Environmental Applications of Chromatography-Mass Spectrometry

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ENVIRONMENTAL APPLICATIONS OF CHROMATOGRAPHY-MASS SPECTROMETRY: BIODEGRADATION OF PERMANENT IONS AND IDENTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN THE KALAMAZOO RIVER OIL SPILL

by

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A Thesis submitted to the Graduate College
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Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are powerful analytical techniques that combine the separation power in a stationary phase with the detection power of mass spectrometry. GC-MS and LC-MS have gained dominant positions in identifying and quantifying different analyte species due to their high selectivity, high sensitivity, and high reproducibility.

My research utilizes the analytical power of LC-MS and GC-MS to address two environmental issues: 1) Identification of biodegradation products of the ionic liquid 1-butyl-3-methylimidazolium chloride (BMIMCl) by an activated sludge microbial community using LC-MS and 2) identification and quantification of polycyclic aromatic hydrocarbons (PAHs) in the Kalamazoo River oil spill using GC-MS. Three major metabolites from BMIMCl biodegradation were identified, and their structures were confirmed using MS/MS and $^1$H-NMR techniques. Many PAHs were identified, and their concentrations were quantified in the Kalamazoo River oil spill area using GC-MS.
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Wisam Abdulabbas Flayyih Al-Isawi
CHAPTER

1. INTRODUCTION ........................................................................................................ 1

1.1 Biodegradation of Ionic Liquids (ILs) in Vitro .................................................. 2

1.1.1 A Brief Background ......................................................................................... 2

1.1.2 Applications ...................................................................................................... 3

1.1.3 Potential Environmental Impact ......................................................................... 4

1.1.4 Identification of Biodegradation Products of the Ionic Liquid
1-butyl-3-methylimidazolium Chloride (BMIMCl) Using LC-MS ....................................... 5

1.2 Polycyclic Aromatic Hydrocarbons (PAHs) in Kalamazoo River after
the 2010 Oil Spill Accident ......................................................................................... 6

1.2.1 Brief Background about the 2010 Kalamazoo River Oil Spill
Accident .................................................................................................................. 6

1.2.2 Identification and Quantification of PAHs Using GC-MS ............................. 7
CHAPTER

1.3 Thesis Structure ........................................................................................................ 7

1.4 References.................................................................................................................. 8

2. CHROMATOGRAPHIC SEPARATION OF PERMANENT IONS ................ 11

2.1 A Brief History of Chromatography.............................................................................. 11

2.2 Chromatographic Methods for the Separation of Ionic Liquids (ILs) .... 12

2.2.1 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC).......................................................................................... 14

2.2.1.1 Biphenyl Column ...................................................................................... 15

2.2.2 Ion-Pairing Reversed-Phase High-Performance Liquid Chromatography (IP-RP-HPLC) ........................................................................... 16

2.2.2.1 Post Column Addition............................................................................. 19

2.2.3 Hydrophilic Interaction Liquid Chromatography (HILIC) ......... 19

2.2.4 Ion Chromatography (IC)................................................................................... 22

2.3 Detectors ...................................................................................................................... 23

2.3.1 Mass Spectrometry ............................................................................................. 23
Table of Contents-Continued

CHAPTER

2.3.2 Ultraviolet-Visible (UV-Vis) Detectors ........................................ 24

2.3.3 Conductivity Detectors ................................................................. 25

2.4 Discussion ........................................................................................... 27

2.5 References ........................................................................................... 28

3. SEPARATION AND IDENTIFICATION OF BIODEGRADATION
PRODUCTS OF THE IONIC LIQUID 1-BUTYL-3-
METHYLIMIDAZOLIUM CHLORIDE (BMIMCl) BY LIQUID
CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) ....................... 32

3.1 Introduction ......................................................................................... 32

3.2 Experimental Procedures ...................................................................... 35

3.2.1 Biodegradation of 1-butyl-3-methylimidazolium chloride
(BMIMCl) by Activated Sludge Microbial Community ............... 35

3.2.2 Characterization of Microbial Community .................................. 36

3.2.3 Identification of BMIMCl Metabolites Methods ...................... 37

3.2.3.1 High-Performance Liquid Chromatography Coupled
with UV Detector and Mass Spectrometer (HPLC-
UV-MS) .................................................................................... 37

3.2.3.2 Extraction of Product Ion .............................................. 38
### Table of Contents-Continued

#### CHAPTER

**3.2.3.3**  Proton Nuclear Magnetic Resonance ($^1$H-NMR) ...... 39

**3.3**  Results and Discussions................................................................. 40

**3.3.1**  The Microbial Community.......................................................... 40

**3.3.2**  Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis ................................................................. 41

**3.3.3**  Biodegradation of 1-butyl-3-methylimidazolium Cation (BMIM) ................................................................................. 43

**3.3.4**  Characterization of Microbial Community .................................... 45

**3.3.5**  Biodegradation Products of BMIMCl.............................................. 47

**3.3.5.1**  Appearance and Disappearance of Metabolites....................... 47

**3.3.6**  Identification of BMIM Metabolites .............................................. 51

**3.4**  Conclusion .......................................................................................... 59

**3.5**  References............................................................................................ 60

**4.**  IDENTIFICATION AND QUANTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN THE KALAMAZOO RIVER OIL SPILL BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) .......................................................................... 63
CHAPTER

5. CONCLUSIONS AND FUTURE WORK................................................................. 90

5.1 Identification of BMIMCl Breakdown Products ...................................... 90

5.2 Impact of BMIMCl Breakdown Products on the Environment................. 91

5.3 Identification and Quantification of PAHs in the Kalamazoo Oil spill... 92

5.4 References................................................................................................... 94
LIST OF FIGURES

1.1 Common cations and anions of ionic liquids........................................................... 3

2.1 Schematic diagram of a typical LC system................................................................. 13

2.2 Biphenyl stationary phase....................................................................................... 16

2.3 A typical LC-MS post column addition system...................................................... 19

3.1 MS spectrum of BMIM cation standard solution..................................................... 41

3.2 (a) Extracted ion chromatogram (EIC), (b)Total ion chromatogram (TIC) and (c) UV trace of BMIM standard ................................................................. 42

3.3 Biodegradation of BMIM cation over 46 days of the treatment followed by LC-MS and $^1$H-NMR. .............................................................43

3.4 The percentage of biodegradation of BMIM compared to day three over 46 days of the treatment. ................................................................. 44

3.5 Average absorbance of BMIM cation at 210 nm from the three treatment flasks (diamonds) and the two abiotic control flasks (triangles) over the 46-day incubation period. ................................................................. 45

3.6 The percent relative abundance of bacterial phyla represented as the average of triplicate extraction and sequence reactions prepared from the initial inoculate pellet. ................................................................. 46

3.7 LC-UV-MS of Day 21 showing extracted ion chromatograms for m/z 139 BMIM and breakdown products of m/z 155 BMIM+O, m/z 141 BMIM-CH$_3$+OH, m/z 137 BMIM-2H and UV absorbance at 212nm trace............... 48
List of Figures-Continued

3.8 Biodegradation of BMIM over 46 days followed by LC-MS................................. 50

3.9 Monitoring of BMIM metabolites by $^1$H-NMR spectroscopy ......................... 51

3.10 Structures of BMIM cation and breakdown products predicted from experimental results and literature studies of related compounds and confirmed by MS/MS and $^1$H-NMR studies. .............................................................. 52

3.11 Confirmation that 1-methylimidazolium is the major metabolite produced by degradation of BMIM was obtained by adding 55 $\mu$mol (red) and 110 $\mu$mol (blue) of 1-methylimidazolium to the Day 17 sample. .................................................. 54

3.12 Chemical structure and possible fragmentation pattern of: (a) 1-butyl-3-methyl-1H-imidazol-3-ium (m/z 139, BMIM), (b) (Z)-1-(but-1-en-1-yl)-3-methyl-1H-imidazol-3-ium (m/z 137) and (c) 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium (m/z 155), and (d) An equimolar concentration mixture of 10$\mu$M methylimidazolium standard and BMIM analyzed by ESI-MS in positive ion mode ................................................................................................... 55

3.13 Monitoring of BMIM biodegradation over 46 days by solution $^1$H-NMR spectroscopy. ........................................................................................................... 58

3.14 $^1$H NMR spectrum of the Day 46 sample reveals that other metabolites exist in the sample. .............................................................................................................. 59

4.1 Enbridge’s liquids system and the 1,900-mile lakehead system (the U.S. portion). ......................................................................................................................... 64

4.2 The ruptured segment of Line 6B in the trench following the July 25, 2010, rupture. ....................................................................................................................... 65

4.3 Aerial view of the accident location showing the rupture site to the left and the Talmadge Creek flowing west toward the Kalamazoo River........................................ 66
List of Figures-Continued

4.4 Chemical structure of the selected 16 PAHs, * on the EPA list of frequently monitored PAHs; ** on the EPA and EU lists of frequently monitored PAHs ..... 67

4.4 Continued Chemical structure of the selected 16 PAHs, * on the EPA list of frequently monitored PAHs; ** on the EPA and EU lists of frequently monitored PAHs ................................................................. 68

4.5 Schematic diagram of a typical GC-MS system................................................. 72

4.6 Map showing rupture location, sample locations (white stars), and affected waterways from Talmadge Creek to Morrow Lake (Source: NTSB) .............. 74

4.7 Dr. Barcelona sampling at the Olds Farm, Michigan ........................................ 75

4.8 Soil sampling procedure for the six locations of the study area .................... 76

4.9 A split spoon sampler......................................................................................... 77

4.10 GC-MS spectrum of 20 μg/mL of PAHs standard........................................... 79

4.11 Trend of total PAH concentrations along the study area.............................. 80

4.12 Concentrations of total PAHs at the six sampling sites in the study area......... 82

4.13 Concentrations of individual PAHs at the upper and lower layers of the oil spill accident site. ......................................................................................................................... 83

4.14 Concentrations of individual PAHs at the upper and lower layers at the upstream river, at the oil spill accident site, and downstream river........................................ 85
LIST OF ACRONYMS

ACN ................................................................................................................... Acetonitrile

BMIM .............................................................................................................. 1-butyl-3-methylimidazolium

BMIMCl ....................................................................................................... 1-butyl-3-methylimidazolium chloride

CH₂Cl₂ ............................................................................................................ Dichloromethane

CID ............................................................................................................. Collision Induced Dissociation

CZE ............................................................................................................. Capillary Zone Electrophoresis

EIC ............................................................................................................. Extracted Ion Chromatogram

ESI-MS ............................................................... Electrospray Ionization-Mass Spectrometry

EU ............................................................................................................. the European Union

GC ............................................................................................................ Gas Chromatography

GC-MS ....................................................................................................... Gas Chromatography-Mass Spectrometry

HCl ............................................................................................................. Hydrochloric Acid

HILIC ........................................................... Hydrophilic Interaction Liquid Chromatography

¹H-NMR ................................................................................................ Proton-Nuclear Magnetic Resonance

HPLC ............................................................... High Performance Liquid Chromatography
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>HPLC-UV-MS</td>
<td>High Performance Liquid Chromatography-Ultraviolet-Mass Spectrometry</td>
</tr>
<tr>
<td>IC</td>
<td>Ion Chromatography</td>
</tr>
<tr>
<td>IL</td>
<td>Ionic Liquid</td>
</tr>
<tr>
<td>IPC</td>
<td>Ion Pairing Chromatography</td>
</tr>
<tr>
<td>IPR</td>
<td>Ion Pairing Reagent</td>
</tr>
<tr>
<td>IP-RP-HPLC</td>
<td>Ion Pairing-Reversed Phase-High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to Charge Ratio</td>
</tr>
<tr>
<td>MALDI-MS</td>
<td>Matrix Assisted Laser Desorption Ionization-Mass Spectrometry</td>
</tr>
<tr>
<td>MeIM</td>
<td>Methylimidazolium</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>NIR</td>
<td>Near Infrared</td>
</tr>
<tr>
<td>NP</td>
<td>Normal Phase</td>
</tr>
<tr>
<td>NP-LC</td>
<td>Normal Phase-Liquid Chromatography</td>
</tr>
<tr>
<td>NTSB</td>
<td>National Transportation Safety Board</td>
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</tbody>
</table>
List of Acronyms-Continued

OECD............................ the Organization for Economic Co-operation and Development

OUT ................................................................. Operational Taxonomic Unit

PAH ................................................................. Polycyclic Aromatic Hydrocarbons

