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Application of Electrospray Ionization Mass Spectrometry for Ionic Liquids and their Biodegradation Products

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APPLICATION OF ELECTROSPRAY IONIZATION MASS SPECTROMETRY FOR IONIC LIQUIDS AND THEIR BIODEGRADATION PRODUCTS

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Ionic liquids (ILs) are organic salts with unique physico-chemical properties and are used as green alternatives to volatile organic solvents. ESI-MS is a sensitive technique to detect these stable, permanent ions. My research focused on (1) Identification of biodegradation products of ionic liquids and (2) Multiplexed tandem MS by modulation of ionization efficiency.

Studies, have demonstrated that ILs are toxic to the environment, which makes their biodegradability an important consideration. In this work, metabolites generated during the biodegradation of *1-butyl-3-methylimidazolium chloride* (BMIM), a degradation resistant and commonly used ionic liquid, are identified using high performance liquid chromatography-mass spectrometry (LC-MS).

Multiplexed tandem MS is a method to increase sample throughput for identification of complex mixtures. However, previous multiplexed tandem MS methods mostly require expensive mass spectrometers. In this study, we demonstrate a novel method of multiplexing MS where correlation of the fragments to their respective precursors is achieved by modulating the ionization efficiencies of analytes by changing the solvent composition. This method has the potential to do powerful MS/MS experiments on inexpensive single stage mass analyzers.

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CHAPTER I

INTRODUCTION

1.1 Chapter Introduction

This thesis covers two main topics, the identification of biodegradation products of ionic liquids using chromatography coupled to ESI-MS, and multiplexed tandem ESI-MS of ionic liquids and other compounds by modulation of ionization efficiency. These two main topics are briefly introduced in this chapter, before detailed discussion of background material and experimental results in subsequent chapters. Both projects depend intimately on electrospray ionization mass spectrometry. Therefore ESI is briefly introduced first.

1.2 Electrospray Ionization Mass Spectrometry

Mass spectrometry (MS) is a selective and sensitive analytical technique that can elucidate structural information of chemical compounds according to the behavior of their ions in magnetic and electric fields [1].

Electrospray ionization mass spectrometry (ESI-MS) is a soft ionization technique that produces intact ions with no fragmentation. It was established by Fenn et al in 1984 [2, 3], for which he received the Nobel Prize in 2002 [37]. With this technique, solutions pass through a capillary with a 2-5 kV potential difference relative to the grounded atmospheric pressure interface of the mass spectrometer. When applying a positive potential difference (positive ion mode) the spray solution is oxidized, while a negative voltage (negative ion mode) results in reduction of the

solution. Therefore polar solvents such as water, methanol and acetonitrile, which readily undergo electrochemical reactions are typically used to protonate or deprotonate the polar analytes for ESI-MS experiments [4].

ESI is capable of the analysis of complex, fragile, nonvolatile organic macromolecules. Before the invention of ESI, conventional mass spectrometry methods were not able to ionize molecules without fragmentation. ESI-MS has gained extensive applicability in the analysis of amino acids and lipids [12-15] and different types of macromolecules such as oligonucleotides [6], polymers and proteins [7-11]. Although ESI-MS has applications in biomolecular studies, it is also used for analysis of a broad range of compounds including polar organic [11], inorganic [12], and organometallic complexes [13].

Electrospray ionization occurs in three main steps (Figure 1.1). Firstly, by applying an electric potential and increasing the charge density at the solution meniscus at the capillary tip, the columbic repulsion forces increase. Attraction of the charges to the inlet by the electrical field changes the shape of the solution meniscus to an elongated cone in order to relieve the repulsion forces. This is known as the Taylor cone [19]. The Taylor cone reaches the Rayleigh limit when the Coulombic repulsion of the surface excess charge equals the solution's surface tension. At this point the solution nebulizes to charged droplets, which are accelerated to the grounded MS inlet [20]. In a second step, the released charged droplets evaporate and become smaller as they move through a dry bath gas. Solvent evaporation leads to an increase of the density of the excess charge on the surface of the droplet. Increased surface charge density and decreased droplets radius result in a second coulombic

fission and breaking apart of the primary droplet into many smaller charged droplets [21]. Finally in the last step, analyte molecules are ionized and become free gas-phase ions. Part of the resulting gas phase ions passes through a small skimmer orifice into vacuum, before being transferred to the mass analyzer. [2, 5]

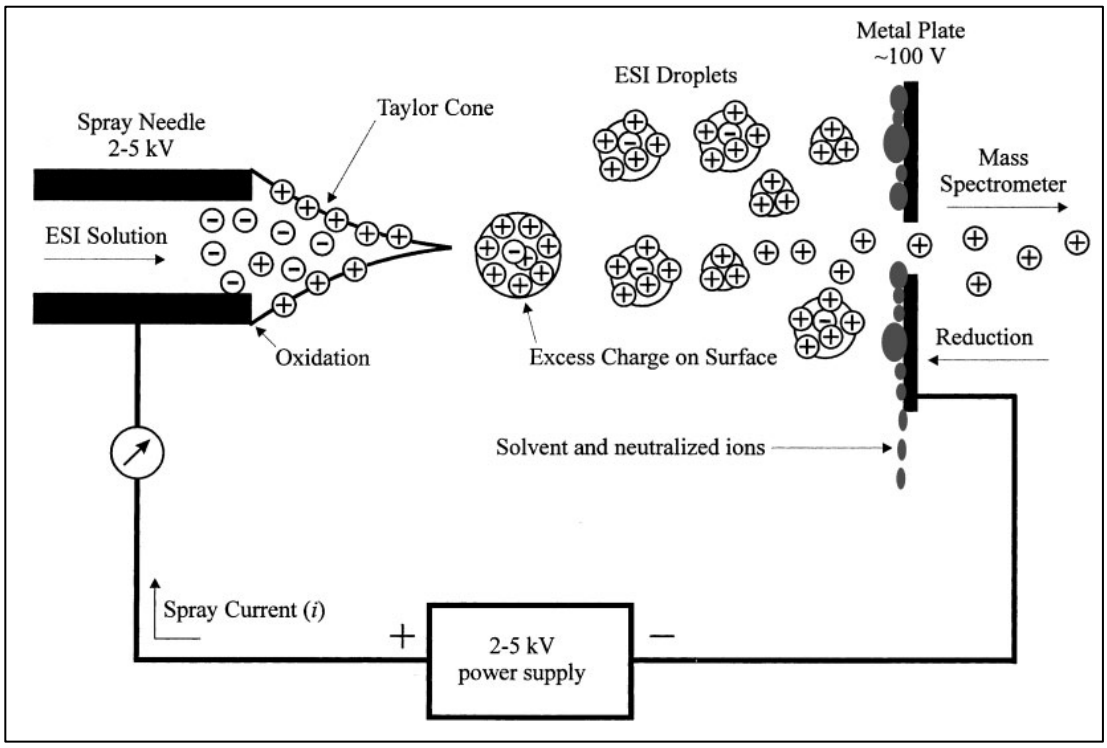


Figure 1.1. Schematic representation of the electrospray ionization process in the positive ion mode. Cech, N. B.; Enke, C. G. Mass Spectrometry Reviews, 2001, 20, 362 – 387. (With permission of John Wiley & Sons, Inc)

Early conventional MS techniques were limited to small, volatile, thermally stable compounds. Malcolm Dole at Northwestern University, IL, in the late 1960s applied ESI-MS to ionize and analyze non-volatile macromolecules of polystyrene [16]. Dole proposed the Charge Residue Model (CRM) to explain how these molecules are changed to gas phase ions. The CRM model suggests that during

electrospray ionization, the electrospray droplets continue to breakup until each small, charged droplet contains a single analyte molecule. Solvent evaporation eventually results in the transfer of the charges present on the droplet surface to the analyte. Although the CRM is the first and most widely adopted model of analyte charging by ESI, other ionization models such as Iribarne and Thomson's Ion Evaporation Model (IEM) [17] and Kaltashov's emission model [18] have been proposed and developed. The IEM model suggests that solvent evaporation and shrinkage of the droplets increases the charge density. Eventually, coulombic repulsion overcomes the solution's surface tension, which results in release of ions from droplet surfaces.

There are many advantages to electrospray ionization (ESI) compared to other mass spectrometry methods. The analyte does not need to be transferred to the vacuum before ionization because the ionization takes place at atmospheric pressure in the source. Applying a nebulizing gas removes the solvent, neutrals and other contaminants before the analyte ions are sampled in the mass analyzer. Since the applied sample is in solution, ESI can be coupled with powerful separation techniques such as liquid chromatography.

ESI is able to ionize a wide range of Brønsted acid or base compounds by protonation or deprotonation. In addition, other less polar compounds can form ions by the formation of ammonium or chloride adducts. Permanent ions, such as the ionic liquids that are analyzed in Chapter 3, simply need to be separated from their counter ions.

ESI is not very energetic and mostly occurs with no fragmentation of the

parent molecule, which implies that molecular masses can be determined before the ion is fragmented to get structural information, as described in Chapter 5. It also can lead to multiple charging of the compounds with multiple potential charging sites. Therefore it's a great choice for the ionization of large biological macromolecules with mass values outside of the detectable mass range of the analyzer.

1.3 Ionic Liquids

Ionic Liquids (ILs) are organic salts consisting of a bulky organic cation and an inorganic or organic anion [22]. In recent years, ionic liquids have been widely used as solvents because of their desirable physico-chemical properties such as their high thermal and electrochemical stability, low volatility and high conductivity [23-26]. Despite the ionic liquid's vast applicability, different studies have demonstrated their toxicity and adverse effects on the environment, which makes their biodegradability of great importance [27-34]. The degradation products could be less or more harmful to the environment and therefore, investigating the biodegradation products and pathways would be imperative.

Chapter 2 introduces ionic liquids and their uses, followed by a detailed literature study of their biodegradability. Chapter 2 also reviews methods to separate ionic liquids and discuss background to the various methods used to detect them, used in the subsequent experiments described in Chapter 3.

Chapter 3 describes investigations into the biodegradation of 1-butyl-3-methylimidazolium chloride (BMIMCl), one of the most widely used ionic liquids. In the work presented in this thesis, biodegradation of BMIMCl by an activated sludge

microbial community was investigated. The applied microbial community was isolated from the wastewater treatment plant in South Bend, IN. The treatment was done within a 46-day period. Since the BMIM cation is a non-volatile permanent ion, ESI-MS is a great choice for its investigation. Therefore, the samples were characterized by reversed-phase high performance liquid chromatography (RP-HPLC) and the combination of UV and MS detection in an attempt to identify the degradation products and pathway. The identification of several metabolites was performed with a combination of tandem mass spectrometry (MS/MS) and NMR techniques. To the best of our knowledge, this study is the first successful biodegradation of the BMIM Cl using a microbial community, which can provide further insight into imidazolium-based ILs biodegradability and provides useful information for studying the toxicity of BMIM metabolites.

1.4 Multiplexed Tandem Mass Spectrometry

The second topic of my studies is discussed in Chapters 4, 5 and 6. Tandem mass spectrometry is a common technique to identify molecules and their chemical structure [35]. In conventional tandem mass spectrometry, each precursor ion is individually selected and dissociated to its fragment ions. Therefore, fragmentation of the complex mixtures of samples require high throughput of MS/MS experiments and long analysis time to be performed. This can be achieved with multiplexed tandem mass spectrometry. As described in Chapter 5, multiplexed tandem MS is an alternative method to increase the MS/MS experiment throughput. In multiplexing tandem MS, all precursor ions are fragmented simultaneously and the fragments are

decoded to their corresponding precursors [36]. The applications of multiplexed tandem MS methods are also briefly discussed in Chapter 5.

While most current methods of multiplexing tandem MS require high accuracy mass analyzers, the novel method of multiplexed tandem MS, by modulation of ionization efficiency, described in Chapter 6, can be achieved on any mass analyzers, even very basic ones.

In order to follow the discussion on modulation of ionization efficiencies in ESI-MS a detailed discussion on the mechanisms of ESI is provided in Chapter 4 of this thesis. This chapter introduces the theories of equilibrium partition model and surface activity which are needed for understanding the novel method of multiplexing tandem MS. Ionic liquids are good examples of molecules with tunable surface activities due to their specific chemical structures with different length carbon chains. Therefore, they were used as model compounds to investigate our novel multiplexed tandem ESI-MS method.

1.5 References

1. Hoffmann, E. d., Stroobant V., *Mass spectrometry: principles and applications*; 3rd ed., J. Wiley: Chichester, West Sussex, England; Hoboken, NJ, 2007.
2. Yamashita, M., Fenn, J. B. J. Phys. Chem. 1984, 88, 4451-4459.
3. Yamashita, M., Fenn, J. B. J. Phys. Chem. 1984, 88, 4671-4675.
4. Cole, R. B., Electrospray and MALDI Mass Spectrometry, John Wiley & Sons, New Jersey, NJ, USA, 2010.
5. Whitehouse, C. M., Dreyer, R. N., Yamashita, M., Fenn, J. B. J., Anal. Chem. 1985, 57, 675-679.
6. Beck, J. L., Colgrave, M. L., Ralph, S. F., Sheil, M. M., Mass Spectrom. Rev.

2001, 20, 61-87.

7. Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C.M. Science 1989, 246, 64-71.

8. Loo, J. A., Mass Spectrom. Rev. 1997, 16, 1-23.

9. Katta, V.; Chait, B. T. J. Am. Chem. Soc. 1993, 115, 6317-6321.

10. McEwen, C. N., Simonsick, W. J., Jr., Larsen, B. S., Ute, K., Hatada, K. J., Am. Soc. Mass Spectrom. 1995, 6, 906-911.

11. Hop, C. E. C. A., Bakhtiar, R. J., Chem. Educ. 1996, 73 (A162), A164-A169.

12. Brugger, B., Erben, G., Sandhoff, R., Wieland, F. T., Lehmann, W. D. Proc. Natl. Acad. Sci. U.S.A. 1997, 94, 2339-2344.

13. Hsu, F. F., Bohrer, A., Turk, J. J., Am. Soc. Mass Spectrom. 1998, 9, 516-526.

14. Lu, W., Yang, G., Cole, R. B., Electrophoresis 1995, 16, 487-492.

15. Schultz, C. L., Moini, M., Anal. Chem. 2003, 75, 1508-1513.

16. Dole, M., Mack, L. L., Hines, R. L., Mobley, R. C., Ferguson, L. D., Alice, M. B., Molecular Beams of Macroions. J. Chem. Phys. 1968, 49 (5), 2240-2249.

17. Iribarne, J. V., Thomson, B. A., J. Chem. Phys. 1976, 64 (6), 2287-2294.

18. Kaltashov, I. A.; Mohimen, A., Anal. Chem. 2005, 77 (16), 5370- 5379.

19. Taylor, G. I.; McEwan, A. D., J. Fluid Mech. 1965, 22 (01), 1-15.

20. Taflin DC, Ward TL, Davis EJ. Electrified droplet fission and the Rayleigh limit. Langmuir 1989, 5, 376–384.

21. Cloupeau, M., Prunet-Foch, B., J. Aerosol Sci. 1994, 25 (6), 1021-1036.

22. Steudte, S., Bemowsky, S., Mahrova, M., Bottin-Weber, U., Tojo-Suarez, E., Stepnowski, P., Stolte, S. RSC Adv. 2014, 4.

23. Neumann, J., Steudte, S., Cho, C. W., Thöming, J., Stolte, S., Green Chem. 2014, 16, 2174–2184.

24. Deng, Y., Beadham, I., Ghavre, M., Gomes, M. F. C., Gathergood, N., Husson, P., Légeret, B., Quilty, B., Sancelme, M., Besse-Hoggan, P., Green Chem. 2015.

25. Zhou, H., Shen, Y., Lv, P., Wang, J., Li, P. J., Hazard. Mater. 2015, 284, 241–

252.

26. Pham, T. P. T., Cho, C. W.l Jeon, C. O., Chung, Y. J., Lee, M. W., Yun, Y. S., Environ. Sci. & Technol. 2009, 43, 516–521.

27. Ventura, S. P. M., Marques, C. S., Rosatella, A. A., Afonso, C. A. M., Gonçalves, F., Coutinho, J. A. P. Ecotoxicol. Environ. Saf. 2012, 76, 162–168.

28. Pham, T. P. T., Cho, C. W., Yun, Y. S., Water Res. 2010, 44, 352–372.

29. Romero, A., Santos, A., Tojo, J., Rodríguez, A., J. Hazard. Mater. 2008, 151, 268–273.

30. Stolte, S., Matzke, M., Arning, J., Böschen, A., Pitner, W. R.; Welz-Biermann, U., Jastorff, B., Ranke, J. Green Chem. 2007, 9.

31. Cho, C. W., Pham, T. P. T., Jeon, Y. C., Vijayaraghavan, K., Choe, W. S., Yun, Y. S., Chemosphere 2007, 69, 1003–1007.

32. Kulacki, K. J., Lamberti, G. A., Green Chem. 2008, 10.

33. Docherty, K. M., Charles F. Kulpa, J. Green Chem. 2005, 7.

34. Pretti, C., Chiappe, C., Pieraccini, D., Gregori, M., Abramo, F., Monni, G., Intorre, L. Green Chem. 2006, 8.

35. Hoffmann, E., de. J. Mass. Spec. 1996, 31, 129-137.

36. Chapman J. D., Goodlett D. R., Masselon C. D., Mass Spectrometry Reviews 2014, 33, 452–470

37. Fenn, J. B. Electrospray Wings for Molecular Elephants 2002. http://nobelprize.org/nobel_prizes/chemistry/laureates/2002/fenn-lecture.pdf, (accessed March 4, 2016).

CHAPTER II

IONIC LIQUIDS

2.1 Introduction

In this chapter, the general properties of ionic liquids and the specific properties of imidazolium based ionic liquids (specific ionic liquid investigated in this study) are discussed. Ionic Liquids (ILs) are low melting points salts which are in a liquid state below a temperature of 100° C. Their desirable physicochemical properties, such as low vapour pressure, non-flammability, high conductivity, and high thermal and chemical stability make them a great alternative for volatile organic solvents as well as their many other applications [1-4]. Ionic liquids consist of a bulky organic cation such as imidazolium, pyridinium, pyrrolidinium, ammonium or phosphonium, and an inorganic or organic anion [5]. The combination of cationic head group, side chain and anion determines the properties of the ionic liquid; therefore ionic liquids can be designed and tuned for specific applications [6].

Ionic liquids have been used in both small-scale research and large scale industrial applications. They have been used for various applications, including mobile phase additives and stationary phases in high performance liquid chromatography [7], solvents [3], catalysts [4], enzyme stabilizers [8], dye-sensitized solar cells and lithium ion batteries [9,10], sensors [11,12], agents for analytical measurements [13-15], potential pharmaceutical ingredients [16,17], gel production [18], oil extraction [19], metal deposition [20], recovery solutions [21] and many

other applications. Many industries have found them as excellent candidates for their applications due to their negligible vapour pressure [22, 23]. Some of the most widely used and studied ILs are imidazolium salts, since they are excellent solvents for a broad range of organic and inorganic materials and they have green properties, like non-volatility, incombustibility and high thermal stability [24-26].

2.2 Biodegradation of Ionic Liquids

The pervasive applications of ILs in both academic and industrial fields make them more likely to be released into the environment as process effluents or consumer products, or via accidental spills [67]. This makes the toxicity, biodegradability and environmental fate of ILs of great importance. The toxicity of several ILs towards microorganisms, aquatic and terrestrial ecosystems has been intensively investigated [43-53]. Their high water solubility and stability make them potential persistent water pollutants. Biodegradation is the most effective method of the degradation of organic pollutants in the environments. Therefore, their biodegradability and their fate in the environment need to be investigated. Many studies have investigated the biodegradability of ILs and identification of their metabolites and degradation pathways [1, 3-5, 60-63]. The biodegradability of ILs significantly depends on the ecosystem of microorganism and the properties of the ILs anion and cation. It has been shown that the biodegradation of ILs can be improved by inserting an ester side-chain into the pyridinium cation [54, 55, and 65]. Also, the length of the substituted alkyl chain has been found to have an effect on the biodegradability of ILs. Microorganisms can decompose the compounds with longer substituted alkyl chains

more easily, despite the fact that their toxicity may increase with an increase in the alkyl group chain length [44]. Harjani et al. demonstrated that the C_4 alkyl chain pyridinium ILs were poorly biodegraded, even after 28 days [55, 56]. Stolte et al confirmed this and showed that the ethyl and butyl pyridinium ILs do not undergo much biodegradation while the N-octylpyridinium cations could be degraded in 31 days [57]. Therefore, short substituted alkyl chain ILs may persist in the environment [58,59]. Furthermore, the biodegradability of ILs is also dependent on the structure of their anion [68]. Organic anions, such as octyl sulfates, acetate and naphthenic acids like 3-cylcohexylpropionate are usually biodegradable [68, 69]. Biodegradation efficiency is effected by inorganic anions and decreases in the order of $PF_6^- > BF_4^- > Br^- > Cl^-$ [70]. In general, common ILs are not readily biodegradable [57]. According to Organization for Economic Cooperation and Development (OECD) standards (U.S. EPA, 1998), compounds can be considered readily biodegradable if 60–70% or greater is biodegraded, by activated sludge microbial inoculate within any 10-day window in a 28-day period.

