Liquid Extraction Based Surface Sampling: Liquid Microjunction Surface Sampling Probes Coupled with Mass Spectrometry

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Matthew John Walworth
August 2011

Copyright © 2011 by Matthew John Walworth.

All rights reserved.

This work is dedicated to my best friend and only brother Patrick K Walworth who passed away October 24, 2004 after a motorcycle accident. There have been times during my graduate career that I have thought the following: *this is not me, but him, and he is dreaming.*

ACKNOWLEDGEMENTS

I would like to first thank Professor Michael J Sepaniak for being more than a research advisor to me. You are a great scientist and mentor. It has been my honor to know you and have the opportunity to work under you the last few years. I would like to thank Dr. Gary J Van Berkel for his support and the opportunity to work in the Organic and Biological Mass Spectrometry (OBMS) group at Oak Ridge National Lab. The lessons you have taught me have molded me into a better scientist.

I would like to generally thank the numerous Sepaniak group and OBMS members I have worked with over the years. I cannot mention everyone but I would like to acknowledge three important colleagues. I thank Maggie Connatser for her guidance early in my research career. I could not have made it through graduate school without Kasey Hill. Kasey you define what a true friend is. Lastly Olga Ovchinnikova has been a great friend and excellent teammate during my time at ORNL. Thank you for all you have done for me Olga.

I would like to thank my family for their love and support throughout the last seven years. My wife Anne has been my rock and my son Dillon my inspiration. I thank my parents for always supporting and believing in me. I would also like to thank my wife's parents for their support the last few years.

ABSTRACT

The direct sampling of analytes from surfaces under atmospheric conditions followed by mass spectrometric analysis is an ever expanding area of scientific research. Atmospheric pressure surface sampling and ionization techniques for mass spectrometry (MS) offer the ability to interrogate samples that could not be studied under vacuum conditions required of more traditional MS surface analysis techniques. The geometry and nature of materials or surfaces that can be analyzed has been greatly expanded as a result. This dissertation characterizes and shows applications of liquid microjunction surface sampling probe (LMJ-SSP) electrospray ionization systems. The presented work compares traditional analytical work flows with novel analytical workflows utilizing LMJ-SSP-MS technology. The increase of throughput and/or chemical information without the sacrifice of analytical figures of merit is shown and discussed. The readout of analytical surfaces; surfaces where analyte has ended up on a surface in a traditional work flow and not just placed there, constitutes the focus of what is presented in the preceding work. Finally the prospects for spatial liquid chromatography-mass spectrometry (LC-MS) as a powerful analytical technology 'in wait' is discussed and supported by the presented data.

TABLE OF CONTENTS

Chapter Page

1. Introduction.....
   1.1. Atmospheric Pressure Surface Sampling and Ionization Techniques.....1
   1.2. Liquid Microjunction Surface Sampling Probe: Modes of Operation.....10
   1.3. Sealing Surface Sampling Probe: Modes of Operation.....17
   1.4. Factors to consider when employing surface sampling mass spectrometry.....19
   1.5. Defining 'Analytical Surfaces'.....20
   1.6. Spatial Chromatography Coupled with Mass Spectrometry: The Next 'Technology' Holy Grail.....22
   1.7. Dissertation overview.....30
2. Extraction, separation, and fluorometric analysis of selected environmental contaminants...29
   2.1. Introduction.....29
   2.2. Experimental.....32
      2.2.1. Materials and Reagents.....32
      2.2.2. Apparatus.....34
      2.2.3. Methods.....35
   2.3. Results and Discussion.....37
      2.3.1. Characterization of High Performance Extraction Disc Cartridge.....37
      2.3.2. Real Sample Matrices Analysis using hydrodynamic-LIF analysis platform.....42
      2.3.3. Reproducibility and Solvent Study.....45
      2.3.4. Analyte Mixture Separation using CD-modified/MEKC Platform.....46
      2.3.5. HPEDC Extract analysis of mixtures using CD-modified/MEKC/LIF.....50
   2.4. Conclusions.....51
3. Direct Sampling and Analysis from Solid Phase Extraction Cards using an Automated Liquid Extraction Surface Analysis Nanoelectrospray Mass Spectrometry System.....52
   3.1. Introduction.....52
   3.2. Experimental.....54

3.2.1. Materials and Reagents 54
3.2.2. Multi-Well Solid Phase Extraction Card 54
3.2.3. Automated Liquid Extraction Surface Analysis 57
3.3. Results and Discussion 59
3.3.1. Solid Phase Extraction Card. 59
3.3.2. Extraction Optimization and Quantitation of Linearity and Reproducibility 59
3.3.3. Combined Capture and Extraction Efficiency 65
3.3.4. Application to Peptides 69
3.3.5. Application to Herbicides 71
3.4. Conclusions 74
4. Hydrophobic Treatment Enabling Analysis of Wettable Surfaces using a Liquid Microjunction Surface Sampling Probe/Electrospray Ionization-Mass Spectrometry System 76
4.1. Introduction 76
4.2. Experimental 78
4.2.1. Materials and Reagents 78
4.2.2. Tryptic Digest 79
4.2.3. Thin-layer Chromatography 79
4.2.4. LMJ-SSP/ESI-MS 81
4.3. Results and Discussion 83
4.3.1. Surface Treatment and Analysis Concept 83
4.3.2. Goldenseal Alkaloids on Silica Gel 60 HPTLC Plates 88
4.3.3. Tryptic Peptides on ProtoChrom® HPTLC Silica gel 60 Plates 94
4.4. Conclusions 99
5. Direct Analysis of Reversed-Phase HPTLC Separated Peptides from Protein Tryptic Digests using a Surface Sampling Probe/ESI-MS System. 100
5.1. Introduction 105
5.2. Experimental 107
5.2.1. Materials and Reagents 107
5.2.2. Tryptic Digestion 108
5.2.3. Thin-layer Chromatography 108
5.2.4. Mass Spectrometry 108
5.3. Results and Discussion 113

5.3.1. Overview of results 113
5.3.2. Cytochrome c. detailed analysis 119
5.3.3. Myoglobin detailed analysis 124
5.3.4. Detailed Analysis of BSA, Beta-Casein and Lysozyme 130
5.4. Conclusions 131
6. Spatial LC-MS: HPTLC- Surface Sampling Analysis of Intact Proteins 133
6.1. Introduction 133
6.2. Experimental 133
6.2.1. Materials and Reagents 135
6.2.2. Thin-layer Chromatography 136
6.2.3. Sealing Surface Sampling Probe Analysis 138
6.3. Results and Discussion 140
6.4. Conclusions 147
7. Total Ion Mapping Scan Function for Direct Infusion and Spatial LC-MS: Chromatographic Peak Parking Study 148
7.1. Introduction 148
7.2. Experimental 151
7.2.1. Materials and Reagents 151
7.2.2. Tryptic Digest 151
7.2.3. Thin-layer Chromatography 152
7.2.4. HPLC/ESI-MS 153
7.2.5. LMJ-SSP/ESI-MS 153
7.2.6. Automated Liquid Extraction Surface Analysis 155
7.2.7. Database Searching 155
7.3. Results and Discussion 175
7.3.1. Column based LC-MS and Direct Infusion Analysis of Seven Protein Digest 157
7.3.2. Spatial LC-MS Analysis of Seven Protein Digest 162
7.3.3. Spatial LC-MS of a Proteome 169
7.4. Conclusions 175
8. Conclusions and Recommendations 176
9. LIST OF REFERENCES 180

10. VITA.....201

LIST OF TABLES

Table Page

Table 1. HPEDC Studies. All data was collected from the analysis of extracts using setup in Figure 11. 39

Table 2. Calibration data related to a real water sample and DI water sample. 49

Table 3. Estimated combined Capture/Extraction Efficiencies. 68

Table 5. MS/MS identified peptides from the LMJ-SSP/ESI-MS/MS analysis of BSA tryptic digests separated on a ProteoChrom HPTLC Silica gel 60 F254s plate. 96

Table 4. Protein sequence coverages obtained by LMJ-SSP/ESI-MS/MS analysis of tryptic digests separated on ProteoChrom HPTLC Silica gel 60 F_{254S} plates and ProteoChrom HPTLC Cellulose sheets. 96

Table 6. Sequence coverages obtained for five protein tryptic digests analyzed by LMJ-SSP-MS/MS for the RP-C8 and RP-C18 HPTLC plates and by DESI-MS/MS for the NP HPTLC plates (NP sequences coverages obtained from reference157). 116

Table 7. Cytochrome c. tryptic digest peptides observed from MS and MS/MS analysis of four types of TLC plates. 123

Table 8. Lists all myoglobin tryptic digest peptides observed from MS and MS/MS analysis of four types of TLC plates. 129

Table 9. Single intact protein solution and application conditions for figure 38. 137

Table 10. Intact protein development progress using ProteoChrom HPTLC Silica gel 60 F(254S) plate and developed using 1-butanol/pyridine/NH3/water 39/20/10/31 (v/v/v/v). 145

Table 11. 7 protein tryptic digest analysis using data dependent scan function with LC-MS and ion mapping scan function with direct infusion chip based nanoESI. 159

Table 12. 7 protein tryptic digest developed on ProteoChrom HPTLC Silica gel 60 F(254S) plates and readout using data dependent scan function with continuous flow-LMJ-SSP-MS and ion mapping scan function LESA chip based nanoESI. 168

Table 13. E. coli digest developed on ProteoChrom HPTLC Silica gel 60 F(254S) plates and readout using data dependent scan function with continuous flow-LMJ-SSP-MS and ion mapping scan function LESA chip based nanoESI. 170

LIST OF FIGURES

Figure Page

Figure 1. Atmospheric Pressure Surface Sampling/Ionization Methods: Examples and forces responsible for desorption or extraction are illustrated. 4