PCB ................................................................. Polychlorinated Biphenyl

RP ................................................................. Reversed Phase

RP-HPLC ........................................ Reversed Phase-High Performance Liquid Chromatography

RP-LC ........................................................ Reversed Phase-Liquid Chromatography

TIC ................................................................. Total Ion Chromatogram

UPLC ........................................................ Ultrahigh Performance Liquid Chromatography

US-EPA ..................................................... the United States-Environmental Protection Agency

UV ................................................................. Ultraviolet

UV-Vis ......................................................... Ultraviolet-Visible

VOC ............................................................. Volatile Organic Compound
CHAPTER 1
INTRODUCTION

This thesis covers two environmental applications of chromatography coupled to mass spectrometry: 1) identification of biodegradation products of the ionic liquid 1-butyl-3-methylimidazolium chloride (BMIMCl) by the activated sludge microbial community using liquid chromatography-mass spectrometry (LC-MS) and 2) identification and quantification of polycyclic aromatic hydrocarbons (PAHs) in the Kalamazoo River oil spill area using gas chromatography-mass spectrometry (GC-MS). These two projects are briefly introduced here as follows:

Ionic liquids (ILs) are a novel class of organic salts that have many potential applications in industry and research due to their green properties such as low volatility and thermal and electrochemical stability.\textsuperscript{1} Despite their green properties, some studies have reported their significant toxic effects on aquatic and terrestrial organisms.\textsuperscript{2-9} This makes biodegradation of ILs a necessary step before release into the environment. Principle number 10 of the 12 Principles of Green Chemistry defines green chemicals as those can be degraded at the end of their function and would not persist in the environment.\textsuperscript{10} Therefore, examining the biodegradation products of ionic liquids and evaluating their impact on the environment is of significant importance.
As demands on fossil fuel increase internationally, oil spill accidents have become a major environmental concern. In the United States alone, over one billion liters of oil have spilled into marine waters since the early 1960. The recent oil spill in Marshall, Michigan in 2010 released over three million liters of crude oil, which entered the Talmadge Creek before flowing into the Kalamazoo River, a Lake Michigan tributary. The oil saturated the spill site and surrounding wetlands and severely affected the environment. The massive quantities of spilled oil released many contaminants into the aquatic environment. Polycyclic aromatic hydrocarbons (PAHs) are among these pollutants that represent significant threats toward aquatic organisms because of their carcinogenic and mutagenic properties and their persistence in the environment. Studies on the levels of PAHs in the Kalamazoo River and surrounding areas after the oil spill accident are limited. The lack of reliable information about the concentrations of the PAH contaminants in the Kalamazoo River after the oil spill accident motivated this investigation to determine the current levels and identities of PAHs in the river.

1.1 Biodegradation of Ionic Liquids (ILs) in Vitro

1.1.1 A Brief Background

Ionic liquids are a class of organic salts made of bulky organic cations and inorganic or organic anions that have a low melting point. The most common cations are those of imidazolium, pyridinium, ammonium, and phosphonium, while common anions are bis-[(trifluoromethyl)sulfonyl]imide (NTf₂⁻), hexafluorophosphate (PF₆⁻), and halides (Figure 1.1). Gabriel and Weiner reported the first ionic liquid back in 1881. At least 10⁸
The unique combinations of cations and anions control the resultant properties of ILs and make them tunable for different applications. ILs exhibit unique physicochemical properties such as negligible vapor pressure, high chemical and thermal stability, good conductivity, high viscosity, good solvation ability, wide electrochemical windows and tunable polarity.\textsuperscript{15,17,18}

**Figure 1.1** Common cations and anions of ionic liquids. (Adopted from Anderson et al. (2012) by permission of the Royal Society of Chemistry)

1.1.2 Applications

The tunable physicochemical properties of ILs have expanded their use into a broad range of different applications. In research, ILs have a great potential in organic synthesis, green chemistry, separations, mass spectrometry, and electrochemistry.\textsuperscript{15,18} In analytical chemistry, ILs have been used as stationary phases and solvents for headspace GC,\textsuperscript{18}
mobile phase additives and surface-bonded stationary phases in LC, for liquid-liquid extractions and solid-phase microextraction (SPME), liquid matrices for MALDI-MS, and solvents for NIR spectroscopy and many other applications. Many “green-focused” industries have found that ILs are excellent candidates for their applications due to their very low vapor pressure. The BASF company for instance commercialized ILs for industrial applications, including the breaking of azeotropes, replacing phosgene, cellulose dissolution, aluminum plating and the BASIL (Biphasic Acid Scavenging utilizing Ionic Liquid) process, which some consider one of the most successful examples of an industrial process using IL technology. The Degussa Corporation is also developing ILs for several industrial applications, such as hydrosilylation, paint additives, and lithium-ion batteries. The French Petroleum Institute (IFP) has commercialized the Dimersol process, when uses ILs as reaction solvents for the dimerization of butane into the more valuable branched octane molecule. Many other companies like Central Glass Co. Ltd. (Japan), IoLiTec (Ionic Liquids Technologies) and SASOL Ltd. have also developed ionic liquids for different applications. So, the use of ILs has been expanding in many different fields due to their desirable properties.

**1.1.3 Potential Environmental Impact**

The rapidly increasing use of ILs in both academic and industrial fields have generated a growing concern about their impact on the environment. Many studies have demonstrated their toxic effects on aquatic and terrestrial ecosystems. Since ILs are highly soluble in water but are not always biodegradable, a release of ILs into the
environment may lead to significant water pollution problems. Moreover, ILs could become persistent pollutants in wastewater effluent due to their high stability in water. For this reason, studies examining IL biodegradability are of great importance.

1.1.4 Identification of Biodegradation Products of the Ionic Liquid 1-butyl-3-methylimidazolium Chloride (BMIMCl) Using LC-MS

The growing role of ILs in industry and research and the increasing concern about their environmental impact have urged a need for the development of fast, reproducible, and reliable methods for the characterization and analysis of ILs. My research employs the analytical power of liquid chromatography-mass spectrometry (LC-MS) to investigate a significant environmental issue, biodegradation of permanent ions in vitro. Metabolites generated during the biodegradation of the ionic liquid 1-butyl-3-methylimidazolium chloride (BMIMCl) by a specially selected, activated sludge microbial community were investigated. Biodegradation products of BMIM were examined using reversed-phase high-performance liquid chromatography (RP-HPLC) and the combination of UV, MS, and $^1$H-NMR detection. The identification of several biodegradation products used a combination of tandem mass spectrometry (MS/MS) and $^1$H-NMR techniques. To the best of our knowledge, we report the first successful biodegradation attempt for the usually biodegradation resistant 1-butyl-3-methylimidazolium chloride.
1.2 Polycyclic Aromatic Hydrocarbons (PAHs) in Kalamazoo River after the 2010 Oil Spill Accident

1.2.1 Brief Background about the 2010 Kalamazoo River Oil Spill Accident

On July 25, 2010, a 30-inch pipeline (Line 6B), owned and operated by Enbridge Incorporated (Enbridge) ruptured in a wetland in Marshall, Michigan. Line 6B transports different types of heavy bituminous crude oil from the oil sand regions of Western Canada that require dilution with lighter petroleum products to enable the crude to flow easier. The fracture in the ruptured segment measured about 2 m long and up to 13.5 cm wide. The rupture was not discovered for over 17 hours. The whole release was estimated to be 3,192,783 litters of crude oil, which entered Talmadge Creek and flowed into the Kalamazoo River, a Lake Michigan tributary. The oil saturated the rupture site and the surrounding wetlands, and seriously impacted the environment.

Spilled oil is expected to degrade with several factors such as evaporation, diffusion, photooxidation, and biodegradation. When an oil spill happens in a water system, the levels of PAHs are significantly higher in water and could have adverse effects on the aquatic environment. PAHs persist in the environment, and their toxicities could represent significant threats towards aquatic organisms. Accurate information about the levels of PAHs in the Kalamazoo River and surrounding areas after the oil spill accident is limited. The lack of reliable information about the levels of PAHs contaminants in the Kalamazoo River after the oil spill accident has urged a need for an environmental investigation to determine the level and identity of PAHs in the river.
1.2.2 Identification and Quantification of PAHs Using GC-MS

Five years following the 2010 Kalamazoo River oil spill accident in Marshall, Michigan, polycyclic aromatic hydrocarbons (PAHs) were investigated in riparian areas, along the Kalamazoo River, between Marshall and Galesburg communities, which were contaminated with spilled oil, using gas chromatography-mass spectrometry. Six locations along the Kalamazoo River were selected and investigated. 16 PAHs were identified, and their concentrations were quantified using gas chromatography-mass spectrometry (GC-MS). Among the 16 PAHs that are examined in this work, 15 PAHs are on the US Environmental Protection Agency (EPA) list of 16 PAHs frequently monitored due to their carcinogenic and mutagenic properties. Seven of the 16 PAHs studied in this investigation are on the European Union (EU) list of 15 PAHs that are of major concern for human health because of their toxic properties. The concentration of total PAHs in the Kalamazoo River shoreline sediments was at high levels at the accident site. Pyrene, fluoranthene, chrysene, benzo(a)pyrene and phenanthrene were dominant PAHs in the oil spill accident site.

1.3 Thesis Structure

In Chapter 2, chromatographic methods for the separation of permanent ions and ionic liquid cations primarily are described. This chapter covers chromatographic methods such as reversed-phase liquid chromatography (RPLC), ion pairing reversed phase liquid chromatography (IP-RPLC), hydrophilic interaction liquid chromatography (HILIC), and
ion chromatography (IC). Different detection techniques for the chromatographic methods also are described in this chapter.

Chapter 3 discusses the separation and identification of breakdown products produced during the biodegradation of the ionic liquid, biodegradation resistant, 1-butyl-3-methylimidazolium chloride (BMIMCl) by a specially selected, activated sludge microbial community using LC-MS. In this chapter, the characterization of the microbial community used for the biodegradation of BMIM is explained. The identity and structures of three breakdown products are revealed in this chapter.

In Chapter 4, identification and quantification of 16 PAHs in the 2010 Kalamazoo River oil spill accident affected area using GC-MS are discussed. This section includes a description of the oil spill accident and the most dominant PAHs in the shoreline soil five years after the accident.

Chapter 5 summarizes the whole work conducted in this thesis with final conclusions and perspectives into future work.

1.4 References


2.1 A Brief History of Chromatography

Chromatography is a powerful separation technique that was first invented by botanist Michael S. Tswett in 1906 in Warsaw.\textsuperscript{1–3} Tswett successfully separated chlorophyll, xanthophyll, and many other colored species by percolating vegetable extracts through a column of calcium carbonate. These pigments had different adsorptions on the calcium carbonate column, and that yielded colored bands at various positions on the column. Tswett called the colored bands a \textit{chromatogram} and the method \textit{chromatography}.\textsuperscript{2} The word chromatography originated from Greek and means color writing, (\textit{Chroma} color and \textit{Graphein} writing).\textsuperscript{2,4} In Tswett’s method, the calcium carbonate column was referred to as the stationary phase, while the solution of pigments and solvents was called the mobile phase since they moved through the column.\textsuperscript{2}

Significant advances in chromatography have revolutionized science. In the 1930’s, thin layer chromatography and ion exchange chromatography were introduced as new chromatographic separation techniques. In 1941, paper chromatography and partition chromatography were introduced by Archer Martin and Richard Synge.\textsuperscript{2,3,5} Martin and Synge also introduced gas chromatography and jointly won the Noble Prize in Chemistry 1952 for their invention of partition
For many decades, chromatography has been routinely and extensively used as a universal separation method in different fields especially chemistry, biology, and medicine.

\subsection*{2.2 Chromatographic Methods for the Separation of Ionic Liquids (ILs)}

ILs are a class of organic salts with a low melting point, often below room temperature. ILs are composed of bulky organic cations and inorganic or organic anions. ILs possess unique physicochemical properties such as negligible vapor pressure, high chemical and thermal stability, high conductivity, high viscosity, and tunable polarity.\cite{7,8,9} To develop a chromatographic method for the separation of ILs, we initially need to understand the unique physicochemical properties of ILs, and their ability to aid in the separation of different ILs. Since ILs are highly polar compounds, gas chromatography would likely not be the best method to separate ILs.\cite{9} Liquid chromatography, on the other hand, does seem to be a convenient and versatile method to separate ILs.

