltages at all three isopropanol modifier percentages (0.6%, 1.1% and 2.2%) examined, the general trend on the intensity of the product ion is the same (Figure 37A,B,C). Specifically, the product ion intensity value increases initially, peaks off at SV=3500 volts and then starts decreasing with application of higher separation voltage. The initial increase with the application of the DMS field is generally described as a DMS focusing effect, but may also involve enhanced desolvation of the electrospray plume due to RF heating by the DMS field. The transmission of the analyte ion through the DMS electrodes is optimal at the separation voltage value of 3500 volts.

Further increase in separation voltage results in a reduction in analyte ion count which is evident at each modifier concentration, but becomes more pronounced as modifier concentration increases. This has previously been illustrated through the use of a dispersion plot [6], where the drop in signal intensity is shown in 2-D DMS SV-CoV scanning mode. Figure 37D shows the comparison of the product ion intensity at separation voltages of 3500, 4000 and 4500 volts with varying isopropanol modifier percentages of 0.6%, 1.1% and 2.2%. Signal intensity of the product ion of dG-PhIP falls both with increased modifier concentration and with SV above 3500 V. This effect can be understood in terms of two contributions: (1) higher modifier contributions result in more frequent ion-modifier collisions, leading to higher probability of loss of charge from the ion or ion cluster, and (2) higher SV results in higher velocity and more energetic and more destructive collisions between the ion and the transport gas, again leading to a higher rate of charge loss, or even to reactive collisions[91].
Figure 37: Effect of separation voltage and modifier isopropanol (IPA) concentration on the MS/MS signal intensity of the DNA adduct dG-PhIP (A) at 0.6% (B) 1.1% (C) 2.2% (D) Trends of AB SCIEX DMS API 3000 signal intensities at separation voltages of 3500, 4000, 4500 volts with increasing modifier percentages
Referring back to Figure 34, it may be noted that in the case of dG-PhIP, the shifts have been significantly influenced by both IPA and 1-butanol with the latter giving larger CoV shifts. It was thus important in the context of quantitative analysis to also compare the effect of these two modifiers on the signal intensities. Samples of dG-PhIP (20 femtomoles), using isopropanol and 1-butanol at 0.6% were screened at the optimal separation voltage value of 3500 volts (Figure 38). Signal intensities were 830 for isopropanol and 400 for 1-butanol (2.1 : 1). For dG-PhIP, use of isopropanol as the modifier gave much better signal intensity than 1-butanol by more than a factor of 2. For dG-ABP, a sample containing 58 femtomoles of dG-ABP was analyzed in the MRM mode at a separation voltage value of 3500 volts at 0.6% concentration of modifiers isopropanol and ethyl acetate consecutively. Signal intensities were 570 for IPA and 910 for ethyl acetate (1 : 1.60) Thus, for dG-ABP, ethyl acetate as the modifier produced 60% higher signal intensity in the MRM mode when compared to isopropanol. The same experiment was repeated at different concentrations of both the analytes (dG-ABP and dG-PhIP) and the same trend was observed for all concentrations.
Figure 38 Effect of modifiers on the product ion intensity at a fixed separation voltage of 3500 volts: (A) Modifiers Isopropanol (IPA) and 1-butanol on the DNA adduct dG-PhIP & (B) Modifiers Isopropanol (IPA) and Ethyl acetate (EtoAc) on the DNA adduct dG-ABP.
Since generation of ion signal is directly dependent on the abundance of the respective [M+H]+ ions, the simplest assumption is to attribute these differences in signal intensity to a competition for the proton between the analyte ion or ion-neutral clusters and the modifiers. The proton affinity of ethyl acetate is 835.7 kJ/mol, isopropanol is 793.0 kJ/mol and 1-butanol is 789.2 kJ/mol respectively [92]. Had the difference in modifier proton affinity been the dominant factor, then ethyl acetate with a greater proton affinity value than isopropanol would have produced lower signal intensity for dG-ABP. For dG-PhIP, although the proton affinity value of 1-butanol is almost the same as that of isopropanol, the difference in signal intensity between the two modifiers is large, with isopropanol giving higher signal intensity. Methyl groups on the isopropanol introduce some steric hindrance that reduce the contribution of H-bonding to the clustering process, whereas the hydroxyl group is more exposed in 1-butanol which may have facilitated clustering. In an alternative model, the fact that isopropanol and 1-butanol have very similar dipole moments, while ethyl acetate dipole moment is much larger could lead to alternative conclusions. None of these approximations can lead to a full and accurate prediction of modifier behavior; only accurate thermochemical free energy values based on all these effects can be expected to be of help. Thus, screening of potential modifiers against the analyte(s) of interest is necessary and can provide users a better understanding of the modifier selection process and optimization of the DMS parameters for analysis. Signal intensities in detail must be examined experimentally, although the general trends with modifier concentration and with SV amplitude are understood.
3.9 Thermochemical kinetics of modifier-assisted DMS

Ion-neutral clustering has been the subject of considerable study, although mechanisms are complex and not yet fully elucidated. A useful early review is that of Castleman and Keese, but there are a number of more recent studies, including those from the Leone group[93], and others. A quantitative analysis of comparative modifier effects appears possible based on thermochemical free energy values from computational chemistry, optimized conformations considering energetics and steric hindrance, combined with computations of mobility [90, 94, 95] from predicted structures, and further investigations are in progress.

The mobility coefficient, $K(E)$, acquires a dependence on field strength, $E = |\vec{E}|$, as a result of interactions of the ion with its chemical environment [10, 66, 78], and also varies with pressure and temperature in the DMS analytical region [96, 97]. Ion mobility loses its dependence on pressure if the field used in ion mobility is expressed in density-normalized Townsend units for the electric field [70]:

$$E_{Td} = \frac{E}{N},$$

$$K_{Td}(E_{Td}) = NK(E) = \frac{v_d(E_{Td})}{E_{Td}},$$

*Equation 5*

where $E_{Td} = E / N$ is the ion mobility field in Townsend units [96](1 Td = $10^{-17}$ volt/cm²).

Density scaling provides pressure correction for analytical instruments such as the AB SCIEX SelexION system [98], but is not useful for temperature variation. The ion mobility coefficient, even under cluster-free conditions, still retains a dependence on temperature that can be minimized but not eliminated by temperature scaling. Temperature scaling leads to a reduced mobility, $K_0$ (corrected to 0°C, 1 atm) that still depends weakly on temperature, but that variation
is dwarfed in DMS by dynamic cluster size variation. Although it is not important in low pressure IMS, or in traveling wave IMS [99, 100], the importance of clustering is well known in atmospheric pressure IMS, where a cluster-free mass-mobility correlation can only be obtained by the use of high drift tube temperatures, typically 200°C or higher [69, 94, 101-106]. Except in special cases, low pressure IMS, and travelling wave IMS, operate with pure, non-polar drift gases and determine accurate cross-sections for a wide range of molecules, while DMS modifier effects depend on polar molecules with a long range attractive potential [107].

In order to understand the observed variation in compensation voltage with DMS field strength and modifier concentration, it is necessary to consider three effects of the DMS field on the ion and on ion-modifier clusters: the heating of the ion, the change in the frequency of collision between the ion and the bulk gas, and the change in collision energy. The rapidly varying DMS field does not affect the transport gas because the ions are very dilute in the total gas mixture. However, the DMS field does change the internal and translational energy of the ion / ionic cluster, as well as the collision energy and collision rate for collisions between the ion or ion cluster and the transport gas composition. The heating effect on the ion can be written in terms of an ion effective temperature, $T_{\text{eff}}$, which is different from the bulk gas temperature, $T$, as follows [10, 70, 97].

$$\Delta T_{\text{DMS}} = T_{\text{eff}} - T_{\text{bulk}} = \frac{\zeta}{3k} \frac{M}{N^2} K^2 \left( \frac{E}{N} \right)^2$$

Equation 6

This expression describes heating of the ion by the DMS electric field at fixed bulk gas temperature, $T_{\text{bulk}}$, and pressure, $P$ [70]. For our purposes, it is only important to note that the heating, the increase in the ion energy, increases rapidly, as the square of the DMS field,
neglecting the constants in square brackets. The zero-subscripted gas density, \( N_0 \), and ion mobility, \( K_0 \), are reduced values, scaled to 760 Torr and 273 K. The relationship, \( NK = N_0K_0 \), is exact when comparing values at the same bulk gas temperature, and is approximate when different bulk temperatures are compared [108].