Figure 2. Liquid microjunction surface sampling probe modes of operation. In *continuous flow* mode, sampled material is either directly infused or directed into a solvent plug. In a autonomous 'pipette' mode only a solvent plug of extracted material is created. 6

Figure 3. Parameters and thermodynamic equilibrium relationships to consider when extracting material followed by gas phase liberation prior to mass spectrometric detection. 9

Figure 4. *Continuous flow* LMJ-SSP Diagram. 11

Figure 5. *Continuous flow* LMJ-SSP probe sampling from MALDI plate. 12

Figure 6. Auto sampler LMJ-SSP tissue analysis. In this work extracted material included a pharmaceutical, propranolol, and known phase I and II metabolites. 16

Figure 7. Sealing surface sampling probe modes of operation. 18

Figure 8. Spatial chromatography modes of operation. 26

Figure 9. Arpino depiction of compatibility of liquid chromatography with mass spectrometry. 27

Figure 10. Target environmental contaminate chemical structures. 33

Figure 11. For 100 fold concentration enrichments 5 mL of spiked water is passed through the HPEDC followed by 50 µL organic solvent for subsequent extraction. T 38

Figure 12. The above calibration data shows both a linear fit of Pyrene methanol standards and a calibration fit based on methanol extracts from spiked Second Creek samples over the same concentration range as the standards. 44

Figure 13. Separation of Second Creek Sample spiked with analytes at ppb concentration range prior to SPE. 48

Figure 14. A) A SPE Card is shown with two Teflon gaskets. 56

Figure 15. Structure and mass-to-charge ratio observed for the compounds investigated. 58

Figure 16. Scheme illustrating individual steps of LESA process. 60

Figure 17. The relative SRM signal level for propranolol (*m/z* 260 → 116, CE 27 eV) was recorded from each of six consecutive extractions of a single 2 mm diameter SPE well card that had been conditioned and loaded with 100 ng propranolol.. 63

Figure 18. Average of the ratio of the integrated SRM signal of propranolol (1 – 1000 ng/ml) and that of propranolol-d7 (500 ng/mL) (Avg. Intensity$_{propranolol}$/Avg. Intensity$_{propranolo-d7}$) as a function of the loaded mass of propranolol ($c_{propranolol}$) in the solution loaded (100 µL) onto the SPE card spot analyzed. 64

Figure 19. (a) µFocus MALDI plates were spotted with 1 µL volumes of propranolol in 80/20/0.1methanol/water/formic acid (v/v/v) and left to dry such that 1 – 1000 picograms was deposited. 66

Figure 20. Angiotensin II from a solution (1 µL of 5 µM (5.9 ng)) containing 5 mM NaCl was spotted on separate 1 mm diameter SPE card wells. 71

Figure 21. The analysis of a herbicide mixture at 2 ppb using the SPE card read out of 1 mm diameter wells with LESA is shown. 72

Figure 22. A 200 µL herbicide mixture at 2 ppb concentration level was concentrated onto a 1 mm extraction well (0.4 ng). 73

Figure 23. Hydrophobic surface treatment examples. 85

Figure 24. LMJ-SSP spot sample of ink on treated kimwipe. 86

Figure 25. Surface treatment process developed and the subsequent surface sampling procedure schematically illustrated for a wettable HPTLC plate. 87

Figure 26. (a) Base peak chromatogram of LMJ-SSP silica gel 60 HPTLC lane scan of Cytochrome C tryptic digest. A 100 µms-1 scan rate was used with an extraction solvent of 60/40 H20/ACN 0.1% formic acid. 89

Figure 27. (a) UV (366 nm) image of Goldenseal root extract developed on a HPTLC glass backed Silica gel 60 F254s plate using 80/10/10 ethyl acetate/water/formic acid (v/v/v). . 90

Figure 28. (a) UV (366 nm) image of berberine standard developed on a HPTLC glass backed silica gel 60 F_{254S} plate using 80/10/10 ethyl acetate/water/formic acid (v/v/v). 93

Figure 29. (a) Image of BSA tryptic peptides developed on ProteoChrom® HPTLC Silica gel 60 F_{254S} using 2-butanol/pyridine/ammonia/water (39:34:10:26, v/v/v/v) and stained using ninhydrin. 95

Figure 30. Schematic of liquid microjunction surface sampling probe ESI-MS/MS setup (not to scale) on the LCQ-DECA for analysis of HPTLC plates. 110

Figure 31. Shows reversed phase C8 (left) and C18 (right) HPTLC plates that were ninhydrin stained. 114

Figure 32. Extracted ion profiles for all peptides identified by LMJ-SSP-MS\MS of a cytochrome c. tryptic digest separated on a RP-C8 TLC plate. 121

Figure 33. Extracted ion profiles for all peptides identified by LMJ-SSP-MS\MS of a cytochrome c. tryptic digest separated on a RP-C18 TLC plate. 122

Figure 34. The extracted ion profiles for myoglobin peptides identified from a RP-C8 plate via LMJ-SSP-MS analysis. 125

Figure 35. Extracted ion profiles for myoglobin peptides identified from LMJ-SSP-MS on a RP-C18 TLC plate. 126

Figure 36. Sealing-Surface Sampling Probe Modes of Operation..... 139

Figure 37. 3 µL of a myoglobin solution, 5 mg ml-1, was spotted as a 6 mm bands on a ProteoChrom HPTLC Silica gel 60 F(254S) plate and developed using 1-butanol/pyridine/NH3/water 39/20/10/31 (v/v/v/v). 142

Figure 38. Single Protein solutions were spotted as a 6 mm bands on a ProteoChrom HPTLC Silica gel 60 F(254S) plate and developed using 1-butanol/pyridine/NH3/water 39/20/10/31 (v/v/v/v). 143

Figure 39. A S-SSP (Camag TLC-MS interface) coupled with an LTQ-XL MS was used to sample the band. 146

Figure 40. Liquid microjunction surface sampling probe modes of operation. 158

Figure 41. (a) The Total Ion Current (TIC) chromatogram of an ion mapping experiment.. 161

Figure 42. Tryptic peptides developed on ProteoChrom HPTLC Silica gel 60 F(254S) plates using 2-butanol/pyridine/ammonia/ water (39:34:10:26, v/v/v/v) are shown.. 164

Figure 43. . A tryptically digested seven protein mix was analyzed using three different LC-MS methods using an LTQ-XL operated in positive ion mode.. 165

Figure 44. A continuous flow LMJ-SSP was used to readout a seven protein tryptic digest, spotted as a 5 mm band, from a HPTLC development lane at 100 µm min^{-1} using 70/30/0.1 water/ACN/formic acid (v/v/v). 166

Nomenclature

AGC	Automatic gain control
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photo ionization
BSA	Bovine serum albumin
CD	Cyclodextrin
CE	Collision energy
CF	Continuous flow
CID	Collision induced disassociation
DB	Database
DBA	Dibenzo [a,h] anthracene
DESI	Desorption electrospray ionization
ECD	Electron capture disassociation
EDC	Endocrine disrupting compound
EPA	Environmental protection agency
ESI	Electrospray ionization
FAB	Fast atom bombardment
GC-MS	Gas chromatography-Mass spectrometry
HPEDC	High performance extraction disc cartridge
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography

ICP	Inductively coupled plasma
JeDI	Jet desorption ionization
LC	Liquid chromatography
LDI	Laser desorption ionization
LESA	Liquid extraction surface analysis
LIF	Laser induced fluorescence
LLOQ	Lower limit of quantitation
LMJ	Liquid microjunction
LMJ-SSP	Liquid microjunction surface sampling probe
LOD	Limit of detection
LSC	Liquid solid chromatography
LSE	Liquid solid extraction
m/z	Mass to charge value
MALDI	Matrix assisted laser desorption ionization
MEKC	Micellar electrokinetic chromatography
MP	Mobile phase
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
NP	Normal phase
PAH	Poly aromatic hydrocarbon
PCP	Personal care products
PMT	Photo multiplier tube

R_f	Retention value
RP	Reversed phase
RSD	Relative standard deviation
SIMS	Secondary ion mass spectrometry
SPE	Solid phase extraction
SRM	Selected reaction monitoring
S-SSP	Sealing surface sampling probe
TD	Thermal desorption
TLC	Thin layer chromatography
TV-NM	TriVersa NanoMate
UV	Ultraviolet
Vis	Visible

Introduction

1.1 Atmospheric Pressure Surface Sampling and Ionization Techniques

Atmospheric pressure surface sampling and ionization techniques for use with mass spectrometry (MS) are comprised of instrumentation that can operate in the open atmosphere and are, in most cases, relatively simple in design. Although MS as a technique will not be discussed at length in this dissertation, the study and design of sample introduction to the MS system is an active area of current research and is of direct relevance to all atmospheric pressure surface sampling and ionization techniques. The simplicity of design and the ability to operate outside the confines of vacuum sealed containment (i.e. at atmospheric pressure) allow the analyst more freedom in experiment design and ease of operation. Surface analysis techniques, in general, can offer the ability to increase throughput and/or simplify many types of analytical workflows or measurements. The analysis of samples or systems in an ambient state with no sample preparation is attractive. The ability to attain spatially resolved chemical information, in both dynamic and static biological/chemical systems, can allow for observations or understanding of said systems not previously possible. Such information could have far reaching impacts in fields such as catalytic materials research, pharmacological drug design, and numerous others. The coupling of MS with surface analysis methods allows for the analysis of complex samples. MS offers high selectivity and sensitivity in the analysis of a broad range of compounds. The ability to elucidate chemical structure of single chemical species in the presence of a complex sample matrix is perhaps only rivaled by a few techniques, including NMR. Therefore the coupling of MS with atmospheric pressure surface sampling/ionization represents a technique or system that

can be universally applicable to a variety of analytical problems/research much in the same way the coupling of column based liquid chromatography and mass spectrometry is presently used for a variety of applications in academia and industrial settings.