Liquid chromatography (LC) is an analytical technique in which species in the liquid state are separated based on their interaction with a stationary phase and then introduced into a detector, such as a mass spectrometer or UV-Vis spectrophotometer. The mobile phase is typically an aqueous solution of organic solvents such as methanol, acetonitrile, propanol or hexane, while the stationary phase is bare or alkylated silica particles or polymer beads such as polysaccharide or polystyrene, housed in a stainless steel column.\cite{2} A schematic diagram of a typical LC system is shown in Figure 2.1. LC operates in different modes such as normal phase (NP), reversed-phase (RP), and
hydrophilic interaction liquid chromatography (HILIC). In NP mode, the stationary phase is more polar than the mobile phase.\textsuperscript{10,11} The retention is increased by increasing the polarity of the sample and the stationary phase, and by decreasing the polarity of the mobile phase. Opposite to NP, in a reverse phase system, the stationary phase is non-polar while the mobile phase is a polar mixture of organic solvents and water or a buffer. The retention in RP mode is opposite to NP. Polar species are less retained than non-polar ones. HILIC employs an NP stationary phase in combination with an RP mobile phase, which contains more than 50% organic solvent in water. HILIC sufficiently retains and has different selectivity for strongly polar compounds relative to NP, and better separation efficiency of strongly polar compounds than classical RP.\textsuperscript{8,10–12} The uses of LC for many analytical applications continues to grow exponentially in different fields. LC is suitable for analyzing a broad range of samples over a wide polarity range and no upper molecular weight limit. It is appropriate for the analysis of liquid volatile and nonvolatile organic, inorganic, biological, polymeric, and chiral compounds.\textsuperscript{2,13,14}

\textbf{Figure 2.1} Schematic diagram of a typical LC system
Many chromatographic methods have been reported in the literature for the separation and determination of ILs. These methods include reversed-phase high performance liquid chromatography (RP-HPLC), ion pairing reverse phase high performance liquid chromatography (IP-RP-HPLC), hydrophilic interaction liquid chromatography (HILIC), capillary zone electrophoresis (CZE), and ion chromatography (IC). In this section, the four most commonly used chromatographic methods will be discussed briefly.

2.2.1 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

RP-HPLC is one of the most widely used chromatographic methods for the analysis of different ionic liquids. A wide variety of stationary phases and different detection techniques have been used. One of the earliest RP-HPLC methods for the separation of the most commonly used class of ILs, differently substituted imidazolium cations-based ILs, was reported by Stepnowski et al. (2003). Stepnowski developed a gradient method for the separation of 1-alkyl- and 1-aryl-3-methylimidazolium-based room temperature IL cations, first on a C\textsubscript{18} column using methanol-water, and subsequently on a 5 μm C\textsubscript{8} MetaSil Basic column. Better resolution, especially for more polar cations, was obtained using the RP C\textsubscript{8} column. Moreover, peak shape and reproducibility of retention values were improved significantly by replacing the mobile phase with acetonitrile/water (1% acetic acid) and by buffering the mobile phase with 20 mM ammonium acetate.

Many studies have reported the use of RP-HPLC for the separation of ILs. Table 2.1 includes typical mobile phases, stationary phases, and detection methods used for the
separation of ILs using RP-HPLC. However, studies have shown that classical reversed-phase liquid chromatography has clear limitations for the separation of short side chains IL cations. Retention and selectivity are weaker for cations with alkyl side chain of less than four carbon atoms.\textsuperscript{8,12,18,19,21,22} The analytes don’t adsorb onto the non-polar stationary phase of RP chromatography columns since they are permanently ionic. RP-HPLC has been widely used for the separation of different ILs cations using different stationary phases, and while the mobile phases often contain additive anionic species like ammonium acetate/acetic acid, potassium phosphate/phosphoric acid mixtures or trifluoroacetic acid for better reproducibility and better peak shape. These additives often act as ion pairing reagents, as described below.

\textbf{2.2.1.1 Biphenyl Column}

The biphenyl column is a high-efficiency reversed-phase liquid chromatography stationary phase that offers a high separation power for non-polar and polar compounds (Figure 2.2).\textsuperscript{23} The biphenyl stationary phase provides enhanced retention, higher sensitivity, and overall better results than the traditional C18 column, especially for aromatic compounds. Biphenyl column can withstand a 100% aqueous mobile phase without experiencing the hydrophobic collapse of the stationary phase, and allows for excellent reversed-phase retention and aromatic selectivity. Biphenyl columns have been applied to the separation of different categories of compounds such as mycotoxins, opiate isomers, polar therapeutic drug, benzodiazepines, and steroids. No reports for the use of biphenyl columns for ionic liquid separation could be found.
2.2.2 Ion-Pairing Reversed-Phase High-Performance Liquid Chromatography (IP-RP-HPLC)

Ion-Pairing Chromatography (IPC) is an analytical technique that utilizes lipophilic ions to improve the separation of organic and inorganic ionic analytes and has better retention, efficiency, and resolution relative to classical reversed-phase liquid chromatography columns. Many organic and inorganic ionic analytes result in poor separation with traditional reversed-phase liquid chromatography, but the addition of lipophilic ions to the typical reversed-phase mobile phase was found to improve performance. The mobile phase additive is called an ion pairing reagent (IPR). Many IPRs were reported to include alkylammonium, tetraalkylammonium, alkyl sulphate and alkyl sulphonate salts, perfluorinated carboxylic acids, chaotropic ions, ionic liquids and many other ions. The mobile phase pH plays a pivotal role in controlling analyte charge status.
and selectivity. Both IPR concentration and organic modifier content in the mobile phase control selectivity.\textsuperscript{24,26}

IP-RP-HPLC have been used for the separation of IL cations.\textsuperscript{8,25} IP-RP-HPLC involves the formation of neutral ion pairs between analyte cations and IPR anions. The improved retention on the hydrophobic stationary phase results from making the analyte cation temporarily more hydrophobic. An alternative is a dynamic ion exchange between adsorbed counteranions of the mobile phase and eluted species. The choice of IPR depends on the nature of analytes that need to be separated.\textsuperscript{29,30}

Rouzo et al. (2007) employed alkylsulfonate salts with long hydrophobic chains as an IPR for the separation of five alkylmethylimidazolium ionic liquids, substituted with different length of alkyl chains. A C8 Metasil Basic column with a buffered acetonitrile/water mobile phase under isocratic conditions was used.\textsuperscript{8} Better retention of polar compounds was obtained, and separation of analytes with four carbon chains or shorter was improved. They observed that retention increased as organic modifier (ACN) content decreased and IPR concentration increased in the mobile phase, and this was consistent with other studies.\textsuperscript{8,29,30}

Another study of the separation of imidazolium and pyridinium ILs cations used IPC on a monolithic stationary phase. Yu et al. (2012)\textsuperscript{25} found that using more hydrophobic anions as IPRs improves retention of imidazolium and pyridinium cations. Increasing IPR concentration improves retention, while increasing organic modifier concentration decreases retention, especially for imidazolium and pyridinium cations with longer alkyl
chains. The concentration of the IPR needs to be optimized because excessive ion pair reagent could damage the column to some degree.

Adding IPRs to mobile phases for the separation of polar species in classical reversed phase liquid chromatography is advantageous because it improves the hydrophobicity of polar compounds through the formation of ion pairs and, as a consequence, promotes separation. However, the lack of volatility of commonly used IPRs causes a major decrease in ESI-MS signal.\textsuperscript{8,31} This makes ion pair chromatography not very compatible with subsequent mass spectrometry analysis.

To make ion-pairing chromatography more compatible with mass spectrometry detection, the volatility of ion pairing reagents, the ionic strength of analytes and the eluent, and the ionizability of the analytes after separation on a stationary phase need to be addressed. Volatile IPRs are more compatible with MS detection. The ionic strength can be mitigated by optimizing the concentrations of additives, buffers, analytes, and mobile phase. Ion-pairing chromatography involves the formation of neutral ion pairs between analyte cations and IPR anions. This improves retention on the hydrophobic stationary phase by making the permanent cation temporarily more hydrophobic. However, this also leads to a significant decrease in the ESI-MS signal. One way to make analytes more ionizable after separation on a column and subsequently detectable by MS is using post-column addition.
2.2.2.1 Post Column Addition

The use of ion-pairing in reversed-phase chromatography is advantageous for better separation of ionic analytes since it increases the hydrophobicity of ionic analytes and subsequently improves retention on traditional reversed-phase columns. However, ion pairing causes a major decrease in ESI-MS signal because it is not easy to separate ion pairs in the gas phase. Post column addition could play a pivotal role in improving ESI-MS signal by breaking ion pairs apart and promoting MS detection. Post column addition is used in liquid chromatography to introduce reagents that modify the analyte charge state or promote volatilization of the eluent system before the MS interface. A post-column addition system is shown in Figure 2.3.

![A typical LC-MS post column addition system](image)

**Figure 2.3** A typical LC-MS post column addition system

2.2.3 Hydrophilic Interaction Liquid Chromatography (HILIC)

Hydrophilic interaction chromatography (HILIC) is a liquid chromatography mode for separating polar compounds that interact with a hydrophilic stationary phase and elute
with a mobile phase made of water-miscible polar hydrophobic eluents, such as acetonitrile, and a small amount of water, which is the stronger eluting solvent. The acronym HILIC was first introduced by Alpert in 1990 when his work on “hydrophilic-interaction chromatography for the separation of peptides, nucleic acids, and other polar compounds” was published in the Journal of Chromatography A. Similar to normal phase liquid chromatography (NP-LC), HILIC uses traditional polar stationary phases, such as silica, amino, or cyano, but the eluent employed is similar to those used in RP-LC. The distinction between HILIC and NP-LC is still being debated in the literature, but the separation mechanism in HILIC is different than that of NP-LC. In HILIC, water is the stronger eluting solvent, and the retention mechanism involves partitioning while the retention mechanism in traditional NP-LC mainly is governed by surface adsorption. The most accepted HILIC retention mechanism was proposed by Alpert. Alpert suggested that the retention mechanism for HILIC is a partitioning between the hydrophobic-rich eluent and a water-enriched layer adsorbed onto the hydrophilic stationary phase. The more hydrophilic the analyte, the more partitioning equilibrium is shifted towards the water-enriched layer, and leads to a long retention time for the analyte.

HILIC mode is used for the analysis of polar molecules, such as biomarkers, nucleosides, nucleotides/oligonucleotides, amino acids, peptides, proteins, saccharides, glycosides, oligosaccharides, hydrophilic drugs, alkaloids, carbohydrates, and small polar or ionizable compounds. HILIC mode can also be used for the separation of polar, organic and inorganic molecules, and ionic liquids cations.
Rouzo et al.\textsuperscript{8} developed an HILIC method for the separation of five alkylimidazolium ILs cations with various hydrophobic characteristics on diol stationary phases. They found that HILIC offers an efficient separation of both polar and non-polar cations, especially short-chain imidazolium cations, regarding retention and selectivity and it is compatible with ESI-MS detection for further determination of polar degradation compounds.

Lamouroux et al. (2011) also found that HILIC stationary phases are the best method to separate a homologous series of alkylimidazolium IL cations, especially the less hydrophobic ones.\textsuperscript{12} They found that the diol stationary phase provided the best results for separating ILs cations with regard to retention and selectivity because diol acquires a thick layer of water that enables polar analytes to partition longer.

HILIC has many advantages over NP-LC, RP-LC, and IP-RP-LC. First, the composition of the HILIC eluent contains more polar organic solvents, and the small portion of water makes eluent preparation less complicated. High organic solvent composition shortens the separation time since the viscosity of the HILIC mobile phase is lower than the traditional reversed-phase mobile phase. High organic content also tends to increase sensitivity in ESI-MS.\textsuperscript{32} Second, HILIC is suitable for the analysis of compounds in complex mixtures that elute near the dead volume in RP-LC.\textsuperscript{10} Third, HILIC is appropriate for the separation of polar compounds since they are soluble in the aqueous mobile phase, as opposed to their poor solubility in NP-LC. Finally, HILIC does not require the use of expensive non-volatile IPRs, which makes it more compatible with ESI-MS analysis.\textsuperscript{10,32}
2.2.4 Ion Chromatography (IC)

Ion chromatography is an analytical technique in which ions (anions or cations) are separated by differences in the rate at which they flow through a stationary phase packed with either an anion- or cation-exchange particles. Cations are separated on a cation-exchange column and anions on an anion-exchange column. Modern IC was first introduced in 1975 by Small et al. for the determination of inorganic anions and cations, and water soluble organic acids and bases. Ion exchange chromatography, with suppressed conductivity detection, has been widely used for the separation of different organic and inorganic ions. In suppressed IC, after ions are separated on an ion-exchange column, together with the eluent, separated sample ions move to suppressor where the conductance of the eluent is suppressed, and conductance of the eluted sample ions is increased, resulting in a big rise in the signal to noise ratio of the detection signal. Development of high-performance electrolytic suppressors was a recent breakthrough in IC. IC is still the method of choice for the separation of inorganic anions and cations.