Imidazolium based ILs are one of the most useful and well characterized ILs among the different types of ionic liquids. High quantities of butyl-methyl imidazolium (BMIM) based ILs are used in industrial applications, despite being persistent and toxic in the environment. Neumann et al., demonstrated that imidazolium-based ILs have the lowest biodegradation potential among 27 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium IL cations under aerobic conditions [1]. A few studies have investigated the effect of

imidazolium ILs alkyl chain length on their biodegradability, and again, it was shown that biodegradation of longer alkyl chain cations is enhanced [54, 64, 66].

Metabolites have their own toxicological characteristics. They can be more or less toxic than their parent compound and this makes the investigation of the degradation pathway of great importance. Stolte et al. proposed the biodegradation pathway of 1-octyl-3-methylimidazolium cation (IM18) based on intermediate products via HPLC-MS analysis after 24 days of incubation with activated sludge [57]. The metabolism of IM18 cation in this proposed pathway appeared to involve a series of oxidation reactions. The oxidation of the alkyl chain starts from the terminal methyl group (ω-oxidation), which is catalyzed probably by mono-oxygenases. Dehydrogenases oxidizes the produced alcohol subsequently oxidized via aldehydes to carboxylic acids, which then can undergo β-oxidation [Figure 2.1].

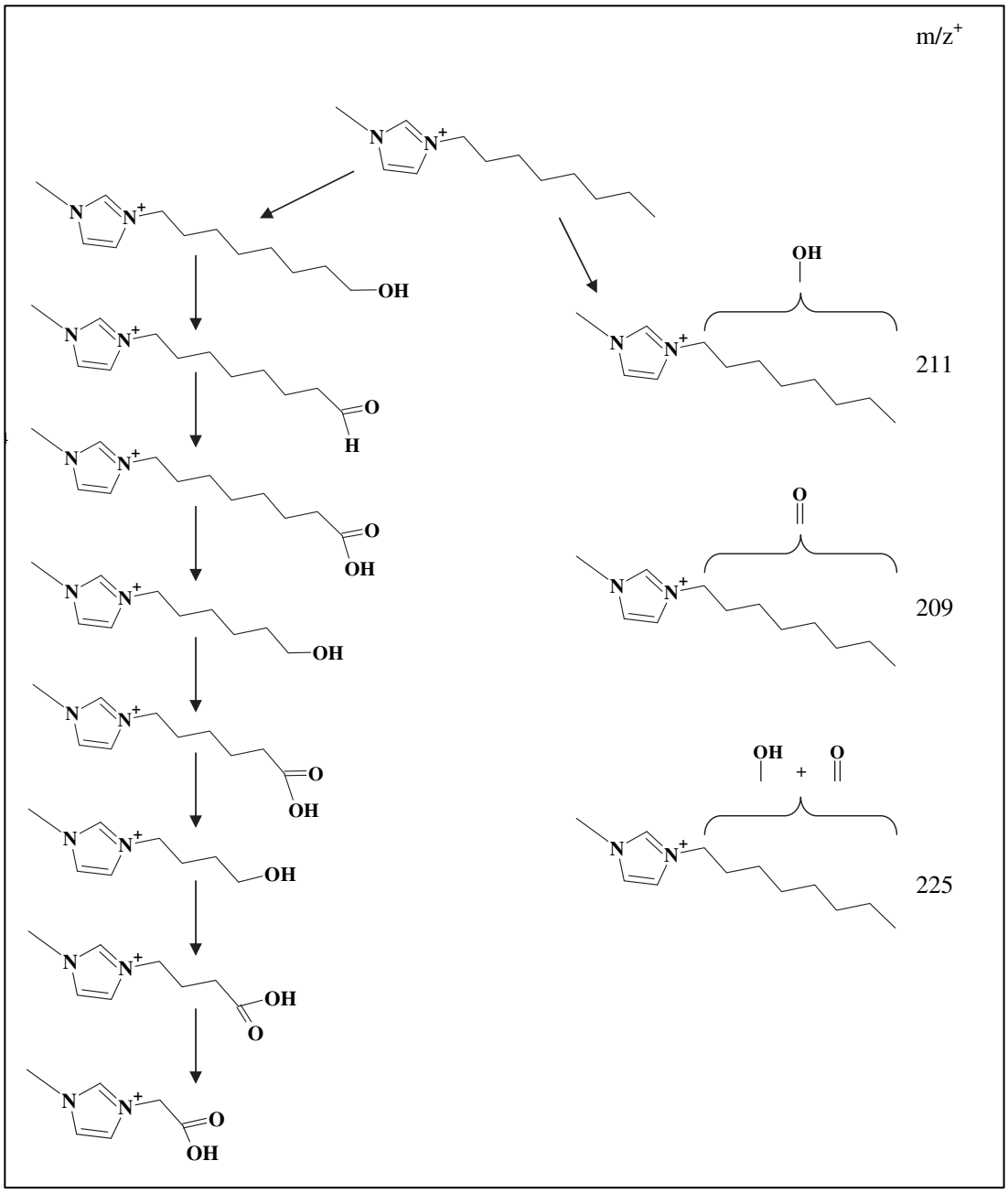


Figure 2. 1. Biodegradation pathways of 1-octyl-3-methylimidazolium by activated sludge microbial community (Adapted from Stolte et al. (2008) by permission of the Royal Society of Chemistry) [44].

2.3 Chromatographic Separation of Ionic Liquids

Considering the high water solubility of ILs and their presence in the aquatic environment and high sensitivity of the ESI-MS, the best technique for identifying and quantifying the degradation products of ionic liquids is high performance liquid chromatography (HPLC) coupled with mass spectrometry. Several studies have reported the separation of imidazolium cations with reversed-phase (RP) HPLC and it has been the most common HPLC mode used for the separation of ionic liquids [27-34]. However, the resolution, retention time and selectivity of very polar short alkyl chain (less than four carbon atoms) cations are weak on conventional RP columns. Moreover, these separations need a high percentage of water in the mobile phase, and electrostatic interactions of cationic solutes with residual silanol groups of the stationary phase leads to significant peak tailing and poor band shape. For these reasons, achieving a good separation of polar and charged ILs in reversed-phase systems requires the usage of ion-pairing agents or high concentrations of non-volatile buffer, which is not a good approach for the coupling to mass spectrometry.

Ion chromatography was also investigated for the separation of ionic liquids [28, 35-37]. Nevertheless, cation exchange chromatography methods still have limitations for the separation of short carbon chain ionic liquids and require using ion-pairing agents or non-volatile salts such as hydrogen phosphate, carbonate to improve retention and selectivity for them. Therefore they are also not convenient for coupling with mass spectrometry techniques. Most of the proposed LC methods for the separation of hydrophilic short alkyl chains IL cations show retention and peak efficiency limitations. The main interactions responsible for the retention of these

cations are firstly the ionic interactions and then hydrophobic interactions, which play a role for selectivity between them.

Polar or mixed-mode stationary phases have also been applied for the separation of ILs [38-40] and hydrophilic interaction liquid chromatography (HILIC) showed the best result. HILIC is the most successful of the HPLC based IL separation methods [39-41]. HILIC is a chromatographic technique that improves retention of very polar compounds, which are poorly retained by RP-HPLC. In HILIC, a layer of water is formed on a polar stationary phase (diol, sulfobetaine, silica, cyano, amino, amide, zwitterionic, etc.) when a high organic solvent content mobile phase is used (usually 70–95% acetonitrile). Le Rouzo et al. first used HILIC for the separation of imidazolium IL cations on diol stationary phase [39]. Buffers employed in HILIC chromatography, such as ammonium acetate or trifluoroacetate, are volatile salts and the lower mobile phase water content and high content of acetonitrile with low viscosity make the HILIC technique more compatible with mass spectrometry in higher sensitivity and faster separation conditions [42].

In the work presented in Chapter 3, a Kinetex Biphenyl column was used for the separation of the short alkyl chain imidazolium metabolites using formic acid and ammonium acetate ion pairing reagents. Although the applied technique was reversed phase chromatography (RP-HPLC), π-π interactions between the phenyl and imidazolium rings, as well as regular hydrophobic interactions provided enough retention for the separation and identification of the metabolites.

2.4 Detection of the Metabolites

The imidazolium ionic liquid cation contains a strong UV absorption group. The maximum UV absorbance wavelength at 212 nm was used by Stolte et al. for the detection of the imidazolium ring [57]. Previous studies on pyridinium-based ILs suggest that after cleavage of the ring, biodegradation occurs rapidly and breakdown products can be incorporated into microbial biomass or released as gas [4,57,63]. Very low concentrations of these breakdown products lead to low signal-to-noise ratios, which prevent the observation of them in the total ion chromatogram obtained by ESI. UV absorbance of the imidazolium ring of BMIM, the IL investigated in this study, allows us to apply UV-Vis for the identification of this IL and its metabolites. After cleavage of the ring the diagnostic absorbance at 212 nm of the imidazolium ring is lost. As shown in Chapter 3 the combination of UV and MS detection of the metabolites was applied. Less sensitive detection of all the ions with intact imidazolium ring was completed with sensitive detection of these permanent ions by ESI-MS.

2.5 Chapter Conclusion

Ionic liquids (ILs) are low melting point salts with bulky organic cations. They have unique physico-chemical properties, which make them potential green alternatives to volatile organic solvents. Nevertheless, studies have demonstrated that they can be toxic to aquatic and terrestrial organisms and can therefore have adverse effects on the environment. Biodegradation is the most effective method of the degradation of organic pollutants in the environments, but degradation products can

be more or less toxic than their parent compounds. This makes identification of the metabolites and their pathway an important consideration. Since ionic liquids are stable water-soluble permanent ions, ESI-MS would be a suitable technique for their identification. Imidazolium salts are excellent solvents for a broad range of organic and inorganic materials and have green properties like non-volatility, incombustibility and high thermal stability, which make them one of the most applicable ionic liquids. BMIM is an imidazolium based IL with a C_4 side chain that high quantities of it are used in industrial applications, although it's persistent and toxic to the environment. The best technique for identifying and quantifying the degradation products of ionic liquids is HPLC coupled with ESI-MS. Break down products with intact imidazolium ring can be detected by UV-Vis technique. Therefore, in this study, the metabolites with intact imidazolium rings were identified by the simultaneous application of HPLC-UV-MS.

2.6 References

1. Neumann, J., Steudte, S., Cho, C. W., Thöming, J., Stolte, S., Green Chem. 2014, 16, 2174–2184.

2. Deng, Y., Beadham, I., Ghavre, M., Gomes, M. F. C., Gathergood, N., Husson, P., Légeret, B., Quilty, B., Sancelme, M., Besse-Hoggan, P., Green Chem. 2015.

3. Zhou, H., Shen, Y., Lv, P., Wang, J., Li, P., J. Hazard. Mater. 2015, 284, 241–252.

4. Pham, T. P. T., Cho, C. W., Jeon, C. O., Chung, Y. J., Lee, M. W., Yun, Y. S., Environ. Sci. & Technol. 2009, 43, 516–521.

5. Steudte, S., Bemowsky, S., Mahrova, M., Bottin-Weber, U., Tojo-Suarez, E., Stepnowski, P., Stolte, S., RSC Adv. 2014, 4.

6. Petkovic, M., Seddon, K. R., Rebelo, L. P. N., Pereira, C., S. Chem. Soc. Rev.

2011, 40.

7. Wang, Y., Tian, M., Bi, W., Row, K. H., IJMS 2009, 10, 2591–2610.

8. Keskin, S.,Kayrak-Talay, D., Akman, D., Hortac¸su, O¨, J. Supercrit. Fluids, 2007, 43(1), 150–180.

9. Kawano, R., Matsui, H., Matsuyama, C., Sato, A., Susan, M. A. B. H., Tanabe, N., Watanabe, M., J. Photochem. Photobiol. A: Chem. 2004, 164, 87–92.

10. Seki, S., Kobayashi, Y., Miyashiro, H., Ohno, Y., Usami, A., Mita, Y., Kihira, N., Watanabe, M., Terada, N., J. Phys. Chem. B 2006, 110, 10228–10230.

11. Pandey, S., Anal. Chim. Acta, 2006, 556(1), 38–45.

12. Wei, D., Ivaska, A., Anal. Chim. Acta, 2008, 607(2), 126–135.

13. Liu, J. f., Jiang, G. b., J. A. Jonsson, TrAC, Trends Anal. Chem., 2005, 24(1), 20–27.

14. Mart´ın-Calero, A., Pino, V., Ayala, J. H., Gonza´lez, V., Afonso, A. M., Talanta, 2009, 79(3), 590–597.

15. Sun, P., Armstrong, D. W., Anal. Chim. Acta, 2010, 661(1), 1–16.

16. Hough, W. L., Smiglak, M., Rodr´ıguez, H., Swatloski, R. P., Spear, S. K., Daly, D. T., Pernak, J., Grisel, J. E., Carliss, R. D., Soutullo, M. D., Davis, J. H. Jr., Rogers, R. D., New J. Chem., 2007, 31, 1429–1436.

17. Moniruzzaman, M., Goto, M., J. Chem. Eng. Jpn., 2011, 44(6), 370–381.

18. Meera, K. M. S., Sankar, R. M., Jaisankar, S. N., Mandal, A. B., Colloids Surf., B, 2011, 86(2), 292–297.

19. Ruivo, R., Couto, R., Simo˜es, P. C., Sep. Purif. Technol., 2010, 76 (1), 84–88.

20. Tsuda, T., Kondo, K., Tomioka, T., Takahashi, Y., Matsumoto, H., Kuwabata, S., Hussey, C. L., Angew. Chem., Int. Ed., 2011, 50(6), 1310–1313

21. Painter, P., Williams, P., Mannebach, E., Energy Fuels, 2010, 24, 1094–1098.

22. Plechkova, N. V., Seddon, K. R., Chem. Soc. Rev. 2007, 37.

23. Tang, S., Baker, G. A., Zhao, H., Chem. Soc. Rev. 2012, 41.

24. Gordon, C.M., Appl. Catal. 2001, A 222, 101–117.

25. Sheldon, R., Chem. Commun. 2001, 23, 2399–2407.

26. Rantwijk, F., Lau, R.M., Sheldon, R.A., Trends Biotechnol. 2003, 21, 131–138.

27. Stepnowski, P. , Müller, A., Behrend, P., Ranke, J., Hoffmann, J., Jastorff, B., Journal of Chromatography 2003, A 993 (1–2) , 173–178.

28. Stepnowski, P., Mrozik, W., Journal of Separation Science 2005, 28 (2), 149–154.

29. Ruiz-Angel, M.J., Berthod, A., Journal of Chromatography 2006, A 1113 (1–2), 101–108.

30. Stepnowski, P., Nichthauser, J., Mrozik, W., Buszewski, B., Analytical and Bioanalytical Chemistry 2006, 385 (8), 1483–1491.

31. Buszewski, B., Kowalska, S., Stepnowski, P., J. Sep. Sci. 2006, 29, 1116.

32. Ruiz-Angel, M. J., Berthod, A., J. Chromatogr. 2008, A 1189, 476.

33. Studzin´ska, S., Buszewski, B., J.Sep.Sci. 2010, 33, 1.

34. Molíková, M., Markuszewski, M. J., Kaliszan, R., Jandera, P., J. Chromatogr., 2010, A 1217, 1305.

35. Villagran, C., Deetlefs, C. M., Pitner, W. R., Hardacre, C., Analytical Chemistry 2004, 76, 2118–2123.

36. Hao, F. P., Haddad, P. R., Ruther, T., Chromatographia 2008, 67, 495–498.

37. Zhou, S., Yu, H., Yang, L., Ai, H. J., Journal of Chromatography 2008, A 1206 (2), 200–223.

38. Kowalska, S., Buszewski, B., J. Sep. Sci. 2006, 29, 2625–2634.

39. Le Rouzo, G., Lamouroux, C., Bresson, C., Guichard, A., Moisy, P., Moutiers, G., J. Chromatogr. 2007, A 1164, 139–144.

40. Lamouroux, C., Foglia, G., LeRouzo, G., J. Chromatogr. 2011, A 1218, 3022–3028.

41. Hawkins, C. A., Rud, A., Guthrie, M. L., Dietz, M. L., J. Chromatogr. 2015, A 1400, 54–64

42. Nguyen, H. P., Schug, K. A., J. Sep. Sci. 2008, 31, 1465–1480.

43. Ventura, S. P. M., Marques, C. S., Rosatella, A. A., Afonso, C. A. M.,

Gonçalves, F., Coutinho, J. A. P. Ecotoxicol. Environ. Saf. 2012, 76, 162–168.

44. Pham, T. P. T., Cho, C. W., Yun, Y. S., Water Res. 2010, 44, 352–372.

45. Romero, A., Santos, A., Tojo, J., Rodríguez, A. J. Hazard. Mater. 2008, 151, 268–273.

46. Stolte, S., Matzke, M., Arning, J., Böschen, A., Pitner, W. R., Welz-Biermann, U., Jastorff, B., Ranke, J. Green Chem. 2007, 9.

47. Cho, C.-W.; Pham, T. P. T.; Jeon, Y.-C.; Vijayaraghavan, K.; Choe, W.-S.; Yun, Y.-S. Chemosphere 2007, 69, 1003–1007.

48. Kulacki, K. J., Lamberti, G. A. Green Chem. 2008, 10.

49. Docherty, K. M., Kulpa, C. F., J. Green Chem. 2005, 7.

50. Pretti, C., Chiappe, C., Pieraccini, D., Gregori, M., Abramo, F., Monni, G., Intorre, L. Green Chem. 2006, 8.

51. Couling, D.J., Bernot, R.J., Docherty, K.M., Dixon, J.K., Maginn, E.J., Green Chem. 2006, 8, 82–90.

52. Studzin´ ska, S., Buszewski, B., Anal. Bioanal. Chem. 2009, 393, 983–990.

53. Kumar, R.A., Papaíconomou, N., Lee, J.M., Slminen, J., Clark, D.S., Prausnitz, J.M., Environ. Toxicol. 2009, 24, 388–395.

54. Docherty, K.M., Dixon, J.K., Kulpa, C.F., Biodegradation. 2007, 18, 481–493.

55. Harjani, J.R., Singer, R.D., Garcia, M.T., Scammelis, P.J., 2008, Green Chem. 10, 436–438.

56. Harjani, J.R., Singer, R.D., Garcia, M.T., Scammells, P.J., Green Chem. 2009b, 11, 83–90.

57. Stolte, S., Abdulkarim, S., Arning, J., Blomeyer-Nienstedt, A.K., Bottin-Weber, U., Matzke, M., Jastorff, B., Thöming, J., Green Chem. 2008, 10, 214–224.

58. Neumann, J.O., Grundmann, J., Thoming, M., Schulte, M., Stolte, S., Green Chem. 2010, 12, 620–627.

59. Coleman, D., Gathergood, N., Chem. Soc. Rev. 2010, 39, 600–637.

60. Peric, B., Sierra, J., Martí, E., Cruañas, R., Garau, M. A., Arning, J., Bottin-Weber, U., Stolte, S., J. Hazard. Mater. 2013, 261, 99–105.

61. Abrusci, C., Palomar, J., Pablos, J. L., Rodriguez, F., Catalina, F., Green Chem.

2011, 13.

62. Quijano, G., Couvert, A., Amrane, A., Darracq, G., Couriol, C., Cloirec, P. L., Paquin, L., Carrié, D., Chem. Eng. J. 2011, 174, 27–32.

63. Docherty, K. M., Joyce, M. V., Kulacki, K. J., Kulpa, C. F., Green Chem. 2010, 12.

64. Liwarska-Bizukojc, E., Gendaszewska, D., J. Biosci. Bioeng. 2013, 115, 71–75.

65. Gathergood, N., Garcia, M.T., Scammells, P.J., Green Chem. 2004, 6, 166–175.

66. Garcia, M. T., Gathergood, N., and Scammells, P. J, Green Chem., 2005, 7, 9e14.

67. Stolte, S., Steudte, S., Igartua, A., Stepnowski, P., Current Organic Chemistry, 2011, 15, 1946-1973.

68. Gathergood, N., Scammells, P. J., Garcia, M. T., Green Chem., 2006, 8, 156–160.

69. Yu, Y. H., Lu, X. M., Zhou, Q., Dong, K., YaoandS., H. W., Zhang, J., Chem.–Eur. J., 2008, 14, 11174–11182.

70. Ranke, J., Stolte, S., Stormann, R., Arning, J., Jastorff, B., Chem. Rev., 2007, 107, 2183–2206.

CHAPTER III

EXPERIMENTAL METHODS AND RESULTS OF IDENTIFICATION OF METABOLITES OF IONIC LIQUIDS

3.1 Introduction

In this study, biodegradation products of the ionic liquid 1-butyl-3-metylimidazolium (BMIM) were identified using HPLC-UV-MS. Aerobic biodegradation of BMIMCl by activated sludge microbial community isolated from South Bend, IN wastewater treatment plant, was done within a 46-day period of treatment. The analytes were separated using a Biphenyl reversed phase column. The HPLC flow was split 50:50 to both UV and MS detectors. Mass spectrometry method of the extraction of product ion identified the metabolites that could not be detected with less sensitive UV detector. Degradation trends of the detected metabolites were plotted on a graph and investigated. Collision induced dissociation (CID) of the detected metabolites provided some structural information. Ultimately, structures of the metabolites were identified using the obtained information and the previous data from the literature.