The coupling of MS with surface sampling techniques has a long-standing history. Techniques such as secondary ion mass spectrometry (SIMS) and different laser desorption ionization (LDI) techniques, including matrix-assisted laser desorption ionization (MALDI), have been used for the better part of three decades.[1,2,3,4] Thermal desorption atmospheric pressure chemical ionization (TD/APCI) use as a surface analysis technique can be traced back to the 1970s, and was, in fact, a commercial product in the 1980s.[5] Atmospheric pressure – Matrix-Assisted Laser Desorption Ionization (AP-MALDI) introduced in 2000 by Laiko et al allowed the analysis of vacuum sensitive samples including gels, tissue, and volatile analytes; however, poorer levels of detection compared to vacuum MALDI are common.[6] Desorption electrospray ionization (DESI), introduced in 2004, sparked renewed interest in MS based surface analysis techniques.[7] Because established MS surface analysis techniques such as those previously mentioned, SIMS and MALDI, must be traditionally used with the sample under vacuum, the ability of DESI to operate under ambient or atmospheric conditions, in the same way AP-MALDI operates, triggered the re-invention of MS based surface analysis and a field of study coined ‘Ambient Ionization MS’ was created. Although the term ‘Ambient’ is meant to convey analysis with no sample preparation or the analysis of an intact dynamic system, the confusion between the established acronym atmospheric pressure ionization (API) is unavoidable. As a result the use of the term atmospheric pressure surface sampling/ionization mass spectrometry is preferred. Figure 1 depicts examples of different atmospheric pressure

surface sampling/ionization MS methods with an emphasis on the mechanism of energy introduction for the removal of material from a surface of interest. The rapid introduction of photons, particles and/or heat to a condensed-phase sample which results in the removal of a species from a surface into the gas phase is called a ‘desorption event’. The use of liquid extraction AP-surface sampling methods may not be grouped by some into classic ‘desorption’ based surface sampling. Energy is however a fundamental mechanism in liquid-solid extraction events where the displacement of material on a surface or the phase changes of a material is governed by kinetic and potential energy barriers. So whether the use of energy can be described as the primary or secondary mechanism to the removal of material is not defined in this field we call atmospheric pressure surface sampling/ionization mass spectrometry.

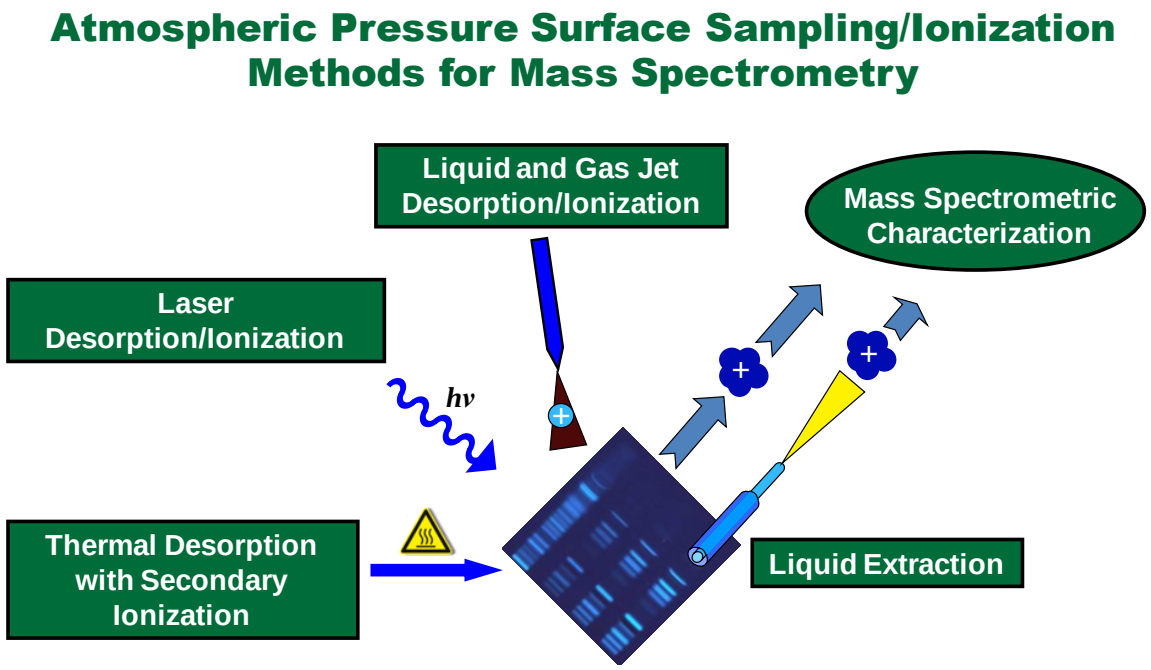


Figure 1. Atmospheric Pressure Surface Sampling/Ionization Methods: Examples and forces responsible for desorption or extraction are illustrated.

In 2002 Van Berkel et al introduced a liquid microjunction surface sampling probe (LMJ-SSP) based on the work and probe design of Wachs and Henion.[8,9] A LMJ-SSP is one of four types of liquid extraction based surface sampling techniques as seen in Figure 2. Other liquid extraction based surface sampling techniques include DESI, jet desorption ionization (JeDI), and a sealing –surface sampling probe (S-SSP). LMJ-SSPs employ an open-air, wall-less liquid microjunction (LMJ) between the sampling end of a solvent delivery apparatus and a surface. Material coming in contact with a liquid microjunction may be extracted into the liquid phase based on inherent chemical properties of the material. The extraction can be influenced by the nature of different chemical phases, different chemical environments, or a combination of both. A S-SSP operates in the same format as a LMJ-SSP; however, the sampling area is physically sealed off and isolated. The S-SSP will be discussed in more detail later. Both DESI and JeDI are generally classified as liquid and gas jet desorption/ionization techniques (as noted in Figure 1) but the sensitivity of both techniques is dictated by the ability to extract material into respective spray solutions prior to a desorption event. Thus they are included in the genre of liquid extraction based surface sampling techniques for MS.

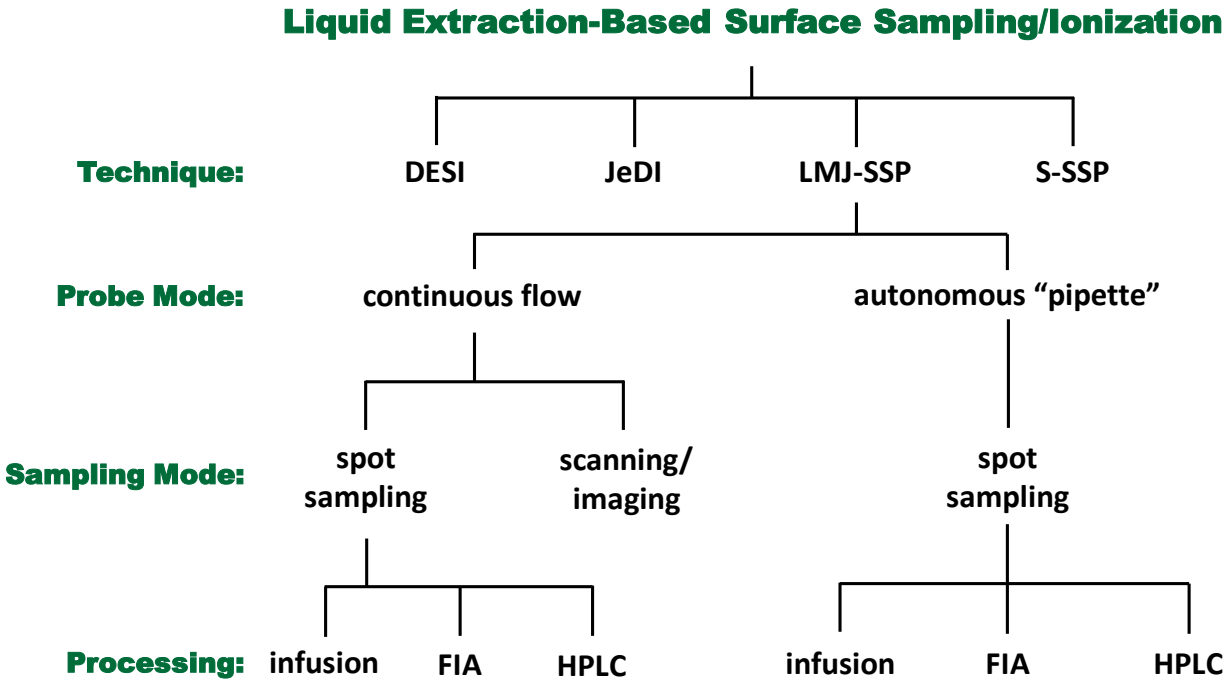


Figure 2. Liquid microjunction surface sampling probe modes of operation. In *continuous flow* mode, sampled material is either directly infused or directed into a solvent plug. In a autonomous 'pipette' mode only a solvent plug of extracted material is created.