IC has been applied to the analysis of ILs because of their ionic properties. Stepnowski et al. used ion exchange chromatography for the separation of 1-alkyl-3-methylimidazolium and 4-methyl-N-butylpyridinium cations on a strong stationary-phase cation exchanger (SCX). Stepnowski et al. reported that IC with SCX can be used for the separation of imidazolium and pyridinium IL cations. Stepnowski et al. observed several modes of interaction governing the chromatographic separation of ILs cations. Cation exchange, nonspecific hydrophobic interactions, and adsorption chromatography behavior were observed. Chromatographic conditions for the separation of IL cations in IC can be
achieved by optimizing the organic modifier, the buffer concentration, and the pH of the mobile phase.

2.3 Detectors

Many studies have reported the use of liquid chromatography coupled with different detection techniques for the separation and analysis of ILs. Three most commonly used detection methods are discussed briefly here.

2.3.1 Mass Spectrometry

Mass spectrometry (MS) is a powerful analytical technique in which ions are generated from either inorganic or organic compounds by a suitable ionization technique and subsequently separated and detected in a high vacuum region by electric and magnetic fields.\textsuperscript{13,14} Mass spectrometry occupies an outstanding position among analytical methods due to its great sensitivity, detection limits, speed and a wide range of applications.\textsuperscript{14} To give a full list of mass spectrometry applications is impossible here because of the enormous number of applications. Mass spectrometry is used in many key applications such as elemental and isotopic analysis, organic and bio-organic analysis, structure elucidation, characterization of ionic species and chemical reactions, mass spectral imaging, and miniaturization. The technique is also easily coupled to separation techniques.
The combination of chromatography and mass spectrometry has introduced formidable hyphenated analytical techniques that offer the separating power of chromatography and the detection capabilities of mass spectrometry. Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS) are the most common examples of these techniques.

2.3.2 Ultraviolet-Visible (UV-Vis) Detectors

Spectrophotometric UV-Vis detectors are widely used in modern chromatography. Many species absorb UV or visible radiation and this absorption characteristic is exploited in their detection. UV-Vis detectors operate under the absorption principle which is governed by the Lambert-Beer law,\textsuperscript{39,43} which states that:

\[ A = \varepsilon \cdot b \cdot C \]

where \( A \) is the absorbance of an analyte, \( \varepsilon \) is molar absorptivity; \( b \) is cell length (usually is measured in cm), and \( C \) is the concentration of the analyte (usually in molar concentration units). UV-Vis detectors can be selectively tuned to the analyte’s maximum absorption wavelength.\textsuperscript{39} UV-Vis detectors can only detect analytes that absorb in the UV or visible range of the spectrum.\textsuperscript{43} The sensitivity of UV-Vis detectors depends on the specific analyte and wavelength. To achieve the maximum sensitivity, the various components of the mobile phase should be transparent at the wavelength of measurement. UV-Vis detectors are less sensitive than mass spectrometer detectors.
2.3.3 Conductivity Detectors

Conductivity detectors are employed extensively in ion chromatography. They measure the ability of a solution containing salt to conduct electricity between two electrodes, which is defined as conductance.\(^\text{39,43}\) Solution conductance is directly proportional to the salt concentration and the mobility of the individual anions and cations. Conductivity increases as the ionic character of molecule increases and/or the size of ions decrease. Conductivity detectors are classified as general detectors because they respond to all or most of the ions that pass through the detector cell. Significant improvement in the conductivity detectors was made after the introduction of “suppressed conductivity detection”. In the suppressed conductivity detection the eluent is treated before detection to make the eluent ions less detectable and the sample ions more detectable. This makes the background signal less conducting and the sample signal more conducting.\(^\text{42}\)
Table 2.1 Common mobile phases, stationary phases, and detection techniques used for the separation of ILs using RP-HPLC

<table>
<thead>
<tr>
<th>No.</th>
<th>Ionic Liquid Type</th>
<th>Mobile Phase (s)</th>
<th>Stationary Phase (s)</th>
<th>Detection Technique</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-alkyl- and 1-aryl-3-methylimidazolium ILs</td>
<td>Acetonitrile/water (1% acetic acid/20 mM ammonium acetate)</td>
<td>RP C₈ MetaSil Basic 250X4.6 mm I.D. 5 μm column</td>
<td>ESI-MS</td>
<td>Ref.²¹</td>
</tr>
<tr>
<td>2</td>
<td>1-alkyl-3-methylimidazolium ILs</td>
<td>Acetonitrile/water (70% v/v)</td>
<td>Kromasol C₁₈ column</td>
<td>UV and Conductivity</td>
<td>Ref.¹⁹</td>
</tr>
<tr>
<td>3</td>
<td>1-alkyl-3-methylimidazolium and 4-methyl-N-butylpyridinium</td>
<td>Methanol or Acetonitrile/water (10-50 mM KH₂PO₄/H₃PO₄)</td>
<td>Kromasil 100-5 RP C₈ 250X4.6 mm ID 5 μm column</td>
<td>UV</td>
<td>Ref.¹⁶</td>
</tr>
<tr>
<td>4</td>
<td>Amino acid ILs containing imidazolium cations and amino acid anions</td>
<td>Acetonitrile/water (heptafluorobutyr-ic acid)</td>
<td>Ultimate ODS column (5 μm, 200 mm X 4.6 mm ID)</td>
<td>ESI-MS</td>
<td>Ref.¹⁸</td>
</tr>
<tr>
<td>5</td>
<td>Quaternary ammonium- and phosphonium ILs</td>
<td>Acetonitrile (0.1% TFA)/water (0.1% TFA)</td>
<td>150 mmX 4.6 mm ID Gemini 5 μm C₁₈ 110 Å column</td>
<td>ESI-MS, UV, and charged aerosol detection (CAD)</td>
<td>Ref.¹⁷</td>
</tr>
<tr>
<td>6</td>
<td>Imidazolium and pyridinium ILs</td>
<td>Acetonitrile/1.0 mmol/L 1-heptanesulfonic acid sodium</td>
<td>Chromolith Performance RP-18e column (4.6 mm i.d. X 100 mm)</td>
<td>UV</td>
<td>Ref.²⁵</td>
</tr>
</tbody>
</table>
Table 2.1—Continued Common mobile phases, stationary phases, and detection techniques used for the separation of ILs using RP-HPLC

<table>
<thead>
<tr>
<th>No.</th>
<th>Ionic Liquid Type</th>
<th>Mobile Phase(s)</th>
<th>Stationary Phase(s)</th>
<th>Detection Technique</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Imidazolium ILs</td>
<td>Acetonitrile/water (different additives)</td>
<td>Acquity UPLC BEH C&lt;sub&gt;18&lt;/sub&gt; (100 mm X 2.1 mm I.D., 1.7 μm)</td>
<td>Acquity UPLC photodiode array detector (PDA)</td>
<td>Ref. &lt;sup&gt;44&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>Imidazolium ILs</td>
<td>Acetonitrile/water (different additives)</td>
<td>Acquity UPLC BEH C&lt;sub&gt;8&lt;/sub&gt; (100 mm X 2.1 mm I.D., 1.7 μm)</td>
<td>Acquity UPLC photodiode array detector (PDA)</td>
<td>Ref. &lt;sup&gt;44&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>Imidazolium ILs</td>
<td>Acetonitrile/water (different additives)</td>
<td>Acquity UPLC BEH Phenyl (100 mm X 2.1 mm I.D., 1.7 μm)</td>
<td>Acquity UPLC photodiode array detector (PDA)</td>
<td>Ref. &lt;sup&gt;44&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>Alkylimidazolium ILs</td>
<td>Acetonitrile/water (10 mM ammonium acetate, 1% acetic acid)</td>
<td>Metasil Basic C&lt;sub&gt;8&lt;/sub&gt;</td>
<td>UV</td>
<td>Ref. &lt;sup&gt;12&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

2.4 Discussion

In developing a reversed-phase liquid chromatography method for the separation of breakdown products of the 1-butyl-3-methylimidazolium chloride ionic liquid, studied in Chapter 3, the combination of stationary phase, mobile phase, and additives plays a pivotal role in obtaining good chromatographic separation. Since IL cations are
permanently charged species, the classical reversed-phase liquid chromatography is not the method of choice because ionic species have poor retention on a traditional non-polar stationary phase. Ion pairing can improve separation of permanent ions in reversed-phase liquid chromatography by making the analyte ion temporarily more hydrophobic, and improve retention on a reversed-phase stationary phase. The choice of ion-pairing reagent should be compatible with MS detection requirements.

While IC, HILIC, and IP on RP stationary phases have frequently been used to study ILs, the π-π interactions of the biphenyl stationary phase with imidazolium ILs has not been studied in the past. This was a major consideration for the use of the biphenyl column discussed in Chapter 3. A 2.1 × 100 mm, Kinetex Biphenyl 2.6μm particle size 100 Å pore size column (Phenomenex, Torrance, CA) was used for the separation of IL cations. The mobile phase consisted of 20 mM aqueous ammonium acetate solution (A) and 0.5% formic acid in acetonitrile solution (B). The gradient was from 0% B (v/v) to 15% B in 15 minutes, followed by step change to 90% B for 10 minutes to elute all the hydrophobic compounds remaining on the column. The HPLC flow rate was 0.2 mL/min. Mass spectrometry was performed in positive ion mode electrospray ionization-MS. The MS conditions were: sheath gas flow rate 25 a/u, auxiliary gas flow rate 10 a/u, spray voltage 3.8 kV, capillary temperature 300 °C, capillary voltage 30 V and tube lens 65 V.

2.5 References


CHAPTER 3

SEPARATION AND IDENTIFICATION OF BIODEGRADATION PRODUCTS OF THE IONIC LIQUID 1-BUTYL-3-METHYLIMIDAZOLIUM CHLORIDE (BMIMCl) BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

Reprinted (adapted) from

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Identification of Metabolites Generated during the Biodegradation of the Ionic Liquid 1-butyl-3-methylimidazolium Chloride by Activated Sludge Microbial Community

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3.1 Introduction

One of the most widely used classes of ILs, as examined in Chapter 2, are the 1-alkyl-3-methylimidazolium based ionic liquids because of their green properties like non-volatility, non-flammability, high thermal stability and the ability of these compounds to be an excellent solvent for a broad range of inorganic and organic materials.¹⁻³
The rapidly increasing use of ILs in both academic and industrial fields have generated a growing concern about their impact on the environment. Many studies have demonstrated their toxic effects on aquatic and terrestrial ecosystems.\textsuperscript{4–11} Since ILs are highly soluble in water, but are not always biodegradable, a release of ILs into the environment may lead to significant water pollution problems. Moreover, ILs could become persistent pollutants in wastewater effluent due to their high stability in water. For this reason, studies examining IL biodegradability are of great importance. In particular, a life cycle assessment of the IL 1-butyl-3-methyl-imidazolium tetrafluoroborate (BMIMBF\(_4\)) has shown that it is more efficient than conventional solvents, but it could have an extremely detrimental impact when it is released into the environment.\textsuperscript{12} Many studies have been conducted to investigate the biodegradability of ILs and identify their breakdown products and pathways.\textsuperscript{13–20} Recent work by Neumann et al.,\textsuperscript{13} found imidazolium-based ILs to have the lowest biodegradation potential among 27 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium IL cations under aerobic conditions. Another study of the biodegradability of imidazolium and pyridinium ILs by an activated sludge microbial community by Docherty et al.,\textsuperscript{21} reported that biodegradation rates increase with longer alkyl chain length. In their study, hexyl and octyl substituted imidazolium-based ILs were only partially mineralized while butyl substituted imidazolium-based ILs were not biodegradable. A study of the removal of the imidazolium ILs by activated sludge microbial consortia investigated the biodegradability and kinetics of selected imidazolium ILs using several standard tests.\textsuperscript{22} These tests revealed that 1-alkyl-3-methylimidazolium bromide ILs are poorly biodegradable compounds, but their biodegradability increased with longer alkyl chain length. A recent study on the
biodegradation of seven imidazolium ionic liquids with different chemical structures by activated sludge microorganisms found that fully substituted ionic liquids and 1-ethyl-3-methyl-imidazolium bromide were barely biodegradable.\textsuperscript{23} Elongation of the alkyl side chain made ILs more susceptible to biodegradation, but cleavage of the imidazolium ring was not observed.