The ion-neutral collision frequency, \( \nu_{\text{coll}} \), and collision energy, \( \varepsilon_{\text{coll}} \), also increase, both dependent on the ion effective temperature[70].

\[
\nu_{\text{coll}} \propto \sqrt{T_{\text{eff}}} = \sqrt{T_{\text{bulk}} + \Delta T_{\text{DMS}}},
\]

\[
\varepsilon_{\text{coll}} = \frac{3}{2} k T_{\text{eff}}.
\]

Equation 7

Thus we see that the DMS field causes heating of the ion, and that the rate of collisions and the energy of those collisions go up. There is also a secondary effect amplifying the temperature differences when low and high field values are compared: the reduced mobility can be lower at low field because it corresponds to a larger ion-neutral cluster, so that when the field increases, \( K_0 \) may also jump due to declustering, increasing \( \Delta T_{\text{DMS}} \). Even without this extra factor, it is possible for \( \Delta T_{\text{DMS}} \) to be in the range of 100K to 450K.

As mentioned above, for an understanding of the modifier effects in DMS, the most important aspect of \( T_{\text{eff}} \) is the DMS heating term \( (T_{\text{eff}} - T) \) which is quadratic in both bulk temperature, \( T \), and applied electric field, \( E \); these effects jointly allow ions in DMS to be strongly heated and become unclustered.

The ability of the internal energy of the ion and its clustered state to remain in dynamic equilibrium with the DMS effective temperature, as determined by the varying electric field, bulk pressure and temperature, is based on the following considerations:
1. Cluster formation rate. Ion mobility is inversely related to the ion-neutral collisional cross-section. The ion mobility cross-section was first derived by Langevin [109] and more fully developed for ion-polar molecule interactions by Su and coworkers using trajectory calculations and dipole orientation [110-113], and by Turulski and coworkers using transition state theory and other methods [107]. The long-range interaction between an ion and a polar molecule is through the ion-dipole interaction and is strongly attractive \((r^{-2}, [114, 115])\). Cluster formation, when the free energy of association is negative, can occur in a very small number of collisions, or even in a single collision, as long as the cluster contains more than a few atoms so that the rovibrational state density allows the collision energy to be retained as internal energy (see especially Lias and Ausloos [116]). Thus, cluster formation between an ion and a polar modifier can proceed at a nearly gas-kinetic rate. Redistribution and stabilization of the internal degrees of freedom within the cluster [117-119] is enhanced by hydrogen bonding between the polar modifier and the ion after they have been attracted by the long range forces.

2. Thermal equilibration rate. The cluster ion acquires kinetic energy through field acceleration between collisions but loses this energy in collisions, largely with the predominant non-polar bulk gas \((\text{N}_2\) in our case). While modifier collisions can lead to cluster formation, the more frequent bulk-gas collisions lead to modifier loss and thermal equilibration at the effective temperature, affecting both the internal state and the size of the cluster ion. This effective temperature is determined with reasonable accuracy by momentum transfer theory, as modified for polyatomic ions by Krylov [10]. The theory developed by Goeringer and Viehland [120, 121] for an analysis of ion swarms in an ion trap can be applied to follow the oscillatory motion of the dynamic cluster ion. Thus the
collision rate that determines equilibration of the cluster at the effective temperature is approximately 100 times faster than the rate that controls cluster formation (for 1% modifier). Thermal equilibration of an existing cluster ion at the effective temperature corresponding to the electric field, bulk temperature and pressure occurs rapidly because it is controlled by bulk gas collisions. Because collisions with the polar modifier occur much more infrequently, the dynamic change in cluster number from the DMS low-field period to the DMS high field can be limited by kinetic effects.

3. Saturation behavior. After the first modifier is in place, modifier shell development is controlled and limited by geometric compatibility, charge and dipole shielding, polarity localization, and hydrogen bonding. It is possible for a second strongly dipolar modifier to add to an existing cluster canceling the dipole moment of the first, but this behavior is dependent on the lock and key relationships between ion and modifier. We have observed such saturation behavior with ethyl acetate, but have not yet performed any related calculations on the decrease in cluster free energy with size. Multiple clustering makes performance less predictable, and appears to be a primary cause of the need for compound-specific method development.
As an example of the cooperative effect of gas temperature and DMS separation field in modulating the cluster number, one result from an extensive set of ab-initio calculations done by Dr. Steve Coy in collaboration with Dr. Bryan Wong for the change in free energy, enthalpy, and entropy on cluster formation. The full results with additional examples are being reported in detail in a future publication. Ab-initio structural optimizations and thermochemical calculations were performed for R-alpha-methylhistamine with and without the modifiers isopropanol or ethyl acetate. From the study, ΔG₀ values show that isopropanol is less strongly bound than ethyl acetate, much as might be expected from the difference in dipole moment values (IPA (1.560 D), EtOAc (4.325 D)). For R-alpha-methylhistamine (C₆H₁₁N₃, mw 125.17164, PubChem 156615), thermochemical values for clustering of the protonated methyl-histamine molecule with IPA were determined to be (ΔG₀ = -7.45 kcal/mol, ΔH₀ = -16.62 kcal/mol, ΔS₀ = -30.76 cal/mol/K). This compound has some structural similarity to deoxyguanosine in that it has nitrogen heteroatoms both in a 5-membered ring and in an amino group. In order to examine access to the unclustered ion configuration, and in recognition of the kinetic limit discussed above, a limit of Nₐ=3 for the maximum IPA count. Assuming K₀=1.5 cm²/V·sec, 1.5% isopropanol, ζ=0.7, the cluster number dependence both on applied electric field and on bulk gas temperature for (methyl histamine·H⁺·isopropanol) can be calculated with the very interesting result of Figure 38. The requirement of high combined field and/or bulk gas temperature for ion declustering is not unexpected because of the earlier experience with atmospheric pressure drift-time IMS [94, 105, 106, 122], but it indicates that both bulk gas temperature and DMS field are critical control parameters.
Figure 39: Using ab-initio thermochemical values, the mean number of bound neutrals is shown as a function of DMS field and transport gas temperature at 1 atm. The maximum neutral count is limited to 3 and the range to 2 shown because shell thermochemistry has not been calculated, and to direct our investigations first to the most accessible situation. These results for R-alpha-methylhistamine, which has a polar core similar to dG, with 1.5% isopropanol show that bulk gas temperature is an essential controlling parameter in modifier DMS selectivity.
We can also use these results to interpret intensity data. In Figure 37D, we saw that the DMS intensity fell off at higher DMS field values. An additional mechanism for signal loss in the higher SV range is collision-induced chemistry, either dissociation (CID) or charge detachment. CID in DMS has been reported in DMS [91] and charge detachment in systems like SF$_6$ is well known[123]. At lower DMS field, the heating is quite a bit less so that less declustering will occur, preserving the ion intensity regardless of the modifier concentration. At very high fields very high energy collisions are effective at declustering but can also be destructive, more so for larger clusters because the proton is more likely to go with the cluster of modifiers. Thus, both DMS field and modifier concentration affect the signal intensity in similar ways, as our data shows.

3.10 Conclusions

In the past few years, efforts have been made to understand and characterize the gas phase cluster formation between the analyte ion and the modifiers. However, an explicit method of characterizing the interactions and predicting the CoV shifts is not available to date. Screening modifiers against the analyte of interest at different conditions has been the most feasible way to perform method development using differential mobility spectrometry and contribute to the understanding on the use of modifiers.

Theoretical considerations for the phenomena associated with compensation voltage shifts in DMS that explain step-wise clustering of modifiers including “saturation effect” and variations in signal intensities have been proposed in this work. These results show that high transport gas temperatures increase the DMS effect and that some combinations of temperature and DMS field lead to sharp changes in cluster number with DMS SV field. Conversely, with moderate DMS
temperatures, DMS is modulating an already clustered ion, making the effect less predictable and requiring a method development phase. This process is illustrated by the approach we have taken for the selective analysis of modified nucleosides, dG-ABP and dG-PhIP, used as model analytes, from a mixture of normal nucleosides using a differential mobility-mass spectrometry-based platform. Modifier DMS at lower temperatures is generally modulating the size of an already clustered ion species for which thermochemical information is not available and few calculations have been done. We believe that further progress can be made in understanding and predicting DMS modifier performance by approaching future experimental work from the high temperature limit with modifiers of differing dipole moments and structure, and by performing calculations on the development of solvent shell clusters.
Chapter 4:

Rapid detection and quantitation of DNA adducts dG-ABP and dG-PhIP using Differential Mobility-Mass Spectrometry
4. Introduction

Exposure to harmful chemicals can cause these chemicals or their electrophilic metabolites to bind covalently to DNA forming DNA adducts. Failure of the cells to repair the chemical modifications can cause accumulation of these adducts leading to mutations which may have severe biological consequences. The linkage between DNA adduct formation and carcinogenicity has been suggested by several studies [124-129] and DNA adducts provide the direct evidence of exposure and genetic damage in cells. The genotoxic damage can be monitored by quantifying the level of these adducts which also serve as a biomarker for health risk assessment.