In many instances the sampling or extraction of material into a liquid microjunction can be described in the same way as adsorption based chromatography or liquid-solid chromatography (LSC). The use of classic chromatographic optimization models, such as the Snyder solvent triangle, can be readily employed in the optimization of extraction efficiency and characterization of results.[10] S-SSPs and LMJ-SSPs in combination with mass spectrometric detection decouple extraction and ionization events. This is illustrated in Figure 3. An analyte *A* will exist as an ion or neutral on a given surface. The ability to extract or remove the analyte from the surface is governed by the analyte's solubility in the solution. Solubility is dictated by the analyte pka and log P (polarity), and the extraction solvent's strength defined as ε°. Perhaps a more appropriate chromatographic field to use as a guide in liquid extraction based surface sampling is 'sample preparation', more pointedly liquid solid extraction (LSE). Again the sampling and ionization events are decoupled, therefore the extracted material is more or less being prepped prior to MS analysis. The analyst could directly analyze extracted material in solution, but the ability to process extracted material using traditional analytical methods becomes possible as well. The most obvious sample processing is liquid column chromatography where a solvent plug of extracted material can be simply injected onto a column prior to detection. Many analytical methods involve some form of sample preparation sometimes called preliminary treatment or sample cleanup. Some sample preparation methods are actually chromatography methods that share common aspects with the later. A detailed discussion on extraction methods is not warranted in the presented research; however, future embodiments of LMJ-SSP systems could parallel with many well studied sample preparation techniques. The most obvious is the enhancement of extraction efficiency in LSE by heat, sonication, or pressure.

This is called assisted liquid-solid extraction in some texts. The use of ultrasound and radiation (in most cases microwaves) has also been used extensively in LSE. Worth noting is the use of energy as an assisting or secondary mechanism, in much the same way other AP-surface sampling MS techniques operate, can be utilized and easily incorporated into LMJ-SSP technology for enhancement in sensitivity. In this way the probe itself is only a liquid transport to an ionization source, with respect to MS analysis, or other preparation mode.

When considering ionization efficiency and the liberation of the analyte into the gas phase for mass spectrometric detection the same properties that governed the liquid solid extraction as well as gas phase proton affinity and basicity will dictate sensitivity and selectivity. The use and compatibility of surface analysis MS techniques with different API techniques is critical in their applicability as global analytical tools. A distinct advantage of LMJ-SSPs is their compatibility with many API modes including: electro spray ionization (ESI), microESI, nanoESI, atmospheric pressure chemical ionization (APCI), atmospheric pressure photo ionization (APPI), and inductively coupled plasma (ICP)-MS. LDI techniques can also be coupled with LMJ-SSPs where desorbed plumes of material are captured in the sampling end of LMJ-SSPs. Ablated material that is captured can be processed and prepped further, or be directly analyzed.[11] Thus the use of LMJ-SSPs as capture devices or as a bridge to API based instruments may allow for even broader applicability of this simple and robust technique.

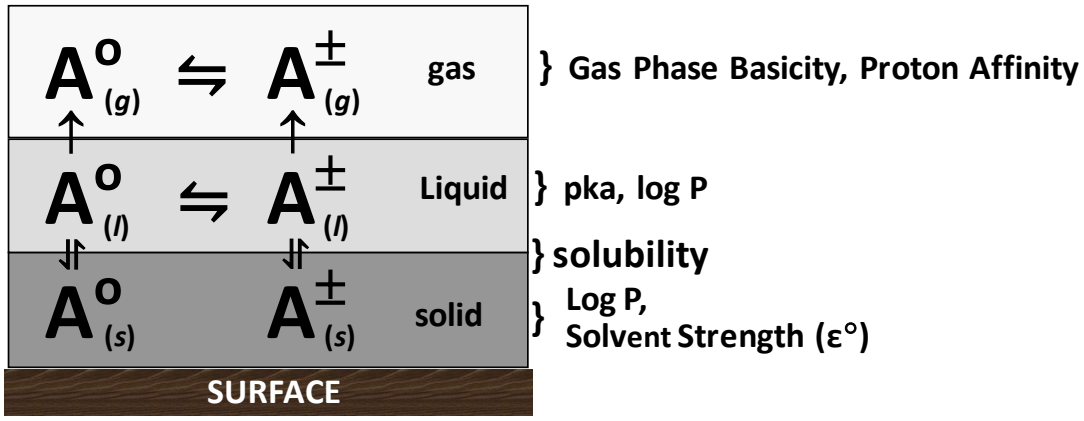


Figure 3. Parameters and thermodynamic equilibrium relationships to consider when extracting material followed by gas phase liberation prior to mass spectrometric detection.

1.2 Liquid Microjunction Surface Sampling Probe: Modes of Operation

LMJ-SSPs can be categorized by the way in which the solvent is introduced in the extraction process. There is continuous flow operation, where the sampling volume is constant, but solvent with extracted material is continually being directed toward a detector or ionization source. There is a discontinuous flow operation, where the solvent is not being replenished, i.e. a static drop of liquid. When extracted material is subjected to a discontinuous flow a sample plug is generated that is not being continuously infused or directed towards a detector. The generation of sample plugs can be used as a means to concentrate analyte into a given volume over a certain period of time, a few seconds, minutes, or perhaps hours. As such LMJ-SSPs can be categorized as operating in a surface sampling plug generation mode or a direct infusion mode.

In the operation of a continuous flow liquid microjunction surface sampling probe (continuous flow-LMJ-SSP) a liquid extraction solvent is brought to a surface through the annular space between two coaxial tubes at the sampling end of the probe and is then carried on through an inner tube to the ionization source through a self-aspirating electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) emitter. Figure 4 illustrates the operation and configuration of a continuous flow-LMJ-SSP. Figure 5 shows a real time image of a spot sampling experiment using a continuous flow-LMJ-SSP for the readout of a MALDI plate. The LMJ-SSP-MS approach to surface sampling/ionization can be applied to all species that can be dissolved and conducted into the probe and subsequently ionized by the respective ionization method being used.