Since BMIM-based ILs are already used in several industrial applications in high quantities, but have also been shown to have a high potential for recalcitrance and toxicity in the environment, treatment steps of ILs before their release into waste water treatment facility is necessary to prevent its impact on the environment. In this study, the biodegradation of the 1-butyl-3-methylimidazolium chloride (BMIMCl IL) by a specialized microbial community, enriched by repeated isolation and transfer techniques, was investigated. The microbial community used was isolated by Docherty et al.\textsuperscript{24} (2015) from the aeration tank at the South Bend, IN wastewater treatment plant, and is capable of biodegrading BMIMCl within a 46-day period. The microbial community composition was examined using high-throughput 16S rRNA-based amplicon sequencing approaches. Biodegradation of BMIM was investigated using reversed-phase high-performance liquid chromatography (RP-HPLC) and the combination of UV, MS, and $^1$H-NMR detection. The identification of several biodegradation products used a combination of tandem mass spectrometry (MS/MS) and $^1$H-NMR techniques.
3.2 Experimental Procedures

3.2.1 Biodegradation of 1-butyl-3-methylimidazolium chloride (BMIMCl) by Activated Sludge Microbial Community

Samples for LC-MS analyses were received from the Docherty lab at WMU and used without further purification. The procedure used by the Docherty lab to isolate the microbial community and biodegrade BMIM is as follows:

The microbial community used for this study was isolated as described in Docherty et al. 2015. In this study, the enriched microbial community was transferred into 1L of new sterile minimal media containing 130 mg/L of BMIMCl. Minimal media was prepared according to instructions for the Dissolved Organic Carbon Die-Away test for ready biodegradability (OECD 2006). After three weeks of constant shaking at 1000 rpm at room temperature, concentrated inocula were prepared from these cultures for this study. All 3 L cultures were centrifuged in 50 mL aliquots at 2000 rpm for 5 minutes. Then the aliquots were resuspended and combined into a single microbial pellet. The pellet was resuspended in 200 mL of minimal media without a carbon source, and then 35 mL of the specialized microbial inoculate was added into duplicate, sterile 2 L bottles containing minimal media spiked with 130 mg/L BMIM. One 2 L bottle also contained the abiotic control, which had the microbial inoculate and minimal media without BMIMCl added. Finally, one 2 L bottle was prepared as an abiotic control, which received no microbial inoculate and was monitored for the non-biotic degradation of BMIMCl. Bottles were shaken in the dark at 2000 rpm at room temperature. The biodegradation of BMIMCl by the specialized microbial community was monitored twice per week for 46 days. At each
time point, the sample bottle was inverted, and 30 mL of sample was aseptically removed using a Pasteur pipette. Samples were filtered through a 0.22 µm pore size syringe filter (Whatman). At the time of collection, the absorbance of the BMIM cation in each treatment and control was measured at 210 nm using an Epoch 96-well plate reader (Biotek). These measurements indicated whether or not the imidazolium ring remained intact. Then, 15 mL aliquots were prepared in sterile plastic tubes. All the samples were kept in a -80 °C freezer after the collection.

### 3.2.2 Characterization of Microbial Community

Characterization of the microbial community was done by Dr. Kathryn Docherty, Assistant Professor of Biological Sciences, Western Michigan University, at Notre Dame University. The microbial community characterization procedure is explained as follows:

At the start of the experiment described above, genomic DNA was extracted in triplicate from the initial microbial inoculate pellet. 20 mL of resuspended pellet was filtered onto a sterile 0.22 µm membrane filter (Whatman), and the filter was placed into the initial tube of a PowerSoil DNA Isolation Kit (MoBio Labs, Inc.). Genomic DNA was extracted according to the manufacturer’s instructions. Following extraction, DNA was quantified from each triplicate extraction using a Qubit 2.0 Quantitation System (Life Technologies, Inc.). DNA concentrations were within the range of 104 ng m/L DNA was sent to the Michigan State Genomics Core Facility (East Lansing, MI) for 16S rRNA amplicon library preparation and high throughput sequencing. The 16S V4 rDNA region in bacteria was amplified using primers described by Schloss et al. Amplification
products were normalized using an Invitrogen SequalPrep DNA Normalization plate and normalized products were pooled. After quality control and quantitation the pool was loaded on an Illumina MiSeq flow cell v2 and sequenced with a 500 cycle (PE250) reagent kit. Online base calling was performed by Real Time Analysis (RTA) software v1.18.54. The output of RTA was demultiplexed and converted to FastQ files with Illumina Bcl2Fastq v1.8.4. Sequences were trimmed and processed using mothur v.1.33.3 (Schloss et al. 2009)\textsuperscript{27} according to standard operating procedures. Following processing, 65,356 sequences per each replicate were rarefied to, which was the lowest number of high-quality amplicon sequences across all samples. Sequence Taxonomy was assigned by comparison to the Silva version 4 database (Quast et al. 2012).\textsuperscript{28} All sequences per sample were grouped according to Phylum-level identification to describe the composition of the bacteria present in the initial inoculates used to degrade BMIMCl.

3.2.3 Identification of BMIMCl Metabolites Methods

3.2.3.1 High-Performance Liquid Chromatography Coupled with UV Detector and Mass Spectrometer (HPLC-UV-MS)

A BMIM solution, after exposure to the activated sludge microbial community for 3, 7, 10, 14, 21, 28, 35, 38, 42 and 46 days, was assayed using HPLC-UV-MS. Samples were analyzed using an Agilent 1100, G1312A Bin Pump, Waters 2487 Dual λ Absorbance Detector and a Thermo LTQ ion trap mass spectrometer. Samples were injected through a 2 μL loop onto a 2.1 × 100 mm, Kinetex Biphenyl 2.6μm particle size 100 Å pore size column (Phenominex, Torrance, CA). The mobile phase consisted of 20 mM aqueous
ammonium acetate solution (A) and 0.5% formic acid in acetonitrile solution (B). The gradient was from 0% B (v/v) to 15% B in 15 minutes, followed by step change to 90% B for 10 minutes, in order to elute all the hydrophobic compounds remaining on the column.

The HPLC flow rate was 0.2 mL/min, which was split 50:50 to the UV and mass spectrometer detectors. The UV wavelength was set at 212 nm (λmax for the imidazole ring), and mass spectrometry was performed in positive ion mode electrospray ionization-MS. The MS conditions were: sheath gas flow rate 25 a/u, auxiliary gas flow rate 10 a/u, spray voltage 3.8 kV, capillary temperature 300 °C, capillary voltage 30 V and tube lens 65 V.

The test compound 1-butyl-3-methylimidazolium chloride used in the biodegradation study was purchased from IoLiTec (Tuscaloosa, AL). The 1-methylimidazole standard for 1H-NMR and LC/MS analyses was purchased from TCI America, and its purity was 99%.

3.2.3.2 Extraction of Product Ion

To search for additional biodegradation products below the UV detector limit of detection, an extraction of product ion methodology was applied using the Thermo LTQ ion trap mass spectrometer, since ion trap mass spectrometers are unable to perform precursor ion scans. Collision-Induced Dissociation (CID) of BMIM leads to a very stable fragmentation product with a mass to charge ratio (m/z) value of 83, corresponding to methyl-imidazolium. Extraction of this product ion under non-selective in-source
fragmentation conditions resulted in the identification of a larger set of biodegradation products with the intact imidazolium ring. In-source fragmentation with a collision energy of 25 kV was used to obtain extracted product ion peaks for the fragment at m/z 83 in the chromatogram. These peaks represent potential metabolites, which were identified in the corresponding full scan mass spectrum and analyzed by CID MS/MS.

3.2.3.3 Proton Nuclear Magnetic Resonance (¹H-NMR)

The BMIM solutions described above that were exposed to the activated sludge microbial community for varying lengths of time were also analyzed by 1-dimensional ¹H-NMR spectroscopy. Collection of NMR spectra and data interpretation were conducted by Dr. Blair Szymczyna, Assistant Professor of Chemistry, Western Michigan University. ¹H-NMR samples were prepared by adding D₂O to the filtered samples to a final concentration of 5%. One-dimensional ¹H-NMR spectra were acquired on an 800 MHz Bruker Avance II spectrometer equipped with a 5 mm TCI cryoprobe with Z-axis gradients and used the WATERGATE pulse sequence to suppress the water signal. Time course spectra were collected using identical parameters and were referenced to an external 2,2-dimethyl-2-silapentane-5-sulfonate standard. The NMRPipe program package was utilized to process and analyze the spectra. Resonances to protons in the molecule were assigned based upon signal multiplicity and previous work (Docherty et al. 2007). Chemical shift predictions were obtained from ChemBioDraw.

The metabolites in the sample were identified from the one-dimensional data. The volumes of each observed resonance in the time course spectra were determined by
integration and the change in intensity was plotted as a function of time. Resonances that have similar rates of appearance and disappearance were ascribed to the same molecule, and the correlation was confirmed using multiple regression analysis. For direct comparison of the resonances associated with one molecule, the volume at each time point was normalized to the sum of all time point volumes associated with the resonance. Based on the starting material and the associated chemical shifts, the identity of the major metabolite was predicted to be 1-methylimidazolium. Confirmation of the molecule’s identity was achieved by doping the Day 17 sample with 55 μmol and 110 μmol of 1-methylimidazole (TCI America).

3.3 Results and Discussions

3.3.1 The Microbial Community

The bacterial community described is distinctly different from communities that are typically collected directly from aeration tanks at a wastewater treatment facility. These communities are typically dominated by taxa within the Proteobacteria, Firmicutes and Actinobacteria phyla, and are not dominated by taxa within Bacteriodetes (e.g. Xia et al. 2010. Yu and Zhang 2012). Bacteroidetes are typically characterized by slow-growing members capable of using recalcitrant carbon substrates, which can be selected for by reducing the amount of other faster-growing phyla that are adapted to labile substrates. It follows that a disproportionate amount of Bacteroidetes must be selected for to degrade the normally recalcitrant BMIMCl. Interestingly, while Bacteroidetes is the predominant phylum, the inoculate still contains a high level of diversity. It is possible that
Bacteroidetes are responsible for metabolizing BMIM, but that other community members are responsible for metabolizing the break-down products. Similarly, other community members may also be necessary to provide necessary co-factors for the primary bacterial degraders.

3.3.2 Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

The BMIM cation is dissociated from its chloride counter ion in aqueous solution and detected in positive ion mode ESI-MS at m/z 139.1 with a retention time of 9.95 minutes and at 212 nm using the UV-absorbance detector (Figure 3.1 and 3.2).

Figure 3.1 MS spectrum of BMIM cation standard solution
Figure 3.2 (a) Extracted ion chromatogram (EIC), (b) Total ion chromatogram (TIC) and (c) UV trace of BMIM standard

Area counts of BMIM cation peaks obtained from Extracted Ion Chromatograms (EIC) over 46 days of treatment were plotted to measure the biodegradation over time as shown in Figure 3.3. BMIM cation area counts correspond to the concentration of BMIM cation over the 46 days of the microbial treatment. The $^1$H resonances observed in the NMR spectra for the BMIM molecule were integrated, normalized to the resonance volume at Day 3 and also plotted as a function of time, as shown in Figure 3.3.
3.3.3 Biodegradation of 1-butyl-3-methylimidazolium Cation (BMIM)

Biodegradation of BMIMCl was monitored throughout the 46 day incubation period using LC-MS, $^1$H-NMR, and the absorbance at 212 nm chromatographic peak area counts. Complete biodegradation of BMIM, including the imidazolium ring, was completed by a selected microbial consortium. Both MS and $^1$H-NMR data showed that the concentration of BMIM cation reached the lowest detectable concentration after 31 days of the treatment, as shown in Figure 3.3. These results indicate that the activated sludge microbial community used in this study is capable of effective biodegradation of BMIM in 30-35 days. This is in contrast to previous studies in which BMIM was shown to be a poorly biodegradable compound.$^6,21,23,34,35$

![Figure 3.3 Biodegradation of BMIM cation over 46 days of the treatment followed by LC-MS and $^1$H-NMR. The orange triangles represent the results obtained by LC-MS while the blue dots represent the resonance volume change observed by $^1$H-NMR spectroscopy. The $^1$H-NMR resonance values are the average of all normalized volumes, and the error bars correspond to the variability between resonances (±1 SD, n=7). All data points are normalized to day 3.](image-url)
The percentages of biodegradation are calculated relative to Day 3 of the treatment. BMIM cation biodegradation was complete by the end of the treatment time course as shown in Figure 3.4.