Over the past decades, significant efforts have been made towards the analysis of DNA adducts. Different sensitive analytical methods such as nuclear magnetic resonance, circular dichroism, fluorescence spectroscopy, laser induced fluorescence, immunoassay, electrochemical detection and capillary electrophoresis with laser induced fluorescence detection have been employed to monitor the level of DNA adducts.[130-136] Notably $^{32}$P-postlabeling[137] has been widely used for the detection of DNA-adducts because of its high sensitivity. However, this method is criticized as being non-specific and internal standards are not used to account for analyte losses during sample processing or due to other procedural inefficiencies. Mass spectrometry based methods with the ability to provide structural information has been the platform of choice for DNA adducts analysis. Our laboratory [138, 139]along with those of several other investigators[140-142] have successfully applied HPLC-MS/MS for the analysis of DNA adducts with high sensitivity and reliability.

There remains continued interest in exploring new analytical technologies for the detection and quantitation of DNA adducts, in particular with the goal of reducing clean-up steps and
improving sample throughput. Differential Mobility Spectrometry – Mass Spectrometry, which has been an area of considerable activity in our laboratory in recent years, has proved to be effective in a variety of applications that have included analysis of peptides and drugs of forensic interest. Hence investigation of its capabilities for DNA adducts analysis is a logical extension of these efforts.

Since its inception in early 1990s [72], Differential Mobility Spectrometry (DMS) has been used as a rapid gas phase ion separation/ filtration technique. Although DMS has been primarily used for separations, it has been demonstrated that its unique capability allows pre-filtering of ion species prior to mass analysis resulting in reduced noise and enhanced sensitivity.[143] Two different types of differential mobility ion filters are commonly used: the cylindrical FAIMS (field-asymmetric waveform ion mobility spectrometry) and the planar DMS. Both of them are continuous ion filtering devices and utilize high frequency asymmetric waveforms to separate ions. Schneider et al. have pointed out that FAIMS provides the potential advantage of ion focusing at atmospheric pressure because of radio frequency (RF) fields and analyzer curvature, whereas, DMS provides faster ion transit times and offers a transparent mode of operation which allows all ions to be transmitted without discrimination.[143]

CoV scans at varying SVs with different potential modifiers introduced at various rates in the transport gas are normally performed in order to identify the optimal parameters for DMS ion transmission as demonstrated in Chapter 3. Once the peak values are identified and set as the DMS parameters, ions of interest can be separated/ filtered through the DMS cell in milliseconds which makes this method very suitable for coupling to mass spectrometers. In practice, DMS provides an extra dimension of orthogonal separation/ filtration without adding to the overall analysis time.
With the stated aim to improve sample throughput by by-passing the time consuming chromatographic analysis step and possibly eliminating, or at least reducing sample cleanup steps, we report here on the application of DMS – MS toward the analysis of DNA adducts. \(N\)-(2-deoxyguanosine-8-yl)-4-ABP (dG-ABP), the deoxyguanosine adduct of the bladder carcinogen 4-aminobiphenyl (4-ABP), found in cigarette smoke, paints, food colors, hair dyes and fumes from heated oils and fuels and [79-82] Heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5]pyridine (PhIP) found in grilled meats[83] are used as model compounds. dG-ABP has been identified as major adduct in biopsy samples of human urinary bladder[144]. PhIP is a known foodborne carcinogen which has been implicated in mammary and colon gland tumors in rodents [145, 146]. These two adducts are related to lifestyles of people and monitoring their levels to determine their plausibility as cancer causing agents is important. A comparison of the DMS-MS approach with the results obtained using our current LC-MS/MS protocol is presented.

4.2 Experimental

Chemicals and Standards:

Caution: 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5]pyridine and 4-Aminobiphenyl and its derivatives are carcinogenic and should be handled carefully.

Calf-thymus DNA, nuclease p1 from \textit{Penicillium citrinum}, deoxyribonuclease 1 (DNase I) type 2 from bovine pancreas, alkaline phosphatase from \textit{Escherichia coli} (type IIIIs), ethanol, dimethyl sulfoxide (DMSO), 4-Aminobiphenyl (4-ABP) and Benzonase were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Snake venom phosphodiesterase was purchased from USB Corporation (Cleveland, OH). 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5]pyridine (N-OH-PhIP) was purchased from the Midwest Research Institute’s Chemical Carcinogen
Repository (Kansas City, MO). N-(2-Deoxyguanosine-8-yl)-4-ABP (dG-ABP) and N-(Deoxyguanosine-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (dG-PhIP) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). dG-ABP-d9 was previously synthesized and characterized in our laboratory[147]. Crude N-(Deoxyguanosine-8-yl)-2-amino-1-(trideuteromethyl)-6-phenyl-imidazo[4,5-b]pyridine (dG-PhIP-d3) was previously characterized in our lab. Formic acid solution was purchased from Fluka Analytical. HPLC grade water and methanol were purchased from Fisher Scientific.

4.3 Modification of calf-thymus DNA with 4-nitrobiphenyl
The synthesis of ABP modified calf thymus DNA was previously reported.[147] Briefly, 200 mg of 4-nitrobiphenyl (Toronto Research Chemicals (Toronto, ON, Canada)) was reduced to N-hydroxy-4-aminobiphenyl in the presence of 50 mg 5% Pd/C at 0°C. Next, 3.8 ug of N-hydroxy-4-aminobiphenyl dissolved in argon purged ethanol was reacted overnight with mild agitation at room temperature with 3.6 mg of calf thymus DNA dissolved in 5.0 ml of 10 mM sodium citrate buffer, pH 5. The aqueous DNA reaction mixture was extracted three times with 5.5 ml distilled phenol (saturated with TE buffer: 40 ml phenol + 10 ml TE buffer) followed by extraction with 5.0 mL of n-butanol three times. The DNA was then precipitated with five volumes of ice-cold ethanol and air dried. The DNA was redissolved in TE buffer to give approximately 1 mg/ml concentration.
4. 4 Cell culture and dosing of MCL-5 cells

Human lymphoblast cells (MCL-5) which are competent of emulating living systems where adduct formation and repair mechanisms undergo were used. MCL-5 cells are and sterile dimethylsulfoxide (DMSO) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). GlutaMAX™-I Supplement (200mM) and horse serum, heat-inactivated, were purchased from Gibco brands, Invitrogen Corporation (Carlsbad, CA). Custom formulated RPMI-1640 media which did not contain l-glutamine and l-histidine was custom prepared by Gibco brands, Invitrogen Corporation (Carlsbad, CA). Stock solution A was prepared by dissolving 192 mg 5-aminolevalinic acid in 5 mL sterile water and Stock solution B was prepared by adding 1 mL sterile water to the 1 g bottle of L-histidinol dihydrochloride. For the preparation of the final cell culture formulation, 500 ul of Stock solution A, 325 μL of Stock solution B, 1.5 mL of the hygromycin B antibiotic solution, 5 mL of the GlutaMAX-I™ solution and 45 mL of horse serum (9% serum in medium) were added to 46 mL of modified RPMI-1640 medium. The mixed formulation was vacuum filtered through a Corning® 500 mL sterile bottle-top 0.22 μm nylon filter system (Lowell, MA).

Cells were cultured in BD Biosciences (San Jose, CA) BD Falcon™ tissue culture flasks with 0.2 um vented caps. The tissue culture flasks were placed flat-side down in a Sanyo Scientific Incu-Safe™ incubator (Bensenville, IL) at 37\(^0\)C, 5% CO\(_2\) and 100% relative humidity. Cells were counted manually for viability on a Fisher Scientific (Pittsburg, PA) Micromaster™ II microscope with a Bright-Line® hemacytometer from Hausser Scientific (Horsham, PA).

For the dosing study, MCL-5 cells were grown in a 300 mL BD Falcon tube to an approximate concentration of 5.0 x 10\(^5\) cells/mL in modified RPMI-1640 medium which was changed every two days. In about a week, the cell density grew to approximately 1.0 x 10\(^6\) cells/mL. At this
point, an aliquot of approximately $1.0 \times 10^5$ cells/mL was saved and the rest was discarded. Variable concentrations of PhIP stock solutions (in $<2\%$ DMSO) were dosed to 50 mL portions. The dosed concentrations corresponded to 0.1 μM, 1 μM, 5 μM and 10 μM PhIP and a no dose control. After 24 hours of incubation with PhIP, the medium was removed and the cells were centrifuged at 4000 rpm. The pellet was washed with 10 mL PBS to remove residual PhIP. The cells were suspended in fresh 5 mL PBS and stored at -80°C.

4. 5 DNA quantification, enzymatic digestion and protein precipitation

DNA quantification was done using an Invitrogen Corporation (Carlsbad, CA) Quant-IT™ double strand (ds) DNA BR Assay kit with a Qubit fluorometer. Aliquots containing 2 μg DNA (dissolved in 5mM Tris-Cl/ 10 mM ZnCl$_2$) were removed for digestion and analysis for each sample point.