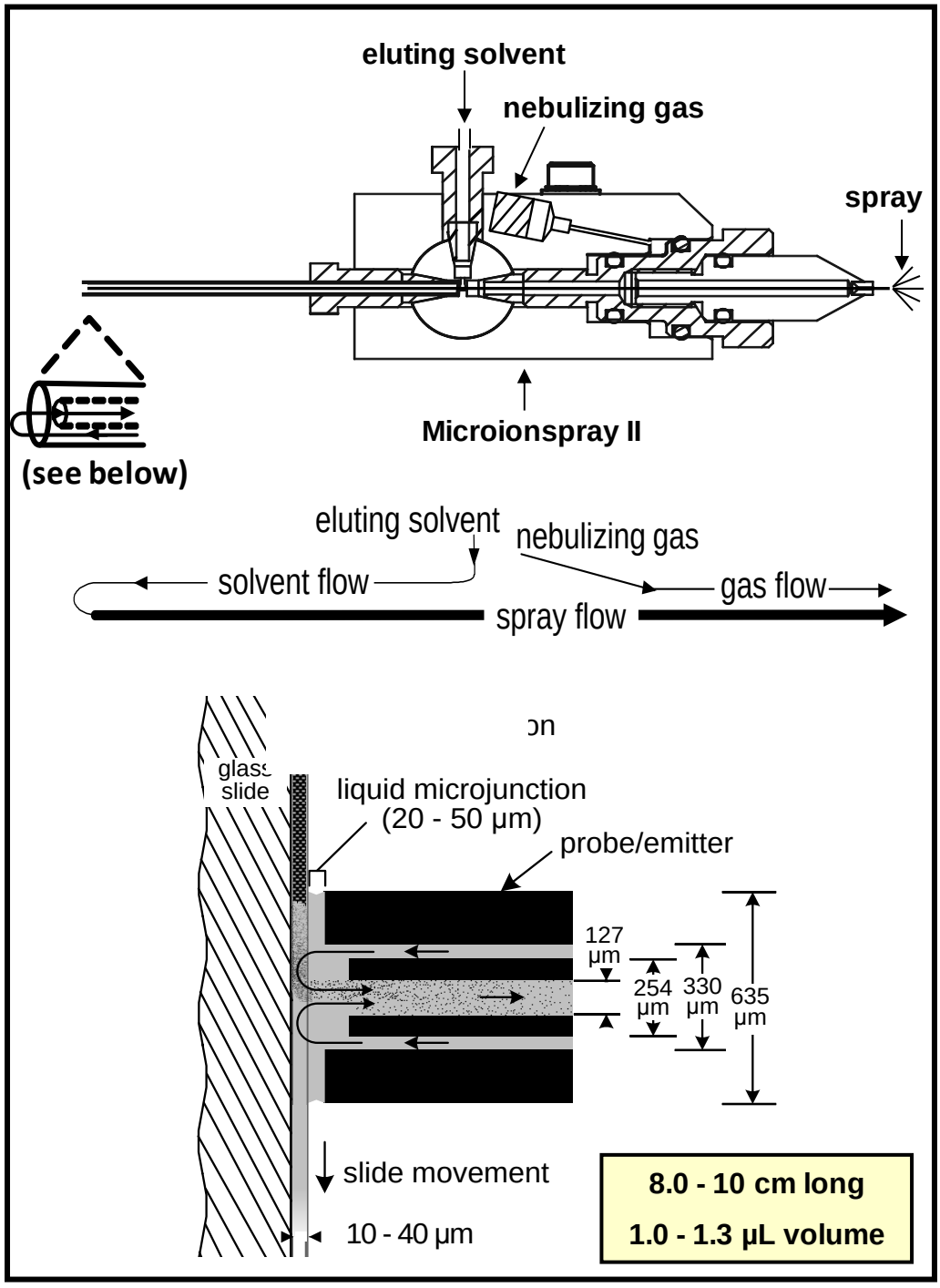


Figure 4. ***Continuous flow*** **LMJ-SSP Diagram.**

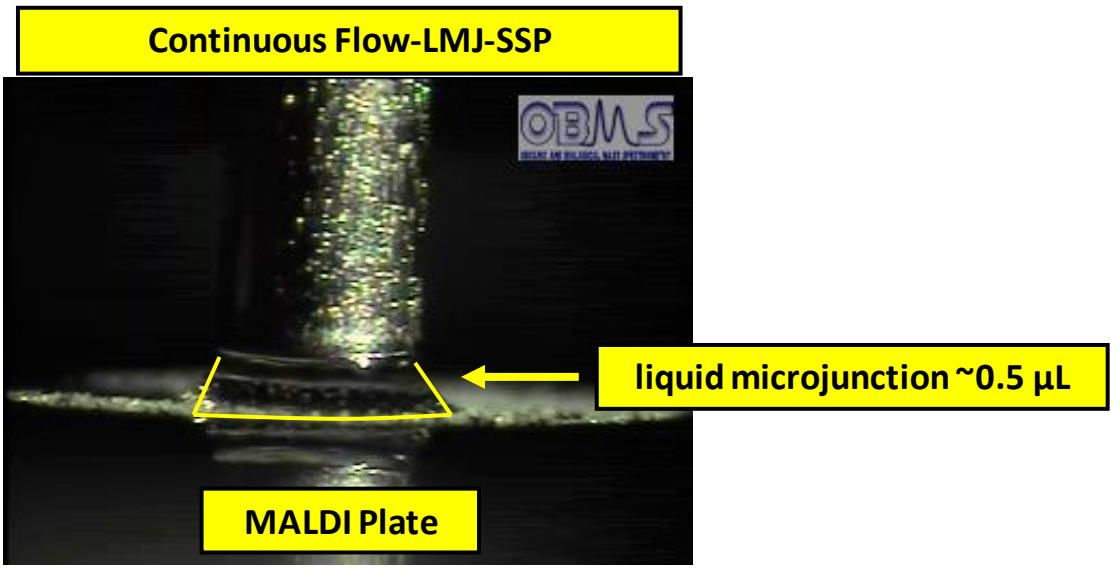


Figure 5. ***Continuous flow*** **LMJ-SSP probe sampling in a 'drool and slurp' mode from a MALDI plate spot.**

The use of APCI and ESI, including nanoESI, has been used with a continuous flow-LMJ-SSP design.[8,12,13] The liquid microjunction is created and maintained by using constant, balancing of solvent and nebulizer gas flow rates and electrospray voltage during the entire analysis. First a contact is made between the sampling probe and the surface (in some cases there is no contact between the probe and surface and protruding liquid makes contact first) creating a liquid microjunction followed by an adjustment of their distance, i.e. the width of the junction to about 20-50 µm. Again in this configuration, analyte is continuously extracted from the surface and transferred for subsequent spraying at the spray end of the probe. This constitutes a direct infusion LMJ-SSP. At the end of the analysis the liquid microjunction is disrupted by increasing the sampling probe to surface distance. Reported applications of the LMJ-SSP device in this mode include sampling and analysis of dried drugs or proteins or solutions thereof from wells on microtiter plates, drugs captured in solid-phase extraction cards,[12] dyes, inks, or pharmaceuticals on paper or separated on hydrophobic reversed-phase (C8 and C18) thin-layer chromatography plates,[8,13,14,15,16] exogenous compounds from thin tissue sections,[19,20] and surface deposited and affinity captured proteins.[18] The LMJ-SSP has also been configured as a two-electrode electrochemical cell to enable beneficial electrochemically initiated analyte modifications.[31] In this research arena, significant efforts were made to understand and to advance liquid extraction based surface sampling techniques.[8,9,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35] A continuous flow-LMJ-SSP can operate in a spot sampling mode where the extracted material can be directly infused, subjected to flow injection analysis, or processed in an HPLC column prior to API-MS. A continuous flow-LMJ-SSP can also scan a surface and produce chemical images

based on several or many scanning events. Chemical imaging with mass spectrometry is a continuously growing field of research but will not be discussed here.[36]

The discontinuous flow LMJ-SSP is referred to as an 'autonomous' pipette where a surface sampling plug is generated. There is much instrumentation available that can perform this type of surface sampling including HPLC auto samplers and any robotic syringe device; however, a hand held pipette that simply is used to dispense liquid on a surface, re-aspirate said solvent, and analyze would be included as an 'autonomous' pipette. In this type of surface sampling format a distribution equilibrium or steady state will be reached after which only outside forces that would drive the equilibrium one way or the other will define the amount of material extracted prior to analysis. First, the sampling probe is positioned at a distance from the surface. A LMJ-SSP 'autonomous' pipette probe can be positioned as far as 200-300 μm from the surface and a liquid microjunction formed by letting the liquid from the sampling end of the probe/pipette tip extend out from the probe/pipette tip to the surface. In case of the Tri Versa NanoMate system (a commercially available nano-ESI direct infusion device to be discussed later) simple dispensation of the extraction solvent onto the surface and subsequent aspiration of the liquid back into a pipette tip results in a sample solution. The use of the LMJ-SSP as an 'autonomous' pipette, including a TriVersa NanoMate system, in the analyses of spotted sample arrays, thin tissue sections, and dried blood spots has been reported.[34,37] Figure 6 shows a conventional PAL auto sampler used as an autonomous 'pipette' in the analysis of a thin tissue section.[38] A continuous flow-LMJ-SSP can also be operated as a pseudo 'autonomous' pipette where a surface sample plug is generated. This is accomplished by reducing the liquid flow rate of the probe to a value less than the volume flow rate pumped into the probe. It is followed by

adjusting the nebulizer gas pressure and/or the electrospray voltage so the extraction solvent protrudes from the sampling end and dispenses extraction liquid on the targeted surface. When the self-aspiration rate of the probe is subsequently increased, analytes on the surface that dissolve at the liquid microjunction are aspirated back into the probe with the liquid that created the liquid microjunction. Another variation of the "changing junction volume" mode was demonstrated for a simple and relatively fast (30 s sample-to-sample) quantitative analysis of spotted sample arrays using the classic continuous flow-LMJ-SSP design.[32]

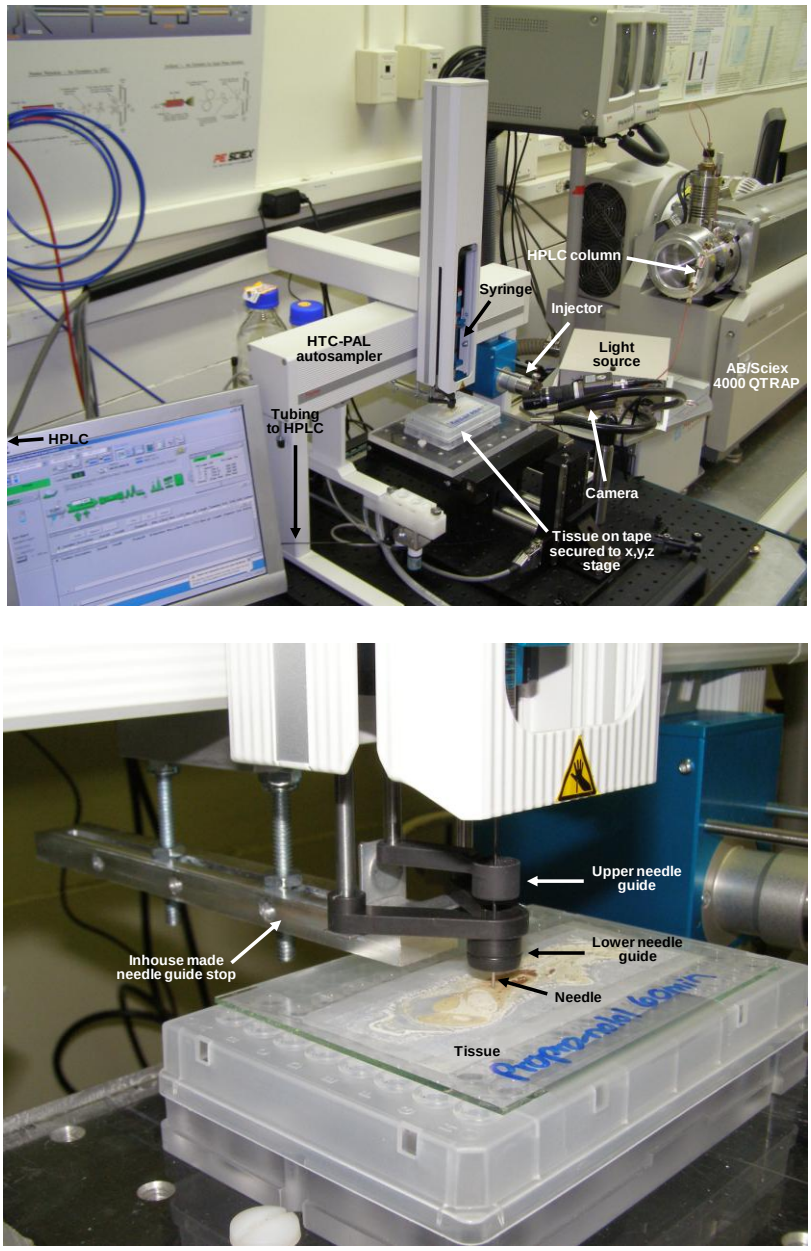


Figure 6. Auto sampler LMJ-SSP tissue analysis. In this work extracted material included a pharmaceutical, propranolol, and known phase I and II metabolites. The extracted material was processed using a HPLC column prior to MS analysis using a 4000 qtrap in SRM mode.