![Graph showing biodegradation over time](image)

**Figure 3.4** The percentage of biodegradation of BMIM compared to day three over 46 days of the treatment.

The absorbance of the BMIM cation at 210 nm was also measured during the growth period using filtered samples at each time point. The results of this time series are shown in Figure 3.5. Absorbance measurements agree with LC-MS results and indicate that metabolism of the core imidazolium ring, which is the chromophore that absorbs at 210 nm, begins on Day 28 and rapidly decrease following this initial drop in absorbance. Specific time points for $^1$H-NMR and LC-MS analysis were chosen based on these measurements.
3.3.4 Characterization of Microbial Community

To determine why this particular community was able to successfully degrade BMIM where others failed, the initial microbial inoculate was characterized using a high throughput Illumina MiSeq approach. The average amount of each bacterial phylum detected across the triplicate samples extracted from the inoculate pellet are described in Figure 3.6. There were 5030 distinct operational taxonomic units (OTUs) identified across the samples, indicating that the enriched bacterial community used for inoculation was highly diverse and did not represent a few selected organisms. There was little variation
between each of the replicates. The predominant bacterial phylum represented in the community was Bacteroidetes, which comprised >33% of the community. Taxa within β-Proteobacteria (8%), Verrucomicrobia (7%) and α-Proteobacteria (5%) also represented significant fractions of the total bacterial community. A large amount of OTUs (22%) were unclassified at the phylum level, and 24% of OTUs were identified as members of phyla that represented less than 5% of the total community.

Figure 3.6 The percent relative abundance of bacterial phyla represented as the average of triplicate extraction and sequence reactions prepared from the initial inoculate pellet. (Data collected and Figure prepared by Dr. Kathryn Docherty)


3.3.5 Biodegradation Products of BMIMCl

Three major biodegradation products were identified in this study by the two complementary NMR and HPLC techniques. HPLC was combined with both UV and MS detections. The MS detected either the intact molecules (BMIM) or was used to search for molecules with the common imidazolium structure by using an extraction of product ion mass spectrometric strategy. \(^1\)H-NMR spectroscopy results suggest that two major metabolites arise from the degradation of BMIM, but the appearance of several less intense peaks at longer incubation times suggest other metabolites exist as well. HPLC-UV and HPLC-MS led to the identification of the m/z 83 biodegradation product while extraction of product ion resulted in the discovery of the m/z 137 and 155 biodegradation products.

3.3.5.1 Appearance and Disappearance of Metabolites

To investigate the breakdown products by LC-MS, the m/z ratios of the three major biodegradation products were extracted from the total ion chromatograms (TICs) over the 46 days of the treatment. An example of the extracted chromatograms is shown in Figure 3.7. The area counts of the extracted ion chromatograms (EICs) for each suspected breakdown product was plotted in Figure 3.8. Extraction of m/z 137 from the total ion chromatogram (TIC) of BMIM samples, however, resulted in three different peaks at three different chromatographic retention times (as shown in Figure 3.7). The peak with a 1 min. retention time maintained a constant concentration over the 46 days of treatment as shown in Figure 3.8. This compound was therefore assumed not to be a BMIM biodegradation product. The other two products increased with longer degradation times, and this was
interpreted as evidence that they were potential biodegradation products. None of these suspected metabolites were observed in the LC-MS chromatogram for a BMIM reference standard, which is further evidence that they are likely compounds produced during biodegradation and not impurities in the BMIM standard initially used in this study.

![Figure 3.7](image)

**Figure 3.7** LC-UV-MS of day 21 of BMIM microbial treatment showing extracted ion chromatograms for m/z 139 BMIM and breakdown products of m/z 155 BMIM+O, m/z 137 BMIM-2H, m/z 83 MeIM and UV absorbance at 212nm trace. (Order from top to the bottom)
Additional ions were identified at m/z 141, 123, 98 and 82 using HPLC-UV and HPLC-MS techniques, but they did not show any significant change over the 46 days of treatment.

LC-MS data suggest that ion with m/z 83 is the most abundant of the metabolite and is an intermediate product in the degradation of BMIM. The metabolite begins to appear in the media immediately after inoculation of the BMIM sample, achieves a maximum concentration at Day 17, and is completely degraded after 46 days. The area under the time-course curve for the m/z 83 metabolite, presented in Figure 3.8, accounts for less than 14% of the BMIM concentration originally present in the sample, even when differences in mass spectrometry sensitivity due to different ionization efficiencies are taken into account (Figure 3.12(d)). Since the microbial community is not lysed before filtration, the increase and decrease in the signals associated with the m/z 83 metabolite suggest that the metabolite is excreted by one or more microbes as a waste product, and subsequently metabolized by other microbes in the culture. According to the previous studies\textsuperscript{15,20,34} on pyridinium-based ionic liquids, after cleavage of the ring, biodegradation occurs rapidly (Docherty et al. 2010), and metabolites are likely respired or incorporated into microbial biomass.\textsuperscript{36} \textsuperscript{1}H-NMR studies confirmed LC-MS results and indicated that the resonances of the most prevalent metabolite (m/z 83) increased in intensity up until Day 17 but was, in turn, degraded. The less prevalent metabolite (m/z 155) increased to a maximum value by Day 24 and was not further degraded as shown in Figure 3.9.
Figure 3.8 Biodegradation of BMIM over 46 days followed by LC-MS. Formation and depletion trends for the major and two minor detectable biodegradation product trends over 46 days of the treatment. The green line represents MeIM: m/z 83 (D); the dark blue BMIM – CH$_3$ + O: m/z 141; the light blue BMIM + O: m/z 155 (B); the grey and orange lines represent BMIM-2H: m/z 137 (C) at 9 and 10 min. retention times respectively. For B, C and D see Figure 6. To have all breakdown products in the same scale, MeIM area count is divided by 5.
3.3.6 Identification of BMIM Metabolites

The identity of the metabolites that result from BMIM degradation, shown in Figure 3.10, were determined by experimental results and validated by literature studies. The three metabolites identified by MS and \(^1\)H-NMR, which have m/z ratios of 83, 137 and 155, were compared to structures that may be derived from BMIM (m/z 139). For the biodegradation product with m/z 155, hydroxylation of the imidazolium ring was proposed and confirmed by MS/MS and \(^1\)H-NMR studies. The breakdown product at m/z 137 has a
mass that is consistent with the introduction of a double bond into the butyl side chain of BMIM cation. Removal of the butyl group from the imidazole group would result in a methylimidazolium cation that has an m/z of 83.

Figure 3.10 Structures of BMIM cation and breakdown products predicted from experimental results and literature studies of related compounds and confirmed by MS/MS and $^1$H-NMR studies. A: 1-butyl-3-methyl-1H-imidazol-3-ium (m/z 139 BMIM); B: 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium (m/z 155 BMIM+O); C: (Z)-1-(but-1-en-1-yl)-3-methyl-1H-imidazol-3-ium (m/z 137 BMIM-2H); D: 3-methyl-1H-imidazol-3-ium (m/z 83 MeIM).
The major primary metabolite that results from the degradation of BMIM has an m/z ratio of 83 and was identified as 1-methylimidazolium, which is in turn degraded by the microbial community. The 1-methylimidazolium product is resistant to CID-fragmentation, and this property was observed for the degradation samples. The 1-methylimidazolium standard and the CID fragmentation of BMIM are shown in Figure 3.12(a) and (d). During CID fragmentation of the BMIM cation, the fragment at m/z 83 had a similar resistance to subsequent fragmentation attempts in MS/MS studies.

Confirmation of the major metabolites identity was obtained by adding pure 1-methylimidazolium directly to the Day 17 NMR sample. Only the four resonances attributed to this molecule increased in intensity upon doping the sample (Figure 3.11).
Figure 3.11 Confirmation that 1-methylimidazolium is the major metabolite produced by degradation of BMIM was obtained by adding 55 μmol (red) and 110 μmol (blue) of 1-methylimidazolium to the Day 17 sample. Only the resonances ascribed to 1-methylimidazolium increased in volume. (Data collected and Figure prepared by Dr. Blair Szymczyna)
Figure 3.12 Chemical structure and possible fragmentation pattern of: (a) 1-butyl-3-methyl-1H-imidazol-3-ium (m/z 139, BMIM), (b) (Z)-1-(but-1-en-1-yl)-3-methyl-1H-imidazol-3-ium (m/z 137) and (c) 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium (m/z 155), and (d) An equimolar concentration mixture of 10μM methylimidazolium standard and BMIM analyzed by ESI-MS in positive ion mode.
Fragmentation of the ion at m/z 137 yielded the three fragment ions shown in Figure 3.12(b). Fragment ions with m/z 122 and 95 correspond to the loss of one and three carbon groups, respectively, from an unsaturated butyl chain of the BMIM cation and the m/z 83 fragment ion. Unsaturation of the carbon side chain is consistent with the suggested degradation pathway for pyridinium based ionic liquids, as shown previously by Docherty et al\textsuperscript{20}. A broad resonance at 5.78 ppm in the \textsuperscript{1}H-NMR spectrum is likely associated with protons attached to carbon atoms involved in a double bond.
Identification of the ion at m/z 155 as 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium was determined using a combination of MS and $^1$H-NMR spectroscopy. Fragmentation of the ion, as shown in Figure 3.12(c), yielded two main fragment ions with m/z values of 83 and 99. The fragment ion at m/z 83 is consistent with 1-methylimidazolium, while the fragment ion at m/z 99 corresponds to methylimidazolium with a hydroxyl group attached to the imidazolium ring. CID is not able to determine the position of the hydroxyl group on the ring, but NMR spectroscopy revealed that the hydroxyl group is at position 2. For the BMIM and 1-methylimidazolium molecules, three resonances exist in the aromatic region of the spectrum. The downfield most resonance appears as a singlet at 7.96 ppm and corresponds to the hydrogen at position 2, while two upfield signals have similar chemical shifts (7.16 and 7.23 ppm) and correspond to the hydrogen atoms at positions 4 and 5. (Figure 3.13) The metabolite detected by $^1$H-NMR that has a similar trend as m/z 155 lacks the upfield resonance but the two downfield resonances are present (Figure 3.14). The chemical shift values obtained by 1D-NMR are nearly identical to the values predicted by ChemBioDraw for 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium. Resonances at 1.61, 3.24 (singlet) and 3.62 ppm (triplet) have trends that correlate with the aromatic peaks. The triplet is consistent with the methylene group adjacent to the imidazole ring, but other resonances are difficult to assign unambiguously and identify due to overlapping signals. Hydroxylation of the ring is analogous to the previously suggested degradation pathway for pyridinium based ionic liquids. In the time frame used in this study, though, 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium is a metabolite that cannot be further degraded by the microbial community tested.
Figure 3.13 Monitoring of BMIM biodegradation over 46 days by solution $^1$H-NMR spectroscopy. The signals assigned to BMIM (solid arrows) disappeared over the course of the experiment while signals corresponding to metabolites appeared (open arrows). Two metabolites (lower case and Greek letters) could be identified by monitoring the signal intensity trends. The observed resonances aided in the identification of the metabolites produced by the microbial cultures. (Data collected and Figure prepared by Dr. Blair Szymczyna)
Figure 3.14 $^1$H NMR spectrum of the Day 46 sample reveals that other metabolites exist in the sample. Weak signals at 5.78 and 3.5 ppm could be associated with hydrogen atoms adjacent to double bonds and hydroxyl groups, respectively. The labeling scheme used is identical to that in Figure 4. (Data collected and Figure prepared by Dr. Blair Szymczyna)

3.4 Conclusion

This investigation demonstrated that biodegradation resistant BMIM could be fully degraded by the Bacteroidetes-enriched microbial community used in this study, and indicates that selected microbial consortia could be effectively used to increase biodegradability of different ionic liquids. Three major metabolites from BMIM biodegradation were identified, and their structures were confirmed using MS/MS and $^1$H-
NMR techniques. The main metabolite identified and confirmed in this study was produced by loss of the butyl side chain from the initial compound, which will make it less lipophilic and, potentially, less toxic to microorganisms. Further investigation of the toxicity of the obtained metabolites is necessary as future work. A side product that forms to a lesser extent, 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-iium, is resistant to further biodegradation by the microbial community described here. To the best of our knowledge, we report the first complete biodegradation of the imidazole ring in BMIM by a specially selected microbial community.