3.2 Experimental Section

This section consists of sample preparation details and the experimental procedures applied for identification of the BMIM breakdown products using HPLC-UV-MS.

The specialized microbial community used in this study was selected by

repeated isolation and transfer techniques from the aeration tank at the South Bend, IN wastewater treatment plant, as described in Docherty et al. 2015. The microbial community composition was examined using high throughput 16S rRNA-based amplicon sequencing approaches. The results were confirmed by NMR results performed on the same samples obtained by Dr. Szymczyna's group at Western Michigan University and are occasionally referred to, since they collaborate in the interpretation of the MS findings.

3.2.1 Sample Preparation for Degradation

The test compound 1-butyl-3-methylimidazolium chloride used in this study was purchased from IoLiTec (Tuscaloosa, AL). The 1-methylimidazole standard was purchased from TCI America. The microbial community used for this study was isolated as described in Docherty et al. 2015. The enriched microbial community was transferred into 1L of media containing 130 mg/L of BMIM Cl, which was then be used to prepare concentrated inoculate after 3 weeks. The biodegradation of BMIM Cl by the microbial community was monitored twice per week for 46 days. All the samples were filtered through a 0.22 μm pore size syringe filter and were kept in a -80 ˚C freezer after collection. The BMIM samples collected at days 3, 7, 10, 14, 21, 28, 35, 38, 42 and 46 were assayed using HPLC-UV-MS

3.2.2 Experimental procedure for HPLC-UV-MS analysis

Samples were analysed using an Agilent 1100, G1312A Bin Pump, Waters 2487 Dual λ Absorbance Detector and a Thermo LTQ ion trap mass spectrometer and 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 0.5% formic acid in acetonitrile (B) and 20 mM aqueous ammonium acetate. 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 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 (λ_{max} 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.

3.2.3 Mass Spectrometry Scan Modes

Tandem MS is explained in detail in Chapter 5. Here two modes of tandem MS are used, CID and Extraction of Product Ion.

3.2.3.1 Collision Induced Dissociation (CID)

Precursors of BMIM, and potential precursor ions are selected in the ion trap and then fragmented using CID. The molecules were excited with radio frequencies in the ion trap at an excitation energy of 30 kV and then fragmented by collisions with helium.

3.2.3.2 Extraction of Product Ion

To identify the breakdown products below the limit of detection of the UV detector, the precursor ion scan mode of mass spectrometry was implemented. Precursor ion scan is a mode of mass spectrometry in which a product ion is selected and all the precursor ions producing this specific fragment are then determined, as described in section 5.1. Given that CID of BMIM results in a stable fragment for the

methyl-imidazolium ion at m/z 83, which is resistant to further fragmentation, this mode of mass spectrometry can be used to identify all the ions in the sample that contain the common methyl-imidazolium fragment. These compounds were then further investigated as possible breakdown products. This scan mode cannot be implemented using time-based mass spectrometers, such as ion traps, because this mode requires the focusing of the second spectrometer on a selected product ion while scanning all the precursors using the first spectrometer. Unfortunately ion trap mass spectrometers are not able to perform precursor ion scans, however, we implemented a related method *extraction of product ion* methodology [1] to find additional biodegradation products below the UV detector limit of detection. With the *extraction of product ion* methodology a common fragment, produced under non-selective in-source fragmentation conditions, results in the identification of related compounds. In our experiments, the biodegradation products with the intact imidazolium ring with m/z value of 83 were screened for during a chromatographic run. In-source fragmentation was performed with collision energy of 25 kV. m/z 83 product ion peaks were extracted in the chromatogram, which then were identified in the corresponding full scan mass spectrum. These obtained m/z values correspond to potential metabolites and were then confirmed by CID MS/MS data. Using this method resulted in the identification of two additional metabolites with m/z values of 137 and 155.

3.3 Results and Discussion

3.3.1 Biodegradation of BMIM

The BMIM cation is released from BMIMCl salt in aqueous solution and can be detected in positive ion mode ESI-MS at m/z 139.1. Figure 3.1 shows the simultaneous detection of the BMIM standard in both MS chromatogram and UV trace at the retention time of 9.95 minutes. The bottom trace shows the BMIM UV absorbance peak at the wavelength of 212nm. The middle chromatogram in Figure 3.1 is the total ion chromatogram (TIC) of the BMIM standard sample in which the peak of BMIM is observed at the same time as the UV trace. The extracted ion chromatogram of BMIM is also observed in the top figure.

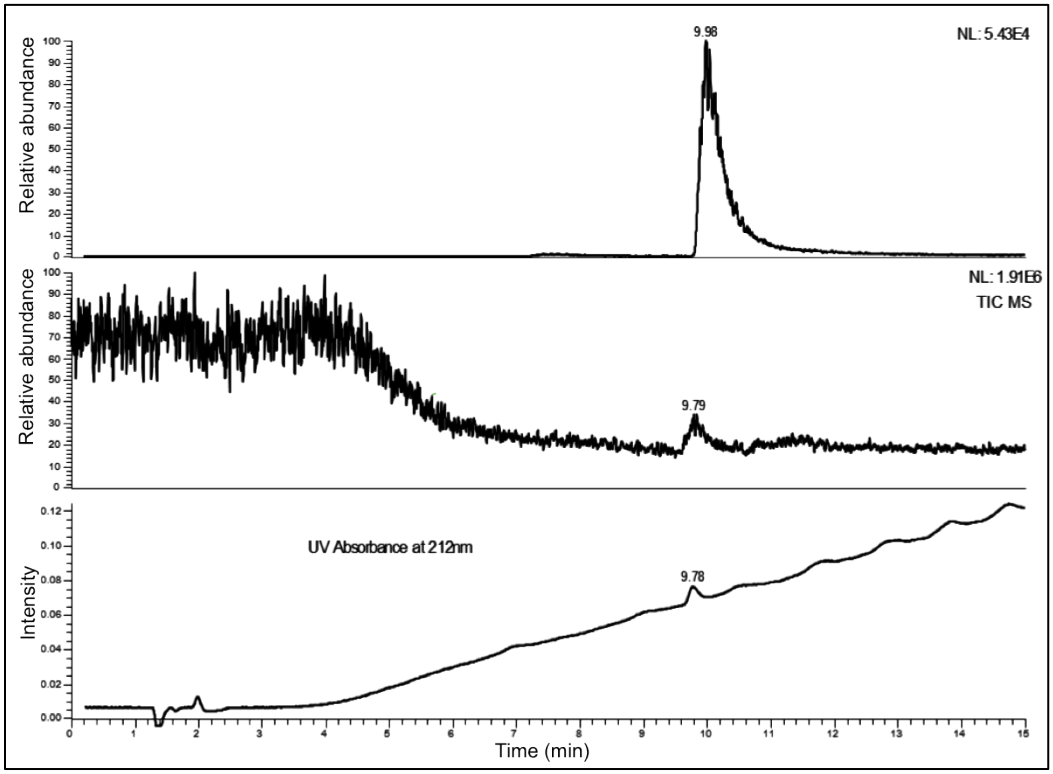


Figure 3. 1. Extraction ion chromatogram (EIC), total ion chromatogram (TIC) and UV trace of BMIM standard.

The area counts obtained from extracted ion chromatograms (EIC) of BMIM cation peaks are proportional to the concentration of the BMIM cation. Plotting these area counts over 46 days of the microbial treatment demonstrates that BMIM degradation is nearly complete after 31 days (Figure 3.2).

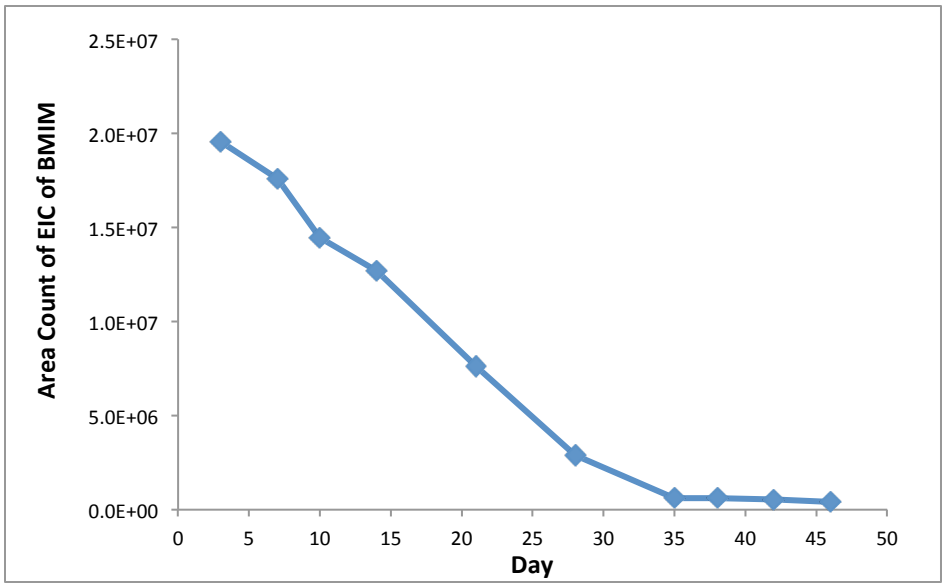


Figure 3. 2. Biodegradation of BMIM cation over 46 days followed by LC-MS.

Due to the lack of data for the first two days of the treatment, the percentages of biodegradation were calculated relative to day 3 and were also plotted on a graph (Figure 3.3). Percentage of BMIM biodegradation reached complete degradation (97%) within the 30-35 days of microbial treatment time, indicating that the microorganisms used in this study are capable of effectively biodegrading of BMIM in 30-35 days, while previous studies have shown that BMIM is a poorly biodegradable compound [2-5].

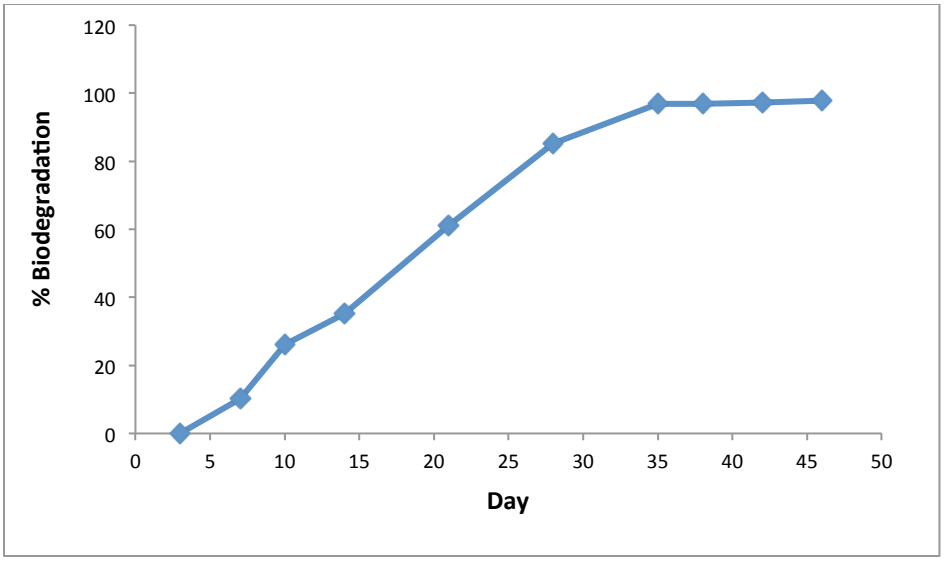


Figure 3. 3. Percentage of biodegradation compared to day 3 over 46 days of the treatment.

3.3.2 Identification of Biodegradation Products and Pathways

In this study, four major biodegradation products were identified. The more abundant m/z 83 and 141 biodegradation products could be observed by the less sensitive UV detector, but were identified by HPLC-MS methods. Two additional metabolites with m/z 137 and 155 were discovered using the extraction of product ion method. The changes in concentration for the identified breakdown products were investigated over the 46 days of the treatment. This was achieved by extraction from the total ion chromatograms (TICs) the mass to charge ratios (m/z) of these four major metabolites over the 46 days of the treatment (Figure 3.4). The area counts of the extracted ion chromatograms peaks were then plotted over time (Figure 3.5). Extraction of m/z 137 from the TIC resulted in three different peaks at three different retention times (Figure 3.4D). The peak with the 1.4 minute retention time kept a constant concentration over the entire course of treatment, therefore it is likely not a

BMIM biodegradation product. None of the identified major identified metabolites were observed in the LC-MS chromatogram of BMIM reference standard. This indicates that they were not impurities in the BMIM standard and were more likely compounds produced during biodegradation. Additional m/z values of 123, 98 and 82 were also recognized using HPLC-UV-MS technique, but they did not show any significant change in concentration over the 46 days of treatment which makes them less likely to be biodegradation products.

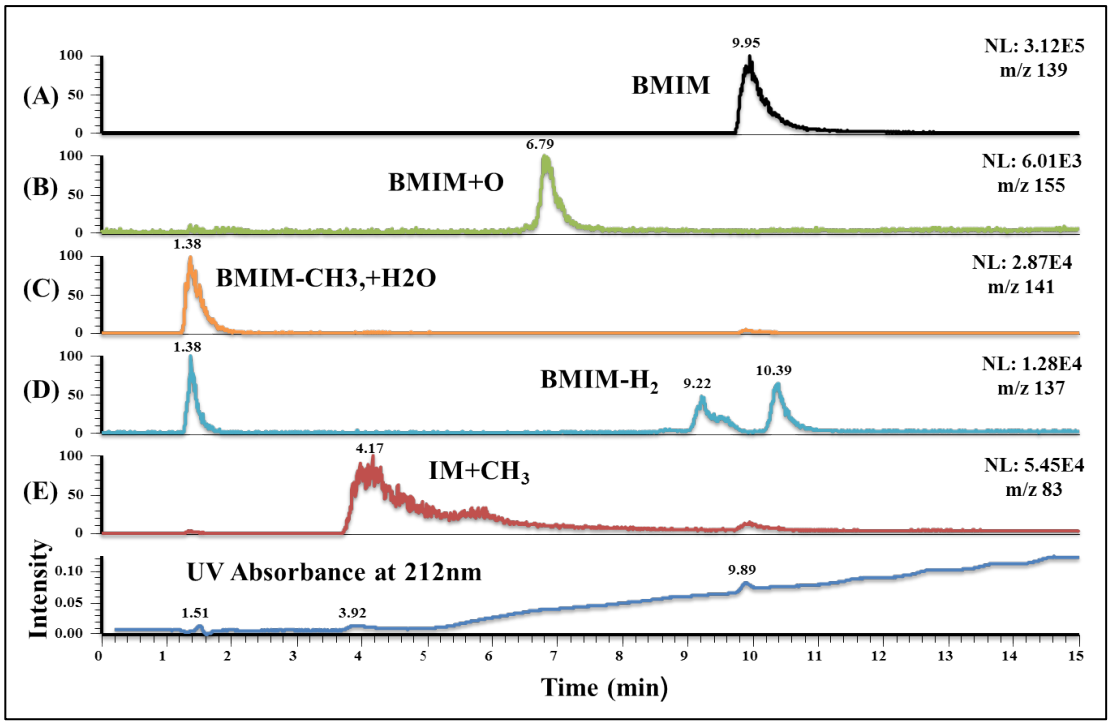


Figure 3. 4. LC-UV-MS extracted ion chromatograms for m/z 139 BMIM and metabolites of m/z 155 BMIM+O, m/z 141 BMIM-CH3+OH, m/z 137 BMIM-2H and UV absorbance at 212nm trace at day 21 of the treatment.

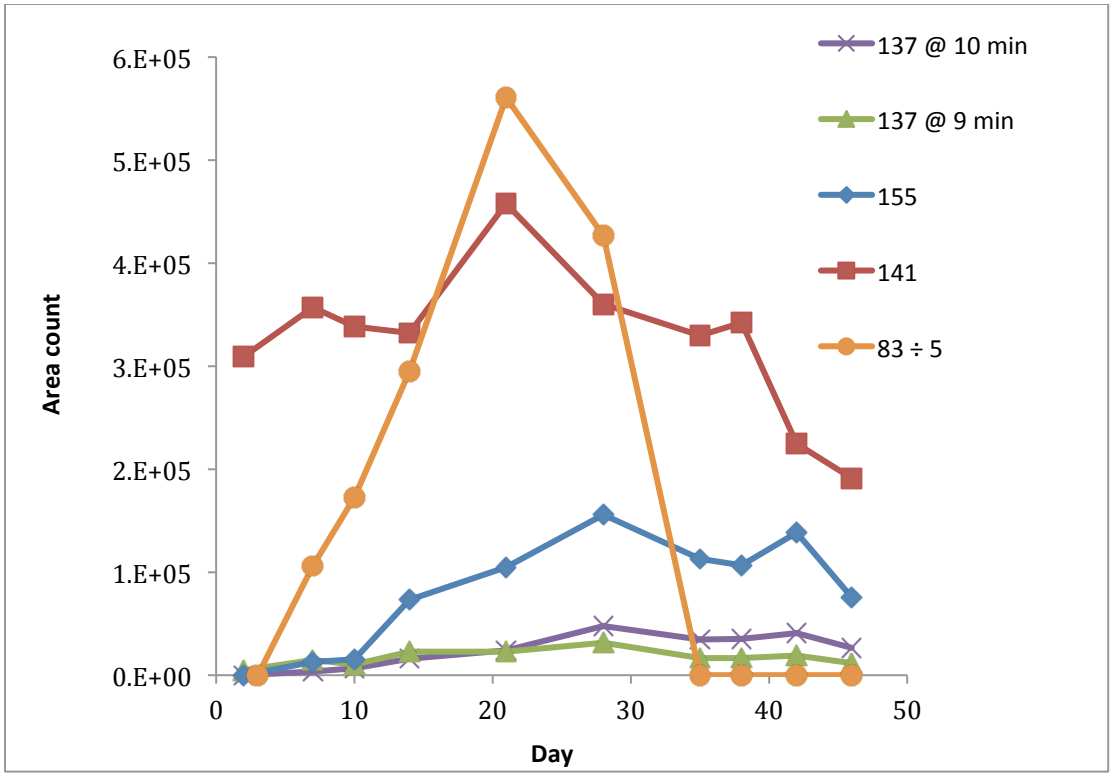


Figure 3. 5. Formation and depletion trends for the major detected biodegradation products over 46 days of the treatment. m/z 155: BMIM+O; m/z 141: BMIM-CH3+OH; m/z 137: BMIM-2H; m/z 83: IM+CH3. m/z 83 area count is divided by 5.

The m/z 83 metabolite is the most abundant intermediate product in the degradation of BMIM. It begins to appear immediately after inoculation of the BMIM, reaches a maximum concentration at day 17 of the treatment, and is completely degraded by the end of the treatment period. (These results were also observed by NMR spectroscopy, using the same samples. Spectra were collected by Dr. Szymczyna's group at Western Michigan University).

The resistance of 1-methylimidazolium product to CID-fragmentation is consistent with the resistance of the 1-methylimidazolium standard and the CID fragmentation of BMIM. CID fragmentation of the BMIM cation results in the fragment at m/z 83 that has a similar resistance to subsequent fragmentation attempts

in MS^n studies (Figure 3.6).