Calf thymus DNA was hydrolyzed similarly to a method previously described[85]. Samples were incubated at 98 °C for 3–5 min and chilled in the freezer down to room temperature. 0.3 units of nuclease P1 (0.3 units μL$^{-1}$ solution of 5 mM Tris–Cl, pH 7.4) and 3.1 KU of DNase I (1 μg μL$^{-1}$ solution in 5 mM TRIS/10 mM MgCl$_2$, pH 7.4) were then added per μg of DNA and incubated in a water bath maintained at 37 °C. After 5 hours, 0.003 units of phosphodiesterase (100 ng μL$^{-1}$ in 5 mM TRIS/10 mM MgCl$_2$, pH 7.4), and 0.002 units of alkaline phosphatase per μg of DNA were added and the mixture was further incubated at 37 °C for 18 h. Protein precipitation was done by adding five volumes of ice cold ethanol and centrifuging at 10000 rpm for 15 minutes. The samples were dried down and stored at -80°C until analysis.

From the MCL-5 cell cultures, DNA was isolated using the Qiagen Inc. Blood and Cell
Culture DNA kit (Valencia, CA). For the digestion of the DNA isolated from the MCL-5 cells, a method similar to the one described by Quinlivan and Gregory was used[148]. The enzyme cocktail consisted of the following for digesting 2 ug of DNA: 625 U of benzonase, 0.75 U snake venom phosphodiesterase (SVP) and 500 U of alkaline phosphatase (AP) diluted in 5 mL of digest buffer consisting of 20 mM Tris-HCl (pH 7.9), 100 mM NaCl and 20 mM MgCl₂. 50 ul of enzyme cocktail was added prior to digestion and another 50 ul aliquot of the same cocktail was added after 12 hours for a total of 24 hours incubation at 37°C.

Protein precipitation was done by adding five volumes of ice cold ethanol and centrifuging at 10000 rpm for 15 minutes. For the DNA extracted from the MCL-5 cells, a second protein precipitation was done with 400 uL ice cold acetonitrile. The samples were dried down and stored in -80°C until analysis.

4. 6 Instrumentation
The fundamental goals of the proposed approach to the analysis of DNA adducts by DMS-MS was tested using two different planar DMS systems, one attached to a 3-D ion trap (Thermo Finnigan LCQ Classic) (San Jose, CA) and the second interfaced to a Sciex 3000 triple quadrupole MS (Toronto, ON, Canada). The DMS filters varied only in terms of their dimensions and this comparison provided an assessment of the general effect and applicability of the DMS configuration to such analyses.
DMS-Ion Trap Mass Spectrometer:

A planar DMS developed by Sionex Corporation (now defunct) with filter gap 0.5mm high x 3.0mm wide x 10.0mm long was used for the work which sat on the heated capillary of a Thermo-Finnigan, LCQ Classic mass spectrometer. Custom software, Expert, manufactured by Sionex Corporation was used to set the DMS parameters. The SV could be varied from 0 to 1500 volts and the CoV could be varied from -43 to +15 volts under this setup.

Electrospray was performed using coated 10 um PicoTip emitter from New Objective (Woburn, MA). Samples were introduced at the rate of 300 nl/min using a Harvard Apparatus syringe pump. The desolvation gas (ultra high purity nitrogen) was introduced at a flow rate of approximately 100 cc/min into the desolvation region. External air flow of approximately 500 cc/min also merged in with the desolvation gas into the orifice of the DMS. Ethyl acetate modifier was introduced into the desolvation region along with the nitrogen gas. The electrospray was held at 2 kV throughout the analysis.

DMS-Triple Quadrupole Mass Spectrometer:

A prototype DMS-Triple Quadrupole mass spectrometer built by AB Sciex which has the removable DMS filter online in front of the first quadrupole was used for this study. The dimensions of the DMS were 1 mm x 10 mm x 15 mm. A modified version of the software Analyst version 1.5 which included parameters for SV and CoV was used. The SV could be varied from 0 to 5000 volts and the CoV could be varied from -100 to +100 volts under this setup. Electrospray was done using stainless steel 30 µm nebulized emitter from Proxeon (Odense, Denmark). Samples were introduced at the rate of 400 nl/min using a Harvard Apparatus syringe pump. Electrospray was held constant at 3500 volts throughout the analysis.
Modifiers were introduced into the curtain gas (nitrogen, 500 cc/min) using a second Harvard Apparatus syringe pump at 0.6% v/v.

4.7 Results and Discussion

The first step in the protocol of DNA adducts analysis involves digestion of the DNA to mononucleosides for the liberation of DNA adducts. Once the digestion is complete, the sample is subjected to a protein precipitation step and the supernatant is dried down. As shown in the left hand portion of the flow chart in Figure 40, after the protein precipitation, solid phase extraction is required before LC-MS analysis in order to remove residual matrix components. This is typically accomplished by trapping of the analyte in the stationary phase and eluting it with high percentage of suitable organic solvent. Solid phase extraction (SPE) of DNA adducts is challenging because of the potential for analyte losses during the cleanup, given the relative proportions of typical adduct levels (pg) to the smallest available SPE bed masses (mg). It has also been pointed out that polypropylene frit material from the SPE cartridges can leach out and cause ion suppression problems during the analysis [149]. These factors alone suggested the consideration of DMS-MS as an alternative option that may at least by-pass some of the limitations imposed by the traditional LC-MS protocol, as described below.
Figure 40: Comparison of the workflow for the analysis of DNA-adducts by LC-MS and DMS-MS
4. 8 Detection of dG-ABP in calf thymus DNA using DMS-Ion Trap MS

While LC-MS has proved to be highly effective in meeting many of the requirements like sensitivity, sample requirements for the analysis of DNA adducts, it is clear from the above discussion that this protocol is time consuming which can create a bottleneck when analysis of multiple samples is involved. Given our previously demonstrated success of the utilization of DMS-MS for the rapid analysis of forensic samples[9], we set as a goal the reduction of the overall analysis time by eliminating at least some of the steps associated with the LC-MS protocol. In accordance with this objective we sought to determine whether dG-ABP could be detected by DMS-MS in a DNA digest immediately after protein precipitation as indicated in the right hand portion of Figure 40. To establish the feasibility of this goal, the initial investigation was conducted using a DMS filter interfaced to an LCQ Classic ion trap. For this purpose, 100 picograms of dG-ABP was spiked into 2 micrograms of a calf thymus DNA digest and the sample was reconstituted to a total volume of 100 microliters (70% MeOH/30% H₂O/0.1% CH₂O₂ v/v/v) and infused at the rate of 300 nl/min. Data were acquired under two different conditions: i) DMS-transparent i.e., no SV and CoV applied and ii) DMS-on, ethyl acetate modifier. For the DMS-on condition, the separation voltage (SV) was set at 1500 volts and the compensation voltage (CoV) was scanned from -43 volts to +15 volts. After the CoV scan had been performed, the parent ion mass (m/z 435) for the analyte dG-ABP was extracted from the total ion chromatogram. The CoV corresponding to the apex of the extracted ion chromatogram was established as the CoV for selective dG-ABP transmission in this system and introduction into the ion trap MS, thereby preventing unwanted matrix ions from getting into the trap.
In Figure 41A, it can be seen that when the DMS is in transparent mode and without the use of modifier, the signal for the analyte of interest, dG-ABP (m/z → 435) is obscured by background ions. The spectrum is dominated by other species from the matrix which are most likely multiply charged species. When the DMS is turned on with ethyl acetate modifier introduced via the drift gas, the dG-ABP adduct can be selectively introduced into the trap with a significant reduction in background chemical noise (Figure 41B). The reduction of these species can be attributed to the introduction of the modifier into the drift gas consistent with phenomena previously observed[7]. In addition, the increase in signal intensity of dG-ABP from 3.25×10^3 to 1.93×10^4 along with the indicated noise reduction are significant and demonstrate the usefulness of DMS to remove matrix ion interferences and improve sensitivity by selectively filling the trap. The dG-ABP adduct detected in these preliminary experiments corresponded to approximately 4 modifications in 10^5 nucleosides based on the use of 2 micrograms DNA for the analysis.
Figure 41: Mass spectra of 100 pg dG-ABP in 2 ug calf thymus DNA under two different conditions: (A) DMS-transparent, No modifier (B) DMS-on, ethyl acetate modifier
4. 9 Quantitation of dG-ABP in Calf Thymus DNA by DMS-triple quadrupole MS

Triple quadrupole mass spectrometers, which can operate in the multiple reaction monitoring (MRM) mode are widely used for quantification purposes. In the case of complex matrices, ion suppression during the electrospray process and possibility of interferences necessitate the use of a clean-up before MS analysis. In the present application addressed here, DMS functions as a post-electrospray ion separation/ filtration technique, analogous to that of an HPLC. Given the successful demonstration of the capability of the DMS to filter out the matrix interferences when combined with an ion trap, we examined next the use of a DMS-Triple Quadrupole platform to explore the possibilities of quantitation of DNA adducts in a matrix of digested DNA after a simple protein precipitation step. Samples were introduced to the DMS-MS platform under nanoelectrospray conditions.