3.5 References


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CHAPTER 4

IDENTIFICATION AND QUANTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN THE KALAMAZOO RIVER OIL SPILL BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

4.1 Introduction

4.1.1 Kalamazoo River Oil Spill Accident

Since the early 1960s, over one billion liters of oil have spilled into the United States marine waters.\(^1\) One of the nation’s worst inland oil spills occurred near Marshall, Michigan, six years ago. On July 25, 2010, at 5:58 p.m., eastern daylight time, a 30-inch pipeline (Line 6B), owned and operated by Enbridge Incorporated (Enbridge) ruptured in a wetland in Marshall, Michigan.\(^2\) Line 6B transports different types of heavy bituminous crude oil from the oil sand regions of Western Canada that require dilution with lighter petroleum products to enable the crude to flow easier (Figure 4.1). The fracture in the ruptured segment measured about 2 m long and up to 13.5 cm wide (as shown in Figure 4.2). The rupture was not discovered for over 17 hours. The estimated 3,192,783 L of crude oil released, entered Talmadge Creek and flowed into the Kalamazoo River, a Lake Michigan tributary (see Figure 4.3). The oil saturated the rupture site and the surrounding wetlands, and seriously affected the environment.
Figure 4.1 Enbridge’s liquids system and the 1,900-mile lakehead system (the U.S. portion). The inset shows Line 6B, the 293-mile extension from Griffith to Sarnia installed in 1969. (Source: NTSB)
Figure 4.2 The ruptured segment of Line 6B in the trench following the July 25, 2010, rupture. The fracture face measured about 2 m long and was 13.5 cm wide at the widest opening. The fracture ran just below the seam weld that was oriented just below the 3 o’clock position. A red circle shows a location where the coating was wrinkled and had separated from the pipe surface. (Source: NTSB)
Five years following the 2010 Kalamazoo River oil spill accident in Marshall, Michigan, polycyclic aromatic hydrocarbons (PAHs) were investigated in riparian areas along the Kalamazoo River, between Marshall and Galesburg communities, which were polluted with spilled oil, using gas chromatography-mass spectrometry. Six locations along the Kalamazoo River were investigated. 16 PAHs (as shown in Figure 4.4) were identified, and their concentrations were quantified using GC-MS. Among the 16 PAHs examined in this work, 15 PAHs are on the US Environmental Protection Agency (EPA) list of 16 PAHs frequently monitored because of their carcinogenic and mutagenic properties (Figure 4.4).\(^3\) Seven of the 16 PAHs were studied in this investigation are on the European Union (EU) list of 15 PAHs that are of major concern for human health because of their toxic properties (Figure 4.4).\(^4\) The concentration of total PAHs in soil at the accident site was high. Pyrene,
fluoranthene, chrysene, benzo(a)pyrene and phenanthrene were dominant PAHs at the oil spill site.

Figure 4.4 Chemical structure of the selected 16 PAHs, * on the EPA list of frequently monitored PAHs; ** on the EPA and EU lists of frequently monitored PAHs
Figure 4.4 - Continued Chemical structure of the selected 16 PAHs, * on the EPA list of frequently monitored PAHs; ** on the EPA and EU lists of frequently monitored PAHs
4.1.2 Environmental Effects of PAHs

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds that are composed of two or more fused benzene rings (Figure 4.4).\(^5,\!^6\) PAHs are an important class of persistent environmental pollutants because of their carcinogenic and mutagenic properties, and their tendency to deposit and accumulate in soil, sediments and water.\(^6,\!^7\) The most environmentally relevant PAHs are those containing two to seven aromatic rings.\(^5\) The major anthropogenic sources of PAHs in the environment are pyrogenic and petrogenic sources. Pyrogenic PAHs come from the incomplete combustion of organic substances such as fossil fuel, wood, asphalt, and industrial waste, while petrogenic PAHs originate from crude oil and petroleum products.\(^7,\!^8\) The most common sources of petrogenic PAHs in the environment are caused by oil spills, leaking engines and fuel oil, dumping of used crankcase oil, and unburned fuel absorbed into street dust.\(^9\)
Spilled oil is expected to degrade with several factors such as evaporation, diffusion, photooxidation, and biodegradation.\textsuperscript{10–14} When an oil spill occurs in a water ecosystem, the levels of PAHs are significantly higher in water and could have adverse effects on the aquatic environment.\textsuperscript{15} PAHs persist in the environment, and their toxicities could represent significant threats towards aquatic organisms. A study of tumor frequencies in walleye and brown bullhead, and sediment contaminants in tributaries of the Laurentian great lakes by Baumann et al.,\textsuperscript{16} found that elevated PAHs in sediments can induce cancer in wild fish. Accurate information about the levels of PAHs in the Kalamazoo River and surrounding areas after the oil spill accident is not readily available. The lack of reliable information about the concentrations of the PAHs contaminants in the Kalamazoo River after the oil spill accident motivated this investigation to determine the levels and identities of PAHs in the river.

4.1.3 Health Effects of PAHs

The presence of PAHs in the atmosphere is mainly due to the incomplete combustion of organic matter, while in aquatic environments PAHs come mostly from atmospheric deposition, municipal or industrial effluents, and oil spills.\textsuperscript{8,17} Exposure to PAHs is a huge concern for human health, and many PAHs are listed by the EPA and EU as carcinogenic and mutagenic.\textsuperscript{3,4} PAHs enter the organism by inhalation, ingestion or penetration, followed by distribution to various organs.\textsuperscript{18,19} PAHs are metabolized to dihydrodiols by hydrocarbon hydroxylases that are present in the liver.\textsuperscript{3,19,20} Dihydrodiols and their epoxide derivatives bind to DNA and proteins and promote mutagenic processes
in the cells. Exposure in utero to PAHs can cause DNA damage, chromosomal mutation and increased the risk of leukemia in childhood. Perera et al, Columbia University, demonstrated that prenatal exposure to PAHs causes genetic changes in newborns that have been linked to leukemia and other cancers.\textsuperscript{21} PAHs such as benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, and indeno [1,2,3 c,d]pyrene caused tumors in laboratory animals when they were inhaled, eaten, or exposed for long periods of time.\textsuperscript{18} An experiment was carried out on mice to study the effect of some PAHs and showed that feeding pregnant mice with high levels of benzo[a]pyrene resulted in decreased fertility, for the mice and their offspring.\textsuperscript{18} Directly feeding the offspring of the pregnant mice with benzo[a]pyrene can lead to harmful effects, such as body defects and decreased body weight. Similar effects could conceivably occur in humans.

4.2 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is a very versatile analytical tool in which substances in the gaseous state are separated based on volatility and interaction with a stationary phase, and then introduced into a mass spectrometer. The mobile phase usually is helium, nitrogen, or hydrogen gasses, while the stationary phase is a viscous liquid such as squalene, polyethylene glycol, or polymethyl siloxane, or an adsorbent solid such as silica, molecular sieves, alumina, and porous polymers.\textsuperscript{22} The stationary phase is housed in a polyimide-coated fused silica or metal column. A schematic diagram of a typical GC-MS system is shown in Figure 4.5. The combination of gas chromatography with mass spectrometry has
numerous advantages when dealing with a broad range of contaminants with different polarities and volatilities. The complexity of environmental samples requires a method that is capable of separating individual components from a mixture before detection in order to identify and quantify them efficiently and accurately. Chromatographic techniques combined with mass spectrometry, especially LC-MS and GC-MS, are intensively used in the environmental sciences to investigate the presence of organic contaminants.  

GC-MS is widely used for the analysis of volatile or semi-volatile compounds sampled from solid, liquid, or gaseous samples. GC-MS enables the identification of many environmental organic pollutants, such as pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxin, and volatile organic compounds (VOCs).

![Figure 4.5 Schematic diagram of a typical GC-MS system](image)

Figure 4.5 Schematic diagram of a typical GC-MS system
4.3 Instrumental Details and Methods

4.3.1 Study Area and Soil Samples Collection

The study was carried out on the Kalamazoo River oil spill area between Marshall, where the oil pipeline rupture occurred, and Galesburg, which is located 2 miles away from Morrow Lake, in Michigan (see Figure 4.6). The study area covered 35.5 miles along Kalamazoo River. The starting point of the study area was set at 6 miles before the oil spill accident site while the end point was 29.5 miles from the accident site. Soil samples were collected by Dr. Michael J. Barcelona, Professor of Chemistry at Western Michigan University, and Pablo Rodriguez (a Brazilian exchange student) in July 2015 five years after the Kalamazoo River oil spill accident (Figure 4.7).
Figure 4.6 Map showing rupture location, sample locations (white stars), and affected waterways from Talmadge Creek to Morrow Lake (Source: NTSB)
Soil samples were collected from six shoreline locations along the Kalamazoo River, which spans the oil spill affected area. Two soil samples were collected from sites upstream to the spill, one sample was obtained from the rupture site, and three samples were collected from downstream sites. Each soil sample was divided into two layers: an upper layer (0-6 inches depth) and a lower layer (6-12 inches depth) as shown in Figure 4.8. The soil samples were collected using a split-spoon sampler. A split-spoon sampler is
shown in Figure 4.9. After collection from sampling sites, soil samples were stored in glass jars and transported to the laboratory to be kept in the refrigerator.

The study area has a humid continental climate with hot summers and no dry season. Over the course of a year, the temperature typically varies from 16°F to 83°F and sometimes is below 2°F or above 89°F. The warm season is between May and September with an average mean temperature above 73°F while the cold season is between November and March with an average mean temperature below 40°F.  

Figure 4.8 Soil sampling procedure for the six locations of the study area
4.3.2 Sample Preparation for GC-MS

After collection, samples were kept in capped glass jars in a refrigerator. To prepare the samples for GC-MS analysis, 10 g of each collected soil sample was placed in a 60 °C drying oven overnight. Then, 10 mL of H₂O and 1 mL of hydrochloric acid (HCl) were mixed with each sample using a stir bar, and further incubated in the oven overnight at 60 °C. Finally, PAHs were extracted from the sample with dichloromethane (CH₂Cl₂) prior to GC-MS analysis.

4.3.3 GC-MS Analysis

4.3.3.1 Apparatus

GC-MS analyses were carried out on an HP 6890 Series GC system (Hewlett-Packard, Palo Alto, CA, USA) connected to an HP 5973 Mass Selective Detector operated
in the 70 eV electron impact mode under standard conditions. A 30 m long, 250 μm I.D and 0.25 μm film thickness ZB-5MS (Phenyl Methyl Siloxane) Phenomenex column (Phenomenex, Torrance, CA, USA) was used with helium as a carrier gas at a linear velocity of 1.3 mL/min. The injector temperature was set at 250 °C. The sample solutions were injected in the splitless mode and the injection volume was 2 μL. The mass spectrometer source temperature was set at 200 °C. The mass range was set at m/z 35-600. The column temperature was programmed to hold at 40 °C for 2 min., then the temperature was changed at 10 °C/min, and then hold at 320 °C for 20 min.

4.3.3.2 Chemicals

A standard solution of 16 PAHs (shown in Figure 4.4) at 2000 μg/mL in dichloromethane was purchased from Sigma-Aldrich (CRM47930, Sait Loius, MO, USA) and used as a calibrant. GC-MS grade dichloromethane was supplied by Fisher Scientific (Hampton, NH, USA) and used as a solvent for sample extraction and GC-MS analyses.

4.4 Results and Discussion

The standard solution of 16 PAHs (as shown in Figure 4.4) in dichloromethane was used as a calibrant to investigate the presence of PAHs qualitatively and quantitively at the six sampling sites. A GC-MS chromatogram of 20 μg/mL of the PAHs standard in dichloromethane is shown in Figure 4.10. The choice of the PAH standard compounds was made based on a survey of the most predominant PAHs in the study area and considering
the EPA list of 16 PAHs that are most frequently monitored because of their carcinogenic and mutagenic properties.