1.3 Sealing Surface Sampling Probe: Modes of Operation

A sealing surface sampling probe (S-SSP), introduced by Luftmann, will be discussed in this dissertation. At least two elution approaches with the same basic type of S-SSP concept have appeared in the literature.[39,40] With this probe, analyte is extracted from a surface by sealing the probe to the surface using a knife edge on the probe that cuts into the surface. By principle, this sealing mechanism does not readily allow analysis of analytes simply deposited on the surface of hard, inflexible, nonporous materials like metal, glass, or various plastics sheets. Using an alternative sealing mechanism such as a plastic or rubber seal versus a knife edge seal might allow for sampling of 'hard' surfaces.[41] For this reason, S-SSPs have been mainly used for the analysis of mixtures and extracts (pharmaceuticals, components of plant extracts, etc) separated on normal phase TLC plates. A modified version of the Luftmann-type S-SSP became commercially available recently which was named the "TLC-MS interface". Soon after its release, this interface was used for analysis of dried blood spots on paper and small animal whole-body thin tissue sections on adhesive tape.[33]

S-SSPs can efficiently operate as spot sampling devices. As indicated in Figure 7 the extracted material can be directly infused, subjected to flow injection analysis or injected onto a LC column.

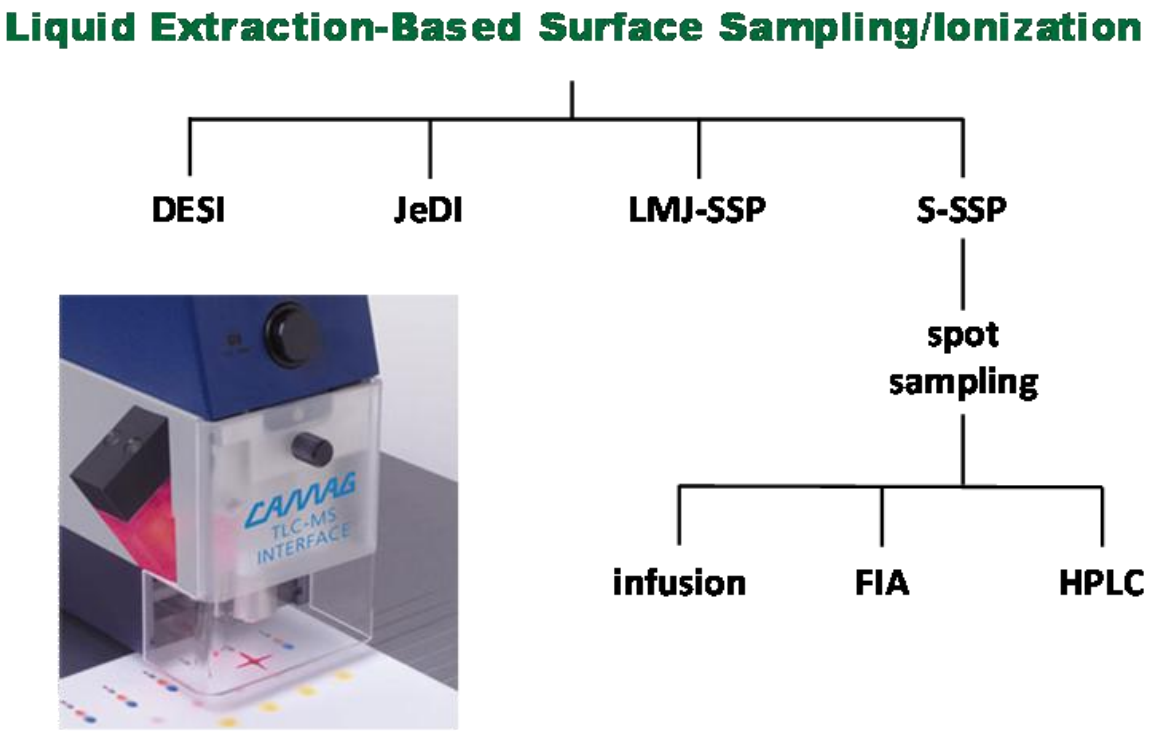


Figure 7. Sealing surface sampling probe modes of operation.

Imaging via sequential spot sampling is possible but resolution and analysis time are greatly hindered so imaging is not suggested. The sealing surface-sampling probe has again been used almost exclusively for the analysis of analytes separated on TLC plates. The applications have been limited to relatively low molecular-mass compounds like caffeine or other pharmaceuticals, or components of plant extracts. The inlet capillary of a stainless-steel plunger which seals to the surface to be sampled is connected to an HPLC pump. The outlet capillary is connected to the ion source of a mass spectrometer. The zone or analyte band of interest on the TLC plate, or other surface, is positioned under the probe, and the surface sealed by compression with the knife edges on the probe. By switching the valve from standby to extraction mode, the solvent that was previously flowing directly to the ion source travels to the surface, extracts the analyte from the surface, and then carries it onto the ion source. The probe typically has a diameter of 2 or 4 mm depending on the extraction head used. A 2 or 4- mm elliptical sampling head has also been used to better match TLC band size and shape for more effective sampling.

1.4 Factors to consider when employing surface sampling mass spectrometry

Many different factors must be considered when employing or deciding whether to employ MS based surface analysis methods/techniques. The analyst must consider whether extraction of material is possible and whether chemical information can be attained. In some cases analytes of interest may be irreversibly bound to the surface as can happen with intact protein chromatography.[42] When using an API mode prior to detection the balance between the highest extraction efficiency and ionization efficiency must be considered. The use of non-volatile salts in column based HPLC methods, phosphates etc., is common and in many cases

allows for higher separation power. The use of non-volatile salts is discouraged in many API methods, most notably ESI because of its detrimental effect on ESI efficiency. Solvent additives, such as micelles, ion-paring reagents, non-volatile salts, and many non-polar solvents, can be detrimental to the API-MS sensitivity (ionization is suppressed) and in the same instance these same additives may enhance the initial extraction efficiency prior to detection. The extraction efficiency is negated because the MS detector will be 'blind' to the material of interest because of signal/ionization suppression. Thus further sample preparation following extraction, at the expense of time and reagents, may be appropriate in some instances. The analyst must consider what and if a minimum readout resolution is required. In some cases high readout resolution is called for such that the information can be related to say pharmacological studies in heterogeneous tissues like the brain that dictate >50 μm resolution for biological significance.[43] Some analyses of surfaces may require a low readout resolution (say < 1 mm). A low readout resolution can boost signal intensity and alleviate in-homogeneity in sampling surfaces at higher resolution.[44] The ability to scan a surface or spot sample may be hindered by surface topology thus the surface itself can interfere with throughput. Addressing or considering the factors presented before implementing or attempting any atmospheric pressure surface sampling/ionization technique is advised.

1.5 Defining 'Analytical Surfaces'

Many atmospheric pressure surface sampling/ionization techniques are also defined as 'ambient sampling/ionization' MS techniques.[45] Ambient can entail many meanings and some choose to focus on the ability to analyze a sample with no or little 'traditional' sample preparation. However, sample preparation knowledge would dictate,[46] and several recent

ambient surface sampling/ionization studies have clearly illustrated[47,48,49,50,51] that, under certain analysis scenarios, the use of sample preparation methods prior to analysis greatly improves the analytical figures of merit. The current state and view of so-called *ambient* sampling/ionization MS methods as a collection of useful analytical techniques is still a reflection of hype that sacrifices sensitivity and selectivity when contrasted with proven technologies. The sacrifice of sensitivity and/or selectivity for speed (and one could argue simplicity) is going to be useful in addressing few analytical challenges now and in the future. One such sample preparation scheme that has been used with these techniques is solid phase extraction (SPE).[52] For example, Takáts' group[47] showed that a SPE-based sample preparation scheme allowed for a lower detection level for desorption electrospray ionization (six orders of magnitude lower LOD compared with direct analysis w/o SPE procedure). Wachs and Henion[53] demonstrated the automated quantitative analysis of analytes captured with a conventional 96-well format SPE plate using a continuous flow surface sampling probe. Surfaces such as SPE cards can be referred to as analytical surfaces. Examples of other analytical surfaces are thin layer chromatographic media, thin biological tissue sections mounted on slides, MALDI plates, affinity arrays, dried blood spot paper, protein gels and blotting membranes. Analytical surfaces in some cases are identified as surfaces that are traditionally part of an analytical workflow. A sample or analyte is deposited on a surface which can be readout with a surface analysis technique. In other cases some form of clean-up, fractionation, or concentration, either singly or concert, happens such that sensitivity and reproducibility are not sacrificed in lieu of throughput. Some surfaces cannot be defined as analytical surfaces and are ambient in nature. Examples of such surfaces are not limited to but include skin *in vivo*, plant leaves, formed tablets, solid

catalytic surfaces, paper, and so on. If material can be removed or extracted from a targeted surface chemical information is attainable in most cases.

In this dissertation work will be presented that shows the use of LMJ-SSPs for the readout of normal phase or 'wettable' analytical surfaces. This type of readout was made possible by the development of an aerosol silicone treatment procedure. As a result of this developed protocol the hydrophobicity of a given surface does not dictate whether the sample or surface can be analyzed by LMJ-SSP based methods. Wettable surfaces are problematic because sampled material of interest is developed out of the sampling region of the probe. The majority of material or analyte in the probe sampling region then becomes somewhat inaccessible during the sampling event. Important to note is this causes inefficient sampling, but does not render the LMJ-SSP unable to detect some material albeit sensitivity is compromised. As a result of the developed protocol the use of LMJ-SSPs for the readout of any analytical surface, as defined above, is inherently feasible.