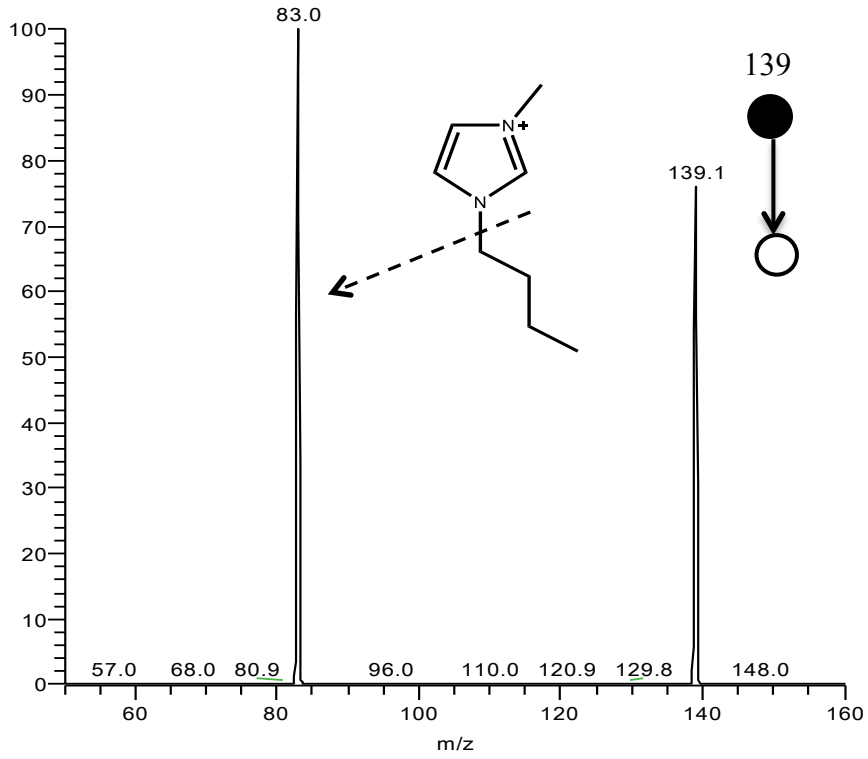


Figure 3. 6. Chemical structure and fragmentation pattern of m/z 139 (BMIM)

The metabolite at m/z 155 was determined to be 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium using a combination of MS and NMR spectroscopy. CID fragmentation of this ion yielded two main fragments with m/z values of 83 and 99 (Figure 3.7). The fragment ion at m/z 83 would be consistent with 1-methylimidazolium, while the fragment ion m/z 99 corresponds to a methylimidazolium, with a hydroxyl group attached to its imidazolium ring. Although determination of the position of the hydroxyl group on the ring was not possible with MS/MS, NMR spectroscopy suggested the hydroxyl group is at position 2 (Dr. Blair

Szymczyna, personal communication). Hydroxylation of the ring is consistent with the biodegradation product suggested for pyridinium based ionic liquids [7]. 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium is a metabolite that was not completely degraded by the microbial community tested in the course of microbial treatment in this study and after reaching a maximum concentration at day 28, persisted at this level for the remainder of the study. However, it was present at a low concentration.

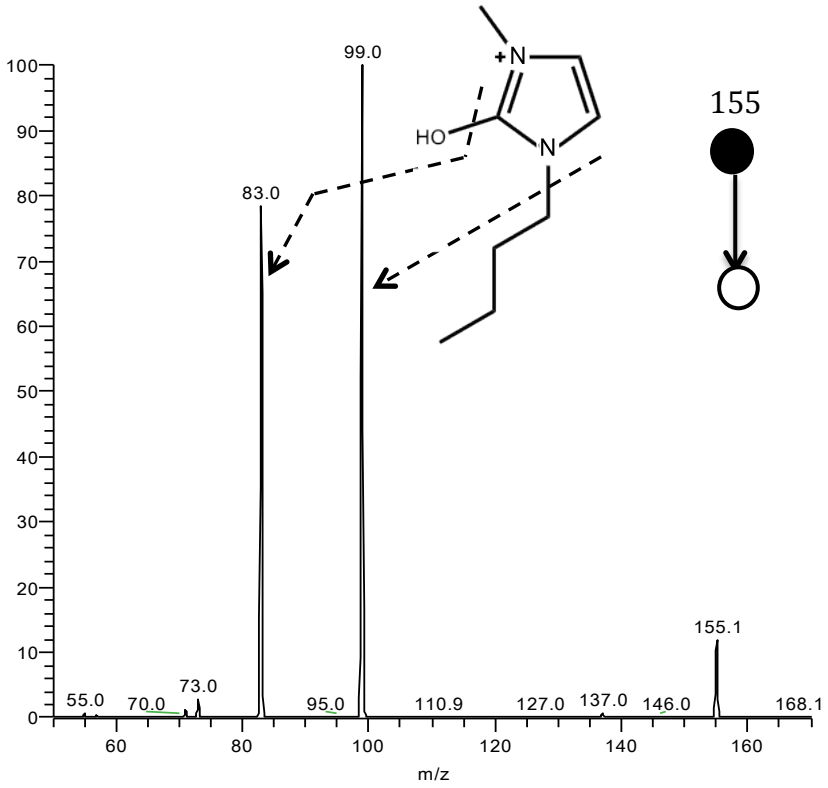


Figure 3. 7. Chemical structure of m/z 155 and its fragmentation pattern

CID fragmentation of the metabolite m/z 141 yielded two main fragment ions with m/z values of 84 and 97. The ion with m/z 84 corresponds to methyl imidazolium and m/z 97 is consistent with 1,3-dimethyl imidazolium (Figure 3.8).

This hydroxylation on the carbon chain is analogous with the degradation pathways suggested for both pyridinium and imidazolium based ionic liquids in previous studies [4, 7, and 8].

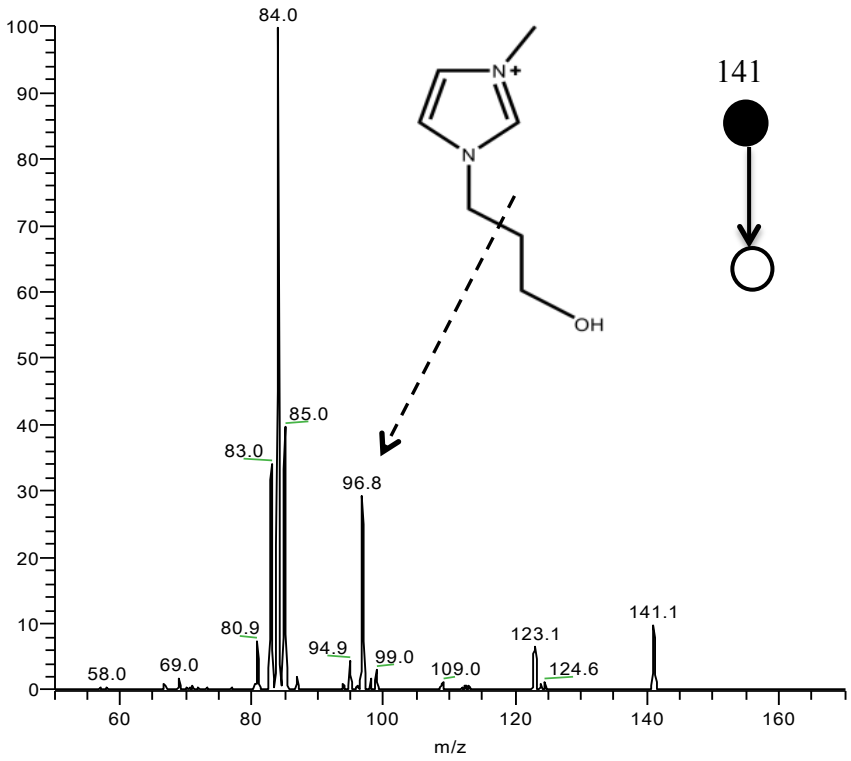


Figure 3. 8. Chemical structure of m/z 141 and its possible fragmentation pattern

Fragmentation of the metabolite m/z 137 yielded three main fragments with m/z values of 83, 95 and 122 (Figure 3.9). Fragment ions with m/z 122 and 95 respectively correspond to the loss of one and three carbon groups from the unsaturated side butyl chain of the BMIM cation. However, the exact location of the double bond is currently unknown. The unsaturation of the side carbon chain is consistant with the suggested structure for a pyridinium based ionic liquids

biodegradation product by Docherty et al. [7].

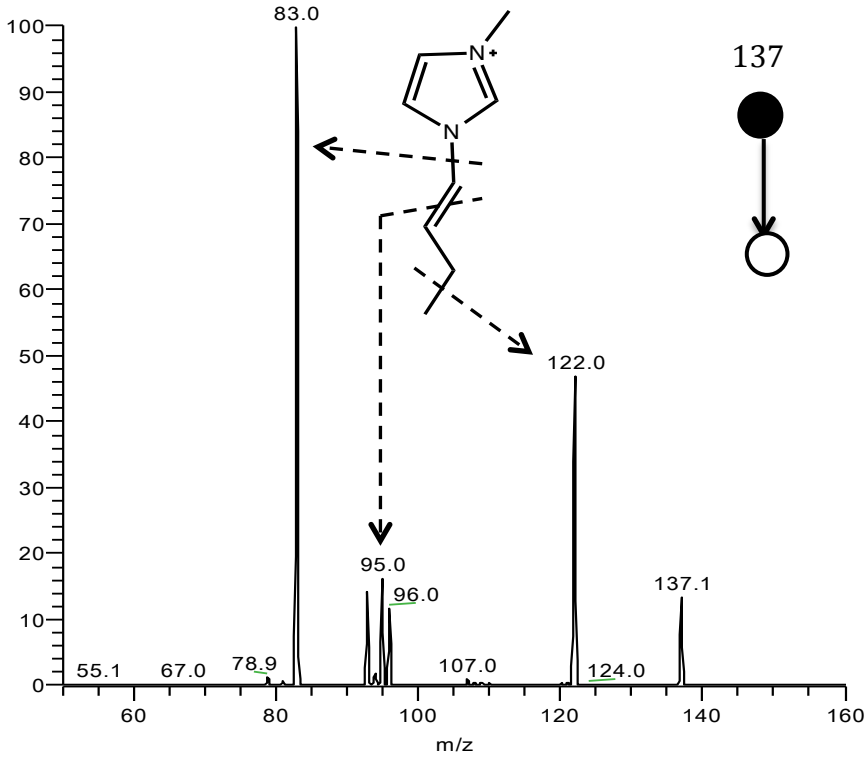


Figure 3. 9. Chemical structure of m/z 137 and its possible fragmentation pattern

The identity of the BMIM degradation metabolites, were determined based on the experimental results and also literature studies [4,6,7]. The four major identified metabolites of m/z 83, 137, 141 and 155, were compared to possible derived structures from BMIM (m/z 139). Hydroxylation, oxidation of terminal carbon to hydroxyl, dehydrogenation and side chain cleavage were observed respectively as described below.

Hydroxylation of the imidazolium ring was proposed for the biodegradation product with m/z 155 and it was confirmed by MS/MS studies (Figure 3.10 B).

Replacement of the terminal methyl group on the butyl chain by a hydroxyl group was proposed for the metabolite at m/z 141 and its presence was also confirmed by MS/MS results (Figure 3.10 C). The m/z 137 breakdown product has a molecular mass consistent with a BMIM cation that has a double bond in the butyl side chain (Figure 3.10 D). And m/z 83 would belong to a methylimidazolium molecule, which is achieved by removal of the butyl group from the imidazolium ring (Figure 3.10 E).

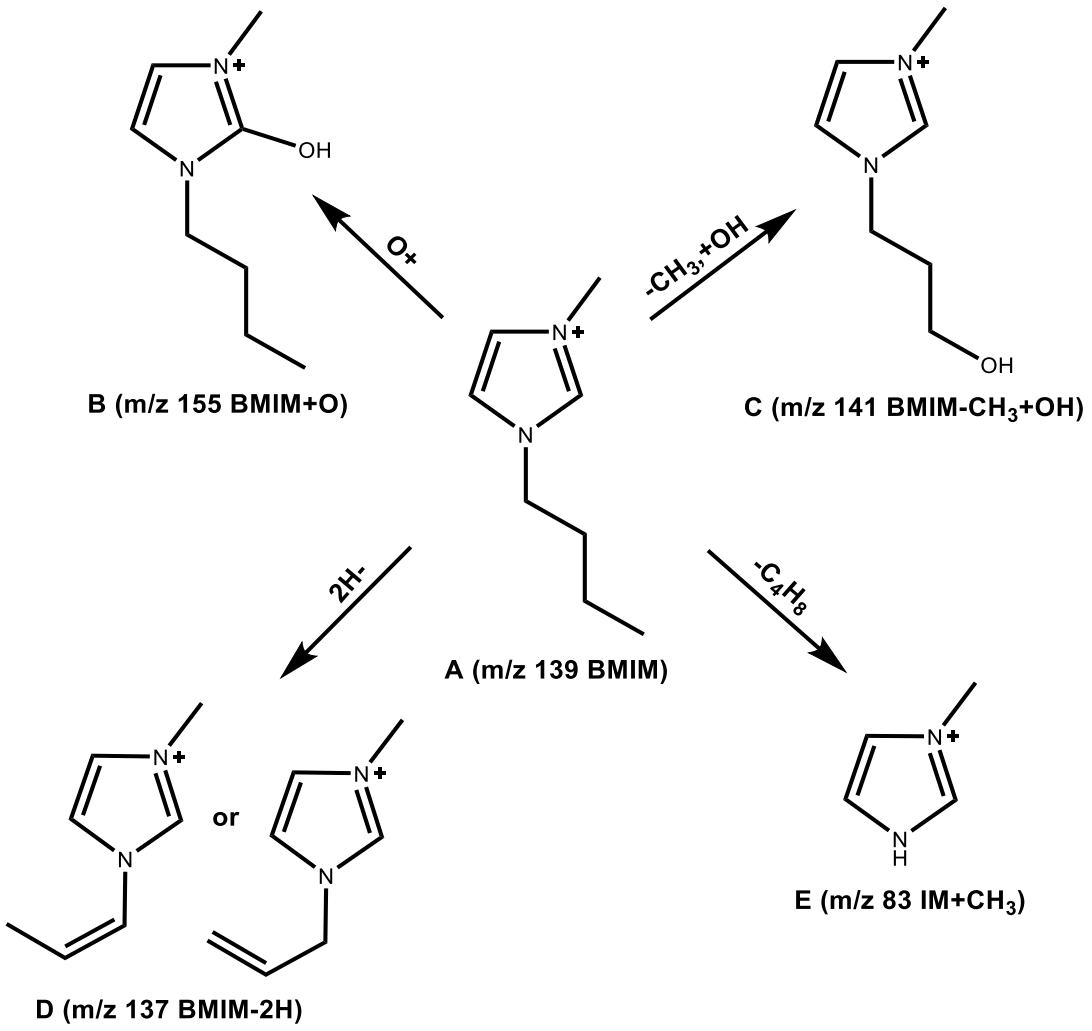


Figure 3. 10. Structures of BMIM cation and identified Breakdown Products. A: 1-butyl-3-methyl-1H-imidazol-3-ium (m/z 139 BMIM); B: 1-butyl-2-hydroxy-3-methyl-1H-imidazol-3-ium (m/z 155 BMIM+O); C: 1-(3-hydroxypropyl)-3-methyl-1H-imidazol-3-ium (m/z 141 BMIM-CH3+OH)

3.3.3 Mass Balance of Degraded BMIM

The area counts obtained from extracted ion chromatograms (EIC) can be related to the concentration of the metabolites. The area under the time trends of the metabolites does not add up to the BMIM trend area. Intensities of the metabolites m/z 137, 141 and 155 are negligible compared to BMIM and the area under the

metabolite with m/z 83 is the most concentrated of the metabolites. To comprise the concentrations of the ions based upon their intensities, ionization efficiencies of the ions need to be considered. BMIM has a C_4 alkyl chain and is more surface active (Surface active molecules preferentially exist on the surface of the electrospray droplet) than methyl imidazolium and therefore has higher ionization efficiency. To investigate the difference of their ionization efficiencies, a mixture of 1-methylimidazolium standard and BMIM were analysed at equimolar concentrations (10μM). The intensity of 1-methylimidazolium standard is almost 38% of the intensity of BMIM due to the lower ionization efficiency of this compound (Fig 3.11). Considering this difference in ionization efficiencies, the area under the time-course curve for the m/z 83 metabolite is less than 14% of the BMIM concentration originally present in the sample.

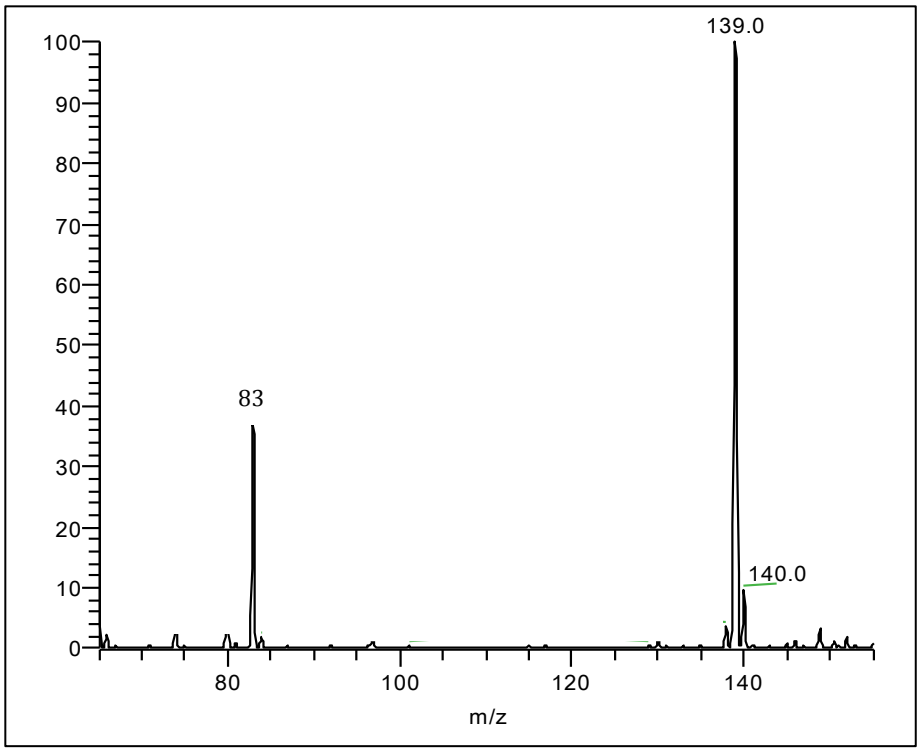


Figure 3.11. A mixture of methylimidazolium standard and BMIM analysed by ESI-MS in positive ion mode at equimolar concentration of 10μM.

The increase and subsequent decrease in the m/z 83 metabolite concentration over the course of the microbial treatment suggests that it's excreted by one or more microbes as a waste product, and subsequently metabolized by other microbes. The previous studies on pyridinium-based ILs suggest that after cleavage of the ring, biodegradation occurs rapidly and breakdown products can be incorporated into microbial biomass or released as gas [4,7,8]. Very low concentrations of these breakdown products leads to low signal to noise ratios, and prevent their ready detection by LC-MS.

3.4 Chapter Conclusion

This work demonstrated that biodegradation resistant BMIM cation could be completely degraded by the activated sludge microbial community used in this study. Since this microbial community was able to decompose BMIM, while previous communities failed, it suggests that different microbial communities may be capable of increasing the biodegradation rates of different ionic liquids. In this study, ESI-MS was used as a technique to identify BMIM biodegradation products and their structures. Four major metabolites were identified for BMIM and were confirmed by the MS/MS technique. The main metabolite was produced by loss of the butyl side chain from the parent compound of BMIM, which can make it less lipophilic and therefore probably less toxic to microorganisms. For the breakdown products observed and the trends in their appearance we could not conclude a particular sequence in their formation, rather it appears that the observed products represent competing pathways.

Two of the minor biodegradation products were resistant to further degradation, and further investigation of the toxicity of these metabolites is necessary as future work.

3.5 References

1. Zhu, X., Kalyanaraman, N., Subramanian, R., Anal. Chem. 2011, 83, 9516–9523.
2. Romero, A., Santos, A., Tojo, J., Rodríguez, A., J. Hazard. Mater. 2008, 151, 268–273.
3. Docherty, K. M., Dixon, J. K., Jr C. F. K. Biodegradation 2006, 18, 481–493.
4. Stolte, S., Abdulkarim, S., Arning, J., Blomeyer-Nienstedt, A. K. Bottin-Weber, U., Matzke, M., Ranke, J., Jastorff, B., Thöming, J. Green Chem. 2008, 10.

5. Garcia, M. T., Gathergood, N., Scammells, P., J. Green Chem. 2005, 7.

6. Petkovic, M., Seddon, K. R., Rebelo, L. P. N., Pereira, C. S., Chem. Soc. Rev. 2011, 40.

7. Docherty, K. M., Joyce, M. V., Kulacki, K. J., Kulpa, C. F. Green Chem. 2010, 12.

8. Pham, T. P. T., Cho, C. W., Jeon, C. O., Chung, Y. J., Lee, M. W., Yun, Y. S., Environ. Sci. & Technol. 2009, 43, 516–521.

CHAPTER IV

BACKGROUND AND THEORY OF ELECTROSPRAY IONIZATION MASS SPECTROMETRY

4.1 The Electrospray Ionization Process

In electrospray ionization, the analyte needs to be solvated. Polar solvents, which readily undergo electrochemical reactions such as water, methanol and acetonitrile are the common solvents for the ESI-MS experiments [1]. A 2-5 kV potential difference is applied to the capillary when the solutions pass through it. Applying a positive potential difference between the capillary and the inlet to a mass spectrometer (positive ion mode) oxidizes the solution and a negative voltage (negative ion mode) results in reduction of the solution. In positive ion mode the electrons removed from the compounds during oxidation, are conducted away and an excess positive charge remains in the liquid. Because of charge-charge repulsion and the force of the electric field, these charges move to the surface of the liquid. Electrospray ionization occurs in three main steps. Firstly, by applying the electric potential, the analyte solution forms a Taylor cone at the capillary tip [2], as the excess charges are pulled along the electric field. When it reaches the Rayleigh limit the solution nebulizes to charged droplets [3]. The Rayleigh limit is reached when the Columbic repulsion of the surface excess charges equals to the solution's surface tension [33]. These droplets have an excess charge with the same polarity as the applied voltage, which reside at the droplet surfaces. The amount of excess charge is fixed and is determined by the flow rate and applied voltage [10]. In a second step,

the released charged droplets get smaller as they move through a dry bath gas. Solvent evaporation increases the density of the excess charge on the surface of the droplet, which results in Columbic fission and the droplet breaking apart into many smaller charged droplets [4]. Finally increasing surface charge density and decreasing the droplets radius result in dispersion of the ions, and the ionized analyte molecules create gas-phase ions. The process by which this occurs is known as the Ion Evaporation Model, one of the theories of ion production by electrospray (Others are explained below) [8]. Part of the resulting gas phase ions passes through a small skimmer orifice into the vacuum. These ions are then transferred into the mass analyzer for analysis [5,6].