Prior to sample analysis, the instrument parameters for the DMS-MS were optimized by infusing 25 pg/ul dG-ABP in 70% MeOH/30% H2O/0.1% CH3O2 v/v/v. The instrument was operated in the MRM mode and the transition from the precursor ion [M+H]+ of dG-ABP (to the product ion [M+H-116]+ (m/z 435 \(\rightarrow\) m/z 319) was monitored at unit resolution in both Q1 and Q3. The electrospray was set at 3500 Volts. Declustering potential (DP) was set at 53 volts, focusing potential (FP) at 190 volts, entrance potential (EP) at 10 volts, focusing lens 1 (IQ1) at -10.5 volts, prefilter (ST) at -18.7, collision energy (CE) at 19 volts and collision cell exit potential (CXP) at 18.8 volts respectively.

For the DMS analysis of dG-ABP adducts, two modifiers: i) isopropanol and ii) ethyl acetate were screened at different modifier concentrations and at different separation voltages (SV) as demonstrated in Chapter 3. For the final analysis, ethyl acetate was established as the modifier of choice and was introduced at 0.6 % of the curtain gas flow rate (v/v). With the separation voltage
(SV) set at 3500 volts, the compensation voltage (CoV) was scanned from -30 volts to +10 volts. As shown in Figure 42, in the presence of 0.6% ethyl acetate modifier the parent ion of dG-ABP was shifted from a CoV=0 to a value of CoV= -3.8V with concurrent removal of the matrix components and this value was used for all subsequent analyses.

**Figure 42**

(A) Normalized extracted ion profile showing CoV shifts for dG-ABP with and without ethyl acetate modifier at SV values of 0 and 3500 volts and (B) MRM transitions monitored for dG-ABP and internal standard
4. 10 Calibration curve of dG-ABP

The calibration curve for dG-ABP was prepared in matrix matched calf thymus DNA. Two micrograms of DNA was used for each point. Isotopically labeled dG-ABP-d9 was used as the internal standard. For the internal standard, the transition from the parent ion [M+H]$^+$ of dG-ABP-d9 to the product ion (m/z 444 $\rightarrow$ m/z 328) [M+H-116]$^+$ at unit resolution was monitored. A seven point calibration curve across a concentration range [10 pg - 250 pg] was generated in triplicate and is presented in Figure 43.

![Calibration curve of dG-ABP in calf-thymus DNA. The error bars represent standard deviations of triplicate analysis (Inset shows linearity across lowest three points in the calibration curve)](image)

**Figure 43:** Calibration curve of dG-ABP in calf-thymus DNA. The error bars represent standard deviations of triplicate analysis (Inset shows linearity across lowest three points in the calibration curve)
Y-axis represents the ratio of the product ion intensities of dG-ABP and dG-ABP-d9 and the X-axis represents the DNA modifications present in the form of dG-ABP per $10^7$ nucleosides analyzed. A linear regression analysis was done in Excel for the seven points used to get a $R^2$ value of 0.9914. To verify the linearity at the lower portion of the calibration curve, a linear regression analysis was performed on the first three points (Figure 43 inset) which gave a $R^2$ value of 0.9973, very much in line with that of the complete curve. The limit of detection (LOD) for the analysis was determined to be 10 dG-ABP adducts in $10^7$ nucleosides with the LOQ represented by the lowest point in the calibration curve that was determined to be 38 modifications in $10^7$ nucleosides.

To assess the ability of the DMS to selectively filter background contaminants, a blank sample of the calf thymus DNA digest was examined in the DMS-transparent mode and, as shown in the first bar of Figure 44, the matrix components produced a strong signal coincident with that of the MRM transition of the adduct. The bulk of this matrix interference was subsequently removed by turning on the DMS filter (middle bar, Figure 44), which reduced the background ion intensity by almost twenty fold. For comparison, the signal for the LOD (5.8 femtomoles) is shown and is based on the time over which the data were acquired during the infusion of the specific sample.
Figure 44: Removal of interference using DMS. The signal from blank matrix CT (calf-thymus) DNA was removed in DMS on mode to produce a linear calibration curve using ethyl acetate as the modifier (A) Signal from blank calf thymus DNA in the DMS transparent (B) Signal from blank calf thymus DNA with DMS on (C) Signal from 5.8 femtomoles of the analyte in calf thymus DNA with DMS on

It should be noted that an attempt to generate a calibration curve for dG-ABP in the DMS-transparent mode using the same sample was unsuccessful. While MRM signals could be seen for analyte and the internal standard, the ratios were skewed throughout the seven points indicative of matrix ion interferences. Linear regression analysis performed in MS Excel, yielded a $R^2$ value of 0.6437 (graph not shown). Clearly, the ability of the DMS to remove the interferences to produce a linear calibration curve for an analyte which is present at the ppm
level in a complex matrix is significant and this opens the possibility of using DMS as a high throughput quantitative platform for adduct quantitation.

To further demonstrate the accuracy and precision of the calibration curve obtained in the DMS-on mode, two quality control points were evaluated in triplicate over a period of two days. The values were calculated from the calibration curve based on the observed analyte to internal standard ratio.

Table 2 Evaluation of quality control points for dG-ABP calibration curve in the DMS on mode

<table>
<thead>
<tr>
<th>Modifications in 10’ nucleosides</th>
<th>Average Analyte/Int. Std</th>
<th>Average precision % RSD</th>
<th>Average % accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>0.67</td>
<td>5.8</td>
<td>98.5</td>
</tr>
<tr>
<td>346</td>
<td>1.62</td>
<td>1.6</td>
<td>106.5</td>
</tr>
</tbody>
</table>

4. 11 Quantitation of dG-ABP in Modified CT-DNA: Comparison of DMS-MS/MS and LC-MS/MS Platforms

Over the past several years LC-MS/MS has established itself as the premier method for the analysis of DNA adducts. It was thus necessary to compare the DMS-MS quantitation approach with existing LC-MS methodology. For the analysis of dG-ABP, a validated method employing a small molecule microfluidic chip interfaced with an Agilent 6330 Ion Trap mass spectrometer has been recently published from our group [139] and used to assess the significance of the presence of this adduct in several chemoprevention studies.[150, 151]

For this comparison, calf thymus DNA was reacted with N-OH-4-ABP as described in the experimental Section. Internal standard was added to a 2 µg portion to account for any losses
during sample preparation and the DNA was digested down to mononucleosides. After protein precipitation, the supernatant was dried down and stored at -80°C until analysis. Just prior to analysis, the sample was reconstituted in 100 microliters of 70% MeOH/30% H₂O/0.1% CH₂O₂ v/v/v and vortexed for one minute. The sample was introduced to the DMS-MS platform at the rate of 400 nl/min by infusion. For the analysis, the MS parameters set were the same as the ones used for the preparation of calibration curve. The product ions for dG-ABP and dG-ABP-d9 were monitored in the MRM mode and the adduct level was calculated based on the ratio observed.

Because preliminary analysis of the sample suggested the presence of a significant level of DNA adduction, three random samples were prepared blindly by diluting the modified DNA with a blank ct-DNA digest. Each sample was then analyzed in triplicate by both DMS-MS/MS and the previously validated LC-MS/MS. The results are presented in Table 3 and show excellent quantitative agreement between the two platforms when data are compared at the same linear segments of their respective calibration curves. This is evidenced by the numbers shown for Samples 1 and 2 which are within 10% of each other. For Sample 3, the variation (176 ± 8 vs. 137 ± 13 per 10⁷ normal nucleosides) may be attributed to the fact that the dG-ABP adduction level computed by LC-MS is based on the extrapolation of the calibration curve; the analyte to internal standard ratio observed for this point was well above the highest point in the LC-MS curve. Most importantly, however, the modifications computed by the two different methods are in the same order of magnitude and are reasonably close to each other. Given the nature of complexity of the samples, we concluded that the observed difference in adduct levels might also be a result of sample preparation steps.
Table 3 Quantitation of dG-ABP in Calf Thymus DNA (# Adducts in 10⁷ Normal Nucleosides) by DMS-MS and LC/MS

<table>
<thead>
<tr>
<th>Sample</th>
<th>DMS-MS/MS</th>
<th>LC-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55 ± 4</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>94 ± 4</td>
<td>99 ± 9</td>
</tr>
<tr>
<td>3</td>
<td>176 ± 8</td>
<td>137 ± 13</td>
</tr>
</tbody>
</table>

After using the DMS-MS platform for the analysis of dG-ABP adduct and comparing the quantitation with existing LC-MS method, we wanted to evaluate this platform further for the analysis of other adducts as well. dG-PhIP was chosen as the model analyte for this purpose.