1.6 Spatial Chromatography Coupled with Mass Spectrometry: The Next 'Technology' Holy Grail

The analysis of complex samples using planar chromatographic media where components are spatially separated and analyzed is attractive. The use of atmospheric pressure surface sampling/ionization techniques for the coupling of planar separations with mass spectrometry has gained much attention.[54,55,56] The efficient coupling of planar chromatography, a branch of liquid chromatography, with mass spectrometry has been called the next technology holy grail.[57] We prefer to call this field *spatial* Liquid Chromatography/Mass Spectrometry (LC-MS). Spatial chromatography takes place in space rather than time. The main advantage of this approach

being the construction of 2 or more separation dimensions becomes more convenient. The time for subsequent separations (2 or more dimensions) happens in parallel such that the exponential increase in serial fractionation analysis time, as seen in multi-dimensional column approaches, is not a detrimental factor to the analysis.[58] There are several advantages to utilizing spatial chromatography coupled with mass spectrometry. Some advantages include the static analysis of fractionated samples such that analysis times and number and type of MS experiments are not dictated by column based chromatographic peak times. Several examples using this advantage will be presented and discussed in this dissertation as they relate to HPTLC plate and SPE card analysis. These advantages arise from a classic advantage of spatial chromatography which is the ultimate in 'peak parking'. Detection and quantitation are static as the analyte bands are not moving after sample fractionation has occurred.

Other advantages of spatial chromatography performed on TLC plates include high sample throughput using multiple samples per plate, two dimensional separations are easy, and all analytes remain on the plate (no sample is in an unknown or inaccessible space). When using TLC plates for spatial chromatography disadvantages must always be considered and have been addressed by others to include limited separation power, no or limited automated or on-line instrumentation systems exists and, in some instances, separations may not be in agreement with column based chromatographic theory. Other disadvantages have been noted by others but are the result of the last mentioned disadvantage that TLC or planar chromatographic separations are not always predictable or in agreement with column based chromatographic theory. This stems from the inherent complexity of performing separations where there is a gas phase thermodynamic equilibrium between the liquid mobile phase (MP) and the stationary phase, that

is either pre-saturated with MP vapor or not. The selectivity of a specific development solution and stationary phase for an analyte can be altered by the use of a saturated or un-saturated TLC development chamber. Some would call a dynamic system such as the one described disadvantageous for obvious reasons. The most important of which is loss of control and understanding of the chromatographic processes. If the system is reproducible, however, this disadvantage can provide an easy means to influence selectivity such that the separation or desired fractionation of mixtures is realized. A more complex discussion of planar chromatographic theory is not warranted.

The successful coupling of mass spectrometry to column based liquid chromatographic systems, commonly called API-LC-MS, was a major milestone reached in the 1980s. Prior to efficient LC-MS coupling, researchers such as Patrick Arpino illustrated how vastly difficult the coupling was thought to be through cartoons such as Figure 9. The research leading up to API-LC-MS was conducted by many, but names that are now synonymous with it are Talrose, Dole, Thompson, McLafferty, Fenn, Covey, and Henion. API-LC-MS has become a mature analysis platform that is the 'work horse' for many industrial and research driven applications. Studies that will be presented in this dissertation point to the fact that spatial liquid chromatography coupled with mass spectrometry may have been erroneously overlooked as fruitful areas of research. The understanding of and technology of column based liquid chromatography (packing materials, the use of monoliths, UPLC, and so on) has seen giant leaps in the last thirty years. One could argue that until recently the largest impact on planar chromatography has been the advent of high performance thin layer chromatography (HPTLC) plates. HPTLC plates are produced from chromatographic packing materials that have narrow pore and particle size

distributions with a layer thickness of 100 – 200 µm. This leads to higher separation efficiency over conventional TLC plates which allows for higher resolution chromatography with an increase in sensitivity. The separation power of HPTLC media is in no way comparable to the peak capacity and efficiency of column LC run in gradient modes. Recent studies though have shown higher separation power through forced flow methods including centrifugal, pressure, and electrophoresis based planar chromatography.[59,60] Figure 8 shows different spatial chromatographic modes of flow and the force governing the flow is indicated. High efficiency spatial separations can be readout using many atmospheric pressure surface sampling/ionization techniques. The ability to statically analyze or prep extracted material for further processing can only be conveniently realized through LMJ-SSP and S-SSP approaches. These AP surface sampling methods coupled to mass spectrometry allow for analysis power that can be on par with or surpass column based LC-MS in theory and practice. In the presented work we will show results that support this claim.

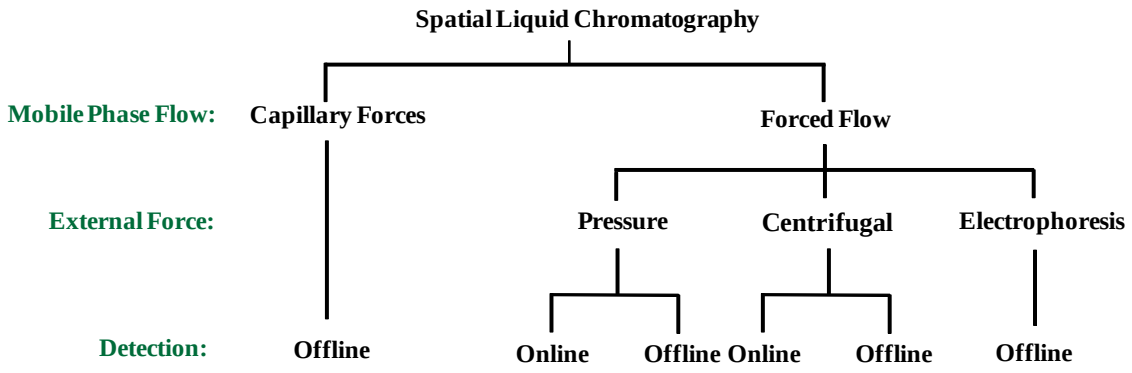


Figure 8. Spatial chromatography modes of operation.

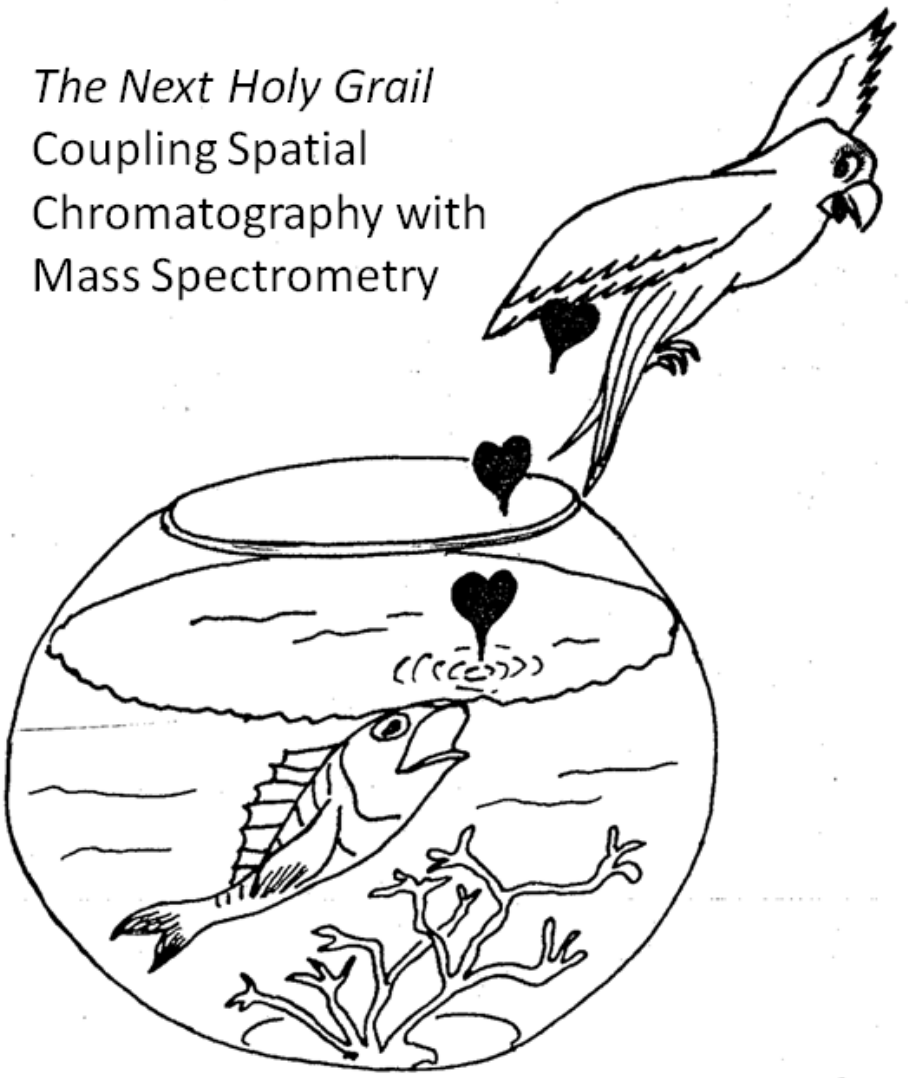


Figure 9. Arpino cartoon depiction of compatibility of liquid chromatography with mass spectrometry.

1.7 Dissertation overview

The second chapter of this dissertation focuses solely on solid phase extraction analysis of multi-class compounds including: suspected endocrine disrupting compounds, personal care products, and pesticides. The second chapter represents work Matthew J Walworth completed and published in the first years of graduate school before beginning research at Oak Ridge National Lab with Dr. Gary J Van Berkel. The third chapter discusses the analysis of solid phase extraction cards using an automated liquid microjunction surface sampling probe system. Chapter 4 details the use of aerosol silicone sprays for the treatment of 'wettable' surfaces which allows for LMJ-SSP analysis. Chapters 5, 6, and 7and part of Chapter 4 discuss and show examples of complex sample analysis using LMJ-SSP and S-SSP readout of tryptic peptides and intact proteins fractionated on planar chromatographic media. Chapter 8 includes some concluding remarks and discussion on the future of LMJ-SSP technology coupled with mass spectrometry.