Early conventional MS techniques were limited to small, volatile, thermally stable compounds. Dole et al. proposed the Charge Residue Model (CRM) to explain how these molecules are changed to gas phase ions [7]. The CRM model suggests that in the electrospray ionization method, the electrospray droplets continue to breakup until each small, charged droplet contains a single analyte molecule and eventually, solvent evaporation results in the transfer of the remaining charges present on the droplet surface to the analyte. Although the CRM is the first and most widely adopted proposed model of analyte charging by ESI, other ionization models such as Iribarne and Thomson's Ion Evaporation Model (IEM) [8] and Kaltashov's emission model [9] have been proposed and developed. IEM is more applicable to small species [11], while the CRM typically is more active for larger species [11, 12].

4.2 Surface Activity

Surface activity (the affinity of an ion for the ESI droplet surface) is one of the

important properties that affects ionization efficiency in ESI-MS [17-26]. According to the Iribarne and Thomson ion evaporation model, ions with large nonpolar regions (surface-active analyte ions) that are less solvated or ion paired prefer the air-liquid interface at droplet surfaces and are more likely to be present on the surface of a droplet rather than in the interior. Consequently, they are more likely to evaporate from the droplet and be present in the spectrum and therefore have higher response [8, 13, 16, 27]. In ESI, there is a good match between concentration and abundance for ions with similar properties but the ESI response differs for ions with different size or polarity [14, 15]. Iribarne et al. demonstrated that at atmospheric pressure ionization mass spectrometry (API-MS), analytes with nonpolar portions have a higher response than highly polar analytes [16]. This was demonstrated experimentally by Tang and Kebarle who showed that tetraalkylammonium ions, which are more surface active than alkali metal cations, have a much higher ESI response [17]. They suggested that not only lower solvation energy and therefore higher ion evaporation rate of tetraalkylammonium ions cause this higher sensitivity but also surface activity has an important role in their higher response. Rundlett and Armstrong explained ion suppression of the analyte in the presence of anionic surfactants using a modified aerosol ionic redistribution (AIR) mode in CE-ESI-MS using this argument. Surface active components, such as surfactants, are more present on the surface of droplets than the analyte and can suppress the analyte ion signals, leading to low ion signals for the molecules of interest [18].

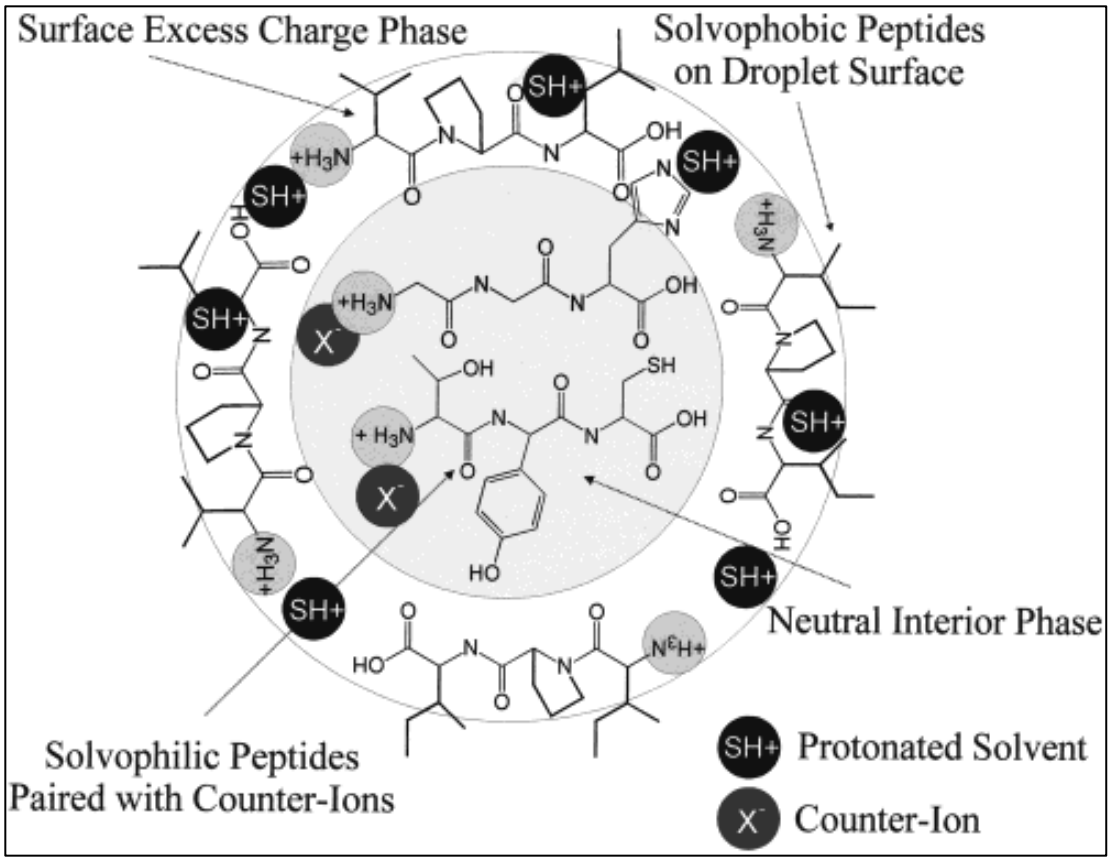


Figure 4.1. More polar peptides remain in the neutral droplet interior where they are neutralized by counter ions. Peptides with nonpolar side chains exist mostly on the droplet surface where these side chains can be desolvated. [Reprinted from Cech & Enke, Anal Chem, 2000, 72:2717–2723 with permission of American Chemical Society]

When ESI droplets fission, a smaller fraction of charge than mass is retained by the parent droplet [3]. Since the surface layer of the parent droplet breaks up into offspring droplets, during this fissioning, the surface active analytes will follow the charge. Consequently, the offspring droplets are enriched in the excess charge and also the surface-active analytes. Tang and Smith, photographed fluorescent species that were deposited on a metal counter electrode by an ESI spray [23]. They demonstrated that the outer ring of the ESI spray is enriched in surfactant in the lighter offspring droplets and non surface-active analytes are equally distributed

throughout the ESI spray and fail to be charged in the droplet interior even with there are plenty of excess charges in the parent droplets.

Figure 5.2 shows a mass spectrum of an equimolar mixture of six peptides with different C-terminal residues [21]. The peaks in the mass spectrum corresponding to the peptides with more nonpolar (hydrophobic) side-chains are more intense.

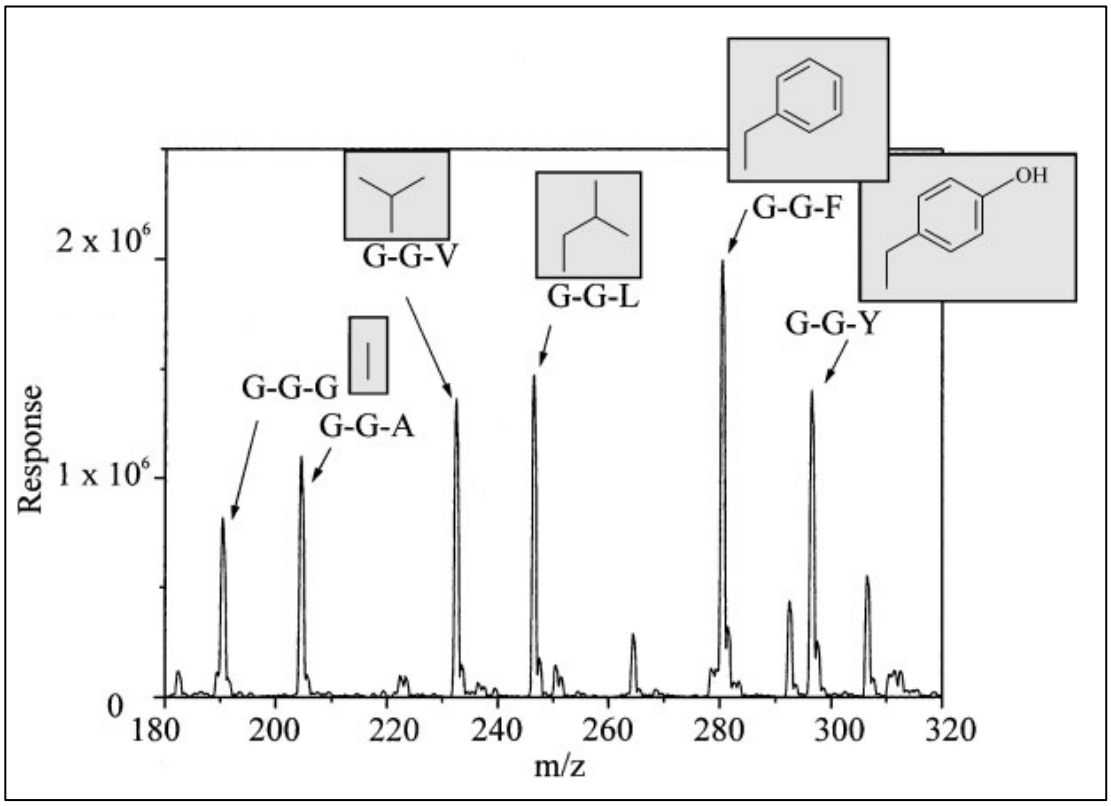


Figure 4.2. Mass spectrum of an equimolar mixture of six tripeptides that have different C-terminal residues. ESI response increases as the side chain on the C-terminal residue becomes increasingly nonpolar. [Reprinted from Cech & Enke, Anal Chem, 2000, 72:2717–2723 with permission of American Chemical Society] [28].

4.3 Equilibrium Partitioning Model

Enke presented a mathematical model for the relationship between concentration and

the ion abundances in ESI for permanently charged analytes. This is known as the equilibrium partitioning model (EPM) [20]. In electrospray ionization (ESI), droplets are created with a constant rate of surface excess charge. The small current of ESI source drives the electrochemical oxidation or reduction at the ESI emitter, which creates the excess charge generated in ESI. The concentration of the excess charge on the initial droplets can be calculated using the equation $[Q] = I/\Gamma F$, where [Q] is the concentration of excess charge, I is the ESI source current, Γ is the volume flow rate of solution into the ESI source, and F is Faraday's constant [20]. Due to electrostatic repulsions, the excess charge resides in the surface layer of the droplet and the droplet interior is electrically neutral and the ions are matched by an equal number of counter ions [20]. On the other hand, molecules on the droplet surface are less well solvated, creating chemically different states of the outer surface layer and the interior of the electrospray droplets [17]. Consequently, the ions partition between the two states of the electrically neutral interior, and the surface of the ESI droplet that carries the excess charge. When an ion goes from the bulk interior phase to the surface phase it replaces an ion that is already on the surface and no new excess charges are created. EPM suggests that ions in solution compete for the limited number of excess charges on the electrospray droplet surface. Thus, increased nonpolar regions of the ions, which enhance their affinity for the droplet surface, results in higher relative ESI signal.

The higher response of the surface active molecules can be explained by their higher equilibrium partition coefficients (K) [20-22]. An equilibrium partitioning coefficient (K) is the ratio of analyte's concentration on the droplet surface to its

concentration in the interior [20]. Factors such as polarity (hydrophobicity), charge density, basicity (ability to carry protons as charge), solvation energy and ion-pairing energy determine the magnitude of *K* for a given ion. Analytes with high *K* values exist mostly on the surface of droplets and are capable of carrying most of the excess charge. Analytes with small *K* exist in the droplet interior and are paired with counter ions, resulting in alower ability to carry the excess charge. Only ions that are present in the surface phase can carry excess charge and show up in the mass spectrum. The ions in the electrically neutral droplet interior are lost as neutral salts. Consequently, the tendency of an ion to exist on the surface of a droplet determines its ESI response. Various aspects of the equilibrium partitioning model have been studied [21,22,29-32] but most of them are limited to simple, permanently charged analytes.

The ESI solution solvent has an influence on ionization efficiency of the analytes. Higher solvent polarity (more water content) increases the distribution coefficient between the droplet surface and bulk solution and the partitioning because the hydrophobic ions tend to avoid the polar bulk solution, therefore their presence on the surface increases. An increased organic solvent composition increases ionization efficiency and therefore the response of the ions. Hydrophobic ions are more difficult to solvate when the solvent is more polar (more water content). The electrospray signal is optimized at a high methanol concentration.

4.4 Chapter Conclusion

Molecules with more nonpolar regions have more affinity to the surface of the ESI droplet (more surface-active molecules) and therefore have greater signals in the

mass spectrum. Surface activities of the molecules can be manipulated by changing the organic content of the electrospray solution. The intensity of the compounds with different surface activities responds in different extents to the changing the organic solvent composition. The intensity ratios of the precursor and fragment ions at high versus low methanol concentrations can be used to correlate the fragments to precursors. In this study, a novel method of multiplexing MS/MS is proposed where correlation of the fragments to the corresponding precursors is performed by modulation of the ionization efficiencies of analytes by changing the solvent composition.

4.5 References

1. Cole, R. B., Electrospray and MALDI Mass Spectrometry, John Wiley & Sons, New Jersey, NJ, USA, 2010.
2. Taylor, G. I., McEwan, A. D., J. Fluid Mech. 1965, 22 (01), 1-15.
3. Taflin D. C., Ward, T. L., Davis, E. J., Electrified droplet fission and the Rayleigh limit. Langmuir 1989, 5, 376–384.
4. Cloupeau, M., Prunet-Foch, B., J. Aerosol Sci. 1994, 25 (6), 1021-1036.
5. Yamashita, M., Fenn, J. B., J. Phys. Chem. 1984, 88, 4451-4459.
6. Whitehouse, C. M., Dreyer, R. N., Yamashita, M., Fenn, J.B., Anal. Chem. 1985, 57, 675-679.
7. Dole, M., Mack, L. L., Hines, R. L., Mobley, R. C., Ferguson, L. D., Alice, M. B., Molecular Beams of Macroions. J. Chem. Phys. 1968, 49 (5), 2240-2249.
8. Iribarne, J. V., Thomson, B. A., J. Chem. Phys. 1976, 64 (6), 2287-2294.
9. Kaltashov, I. A., Mohimen, A., Anal. Chem. 2005, 77 (16), 5370- 5379.
10. Gomez, A., Tang, K., Phys. Fluids 1994, 6, 404-414.

11. Kebarle, P., Peschke, M., Anal. Chim. Acta 2000, 406, 11-35.

12. Cole, R. B., J. Mass Spectrom. 2000, 35, 763-772.

13. Tang, S., Babai, A., Mudring, A. V., Angew. Chem. Int. Ed. 2008, 47, 7631–7634.

14. Amad, M. a. H., Cech, N. B., Jackson, G. S., Enke, C. G., J. Mass Spectrom. 2000, 784–789.

15. Bruins, A. P., J. Chromatogr. 1998, A 794, 345–357.

16. Iribarne, J. V., Dziedzic, P. J., Int. J. Mass Spectrom. Ion Phys. 1983, 50, 331-347.

17. Tang, L., Kebarle, P., Anal. Chem. 1993, 65, 3654-3668.

18. Rundlett, K. L., Armstrong, D. W., Anal. Chem. 1996, 68, 3493-3497.

19. Armstrong, D. W., Hinze, W. L., Bui, K. H., Singh, H. N., Anal. Lett. 1981, 14, 1659-1667.

20. Enke, C. G., Anal. Chem. 1997, 69, 4885-4893.

21. Cech, N. B., Enke, C. G., Anal. Chem. 2000, 72, 2717-2723.

22. Cech, N. B., Enke, C. G., Anal. Chem. 2001, 73, 4632-4639.

23. Tang, K., Smith, R. D., J. Am. Soc. Mass Spectrom. 2001, 12, 343-347.

24. Zhou, S., Cook, K. D., J. Am. Soc. Mass Spectrom. 2001, 12, 206-214.

25. Sherman, C. L., Brodbelt, J. S., Anal. Chem. 2003, 75, 1828-1836.

26. Sherman, C. L., Brodbelt, J. S., Anal. Chem. 2005, 77, 2512-2523.

27. Thomson, B. A., Iribarne, J. V., J. Chem. Phys. 1979, 71, 4451-4463.

28. Cech, N. B., Enke, C. G., Mass Spectrometry Reviews, 2001, 20, 362 – 387.

29. Constantopoulos, T. L., Jackson, G. S., Enke, C. G., Anal. Chem. Acta 2000, 406, 37-52.

30. Sjo¨berg, P. J. R., Bo¨kman, C. F., Bylund, D., Markides, K. E., J. Am. Soc. Mass Spectrom. 2001, 12, 1002-1010.

31. Constantopoulos, T. L., Jackson, G. S., Enke, C. G., J. Am. Soc. Mass Spectrom. 1999, 10, 625-634.

32. Sjoberg, P. J. R., Bokman, C. F., Bylund, D., Markides, K. E. Anal. Chem. 2001, 73, 23-28.

33. Rayleigh, Lord Philos. Mag. 1882, 14, 184.

CHAPTER V

TANDEM MASS SPECTROMETRY

5.1 Introduction

ESI converts the analyte molecules to the gas phase without breaking any covalent bonds and thus is known as a soft ionization method. It can provide information about the molecule's molecular weight, but does not provide much information about the structure of an unknown analyte. Tandem mass spectrometry (MS\MS) is a common technique, in which a precursor ion is mass-selected and fragmented to elucidate information about structure and identity of the molecules [1] or to improve quantitation. MS/MS can greatly improve the detection limits for selected compounds, especially in complex mixtures, even though the total ion current is decreased relative to the normal mass spectrum. This is achieved by increasing selectivity and therefore the signal-to-noise ratios for the detection of compounds present in mixtures [39, 40]. In a tandem MS experiment, a precursor ion is usually selected by a first mass analyzer, focused into a collision cell, where it is fragmented to product ions and neutral fragments. A second mass analyzer then separates and detects the product ions. Neutral fragments, which don't carry a charge, are known as neutral losses and can not be detected. The mass analyzers can be arranged in space (triple quadrupole, sector and hybrid instruments) or in time (ion trap instruments). Inert target gas molecules are introduced into the collision cell, in which collisions between the precursor ion and inert gas analyte molecules occur. The common gases used in the collision stage are inert and neutral gases such as nitrogen,

helium, or argon [3]. These collisions result in increased internal energy of the precursor ions. Ultimately, unstable excited precursor ions decompose into product ions. This process is called “collision-induced dissociation” (CID). CID is the most popular and common ion activation method used in conjunction with ESI-MS. Product ions are then analyzed by a second mass-analyzer [2].

Tandem mass spectrometry was initially developed as a tandem-in-space method in which the analyzers are sequentially coupled together. Sector mass spectrometers were utilized as first analyzers for MS/MS experiments. Different analyzer arrangements were then developed including sectors [4-6], multiple quadrupoles [7-9], time-of-flight (TOF) [10] and various combinations [11-18]. Tandem MS was further elaborated into tandem-in-time instruments in which the selection and dissociation of the ions are performed sequentially within the same space, including radiofrequency quadrupole trap [19] and ion cyclotron resonance (ICR) mass spectrometers [20, 21].

Four main scan modes are available using tandem mass spectrometry [22]:

1. Product ion scan (Figure 4.1 (1)): A precursor ion with a specific mass-to-charge ratio is selected and all of the product ions resulting from collision-induced-dissociation (CID) are determined.

2. Precursor ion scan (Figure 4.1 (2)): A product ion is selected and all the precursor ions producing this specific fragment are determined. This scan mode cannot be implemented with time-based mass spectrometers, such as ion traps. It requires the focusing of the second spectrometer on a selected product ion while scanning all the precursors using the first spectrometer. However, as illustrated in Chapter 3, section

3.2.3, methods such as extraction of product ion can be used to simulate this.

3. Neutral loss scan: A neutral fragment is selected and all the fragmentations leading to the loss of the neutral fragment are determined. This scan mode is also not available with time-based mass spectrometers. The first and second analyzers are scanned at an offset to determine the neutral loss.

4. Selected reaction monitoring (SRM): A fragmentation reaction is selected. Both the first and second analyzers focus on selected masses. The ions selected by the first mass analyzer can only be detected if a given fragment is produced by a selected reaction. Since there is no scanning in this mode, the precursor and fragment ions are detected in more scans, resulting in increased sensitivity.