4.12 Quantitation of dG-PhIP in MRM mode using DMS-MS

For dG-PhIP analysis, the instrument parameters for DMS-MS analysis were optimized by infusing 50 pg/ul dG-PhIP in 70% MeOH/30% H₂O/0.1% CH₃O₂ v/v/v. The instrument was operated in the MRM mode and the transition from the parent ion [M+H]⁺ of dG-PhIP (m/z→490) to the product ion (m/z→374) [M+H-116]⁺ was monitored at unit resolution. The electrospray was set at 3500 Volts. Declustering potential (DP) was set at 76.5 volts, focusing potential (FP) at 300 volts, entrance potential (EP) at 10.4 volts, focusing lens 1 (IQ1) at -10.5 volts, prefilter (ST) at -19.7, collision energy (CE) at 29.8 volts and collision cell exit potential (CXP) at 26 volts respectively.
Three modifiers: i) Isopropanol ii) Ethyl acetate iii) 1-butanol were screened for use in the dG-PhIP analysis at different modifier concentrations and at different separation voltages (SV) as demonstrated in chapter 3. Isopropanol at 0.6% of the curtain gas (v/v) was established as the modifier of choice. The SV was set at 3500 V and the CoV was set at -1.8 V for dG-PhIP analysis.

Figure 45 A) Extracted ion chromatogram showing CoV shifts for dG-PhIP with and without Isopropanol modifier at SV values of 0 and 3500 volts and (B) MRM transitions monitored for dG-PhIP and Internal standard
4. 13 Calibration curve of dG-PhIP

Two micrograms calf thymus DNA was used as the matrix for preparation of the calibration curve for dG-PhIP and the adducts were calculated per microliter infused. Isotopically labeled dG-PhIP-d3 was used as the internal standard. For the internal standard, transition from the parent ion [M+H]$^+$ of dG-ABP-d9 (m/z→493) to the product ion (m/z→377) [M+H-116]$^+$ at unit resolution was monitored. The ratios of the intensities of the products ions of the analyte and internal standard were recorded and a nine point calibration curve was analyzed in triplicate. A linear regression analysis was done in MS Excel to get a $R^2$ value of 0.9965. To verify the linearity at the bottom of the calibration curve, a linear regression analysis was performed on the first four points (Figure 46 inset) which gave us a $R^2$ value of 0.997.

![Figure 46 Calibration curve of dG-PhIP in calf-thymus DNA. The error bars represent standard deviations of triplicate analysis](image-url)
From the quality control perspective, two points were evaluated against the calibration curve prepared.

### Table 4: Evaluation of quality control points for dG-PhIP quantitation

<table>
<thead>
<tr>
<th>Modifications in 10⁷ nucleosides</th>
<th>Average Analyte/Int. Std</th>
<th>Average precision % RSD</th>
<th>Average % accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
<td>2.42</td>
<td>2.52</td>
<td>109.1</td>
</tr>
<tr>
<td>426</td>
<td>11.2</td>
<td>1.59</td>
<td>108.5</td>
</tr>
</tbody>
</table>

### 4.14 Detection of dG-PhIP from procarcinogen PhIP dosing study on MCL5 cells

To demonstrate that the DMS-MS platform is amenable to study DNA-adducts in biological systems as well, a dosing study was conducted using the MCL-5 cell line. MCL-5 cells are metabolically competent human lymphoblast cells and dosing study on this cell line realistically represents metabolic activation for the dosing of procarcinogens under normal physiological conditions. P450 enzymes present in the cells can metabolize procarcinogens like PhIP under normal cell environment and the active metabolite can form covalent adducts with the DNA bases.

The DNA isolation and quantitation were performed as mentioned above. Prior to digestion, internal standard was added and the DNA was digested down to mononucleosides. Protein precipitation was then carried out and the supernatant was dried down in the speed vacuum. Prior to analysis, the samples were reconstituted in 100 microliters 70% MeOH/30% H₂O/0.1% Formic acid v/v/v and vortexed for two minutes. For analysis, SV was set at 3500 V and the CoV was set at -1.8 V. Isopropanol was introduced as the modifier at the rate of 0.6%.
The dG-PhIP modifications from the procarcinogen dosing study on MCL-5 cells were detected using the DMS-MS platform and the results are presented in Figure 47. There were no modifications detected in the no dose control. The dG-PhIP modifications detected in the 1 uM PhIP dosing concentration is almost two times the modifications detected in the 0.1 uM PhIP dosing concentration. There is about a half fold increase in the formation of adducts from 1 uM to 5 uM PhIP dosing concentration. However, there is a decrease in the number of dG-PhIP adducts formed from 5 uM to 10 uM PhIP dosing concentration. The error bars in Figure 47 represent standard deviations of triplicate analyses. From this study, a linearity between dosing concentration of the procarcinogen PhIP and the formation of the dG-PhIP adducts was not established.
4.15 CONCLUSIONS

The data presented demonstrate the successful application of a DMS-MS platform for the quantitation of DNA adducts using as model analytes the deoxyguanosine adduct of the bladder carcinogen 4-ABP and heterocyclic aromatic amine PhIP. DNA adducts formed after biological processes: dG-ABP formed after modification of calf thymus DNA and dG-PhIP formed after dosing experiments were successfully detected and quantified. In the field of quantitative bioanalytical chemistry, the best way to evaluate a new system or method is to directly compare it with an existing validated method. Significantly, in this preliminary investigation there is strong coincidence of the results obtained by DMS-MS/MS with those produced in the analysis of the same samples using a validated LC-MS/MS protocol.

In contrast to the time consuming LC-MS/MS protocol, in the current work, the lyophilized samples (after protein precipitation) were reconstituted and directly analyzed by DMS-MS. With DMS on, the analyte of interest can be selectively introduced into the mass spectrometer by setting the CoV to a unique voltage for that particular analyte which has allowed us to bypass the liquid chromatography step completely. Once the parameters for DMS-MS analysis were set, data were acquired in just 30 seconds and since DMS is a continuous method, subsequent sample can be introduced without the need for re-equilibration. It should be noted that the calibration curve using the DMS-MS platform was completed in approximately 3 hours as opposed to the whole day needed for the construction of the calibration curve by LC-MS. The time savings presented by the use of DMS is significant and offers great potential for the improvement of sample throughput for the analysis of DNA adducts.

It should be noted that in the current work utilizing the DMS-MS platform, we processed only 2 µg of DNA per analysis and in terms of absolute sensitivity, we only utilized the equivalent of 4
ng of DNA during the ca. half-minute period of data acquisition. These detection and quantification levels are already within the range of modifications encountered in routine *in vitro* applications. Future efforts will focus on addressing ion suppression effects, likely introduced by contaminants or other impurities still present in the sample after the protein precipitation. Incorporation of an online sample enrichment/clean-up step prior to electrospray should help reduce the sample complexities encountered in DNA adduct analysis and it should also improve the sensitivity of the DMS-MS platform to detect lower levels of modification. The added benefits of the DMS can be realized with more sensitive mass spectrometers such as commercially available AB Sciex SelexION integrated on a 5500/6500 triple quadrupole. With improvements in sensitivity, DMS-MS holds great potential establishing itself as the high throughput platform for studies in the field of DNA adducts.
Chapter 5:
Conclusions
&
Future Directions
5.1 Conclusions

Chapter 1 of this thesis introduced ion mobility and three different kinds of ion mobility which are predominant in the field today: drift tube ion mobility spectrometry; travelling wave ion mobility spectrometry; and differential ion mobility spectrometry. Out of the three ion mobility techniques, only the differential ion mobility offers continuous ion transmission and is highly orthogonal to the mass spectrometer. Due to the small size of the filter, the DMS can be fitted on any mass spectrometer. The fundamentals of differential ion mobility were presented and the $\alpha$ parameter, which is the main differential ion mobility parameter was introduced. The cluster-decluster model is the accepted working theory for differential mobility in the presence of gas phase modifiers and is widely corroborated by experimental evidence, which was also described in chapter 1.

A historical perspective on the development of DMS as compiled by Dr. Erkinjon Nazarov has been included in the introduction section. Initial works on the DMS were done in the former Soviet Republic and early literatures on DMS are hard to find. Dr. Nazarov has been an important person to the development and commercialization of DMS in the United States and he has been closely involved with the development of DMS-MS platform in the Vouros Lab at Northeastern University as well. A brief history on development of the DMS-MS technology in the Vouros Lab is presented as well. The Vouros Lab has been in the forefront of this technology and important fundamental considerations, theories and many mass spectrometry based applications have been developed as a result of research done here.