Figure 4.10 GC-MS spectrum of 20 μg/mL of PAHs standard.

The distribution of the total PAHs in the study area are shown in Figure 4.11. The trend seemingly indicates that the oil spill accident site has the highest concentration of total PAHs among the six locations of the study area. This results also show that the total PAH concentration in the upper layer (0-6 inches depth) of soil is higher than that of the lower layer (6-12 inches depth). Five years after the oil spill accident, apparently the PAHs did not migrate downstream from the accident site, where they first accumulated, and is only slowly migrating through the sediment layers. PAH deposition at the accident site
could be attributed to the fact that PAHs are poorly soluble in water. Hence, they tend to deposit in the sediments and soils.\textsuperscript{20} Several studies have reported that PAHs concentrate in sediments and soils because of their lipophilic properties.\textsuperscript{20,31,32}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.11.png}
\caption{Figure 4.11 Trend of total PAH concentrations along the study area. The solid blue line represents the concentration of total PAHs in the upper layer samples (0-6 inches-samples) while the dashed line represents the concentration of total PAHs in the lower layer samples (6-12 inches-samples). 0 mile is the oil spill accident site, while negative and positive numbers represent upstream and downstream sites respectively to the accident site.}
\end{figure}
The concentration of total PAHs in the upper layer (0-6 inches-samples) of soil samples in the six sampling locations of the study area is shown in Figure 4.12. The results indicate that the accident site (6 miles from the study area start point) has the highest total PAH concentration of 61,170 ng/g dry weight among the six locations studied in this work. The second highest total PAH concentration is 2,703 ng/g dry weight at sampling site number three, which is half a mile downstream from the oil spill accident site. The other four sampling sites have very low concentrations of total PAHs relative to the accident site.

The concentration of total PAHs in the lower soil sample layer (6-12 inches-samples) from the six sampling locations of the study area is shown in Figure 4.12. The results indicate that the accident site (6 miles from the study area start point) has the highest total PAHs concentration of 32,059 ng/g dry among the six locations investigated in this research. The concentration of total PAHs in the lower layer of the accident site sample is about half that of the upper layer. The results suggest that the PAHs did not penetrate too deeply into the soil, since as you go deeper into the ground, the concentration of PAHs decreases.

The concentration of total PAHs in both the upper layer (0-6 inches-samples) and the lower layer (6-12 inches-samples) of soil samples at the six sampling locations of the study area are shown in Figure 4.12. The results indicate that the accident site (6 miles from the study area start point) has the highest total PAHs concentration of 93,229 ng/g dry weight among the six locations investigated in this research. The concentration of total PAHs in the lower layer of the accident site sample is about half that of the upper layer.
Figure 4.12 Concentrations of total PAHs at the six sampling sites in the study area. The blue segment of the bars represents the concentration of the sum of all PAHs in the upper layer samples (0-6 inches-samples) while the orange segment represents the sum of all PAHs present in the lower layer (6-12 inches-samples). 0 mile is the oil spill accident site, while negative and positive numbers represent upstream and downstream sites respectively to the accident site.

The identities and concentrations of the individual PAHs in the upper layer (0-6 inches-sample) of the oil spill accident site are shown in Figure 4.13 as blue bars. The results indicate that pyrene, chrysene, fluoranthene, and phenanthrene are the dominant PAHs. These findings are consistent with other studies that monitor PAH levels in oil spill-affected areas.\textsuperscript{15}

The identities and concentrations of the individual PAHs in the lower layer (6-12 inches-sample) of the oil spill accident site are shown in Figure 4.13 in orange. Here also pyrene, fluoranthene, chrysene are present at high concentration, as well as benzo(a)anthracene, and benzo(a)pyrene. These findings are consistent with previous
studies on PAHs in oil spill-affected areas where pyrene, phenanthrene, fluoranthene, and chrysene were dominant PAHs in aquatic organisms one month after the *Solar I* oil spill off the coast of the Guimaras Island, in the Philippines.\footnote{15}

The results indicate that the upper layer has a much higher concentration of individual PAHs than the lower layer. The level of many PAHs in the upper layer of the Kalamazoo River accident site is approximately double their level in the lower layer.

**Figure 4.13** Concentrations of individual PAHs at the upper and lower layers of the oil spill accident site. The blue segment of the bars represents the concentration of PAHs in the upper layer sample (0-6 inches-sample) while the orange segment represents the concentration of PAHs in the lower layer sample (6-12 inches sample). Indeno(1,2,3-CD)pyrene and Dibenz(a,h)anthracene are represented in one bar.
The identities and concentrations of the individual PAHs in the upper and lower layers at the upstream river site (before the oil spill accident site), at the oil spill accident site, and downstream river site (after the oil spill accident site) are shown in Figure 4.14. The results indicate that the upper and lower layers near the accident site have a much higher concentration of individual PAHs than the upstream river and downstream river sites. Our data also indicates that many PAHs present at the oil spill accident site in relatively high concentrations are below detectable levels at the upstream and downstream sites. Upstream and further downstream from the spill site, only pyrene and fluoranthene could be identified. In the upstream river site, the concentrations of pyrene and fluoranthene in the upper layer are lower than that in the deeper layer while the opposite is true for the downstream river site.
Figure 4.14 Concentrations of individual PAHs at the upper and lower layers at the upstream river, at the oil spill accident site, and downstream river. Indeno(1,2,3-CD)pyrene and Dibenz(a,h)anthracene are represented in one bar.

The Kalamazoo River basin receives effluent discharges from different sources such as municipalities, industries, and urban areas. However, according to Blasland et al. (2000), the levels of polycyclic aromatic hydrocarbons (PAHs) in the Kalamazoo River are relatively low. Our investigation confirmed that the levels of PAHs in the study area are not significantly high except at the oil spill accident site, which has a much higher concentration of total PAHs among the six sites examined. The Kalamazoo River oil spill accident site has 93,229 ng/g dry weight of total PAHs concentration while the other five
investigated sites have total PAHs ranges between 180-2,700 ng/g dry weight. Our results partially agree with Blasland et al. that the levels of PAHs in Kalamazoo River are not significantly high.

4.5 Conclusion

Five years after the Kalamazoo River oil spill accident, an environmental investigation was conducted on the affected area 35.5 miles along the Kalamazoo River, between Marshall and Galesburg communities in Michigan, to assess the levels of PAHs in the soil using GC-MS. Six locations in the study area were examined. The oil spill accident site has the highest total PAHs concentration among the six sites investigated. The upper layer of oil spill accident site soil has approximately twice the concentration of total PAHs in the lower layer. Pyrene, chrysene, fluoranthene, benzo(a)pyrene and phenanthrene are the dominant PAHs in the oil spill accident site soil. It will be necessary to continue to assess periodically the levels of PAHs in the Kalamazoo River oil spill accident site and the surrounding areas to update the public about the levels of PAHs in the oil spill affected area. Disturbing soils on the river banks, and stream beds at the accident site, future floods, and erosion will eventually lead to a distribution of the PAHs downstream from the accident site. Our study suggests that further cleanup of the accident site is required.

4.6 References


(30) http://www.precisionbalance.net/soil-testing-instrument.html (accessed 3/10/2016)


CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Identification of BMIMCl Biodegradation Products

In this study, BMIM were treated for 46 days with the microbial community isolated by Dr. Kathryn Docherty from the aeration tank at the South Bend, IN wastewater treatment plant. Liquid chromatography-mass spectrometry and $^1$H-NMR techniques were used for the identification of BMIM biodegradation products.

This research demonstrated that biodegradation resistant BMIM could be fully degraded by the Bacteroidetes-enriched microbial community used in this study, and indicates that selected microbial consortia could be effectively used to increase the biodegradability of different ionic liquids. Three major metabolites from BMIM biodegradation were identified, and their structures were confirmed using MS/MS and $^1$H-NMR techniques. BMIM biodegradation products are 3-methyl-1H-imidazol-3-ium (m/z 83), 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium (m/z 155), and (Z)-1-(but-1-en-1-yl)-3-methyl-1H-imidazol-3-ium (m/z 137). The main metabolite identified and confirmed in this study was 3-methyl-1H-imidazol-3-ium. The main biodegradation product was produced by loss of the butyl side chain from the intact BMIM, which will make it less lipophilic and, potentially, less toxic to microorganisms; but further investigation of the toxicity of the obtained metabolites is necessary as future work. Two side products that form
to a lesser extent, 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-i um and (Z)-1-(but-1-en-1-yl)-3-methyl-1H-imidazol-3-i um, are resistant to further biodegradation by the microbial community described here. To the best of our knowledge, we report the first complete biodegradation of the imidazole ring in BMIM by a specially selected microbial community.

5.2 Impact of BMIMCl Breakdown Products on the Environment

The three identified metabolites from BMIM biodegradation and confirmed in this study are 3-methyl-1H-imidazol-3-i um (m/z 83), 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-i um (m/z 155), and (Z)-1-(but-1-en-1-yl)-3-methyl-1H-imidazol-3-i um (m/z 137). The main biodegradation product, 3-methyl-1H-imidazol-3-i um, is fully degraded by the end of the microbial treatment, as the intact BMIM. The two other biodegradation products, 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-i um, and (Z)-1-(but-1-en-1-yl)-3-methyl-1H-imidazol-3-i um, seem to persist and do not degrade further. However, these two biodegradation products are in very low concentrations relative to the main biodegradation product. The effects of the BMIM persisted breakdown products on the environment is not investigated yet, but further research is required to assess their toxicity and compare that to the toxicity of the intact BMIM.
5.3 Identification and Quantification of PAHs in the Kalamazoo Oil Spill

Five years after the Kalamazoo River oil spill accident, an environmental investigation was conducted on the oil spill affected area 35.5 miles along the Kalamazoo River, between Marshall and Galesburg communities in Michigan, to assess the levels of PAHs in the soil using GC-MS. Six locations in the study area were examined. The oil spill accident site has the highest total PAHs concentration among the six sites that were investigated. The upper layer of soil at the oil spill accident site has approximately doubled the concentration of total PAHs relative to the lower layer. Pyrene, chrysene, fluoranthene, benzo(a)pyrene and phenanthrene are the dominant PAHs in the oil spill accident site soil. It will be necessary to assess continually the levels of PAHs in the Kalamazoo River oil spill accident site and surrounding areas to update the public about the levels of PAHs in the affected area. Disturbing of soils on the river banks, and stream beds at the accident site, future floods, and erosion will eventually lead to a distribution of the PAHs downstream from the accident site. Further cleanup of the accident site is recommended.

The use of biological indicators of environmental pollution is a valuable tool to assess the contaminant bioavailability and the ability to investigate compounds that may be present below the analytical detection limit in physical environmental samples. Since PAHs are hydrophobic and lipophilic, PAHs accumulate in sediments, soil, and aquatic organisms. Biological indicators could be used to examine the levels of PAHs in the Kalamazoo River oil spill accident site and the surrounding areas. Using biological indicators could also provide an estimate of the level of PAHs in aquatic organisms tissues.
Toxicity of some contaminants, such as, PAHs increases upon the exposure to ultraviolet radiation (UV) through a phenomenon known as phototoxicity. Phototoxicity becomes critical when large quantities of PAHs are released into the aquatic environment. Our findings showed that significant amounts of PAHs were released into the Kalamazoo River after the oil spill accident. PAHs phototoxicity has adverse effects and represents threats to aquatic environments. Investigating the phototoxicity of PAHs in the Kalamazoo River oil spill accident site and affected area will be of great importance to evaluate the toxicity effect on aquatic organisms.

The U.S. Environmental Protection Agency (EPA) has listed the Kalamazoo River as a Superfund site in the Great Lakes watershed because of the high levels of polychlorinated biphenyls (PCBs). PCBs were mainly coming into the Kalamazoo River from the discharge of paper companies located along the river until the carbonless copying paper production ban back in 1977. While PCBs were not studied in this thesis, some concern exists that PAHs in the water could displace PCBs from sediment and lead to an increase in PCB levels in the water. This indirect consequence of the oil spill should also be investigated further.

Naphthenic acids are toxic compounds in most petroleum sources that are used as a potential indicator for oil contamination. Naphthenic acids represent major contaminants in marine environments following oil spills. Investigating the presence of naphthenic acids as a potential indicator of oil pollution in the Kalamazoo River oil spill site and surrounding areas is an excellent way to monitor the oil contamination in the river.
5.4 References