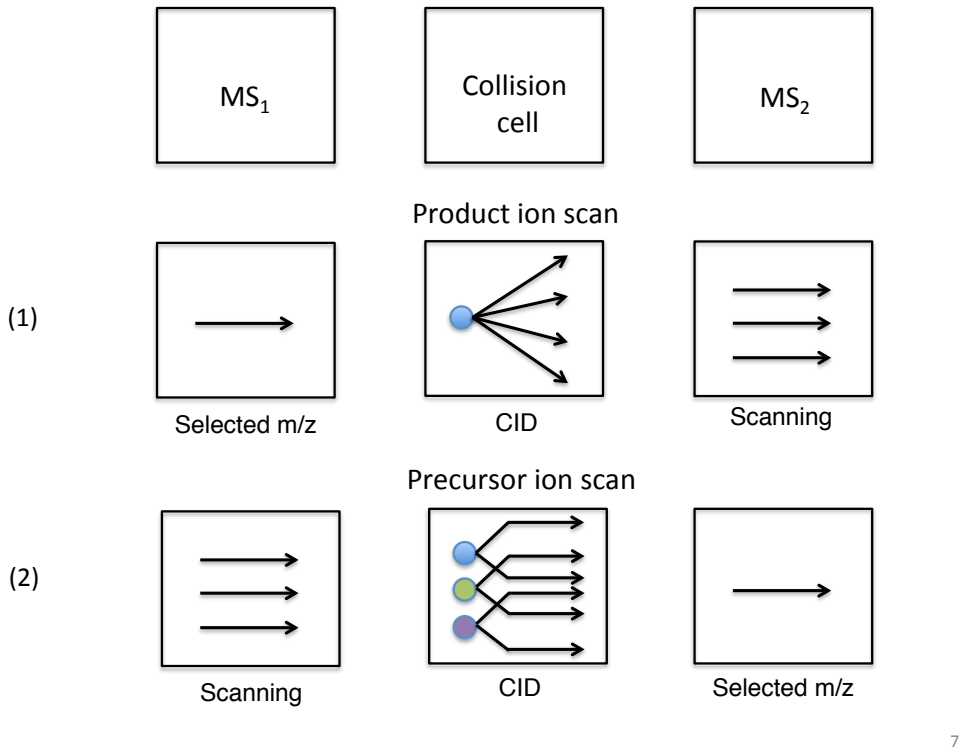


Figure 5.1. (1) Product ion tandem mass spectrometry scan mode, (2) Precursor ion tandem mass spectrometry scan mode [2].

5.2 Multiplexed Tandem Mass Spectrometry

With conventional tandem mass spectrometry, each precursor ion is sequentially selected and dissociated to characteristic fragment ions. For complex mixtures, MS/MS experiments for comprehensive analyses are time consuming and require high-throughput. Multiplexed tandem MS can increase the MS/MS experiment throughput and signal-to-noise ratio. In multiplexed tandem MS, all precursor ions are fragmented simultaneously without any pre-selection and product ions are then decoded to their respective precursor ions [23] (Figure 5.2).

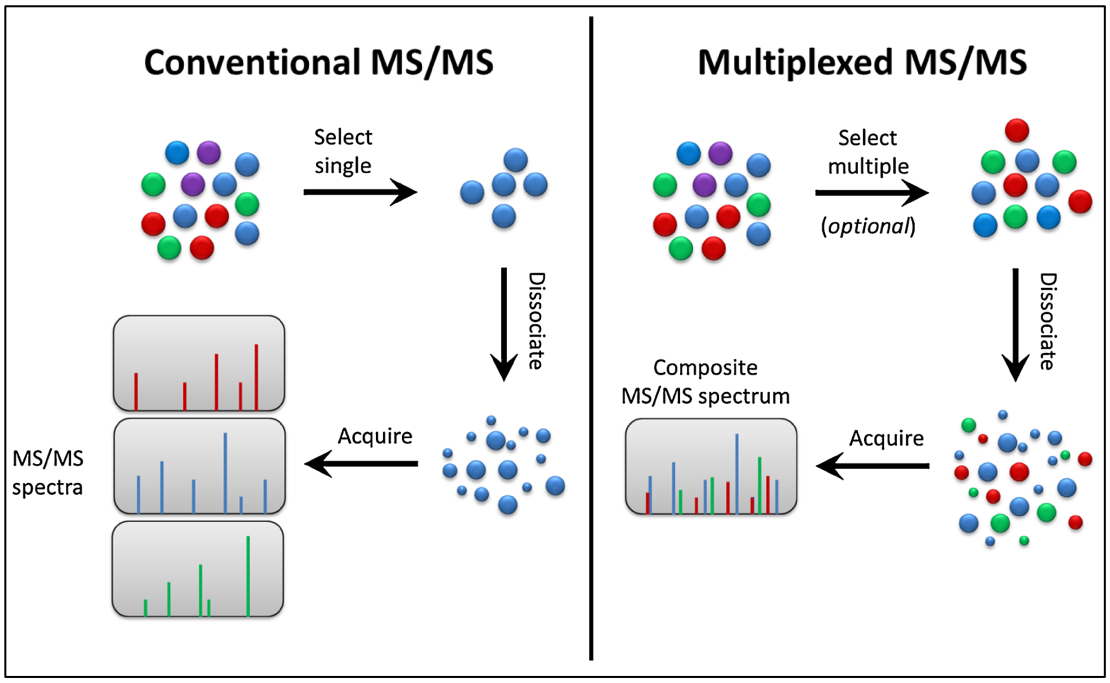


Figure 5.2. Multiplexed MS/MS vs. conventional MS/MS [23] (With permission of John Wiley & Sons, Inc).

Since more than one precursor ion is isolated and dissociated in this approach, matching of product ions to their corresponding precursors is the challenge. Therefore, encoding the precursor ions to their respective product ions is required.

Several multiplexed tandem mass spectrometry methods have been developed for encoding product ions to their respective precursors in mixtures.

McLafferty, et al. developed a multiplexed MS approach using a highly accurate Fourier transform ion cyclotron resonance (FTICR) mass spectrometer [24]. This method requires repeating MS/MS experiments N-times on appropriate subsets of N precursor ions on a high cost FTICR instrument. At each acquisition, N−1 precursor ions are fragmented simultaneously, and each time a new Nth precursor is left intact. The fragments are then deconvoluted, using a Hadamard transform (HT) [24].

A second approach also relies on multiplexing on a FTICR using excitation and de-excitation radio frequency pulses with a specific delay time (*t*d). The abundances of individual precursor ions change according to the phase difference (dependent on the precursor *m/z* value) by the de-excitation pulse. Recording product ion spectra as a function of *t*d, and then FT of this function, results in the determination of the corresponding precursor ions. Therefore, product ions are related to their corresponding precursors by their abundance changes. This technique not only requires a high cost FTICR instrument, but also suffers from low analysis speed for online coupling and requires large amounts of sample for off line implementation [25,26].

Another approach is based on database searching, requires the high mass accuracy of the FTICR and has been applied to known peptides. In this approach, product ion spectra of N precursors can be collected in one spectrum. Hypothetical product ion spectra obtained from the database are then compared to the

experimentally determined product ion spectrum. Ultimately, the product ions are assigned to their respective precursor ions by matching the peptide mass tag, which is derived from experimental data to potential peptide sequence. In addition to the high cost of the required FT-ICR instrument, peptides can only be identified if they exist in a database, and for higher numbers of peptides, sequence matching efficiency drops [27,28].

Multiplexing methods have not only been performed on high mass accuracy instruments but low or intermediate performance mass spectrometers have also been utilized for this purpose. The low cost, ease, speed, and high efficiency of quadrupole ion trap mass spectrometers (QITMS) make them a potential candidate for multiplexed MS/MS experiments.

For example, a multiplexing method has been developed on a QITMS, which relies on the inherent mass bias of ion accumulation in the QIT to encode the intensity of precursor ions to their corresponding product ions. This intensity encoding scheme applies the Gaussian distributions that illustrate the relationship between ion intensities and radio frequency trapping voltages during ion accumulation. One arbitrary waveform is used for isolation and one for dissociation. The intensities of the fragments are encoded using different low mass cut-off settings during ion accumulation. Due to the low resolution and low collision induced dissociation (CID) efficiency, the method is limited to a narrow m/z range [29]. Bushey, et al illustrated this with Iterative Accumulation Multiplexing (IAM) on a hybrid quadrupole FT-ICR instrument. In this method, precursor to fragment ion relationships are encoded by the specific ratio of the accumulation times for each precursor ion in two successive

multiplexed tandem MS acquisitions. Fragment ions that show the same abundance ratio between scans can be related to the particular precursor ion with same ratio of accumulation time. The obtained data in this approach was able to triple the analytical throughput [30].

A recent implementation of multiplexed MS/MS was proposed by Ledvina et al. using advanced ion storage capabilities advantage of a modern hybrid LTQ-Orbitrap instrument. The fragment ions produced from successive dissociation in the linear ion trap were temporarily stored, before their transmission to the Orbitrap detector for simultaneous detection.

This FTMS-based method provided a 2.5 - 3.0 fold greater throughput than a conventional FT tandem MS approach [31].

5.3 The Need for Multiplexed Tandem Mass Spectrometry

Measuring the MS spectrum of each precursor individually results in a sensitivity loss in MS/MS experiments on mixtures with many components. When sample quantity or analysis time is limited, such as on-line trace analysis of toxic materials in incinerator exhaust or peptide sequencing, the use of the tandem MS technique is limited to just a few precursors. For MS^n experiments, even more measurement problems would exist when additional spectra of mass-selected products are required, since the number of possible fragmentation or reactions increases exponentially with *n*.

In a typical high throughput protein identification method, groups of proteins are digested to mixtures of polypeptides, which are then analyzed using an on-line

separation technique, such as capillary LC and tandem mass spectrometry. The resulting peptides and their parent proteins are ultimately identified by sequence database searching. Many polypeptides usually co-elute, and therefore the ability to identify components in complex protein mixtures depends on how many polypeptides can be individually fragmented and how quickly they can be identified [32-34]. Consequently, the throughput of this method is limited by the acquisition time of the MS/MS spectrum for each peptide. Thus, long separation times are required for MS/MS analysis of all detected peptides with an increase in sample consumption, or the complexity of the protein mixtures that can be addressed is limited [37]. Stopping or slowing capillary electrophoresis or LC flow rates have been utilized to increase the number of conducted MS/MS experiments and the number of identified polypeptides in a given sample quantity consumed during the elution time of polypeptides [35,36]. Another method that has been used to overcome this problem is dynamic exclusion (DE), which prevents reselection of the same precursor ion in successive fragmentations, where previously identified peptides are excluded from further fragmentation in subsequent scans. This allows the maximum number of unique precursor ions to be selected and increases the dynamic range of detection and proteome coverage by maximizing the number of selected precursor ions, which are most possible to result in peptide identification [38].

The ability to perform multiple MS/MS experiments simultaneously would enhance both sensitivity and throughput when identifying polypeptides [28].

5.4 Modulated Ionization Efficiency Multiplexed MS

As described in this chapter, most previously developed multiplexed tandem mass spectrometry methods were performed on high resolution and high accuracy mass analyzers (FTICR or FT Orbitrap). Hybrid QIT-FT MS instruments were also used to increase the data acquisition rate. In this work, a novel method of multiplexed tandem MS is implemented by modulation of ionization efficiency of the ions by changing different ESI parameters. This method allows us to perform multiplexing tandem mass spectrometry on less expensive, more accessible single stage mass analyzers.

The differences in ionization efficiencies of the molecules have been studied for many years [41,42], but this is the first time that it's applied for a practically useful purpose. The proposed method has advantages relative to previous multiplexed tandem mass spectrometry methods. The sample throughput is increased using non-selective in-source fragmentation of all the ions, the data points across the chromatographic peaks are increased due to scanning of both full and fragmentation mass spectra and it empowers single MS analyzers to conduct tandem experiments, which are less expensive and more accessible.

5.5 Chapter Conclusion

ESI is an efficient method for producing gas-phase biomolecular ions from solution, but it generally only provides information about the intact molecular weight of molecules. Molecular weight alone does not provide enough information about the structure of an unknown analyte to result in unambiguous identification. Tandem mass spectrometry is a technique to elucidate the structure and identity of unknown

molecules, or to improve quantitation by increasing selectivity and signal-to-noise ratios. However, tandem MS is time-consuming and high-throughput MS/MS experiments are required for complex mixtures. Multiplexing tandem MS can increase the MS/MS experiment throughput and signal-to-noise ratio. Most previously developed multiplexed tandem mass spectrometry methods were performed on expensive high accuracy mass analyzers. In the next chapter we describe the development of a novel method of multiplexing MS/MS, by modulation of the ionization efficiencies of analytes. This method of tandem mass spectrometry has the advantage that it can be implemented on a simple single mass analyzer mass spectrometers.

5.6 References

1. Hoffmann, E. de. J. Mass. Spec. 1996, 31, 129-137.
2. Griffiths, W.J., Jonsson, A. P., Liu, S., Rai, D. K., Wang, Y., Biochem. J. 2001, 355, 545–561.
3. Vachet, R. W., Glish, G. L., J. Am Society for Mass Spectrometry, 1996, 7 (12), 1194–1202.
4. McLafferty, F. W., Todd, P. J., McGilvery DC, Baldwin MA. J. Am. Chem. Soc. 1980, 102, 3360.
5. Vrscaj, V., Kramer, V., Medved, M., Kralj, B., Beynon, J. H., Ast T. Int. J. Mass Spectrom. Ion Phys. 1980, 33, 409;
6. Louter, G. J., Boerboom, A. J. H., Stalmeir, P. F. M., Tuithof, H. H., Kistemaker, P. J., Int. J. Mass Spec- trom. Ion Phys. 1980, 33, 335.
7. Yost, R. A., Enke, C. G., J. Am. Chem. Soc. 1978, 100, 2274.
8. Vestal, M., Futrell, J. H., Chem. Phys. Lett. 1974, 28, 559.

9. McGilvery, D. C., Morrison, J. D., Int. J. Mass Spectrom. Ion Phys. 1978, 28, 81.

10. Schey, K., Cooks, R. G., Grix, R., Wollnik, H., Int. J. Mass Spectrom. Ion Processes 1987, 77, 49.

11. Glish, G. L., McLuckey, S. A., Ridley, T. Y., Cooks, R. G., Int. J. Mass Spectrom. Ion Phys. 1982, 41, 157.

12. Schoen, A. E., Amy, J. W., Ciupek, J. D., Cooks, R. G., Dobberstein, P., Jung, G., Int. J. Mass Spectrom. Ion Processes 1985, 65, 141.

13. Shukla, A. K., Anderson, S. G., Howard, S. L., Sohlberg, K. W., Futrell, J. H., Int. J. Mass Spectrom. Ion Processes 1988, 86, 61.

14. Glish, G. Goeringer, D. E., Anal. Chem. 1984, 57, 2291.

15. Beynon, J. H., Caprioli, R. M., Ast T. Org. Mass Spectrom. 1971, 5, 229.

16. Stults, J. T., Enke, C. G., Holland, J. F., Anal. Chem. 1983, 55, 1323.

17. Pinkston, J. D., Rabb, M., Watson, J. T., Allison J., Rev. Sci. Instrum. 1986, 57, 583.

18. Beynon, J. H., Harris, F. M., Green, B. N., Bateman, R. H., Org. Mass Spectrom. 1982, 17, 55.

19. March, R. E., Todd, J. F. J., (eds). Practical Aspects of Ion Trap Mass Spectrometry, 1995, vols 1, 2 and 3. Eds., CRC Press: Boca Raton, FL.

20. Cody, R. B., Freiser, B. S., Int. J. Mass Spectrom. Ion Phys. 1982, 41, 199.

21. McIver, R. T. J., Hunter, R. L., Bowen, W. D., Int. J. Mass Spectrom. Ion Processes 1985, 64, 67.

22. Hoffmann, E. d., Stroobant, V., Mass spectrometry : principles and applications; 3rd ed.; J. Wiley: Chichester, West Sussex, England ; Hoboken, NJ, 2007.

23. Chapman, J. D., Goodlett, D. R., Masselon, C. D., Mass Spectrometry Reviews 2014, 33, 452–470.

24. Williams, E. R., Loh, S. Y., McLafferty, F. W., Cody, R. B., Anal. Chem. 1990, 62, 698-703.

25. Pfandler, P., Bodenhausen, G., Rapin, J., Walser, M.E., Gaumann, T. J. Am. Chem. Soc. 1988, 110, 5625-5628.

26. Ross, C. W. III., Guan, S., Grosshans, P. B., Ricca, T. L., Marshall, A. G., J. Am.

Chem. Soc. 1993, 115, 7854-7861.

27. Li, L., Masselon, C. D., Anderson, G. A., Pasˇa-Tolic´, L., Lee, S. W., Shen, Y., Zhao, R., Lipton, M. S., Conrads, T. P., Tolic´, N., Smith, R. D., Anal. Chem. 2001, 73, 3312-3322.

28. Masselon, C., Anderson, G. A., Harkewicz, R., Bruce, J. E., Pasa-Tolic, L., Smith, R. D., Analytical Chemistry 2000, 72 (8), 1918-1924.

29. Wilson, J., Vachet, R. W., Analytical Chemistry 2004, 76 (24), 7346-7353.

30. Bushey, J. M., Danell, R. M., Glish, G. L., Analytical Chemistry. 2009, 81 (14), 5623-5628.

31. Ledvina, A. R., Savitski, M. M., Zubarev, A. R., Good, D. M., Coon, J. J., Zubarev, R. A., Anal Chem 2011, 83, 7651–7656.

32. Ducret, A., Van Oostveen, I., Eng, J. K., Yates, J. R., Aebersold, R. Protein Sci. 1998, 7, 706-719.

33. Yates, J. R., McCormack, A. L., Eng, J. Anal. Chem. 1996, 68, 534A-540A.

34. McCormack, A. L., Schieltz, D. M., Goode, B., Yang, S., Barnes, G., Drubin, D., Yates, J. R., Anal. Chem. 1997, 69, 767-776.

35. Goodlett, D. R., Wahl, J. H., Udseth, H. R., Smith, R. D., J. Microcolumn Sep. 1993, 5, 57-62.

36. Moore, R. E., Licklider, L., Schumann, D., Lee, T. D., Anal. Chem. 1998, 70, 4879-4884.

37. Pasˇa-Tolic´, L., Lipton, M. S., Masselon, C. D., Anderson, G. A. et al., J. Mass Spec. 2002, 37, 1185–1198.

38. Wang, N., Li, L., Anal Chem, 2008, 80, 4696–4710.

39. Kondrat, R. W., Cooks, R. G., Anal. Chem. 1987, A81, 50.

40. Kondrat, R. W., Cooks, R. G., McLaughlin, J. L., Science, 1978, 199, 978.

41. Cech, N. B., Enke, C. G., Anal. Chem. 2000, 72, 2717-2723.

42. Sjo¨berg, P. J. R., Bo¨kman, C. F., Bylund, D., Markides, K. E., Anal. Chem. 2001, 73, 23-28.

CHAPTER VI

MULTIPLEXED TANDEM MASS SPECTROMETRY BY MODULATION OF IONIZATION EFFICIENCY

6.1 Introduction

The ESI solution solvent influences the ionization efficiency of analytes. The ionization efficiency of a molecule is determined by the ease of its desorption from the droplet to the gas phase. Different compounds have different ionization efficiencies in ESI [3] and desolvated ions in gas phase are formed to different extents as explained earlier in Chapter 4 [4-6]. Ionization efficiency depends on the analyte identity, solvent properties and electrospray conditions [7,8]. Amad et al. demonstrated that solvents with higher proton affinities in the gas phase decrease the ionization of analytes [9]. Less polar solvents increase the intensity of the compounds according to their surface activity [5,10]. Higher solvent polarity increases the partitioning and more organic solvent content increases the ionization efficiency and therefore the response of the ions. Other electrospray conditions such as position of the sprayer can also affect ionization efficiency of the analyte ions [1,11].

In this novel method of multiplexing MS/MS, encoding the fragments to their corresponding precursor ions was implemented through modulating ionization efficiencies, and was performed by measuring the analytes' responses at high and low ionization efficiencies. Modulation of ionization efficiencies of ions can be accomplished by changing various ESI parameters, such as solvent composition, sprayer position, capillary voltage and the nebulizing gas, etc. Here two methods to

achieve modulation of ionization efficiencies are demonstrated:

a) Solvent composition:

The relative intensities of molecules were changed by changing the percentage of organic solvent (methanol) in the spray solvent. The intensity ratios of molecules at high (80%) versus low (20%) methanol concentration were calculated by dividing the intensities of the ions at 80% MeOH by their intensities at 20% MeOH. All the molecules were fragmented and the intensity ratios were measured again. The fragments intensities tend to increase and decrease to the same extent as the precursors. This property can be used to relate fragments to their respective precursors, since fragments originating from the same precursor increases when the signal for that precursor increases (Figure 6.1).