Some recent DMS-MS examples from leading researchers in the field have been included to demonstrate applications to challenging problems in analytical chemistry and also to present where the development of DMS-MS in the analytical field stands today. To demonstrate the
applicability of the DMS-MS platform for qualitative and quantitative analysis, Chapter 1 ends with two applications performed in the lab with DMS-MS. The first one involves rapid separation of closely related species: harmine and harmaline, analyzed directly from seed extract without any additional sample preparation step. And the second one demonstrates rapid quantitative abilities of the DMS-MS platform as applied to quantitation of Cyclosporin A in media, also done without sample clean-up steps.

Chapter 2 of this thesis presented the use of differential mobility as a pre-filter to remove matrix ion components and improve a trap’s qualitative and quantitative performance. Space charges affect the performance of an ion-trap which can be exacerbated by introduction of matrix ion components during analysis especially when dealing with biological matrices causing premature trap saturation. DMS has emerged as a powerful tool for ion filtration and in combination with an ion trap, it increases the storage capacity of the trap by filtering out the unwanted matrix ions and filling the trap selectively with the analyte(s) of interest. Once the optimal parameters (Separation Voltage, Compensation Voltage, Modifier of Choice) were identified, the DMS-MS platform could be exploited to perform rapid quantitation without the need for lengthy chromatography steps.

Differential mobility spectrometry is an ambient ion separation/ filtration technique which is a post-electrospray continuous method. As emphasized earlier, a stable electrospray is needed for the DMS to carry out its functions. When urine samples were analyzed right after dilution in mobile phase, a stable electrospray profile could not be achieved, so benzoylecgonine (BE) samples were extracted from urine by SPE. When the samples were introduced into the mass spectrometer for analysis, components from the matrix dominated the spectra when the DMS was in the transparent mode. With DMS-on mode, the analyte of interest BE could be selectively
introduced into the trap while removing the matrix ion components. Using DMS as a prefilter, a series of experiments were done using the AGC-on feature and varying the trap fill times.

Considerations were given to signal intensities and resolution. Without the DMS filter, the trap was saturated by matrix ions building up space-charge effects which in turn impacted spectral quality and precursor ion isolation for further MS/MS analysis. In DMS-on mode, the prefiltering allowed the use of an extended fill time of 500 ms without any deleterious effect on the mass spectrum. For quantitative analysis, the use of DMS allowed 5-10 fold lower detection level when compared to DMS-transparent mode and improved the linearity of the calibration curve over the whole concentration range analyzed as well. The quantitation method bypassed traditional chromatography and calibration curves were prepared with DMS-on in under 2 hours.

The role of modifiers to assist DMS-MS analysis has been well established. Different small polar molecules have been screened and successfully used for the same. Cluster-decluster model has been accepted as the working principle for DMS with the use of modifiers. However, a method to effectively predict modifier behavior and effective cross-sectional area is lacking. In Chapter 3, a systematic evaluation of the modifier effects from a method development perspective to enable quantitative analysis has been presented through the use of dG-ABP and dG-PhIP as model analytes. Optimizing the CoV shifts, peak shapes and peak intensities for quantitative applications have been deemed essential. For analytes which are present in higher concentration or for qualitative analysis, a modifier capable of shifting the CoV values to enable separation or moving away the analyte of interest away from the matrix interferences should suffice. However, for quantitative applications and especially for trace quantities of analytes, a rigorous method development phase examining effects of modifiers on peak shapes and signal intensities in addition to CoV shifts is necessary. The method development protocol presented in this chapter
is applicable to other quantitative applications involving the DMS-MS platform and it can serve as a framework for future applications.

In addition to the method development protocol, several theoretical considerations have been developed in Chapter 3 to better understand the modifier and heating effects of the DMS. “Saturation effect” observed in the CoV shifts of dG-ABP with ethyl acetate modifier led to proposal of equilibrium equations as presented in Chapter 3. It has been proposed that the addition of modifiers is a step-wise process where ion-dipole interaction is the primary driving force for cluster formation. Hydrogen bonding may play a role in subsequent additions/arrangements of modifier molecules to the analyte ion cluster. The change of signal intensities at various DMS settings with different modifiers as presented in Chapter 3 led us to consider thermochemical kinetics. It was realized that the heating effect produced by the DMS at high field is significant and it can dramatically affect the effective cluster size. Results from theoretical modeling presented in Chapter 3 are in agreement with the observed behavior. At very high temperatures, the DMS effect can drastically reduce the number of bound neutrals, however, at moderate DMS temperatures, the formation of cluster is more significant and advantages can be taken of this state for DMS based analysis.

Chapter 4 presented application of the DMS-MS platform for the analysis of DNA adducts, which are biomarkers for DNA damage. dG-ABP and dG-Phip were successfully targeted in a matrix of excess unmodified nucleosides after DNA digestions using a cocktail of enzymes. Sample requirements have been reduced to 2 ug of DNA per analysis and detection limits of 10 modifications in $10^7$ nucleosides have been achieved. The ability of DMS to selectively filter a targeted DNA adduct of interest into the mass spectrometer for quantitation without laborious sample clean up steps is a major improvement in the field of adduct analysis. To evaluate if
offline SPE helped in reaching a lower detection limit, the samples were processed through clean-up steps and analyzed with the DMS-MS platform. The yields from offline SPE were not uniform as evidenced by poor linearity in the calibration curves. The modified DNA is a tiny fraction of total starting material of two micrograms and they were likely getting lost in the bed material of the SPE cartridge which have bed weights of several milligrams. As pointed out in Chapter 4, the frit material can elute and interfere with the adduct analysis. We anticipate that an automated online cleanup capable of handling low picograms of analyte should help reduce the sample complexity and also allow sample concentration for achieving lower detection limits.

In comparing the results obtained by the DMS-MS method, it is acknowledged that the current microchip based LC-MS methodology involves a highly sensitive method enabling detection of 2 adducts per 10⁸ normal nucleosides, i.e. a 100-fold higher sensitivity. This comparison however, should also be considered in the context of the information given in Chapter 4 which outlines the entire sample preparation schemes for the respective analyses. As presented in the chapter, after protein precipitation, the sample is lyophilized, purified by SPE, lyophilized for the second time and reconstituted in an appropriate solvent for LC-MS analysis. Solid phase extraction and the second lyophilization procedures can consume well over an hour while the chromatography can take up to twenty minutes which does not include LC re-equilibration that can take an additional 3-5 minutes. However, with DMS the digested samples can be electrosprayed without any further clean-up and the DNA adduct can be selectively introduced by setting the CoV and SV parameters. Subsequent samples can be introduced right after without any delay. With further improvements in the platform through incorporation of an online clean-up step and use of a more sensitive mass spectrometer, the DMS-MS can establish itself as the preferred analysis method for DNA adducts.
**Future Directions**

**5. 2 Application in Radiation Biodosimetry:**

The potential threats from radiological incidents have necessitated development of a rapid, non-invasive assay for detecting radiation exposure. A proposal is being developed between Northeastern University, Georgetown University and Draper Labs which incorporates exploration of DMS-MS for rapid targeted analysis for biomarkers of radiation damage and some preliminary data is presented. The final goal of the project is to develop analytical methods for high-throughput screening for the general public in case of a radiation disaster.

The relationship between radiation metabolomics and the role in biodosimetry has been established[152]. Efforts are underway to identify analytes that can be used as radiation biomarkers for mass screening purposes. A recent study from Fornace Group at Georgetown University using LC-MS performed metabolomic profiling of patients undergoing total body irradiation and has identified metabolites in urine that show a dose response. Prior efforts from the same group have identified several biomarkers that showed dose responses after exposure to radiation in mouse models[153, 154]. LC-MS is the platform of choice for metabolomics studies; however one of the key challenges is the analysis of polar analytes which elute early and are often not well separated in a traditionally used C-18 column. A rapid mass spectrometry based method that requires minimal sample preparation and can selectively target the biomarkers while providing high-throughput can be highly valuable in such applications. Differential Mobility-Mass Spectrometry (DMS-MS) allows rapid quantitation of targeted analytes without the need for lengthy chromatography steps and shows significant promise for biodosimetry studies. Several analytes identified as potential biomarkers in biodosimetry studies are polar compounds which can be rapidly separated and selectively targeted using DMS-MS.
In collaboration with the Fornace Lab from Georgetown, efforts are underway at Vouros Lab at Northeastern to study the feasibility of DMS-MS for biodosimetry studies. In an initial study, a mixture of structurally different compounds identified as potential biomarkers was well separated by DMS and is presented in the figure below.

**Figure 48** Separation of three component biomarkers by DMS in the presence of ethyl acetate as modifier

The corresponding mass spectrum obtained is presented in **Figure 49**. The top panel gives the total ion profile. Creatinine has a prominent signal and is the most dominant peak in the corresponding spectrum. However, without DMS, trimethyllysine and acetylcarnitine have weak signals and are dominated by other peaks in the spectrum making identification difficult.
However, when the DMS is turned on, the analytes of interest can be selectively introduced in the mass spectrometer as shown in Figure 49.