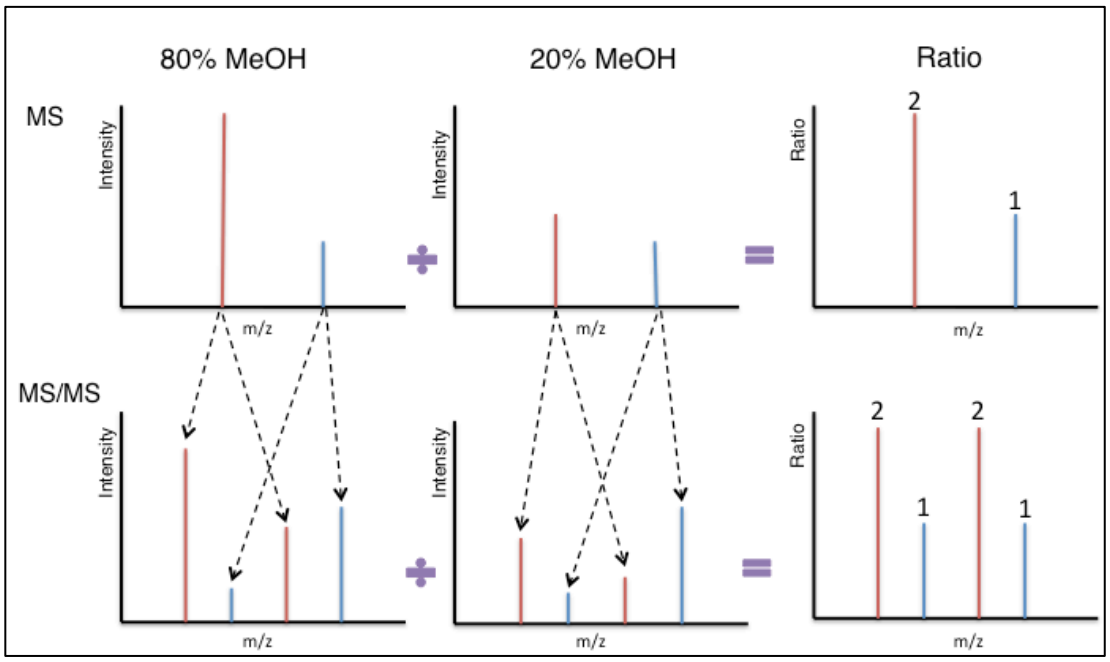


Figure 6.1. Multiplexed MS/MS by modulation of ionization efficiency scheme.

b) Sprayer position:

Modulation of ionization efficiency was also achieved by changing the sprayer position relative to the sampling cone. Changing the sprayer position relative to the inlet of the mass spectrometer was performed while keeping all other conditions the same.

Recognition of two isobaric compounds is also discussed in this chapter as another potentially useful application of the differences in ionization efficiencies of the molecules. The different intensity ratios of the fragments upon solvent modulation could distinguish between two compounds with one m/z value.

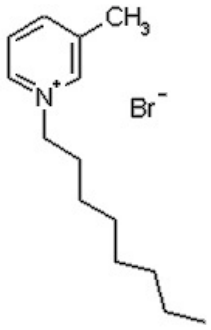
6.2 Experimental Methods

6.2.1 Molecular Probes Used to Study Modulation of Ionization Efficiencies

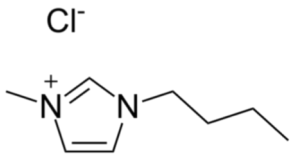
Ionic liquid cations usually consist of an organic ring cation with a carbon chain group attached, as described in Chapter 1. Different lengths of these attached carbon chains cause the cations to have different surface activities, making them a good choice for investigating the novel multiplexing method in this study. Also, one of the most common applications of the multiplexed tandem MS is identification of peptides for proteomics studies. Therefore, in this study, two ionic liquids and two peptides with different surface activities were used for the proof of principle demonstration of the method. The test compounds with their m/z values are shown in the Table 6.1.

Table 6.1. The ionic liquid test compounds used in this study.

Compound	m/z
Valylglycylglycine (Val-Gly-Gly)	232.17
Glycyl-Leucyl-Alanine (Gly-Leu-Ala)	260.17
1-octyl-3-methylpyridinium Bromide (OMPyr)	206.19
1-butyl-3-methylimidazolium chloride (BMIM)	139.12



OMPyr



BMIM

The 1-butyl-3-methylimidazolium chloride (BMIM) and 1-octyl-3-methylpyridinium Bromide (OMPyr) test compounds were purchased from IoLiTec (Tuscaloosa, AL). LC-MS grade methanol and the Valylglycylglycine (Val-Gly-Gly) and Glycyl-Leucyl-Alanine (Gly-Leu-Ala) peptides were purchased from Sigma Aldrich, (Kalamazoo, MI). 10 μM solution of BMIM, OMPyr, Val-Gly-Gly and Gly-Leu-Ala in 20 and 80 % MeOH with 0.1% formic acid were made from the stock solutions of 2mM BMIM, 4mM OMPyr, 4mM Val-Gly-Gly and 4mM Gly-Leu-Ala

in H_2O. The stock solutions were mixed and diluted to 10 μM.

6.2.2 Instrumental Setup

Mass spectrometry was performed in positive ion mode using the Thermo LTQ ion trap mass spectrometer with the following MS conditions: sheath gas flow rate 10 a/u, spray voltage 3.3 kV, capillary temperature 250 °C, capillary voltage 25 V and tube lens 65 V. The sample was infused with the flow rate of 5 μL/min. The sample was analyzed by collecting both full mass spectrum and the non-selective in-source fragmentation spectrum with a collision energy of 27 kV.

6.2.3 Methods

a) Modulation of solvent composition

In this work, a novel method of multiplexing MS/MS is implemented. By changing the solvent composition of the spray solution during ESI-MS analysis, the ionization efficiencies of analytes are modulated. Both a precursor and a fragmentation ion spectra are obtained, first in a solution that contains mostly water (20% MeOH) and then in a solution of mostly methanol (80% MeOH). The compounds with higher surface activities are present in the mass spectrum at higher intensities. When increasing the organic phase composition, the responses of all the compounds in the solution increase to different extents, depending on their relative surface activities. All the molecules are then fragmented together (in source fragmentation) and the response ratios are calculated again. Fragments are then correlated to precursors with the same calculated intensity ratio of consecutive high (80%) versus low (20%) organic mobile phase compositions. Matching the change in

signal associated with fragments and precursors allows for determination of the corresponding precursors.

b) Modulation of sprayer distance

Modulation of ionization efficiency was also achieved by changing the sprayer position relative to the sampling cone. The effect of the position of the sprayer relative to the inlet of the mass spectrometer on ionization efficiency of the analyte ions was investigated before [1]. Here, this effect was used as another possible implementation for multiplexed tandem MS. Changing the sprayer position relative to the inlet of the mass spectrometer was performed while keeping all other conditions the same.

All experiments were conducted with the 10 μM solution of the mixture of the compounds explained above in 6.2.1 in 80% MeOH. The sprayer position was changed in 5 millimeter increments to obtain 5 vertically different positions relative to the inlet, and at 5 horizontally different positions relative to the inlet that differ by 2 millimeter. The ratios between positions were then obtained by dividing the highest intensities of the ions by their lowest intensities.

6.3 Results and Discussion

6.3.1 Modulation by Solvent Composition

OMPyr, which has a long nonpolar carbon chain, is the most surface-active molecule and shows the highest response in the mass spectrum. Surface activities of the analytes are modulated by changing the methanol content of the spray solution. Methanol was used in this study because, compared to acetonitrile it is better able to

enhance the differential surface activity of the ions [2]. This changes the intensity of relative response of the test compounds differently at 20 and 80% MeOH. For example, as shown in Figure 6.2, the response of the BMIM changes from being almost 30% of the base peak of OMPyr at 20% MeOH, to about 20% of the base peak at 80% MeOH, indicating that the response of OMPyr increased relative to that of BMIM. Similarly, the relative responses of the peptides also decrease relative to OMPyr and to each other at low and high methanol compositions.

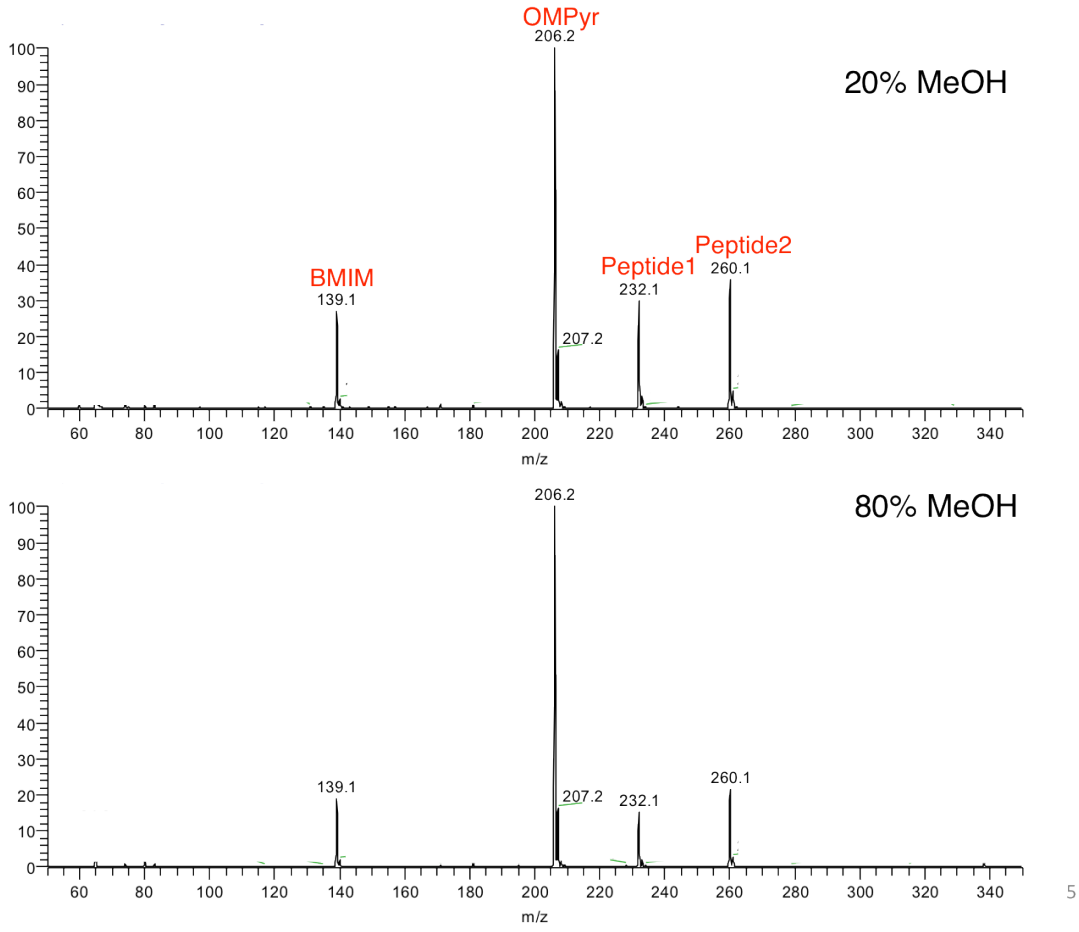


Figure 6.2. Changing relative response of test compounds at 20 and 80% MeOH.

Dividing the intensity of each investigated compound at 80% MeOH by its intensity at 20% MeOH yields a specific ratio. When the precursors are fragmented, the lower concentration of precursor results in decreased fragment intensity. These ratios are well preserved between precursors and fragments, which enables linking the fragments to their respective precursors as shown in Table 6.2. BMIM at m/z 139 produces a major fragment at m/z 83. A ratio of ~7 was obtained for BMIM, which has a C_4 alkyl chain, while a ratio of 7.7 was obtained for the major fragment. This is clearly different than OMpyr, which has 8 carbon atoms in the alkyl chain and a ratio of ~10 was obtained for the precursor at 206, while the ratio for the major fragment was 11. Similarly for the VAL-GLY-GLY peptide at m/z 232 and its two major fragments a ratio ~5 was obtained, while the GLY-LEU-ALA peptide at m/z 260 and its two major fragments have a ratio of ~ 6 or 7.

Table 6.2. The intensity ratios of the test compounds between high (80%) versus low (20%) methanol concentration.

	BMIM		OMPyr		VAL-GLY-GLY		GLY-LEU-ALA	
	m/z	ratio	m/z	ratio	m/z	ratio	m/z	ratio
Precursor	139	6.9	206	9.7	232	5.0	260	5.8
Fragment 1	83	7.7	94	10.9	187	5.1	171	7.1
Fragment 2					157	5.4	143	6.6

6.3.2 Modulation by Sprayer Distance

Less surface active ions change to gas phase ions more slowly which means that, at the end of the ion production zone, they will become predominant at the droplet surface. Therefore, there will be an optimum distance between the sprayer and the inlet for the maximum response of each ion. At lower concentrations, any change in the sprayer position changes the relative intensity and relative intensities of less surface active ions increase by increasing the distance.

The equimolar solution of the mixture of mentioned compounds in 80% MeOH was analyzed at near and far sprayer positions. The ratios were then obtained by dividing the highest intensities of the ions (near sprayer) by the lowest intensities (far sprayer). The obtained preliminary data is shown in Table 6.3. The ratio for BMIM was ~18, while a ratio of 17 was obtained for the major fragment. This is differentiated from OMpyr, for which a ratio of ~20 was obtained for the precursor and 15 for the major fragment. Similarly for VAL-GLY-GLY peptide at m/z 232 the ratio was 20.4 and for it's two major fragments the ratio was obtained ~19 which is differentiated from GLY-LEU-ALA peptide at m/z 260 and it's two major fragments with ratios higher than 22.

The results for these experiments that altered sprayer position were not as clear as the results from modulating the solvent composition. While the ratios obtained for precursor and the fragments are different for each molecule, some fragments produced ratios that were different from their precursors.

Table 6.3. The intensity ratios of the test compounds between near and far sprayer.

	BMIM		OMPyr		VAL-GLY-GLY		GLY-LEU-ALA	
	m/z	ratio	m/z	ratio	m/z	ratio	m/z	ratio
Precursor	139	17.9	206	19.7	232	20.4	260	23.6
Fragment 1	83	16.8	94	15.1	187	18.8	171	27.5
Fragment 2					157	18.7	143	22.2

6.4 Recognition of Two Isobaric Compounds

Another possibly useful application of the differences in ionization efficiencies of the molecules could be for the differentiation of two isobaric compounds. For two compounds with the same m/z value in the mixture, different intensity ratios of the fragments upon solvent modulation could indicate that two compounds with one m/z value exist in the mixture. Two sets of isobaric compounds were investigated in this study, 1,7-dimethylxanthine and theobromine, which have a m/z value of 181, and two α-hop acids with m/z values of 347 and 361 and their isobaric iso-α-acid isomers.

6.4.1 Chemicals and Experimental Conditions

α- and iso-α-hop acid isomers standards were obtained from Kalsec, Kalamazoo, MI. 10 μM solutions of α- and iso-α-hop acid isomers standards in 20 and 80 % MeOH were made. Mass spectrometry was performed in negative ion mode using the Thermo LTQ ion trap mass spectrometer with the following MS conditions: spray voltage 4.1 kV, capillary temperature 255 °C, capillary voltage -20 V and tube lens -90 V. The sample was infused with the flow rate of 5 μL/min. The sample was

analyzed in both full mass spectrum and non-selective in-source fragmentation spectrum with collision energy of 22 kV.

Theobromine and 1,7-dimethylxanhine isobaric compounds were purchased from Sigma Aldrich (Kalamazoo, MI). A 10 μM solution of theobromine and 1,7-dimethylxanhine in 20 and 80 % MeOH were made from the stock solutions of 600 μM in H_2O with 5% formic acid. The stock solutions were then mixed and diluted to 10 μM. Mass spectrometry was performed in positive ion mode using the Thermo LTQ ion trap mass spectrometer with the following MS conditions: spray voltage 4.2 kV, capillary temperature 250 °C, capillary voltage 25 V and tube lens 65 V. The sample was infused with a flow rate of 5 μL/min. The sample was analyzed in both full mass spectrum and non-selective in-source fragmentation spectrum with collision energy of 35 kV.

6.4.2 Results and Discussion

As shown in Figure 6.3, the fragmentation of 1,7-dimethylxanthine yields a main fragment with m/z value of 124. The main fragment obtained by fragmentation of theobromine has a m/z value of 163. The intensity ratios of these main fragments at high versus low methanol concentration were similar to their own corresponding precursors (Table 6.4). The Observation of two different ratios for obtained fragments does indicate the presence of two different isobaric precursor ions (1,7-Dimethylxanthine and theobromine).

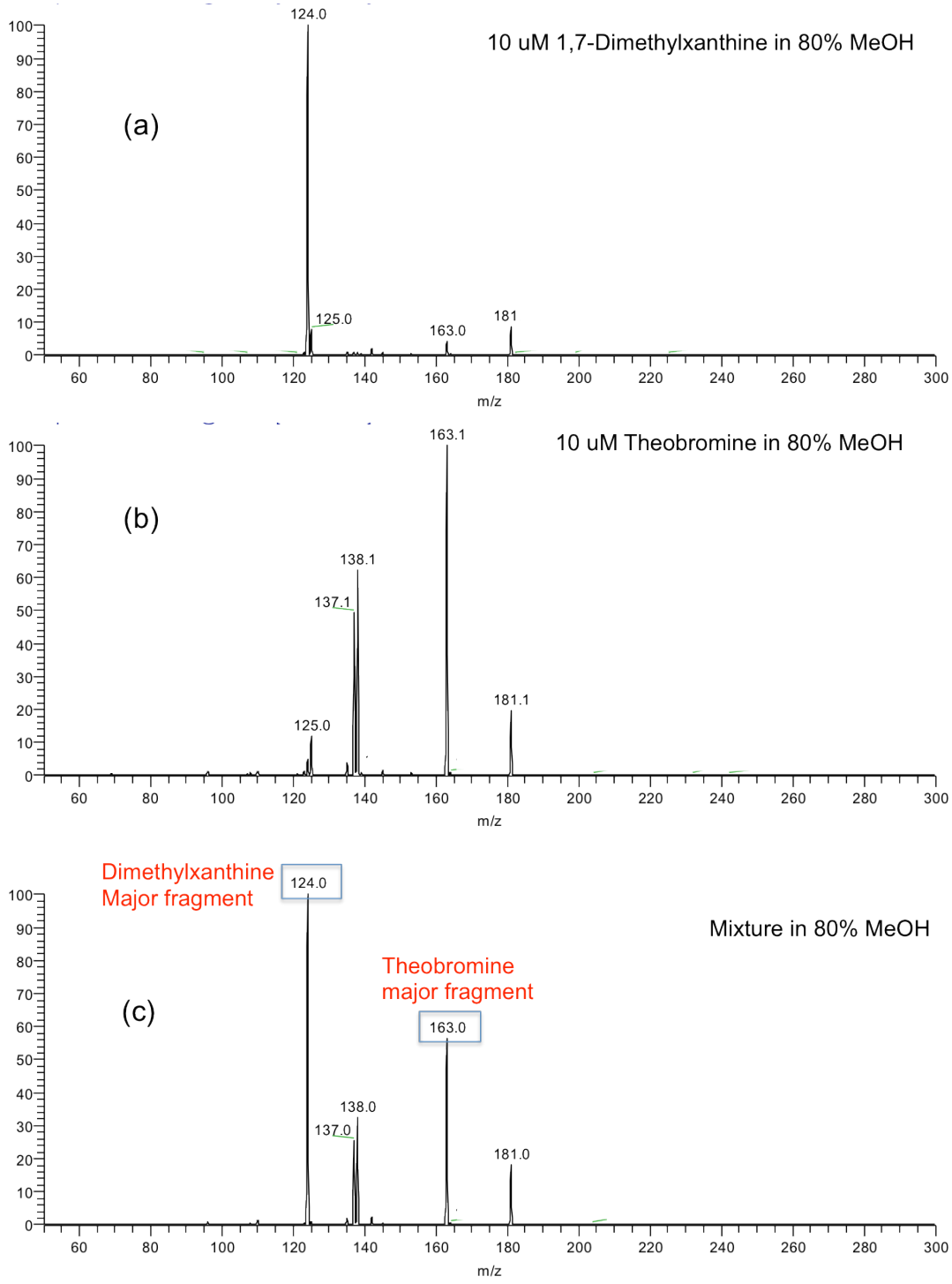


Figure 6.3. (a) 1,7-Dimethylxanthine fragments in 80% MeOH, (b) theobromine fragments in 80% MeOH and (c) fragments of the mixture of 1,7-dimethylxanthine and theobromine in 80% MeOH.

Table 6.4. The intensity ratios of the main fragments of isobaric compounds of 1,7-Dimethylxanthine and theobromine.

m/z	ratio	m/z	ratio
181 (1,7- Dimethylxanthine)	2.5	181 (Theobromine)	1.4
124 (main fragment)	3.3	163 (main fragment)	1.4

Each α- and iso-α-hop acids have two isomers with m/z values of 347 and 361. As shown in Figure 6.4, fragmentation of the m/z 347 alpha acid isomer yields a main fragment with m/z value of 278. The main fragment obtained by fragmentation of the m/z 347 iso-alpha acid isomer has a m/z value of 251. The intensity ratio of the fragments at high versus low methanol concentration showed a similar value to their own corresponding precursor (Table 6.5). Observation of two different ratios for the obtained fragments can indicate the presence of two different isobaric precursor ions.

However, when α- and iso-α-hop acids were analysed in a mixture (Figure 6.5), the ratios obtained were not different enough to differentiate between the two isobaric compounds (Table 6.5). Although we could not determine the reason of this, a few potential reasons are hypothesized which need to be investigated as future work. This can be due to the existence of common fragments between two isobaric compounds. For example, the m/z 278, which is the major fragment of m/z of 347 alpha acid isomer, is also one of the minor fragments of m/z 347 iso-alpha acid isomer and m/z 292, which is the major fragment of m/z 347 alpha acid isomer, is also one of the minor fragments of m/z 347 iso-alpha acid isomer. However it's not

likely, since the intensities of minor fragments are less than 10% of the major ones and can not have significant effect on the ratios. A second possible reason could be that the total concentration of analytes in the mixture were kept at 10μM each in the prepared mixtures, which could result in the presence of too many ions and saturation of the ESI detector. Another possible reason could be that the difference between the surface activities of the tested compounds is not enough to differentiate between the ratios and more different surface active compounds need to be investigated.

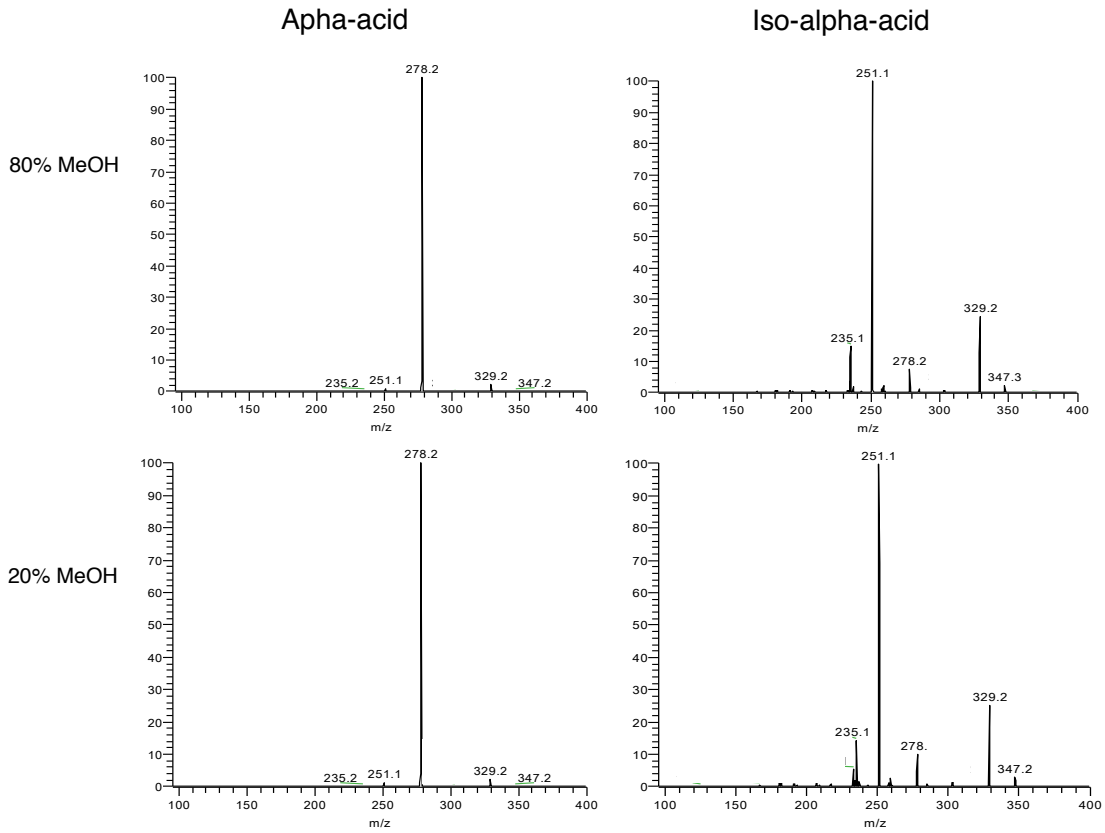


Figure 6.4. Fragments of m/z 347 α and iso-α acid isomers in 80 and 20% MeOH.

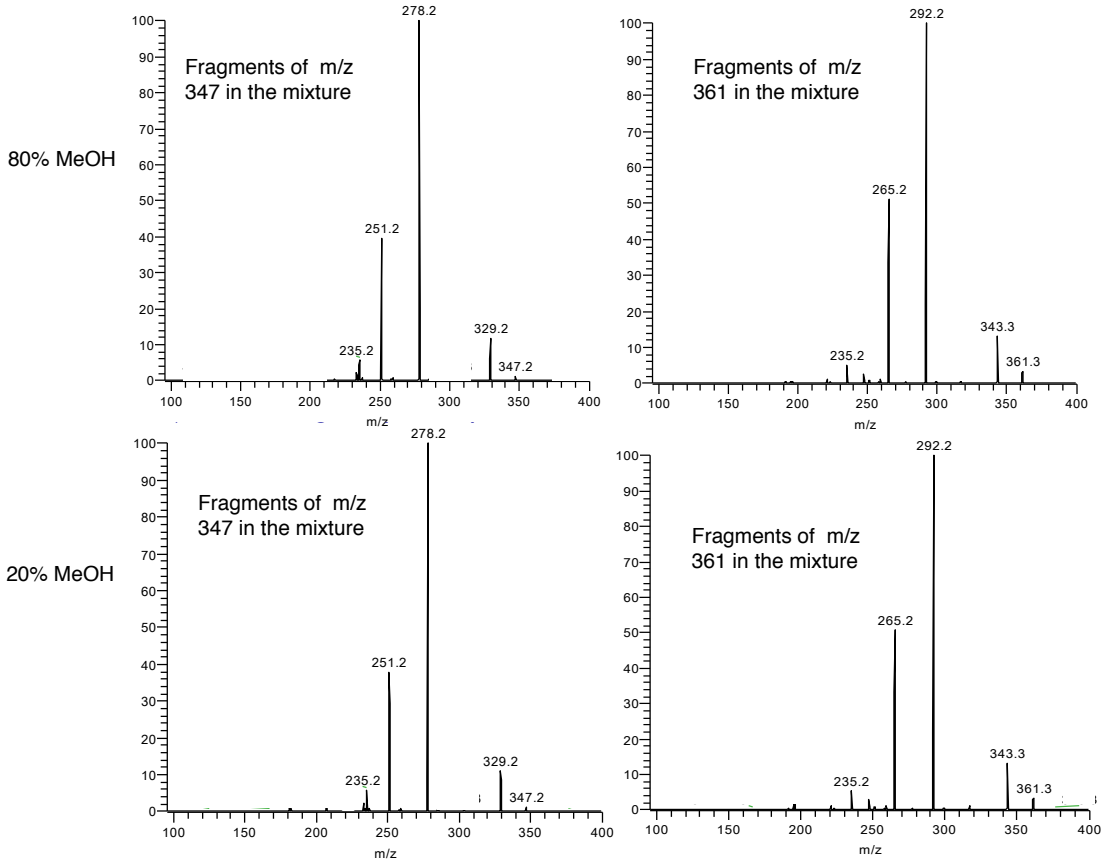


Figure 6.5. Fragments of mixture of m/z 347 and m/z 361 α and iso-α acid isomers in 80 and 20% MeOH.

Table 6.5. The intensity ratios of the fragments of isobaric compounds of m/z 347 α and iso-α hop acid isomers.

	Alpha-acid		Iso-alpha-acid		Mixture	
	m/z	ratio	m/z	ratio	m/z	ratio
Precursor	347	1.58	347	9.81	347	1.09
Fragment 1	235	1.9	235	9.13	235	1.79
Fragment 2	251	1.8	251*	8.67	251	1.82
Fragment 3	278*	1.90	278	6.7	278	1.74
Fragment 4	329	1.88	329	8.5	329	1.84

Fragmentation of the m/z 361 alpha acid isomer yields a main fragment with a m/z value of 292. The main fragment obtained by fragmentation of the m/z 361 iso-alpha acid isomer has a m/z value of 265 (Figure 6.6). The intensity ratio of the fragments at high versus low methanol concentration showed a similar value to their own corresponding precursor (Table 6.6). However, when α- and iso-α-hop acids were analysed in a mixture (Figure 6.5), the ratios obtained were not different enough to differentiate between the two isobaric compounds (Table 6.6), due to the same reasons mentioned above.

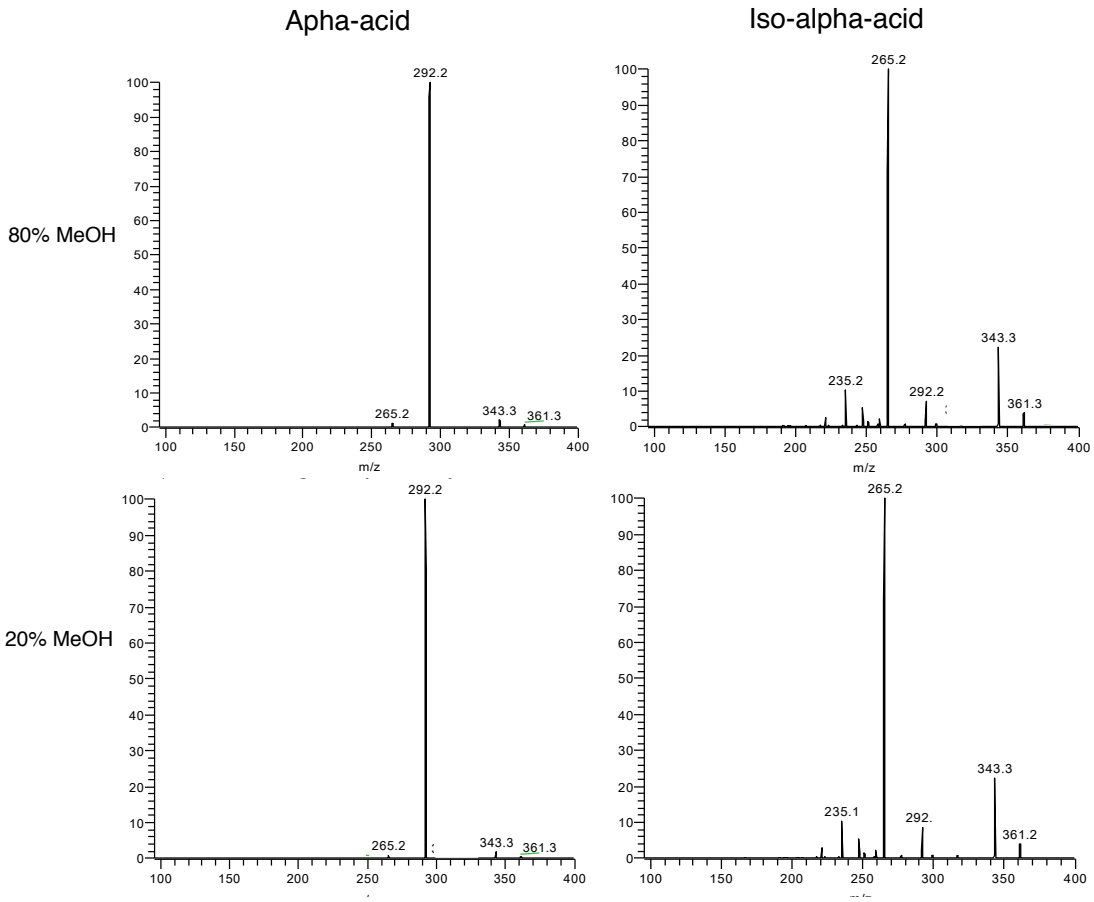


Figure 6.6. Fragments of m/z 361 α and iso-α acid isomers in 80 and 20% MeOH.

Table 6.6. The intensity ratios of the fragments of isobaric compounds of m/z 361 α and iso-α hop acid isomers.

	Alpha-acid		Iso-alpha-acid		Mixture	
	m/z	ratio	m/z	ratio	m/z	ratio
Precursor	361	1.6	361	10.0	361	1.2
Fragment 1	235	4.2	235	6.4	235	2.5
Fragment 2	265	4.7	265*	6.3	265	2.6
Fragment 3	292*	3.3	292	5.3	292	2.6
Fragment 4	343	3.4	343	6.3	343	2.6

6.5 Chapter Conclusion

This investigation demonstrated that modulation of ionization efficiency of ions by changing various ESI parameters, such as organic solvent composition or sprayer position, is a possible option for encoding the fragments to their corresponding precursor ions in multiplexed tandem mass spectrometry. While differences in ionization efficiencies of the molecules have been studied for many years [42,48], this is the first time that it's been applied for a practically useful purpose.

The obtained intensity ratios were well preserved between the fragments and precursors, and each of the analyte ions showed a specific ratio in this study. The results obtained by modulating solvent composition were more similar between the fragments and precursors than the results obtained by changing the sprayer position. These results provide preliminary data for developing a new method of multiplexing mass spectrometry, which is performable on inexpensive, simpler, more accessible

single stage mass analyzers.

The proposed method has some advantages compared to the previous multiplexed tandem mass spectrometry methods. The sample throughput is increased using non-selective in-source fragmentation of all the ions, the data points across the chromatographic peaks are increased due to scanning of both full and fragmentation mass spectrum and it empowers single MS analyzers to do tandem experiments which are less expensive and more accessible. However, implementation of this method has some limitations such as: The identification of precursor ions with similar fragments or identification of molecules in mixtures that have equal responses to changes of ionization efficiency.

Further investigation is needed to develop this into a practical technique, such as determining the minimum required surface activity difference between mixtures of analyte ions, and developing a spray interface able to modulate the solvent composition in real-time.

6.6 References

1. Janusson, E., Hesketh, A. V., Bamford, K. L., Hatlelid, K., Higgins, R., McIndoe, J. S., International Journal of Mass Spectrometry 2015, 388, 1–8.
2. Pape, J., Vikse, K. L., Janusson, E., Taylor, N., McIndoe, J. S., International Journal of Mass Spectrometry 2014, 373, 66–71.
3. Leito, I., Herodes, K., Huopolainen, M., Virro, K., Künnapas, A., Kruve, A., Rapid Commun. Mass Spectrom. 2008, 22, 379–384.
4. Cech, N. B., Enke, C. G., Anal. Chem. 2000, 72, 2717-2723.
5. Cech, N. B., Krone, J. P., Enke, C. G., Anal. Chem. 2001, 73, 208–213.

6. Oss, M., Kruve, A., Herodes, K., Leito, I., Anal. Chem. 2010, 82, 2865–2872 .

7. Yang, X. Y., Qu, Y., Yuan, Q., Wan, P., Du, Z., Chen, D., Wong, C., Analyst 2012, 138, 659–665.

8. Huffman, B. A., Poltash, M. L., Hughey, C. A., Anal. Chem. 2012, 84, 9942–950.

9. Amad, M.a.H., Cech, N.B., Jackson, G.S., Enke, C.G., J. Mass Spectrom. 2000, 784–789.

10. Sjoberg, P. J. R., Bokman, C. F., Bylund, D., Markides, K. E., Anal. Chem. 2001, 73, 23-28.

11. Sjo¨berg, P. J. R., Bo¨kman, C. F., Bylund, D., Markides, K. E., J. Am. Soc. Mass Spectrom. 2001, 12, 1002-1010.

CHAPTER VII

CONCLUSION

In this thesis, electrospray ionization mass spectrometry was studied and two main topics were presented. Firstly, ESI was used in the identification of biodegradation products of ionic liquids, after chromatographic separation. Secondly preliminary data for a novel multiplexed tandem ESI-MS method by modulation of ionization efficiency was demonstrated for the analysis of ionic liquids and other compounds.

7.1 Identification of Ionic Liquids Biodegradation Products

In Chapter 2 and 3 of this thesis, the theory, experimental methods and results for the identification of biodegradation products of ionic liquids using ESI-MS is discussed. Unique physico-chemical properties of ionic liquids make them potential green alternatives to volatile organic solvents, however studies have demonstrated that they can have adverse toxic effects on the environment. The metabolites produced during biodegradation of these compounds can be more or less toxic than their parent compounds in the environment. Therefore, identification of the metabolites and the biodegradation pathway is important. Imidazolium salts are an excellent solvent for a broad range of organic and inorganic materials and high quantities of BMIM are used in industrial applications, despite its persistence and toxicity in the environment. Imidazolium ionic liquid cation contains a strong UV absorption group. Previous studies on pyridinium-based ILs suggest that after

cleavage of the ring, biodegradation occurs rapidly and breakdown products can be incorporated into microbial biomass or released as gas. Very low concentrations of these breakdown products lead to low signal-to-noise ratios, which prevent their ready observation in the total ion chromatogram obtained by ESI. In this study, HPLC-UV-MS was used to identify the metabolites in which the imidazolium ring is intact. This was implemented by simultaneously splitting the HPLC flow equally to both UV and MS detectors.

In this work, the biodegradation resistant BMIM cation was completely degraded by the specially selected activated sludge microbial community. Microbial communities used in previous studies failed to degrade this ionic liquid. This indicates that different microbial communities may be capable of biodegrading different ionic liquids. Four major metabolites were identified for BMIM. Further evidence of their validity as biodegradation products were provided by following their production and degradation over the time-period of the microbial treatment. While we could not identify a particular sequence in their formation, it appears that the observed products represent different biodegradation pathways. Supporting evidence for the proposed structures of the identified metabolites were obtained by using tandem mass spectrometry and NMR spectroscopy. The main metabolite was produced by loss of the butyl side chain from the parent compound of BMIM. This process would make it less lipophilic and potentially less toxic to microorganisms. The minor biodegradation products from competing pathways were resistant to further degradation. As future work, the toxicity of these metabolites should be investigated. Investigating the toxicity of the metabolites can determine if using

microbial communities to biodegrade ionic liquids can be helpful in their disposal.

7.2 Multiplexed Tandem Mass Spectrometry By Modulation Of Ionization Efficiency

In Chapters 4, 5 and 6 of this thesis, the necessary theories for understanding our novel method of multiplexing tandem mass spectrometry and the implemented experiments and their results are discussed. ESI-MS generally provides information about the intact molecular weight of molecules, which does not provide enough information to determine the structure of an unknown analyte to allow for unambiguous identification. Tandem mass spectrometry is a technique to elucidate structure and identity of unknown molecules, but it is time-consuming and high throughput of MS/MS experiments are required for complex mixtures. Multiplexing tandem MS is a potential solution to increase the MS/MS experiment throughput and signal-to-noise ratio, but most previously developed multiplexed tandem mass spectrometry methods were performed on expensive high accuracy mass analyzers. Here we proposed a novel method of multiplexing MS/MS, in which correlation of the fragments to the corresponding precursors is performed by modulating the ionization efficiencies of analytes by changing various ESI parameters, such as organic solvent composition or sprayer position. To the best of our knowledge, this is the first time that differences in ionization efficiencies of the molecules are applied for a practically useful purpose.

The ESI spray solvent has an influence on ionization efficiency of the analytes. Higher solvent polarity (more water content) increases the partitioning. Molecules

with more nonpolar characteristics have more affinity for the surface of the ESI droplet (more surface-active molecules) and therefore they have higher response in the mass spectrum. Surface activities of the molecules can be manipulated by changing the organic content of the electrospray solution. The intensity of the compounds with different surface activities responds to a different extent to the change of organic solvent composition. The intensity ratios of the precursor and fragment ions, at high versus low methanol content can be used to correlate the fragments to precursors. The obtained intensity ratios in this study were well preserved between the fragments and the precursors, and each of the analyte ions showed a specific ratio in this study. In addition to modulating the solvent, other ways to modulate ionization efficiencies could potentially also lead to the decoding of unselected precursors to their fragments. For example we demonstrated the use of changing the sprayer position. The results obtained by modulation of solvent composition were more similar between the fragments and precursors than the results obtained by changing the sprayer position.

The proposed method could have some advantages compared to the previous multiplexed tandem mass spectrometry methods. The sample throughput could be increased using non-selective in-source fragmentation of all the ions, the data points across the chromatographic peaks could be increased due to scanning of both full and fragmentation mass spectra. Most importantly, it will empower inexpensive, simple single MS analyzers to do tandem experiments.

However, implementation of this method has some limitations such as: The identification of precursor ions with similar fragments or the identification of

molecules in mixtures that have equal responses to changes of ionization efficiency.

The results obtained in this study provide preliminary data for development of a new method of multiplexing mass spectrometry as future work.

This method has some limitations and more investigation is needed as future work in different aspects such as the minimum required surface activity difference between the analyte ions.