![Figure 49](image)

**Figure 49** Corresponding mass spectra for the total ion chromatogram and extracted ion chromatograms for three component biomarker mixture

Prior experience in working with biological fluids has suggested that a clean-up step prior to analysis facilitates electrospray. For biological samples in urine, it was soon realized that a SPE step was necessary to generate stable electrospray. Our efforts were then focused on a class of compounds that could be extracted from urine by a single SPE method. Three acylcarnitines that share similar core structure and vary in the length of carbon chains have been identified as an
important reference point for biodosimetry study. The same class of compounds is targeted in urine using the DMS-MS platform as a preliminary study. Demonstration of rapid analysis using DMS-MS in urine represents realistic challenges in utilizing the same for future biodosimetry studies. Use of nanoESI provided optimal sensitivity. Once the appropriate combination of compensation voltage (CoV) and separation voltage (SV) is determined, the targeted analytes along with their isotopically labeled internal standards can be selectively introduced into the mass spectrometry for rapid analysis.

Three acylcarnitines: acetylcarnitine, octanoylcarnitine and decanoylcarnitine were extracted from urine by SPE. The samples were reconstituted in mobile phase and analyzed using the DMS-MS platform. In the transparent mode, the DMS offers no selectivity and the three compounds of interest could not be separated. The extracted ion chromatograms of the three peaks overlapped with each other at zero compensation voltage (CoV) value as can be seen in Figure 50A. When the separation voltage (SV) value was increased and set to 3500 volts, the three analytes shifted away from the zero CoV towards negative CoV values and were separated (Figure 50B).
Figure 50: Analysis of acylcarnitines in urine after SPE using DMS-MS. (A) The three acylcarnitines analyzed: acetylcarnitine, octanoylcarnitine and decanoylcarnitine were not separated under DMS transparent conditions (B) With application of the Separation Voltage (SV), the three analytes moved away from a CoV value of zero and were separated. (C) Mass spectra showing targeted analysis of acetylcarnitine (1ng/ul). Top: Mass spectrum is dominated by components from the matrix. Bottom: DMS allows selective introduction of acetylcarnitine (m/z $\rightarrow$ 204) into the mass spectrometer and the mass analysis was performed in 30 seconds.

To demonstrate the selectivity of the DMS, Figure 50 C shows corresponding mass spectrum for the analysis of 1 ng/ul acetylcarnitine. In DMS transparent mode, the mass spectrum is dominated by matrix ion components and the target analyte signal is surrounded by noise. However, setting the appropriate DMS values of CoV and SV allowed selective introduction of
acetylcarnitine and resulted in significant noise reduction. The total mass analysis time was thirty seconds. Application of the DMS to selectively target biomarkers opens the possibility of applying this platform for biodosimetry studies in the future.

5.3 Proposed improvement in DMS software
Two softwares have been used for controlling the DMS parameters over the research work done. The first one “Expert” software was developed by Sionex Corporation and is a stand-alone controller for the DMS used with the Thermo Finnigan classic ion trap mass spectrometer. For the DMS used with the triple quadrupole, a modified version of the software “Analyst” (Version 1.5) was used which included parameters for the DMS operation as well. Both softwares were designed for use in primarily CoV scanning mode i.e. two CoV points are specified and the DC voltage is ramped at specified intervals across those two points to produce a DMS spectrum.

The software interfaces are presented in Figure 51 and Figure 52. Over the course of this research, it has been identified and demonstrated that CoV stepping mode, i.e. fixed values of SV and CoV can be used to conduct rapid analysis once the optimal parameters are known. In the quantitative work presented in this thesis, CoV scans were performed initially to identify optimal CoV apex values and the value was used for subsequent analysis.
Figure 51 Software interface on the Analyst software (version 1.5) as used on the AB Sciex 3000 DMS-triple quadrupole mass spectrometer used for the research. The DMS parameters are integrated in the Analyst software. Only one CoV value (circled) can be used for normal mode of operation and CoV scanning can be done by specifying two CoV points using the Edit Ramp feature in the software.

MRM (Mutiple Reaction Monitoring) or SRM (Selected Reaction Monitoring) is the preferred method of analysis for quantitative analysis. Monitoring of product ions provides additional confidence in accurate identification and quantitation of analytes. The time spent by the mass spectrometer for individual MRM transition is known as the dwell time. This data acquisition parameter can be specified and increasing the dwell time generally provides better sensitivity. Use of shorter dwell times allows larger number of analytes to be analyzed, however, it comes at the cost of sensitivity; targeting analytes present in low concentrations could be a challenge[155, 156].
Figure 52 Expert Software interface as used on the Sionex DMS-Thermo Finnigan classic ion-trap used for the research. DMS is controlled by the separate software “Expert”. There are two fields for CoV values (circled) which are generally used for scanning mode.

DMS can be used to filter/ separate ion species in milliseconds regime. Given the benefits of the DMS established over the course of this research work such as improvement in signal intensities, reduction in background noise, removal of interfering matrix ions etc, the “DMS dwell time” can be effectively coupled with “mass spectrometric dwell time” to create a powerful analytical platform capable of performing real time analysis in <1 second. An option in the DMS software to enable multiple CoV fields in stepping mode and synchronization with the mass spectrometer software parameters will enable an analyst to target multiple analytes in a mixture and/or complex matrix in seconds’ timeframe.
Epilogue:

The work presented in this thesis demonstrates successful analytical applications of the DMS-MS platform. In the early years of DMS development, most applications were limited to mixtures of compounds and applications involving complex matrices were often done in conjunction with liquid chromatography. In recent years, DMS-MS is being increasingly applied to complex matrices to solve analytical challenges. DMS has emerged as a powerful tool for ion filtration and in combination with a mass spectrometer in MS/MS mode; the DMS-MS/MS platform can be utilized for rapid quantitative purposes. Use of nano-ESI facilitates the desolvation of the ions and provides optimal sensitivity. Once the optimal parameters (Separation Voltage, Compensation Voltage, Modifier of Choice) have been identified, the DMS-MS platform can be exploited to perform rapid quantitation without the need for lengthy chromatography steps for selected applications. The ability to eliminate or reduce sample preparation time and bring down the analysis time to less than a minute makes the DMS-MS the analytical platform of choice for selected applications.

Over the course of this work, it has been realized that a stable electrospray is extremely important to maintain steady stream of ions into the DMS. Use of larger flow rates for electrospray could present challenges in proper desolvation of ions, causing ineffective DMS performance. A nano-splitter, if necessary, can be installed prior to electrospray to address some of the challenges with electrospray. With the flow rates employed during this research work (300-400 nl/min), it is acknowledged that DMS was able to provide ion filtration consistently as demonstrated by good reproducibility in the CoV values.
Different analytes in four different kinds of matrices (seed extracts, media, DNA digest and urine) have been analyzed over the course of this work using DMS-MS. For relatively complex matrices such as seed extracts, media and DNA digest, a stable electrospray could be achieved without any further sample clean-up. However, urine samples, presented a challenge for electrospray. A thousand fold dilution did allow the samples to electrospray to some extent, but the tips used for electrospray would get clogged fast, rendering them unusable. To mitigate the issues in electrospray arising from presence of salts and other larger molecules in biological fluid such as urine or plasma, an additional sample clean-up step is recommended. For the BE samples extracted from urine as presented in Chapter 2 and acylcarnitines samples from urine as presented in Chapter 5, a solid phase extraction step was employed. For samples present in trace quantities, this additional clean-up step can prove beneficial as this allows pre-concentration of samples in addition to removal of matrix components.

Addition of modifiers to the transport gas to enhance DMS analysis is a well-established practice in the field; however an explicit method to compute the effective cross sectional area to predict mobility is not available for DMS. An accomplishment of this research work is contribution to understanding of modifier effects in DMS as presented in Chapter 3. Theoretical considerations for the phenomena associated with compensation voltage shifts in DMS that explain step-wise clustering of modifiers including “saturation effect” and variations in signal intensities have been proposed anticipating that they will serve as a foundation for future work on modifier effects.

Another important achievement presented in this thesis is the comparison of quantitation between DMS-MS and LC-MS in Chapter 4. The close agreement in numerical values achieved between two different platforms performed in biologically processed samples further strengthens the applicability of DMS-MS for rapid quantitative applications. Other advantages of DMS-MS
platform have also been elucidated throughout the work presented in the thesis. Separation of closely related species, removal of background chemical noise, improvement in signal intensity, improvement in linear dynamic range, and application of DMS-MS to different matrices certainly establish the DMS-MS platform as a robust stand-alone analytical platform and this platform is well suited for further analytical applications as well.
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