Chapter 2

2.1 Extraction, separation, and fluorometric analysis of selected environmental contaminants

2.1 Introduction

The pollution of water systems by personal care product (PCP) residue, pesticides, and other pollution have garnered much attention in recent years.[61] The decline of certain aquatic species and changes in water oxygen levels are directly related to water pollution. Trace pollutants in potable water supplies have also been suspected as the cause of numerous adverse health effects in humans from birth defects to reproductive deficiencies.[62] As recently as December 2008, Katsu and Iguchi published findings indicating Tributyltin, a pesticide and a preservative in wood treatment, could actually be promoting obesity.[63] Tributyltin as well as other persistent pollutants can be found in different water systems and can cause harmful effects at low concentrations. In most cases suspect water systems are polluted with numerous compounds including PCPs, pesticides, and pharmaceutical residues. Some of these classes of compounds and specific compounds themselves have been labeled as Endocrine Disruptors.[64] These Endocrine Disrupting Compounds (EDCs) may mimic, block, or cause interferences with normal hormone interactions in the endocrine system. As early as 1996, the Environmental Protection Agency was commissioned by Congress to study EDCs and determine the extent of exposure and subsequent effects on humans. More than ten years later the scope of research has grown to include the study of effects such pollution has on aquatic and terrestrial wildlife as well. Because the scope of research has come to include different ecosystems and also because

broader classes of chemicals are now being screened to determine endocrine disrupting activity, the analytical challenge of dealing with numerous pollutants in complex sample matrices remains very important.

Herein we demonstrate the ability of a 3M High Performance Extraction Disc Cartridge (HPEDC) in the pre-concentration of analyte mixtures containing several classes of compounds including a toxin, pharmaceutical, and suspected EDC class in real aqueous samples. The HPEDC consists of a prefilter, polypropylene microfiber of graded densities, and a bonded C-18 sorbent phase. The HPEDC has been described elsewhere in use with the concentration and extraction of selected analytes from biological media such as plasma, blood, and serum.[65] Moreover, the use of disc Solid Phase Extraction (SPE) has been studied and reported as well.[66,67,68,69] The 'high performance' characteristic of the SPE cartridge stems from the dense particle packing and uniform distribution within 3M Empore disks. As a result there is significant improvement in the efficiency and reproducibility of sample preparation techniques.[70] In this work an SPE procedure is reported including load volume, elution solvent, and sorbent capacity optimization. All compounds included in the study were analyzed separately and as mixtures with recovery percentages at or greater than 90% using only methanol as the eluting solvent. The only instance in which methanol did not efficiently elute the target analyte involved Dibenzo[a,h]anthracene (DBA), the most non-polar analyte in the study. Acceptable extraction recovery of this suspected EDC did take place with the use of ethanol as the eluting solvent. HPEDC extracts were first analyzed using an in house built hydrodynamic loading setup with Laser Induced Fluorescence (LIF) detection. Mixtures of analytes were later spiked into real water samples, subjected to the developed SPE procedure, and subsequently separated and

analyzed using cyclodextrin-modified Micellar Electrokinetic Capillary Chromatography (CD-MEKC) with a confocal LIF detection setup. CD-MEKC is a mode of chromatography that offers the ability to separate highly hydrophobic analytes as well as other classes of compounds.[71,72,73,74] This study shows the applicability and performance of these methods in the interrogation of multi-component mixtures representing three different classes of compounds in real sample matrices. No pre-sample workup was needed prior to SPE and complex elution schemes were also not needed in the presented work.

SPE techniques have been utilized in a variety of applications to enhance sensitivity and allow for sample clean-up prior to analysis. The SPE configuration in a method can be characterized as either on-line or off-line. The former takes place independent of separation and/or detection, while the latter is coupled directly with the separation and/or detection instrumentation. Recent literature has reviewed on-line SPE coupled to LC and outlined comparative features of on-line and off-line SPE configurations.[75] Svoboda and workers used a 6 mL 150 mg Oasis MAX SPE cartridge in the GC/MS analysis of phenols and acidic pharmaceuticals in influent and effluent sewage treatment samples. The SPE cartridges were used in an anion exchanger capacity, with a subsequent elution procedure described to fractionate target compounds. Their procedure produced a cleaner extract and minimized preparation time in comparison to similar methods.[76] Cren-Oliv´e and workers developed GC-MS and LC/-MS/MS methods that incorporated the use of Strata C-18 SPE in the analysis of 33 multi-class pollutants in wastewaters, surface, and ground waters.[77] Optimization to insure suitable recoveries of target analytes in these methods and numerous others requires pre-extraction filtering methods, multi-solvent extraction schemes, and post-extraction derivatization

and /or treatments prior to instrumental analysis. The use of MEKC as a separation platform in the analysis of PCPs, suspected EDCs, and pesticides in various water media has previously been reported.[78,79,80] The use of SPE in conjunction with MEKC has also been reported.[81] Dempsey and workers first addressed the potential of MEKC, cyclodextrin- modified MEKC (CD-MEKC) and electroosmotic flow-suppressed CD-MEKC as novel modes of capillary electrophoretic separations in the analysis of suspected EDCs.[82]

Recent work in the field of microfluidic devices has utilized capillary electrophoresis techniques as a useful means of separation.[83] Due to the efficiency of CE and the characteristically short separation lengths of microfluidic platforms few other separation means can realistically be used in micro fluidic platforms. The modes of detection are perhaps the only obstacle for onsite testing techniques using micro fluidic devices. The presented work here emphasizes the use of the HPEDCs as a tool for significant sample clean up as well as concentration. Matrix interferences problematic for the basic operation and detection in microfluidic devices could easily be overcome by the use of the presented SPE method. Using this developed method of analysis field testing on microfluidic platforms could be realized.

2.2 Experimental

2.2.1 Materials and Reagents

β-cyclodextrin was purchased from Cyclodextrin Technologies Development, Inc. All other buffer components and analytes were purchased from Sigma Aldrich (St. Louis MO, USA). All analytes, structures shown in Figure 10, were purchased from Sigma Aldrich St.Louis MS.

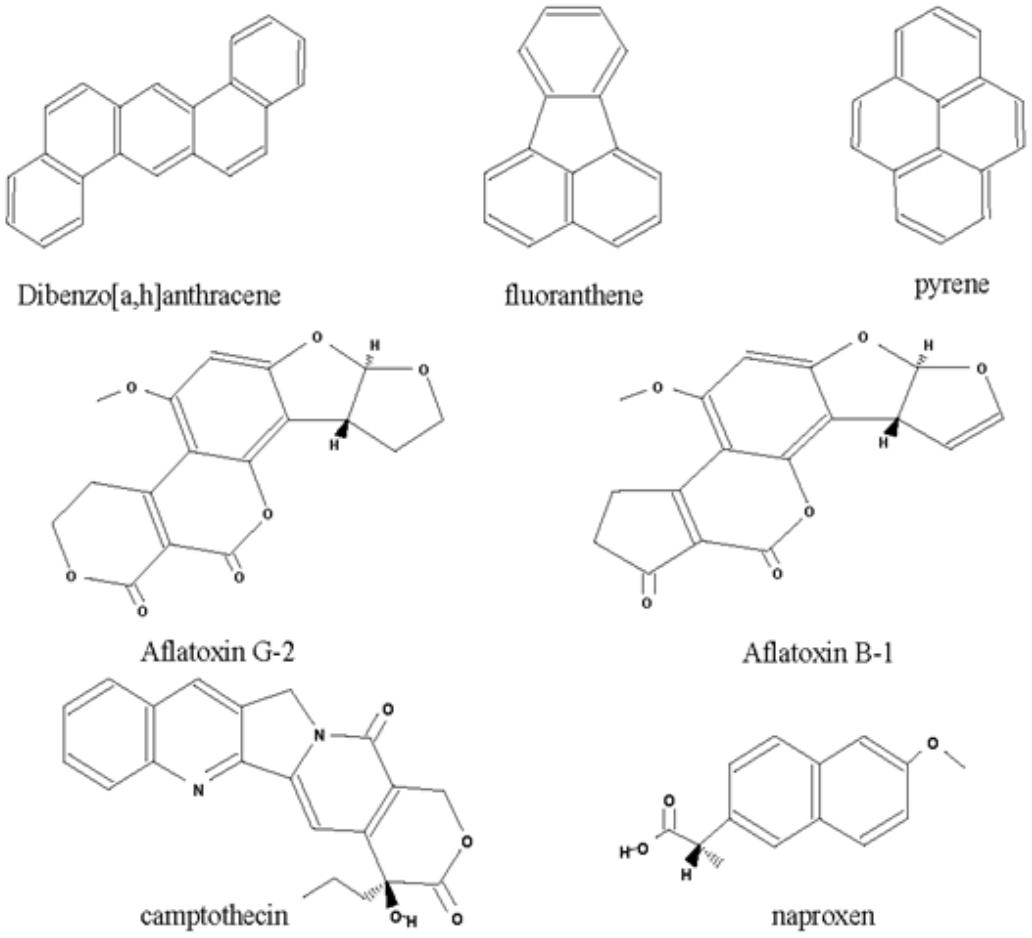


Figure 10. Target environmental contaminate chemical structures.

Analyte stock solutions were prepared in methanol at 0.1 mM concentrations and diluted with appropriate solvent. DBA stock solution was prepared in ethanol at 0.1 mM. In initial characterization of HPDECs analyte solutions were diluted from methanol stock solutions with deionized distilled water from a Barnstead 1800 (18 MΩ resistivity) to the desired concentration. All solutions were filtered through 0.2 μm cellulose acetate syringe filters purchased from Titan Filtration System. All solutions were degassed for 30 minutes prior to analysis. For experiments involving real sample matrices water was collected from both the Tennessee River and Second Creek in Knoxville, Tennessee. Collected samples were analyzed within 24 hours of collection. HPEDCs were purchased from 3M. A variety of dimensions are available with varying load volumes and membrane diameters. For this work the 1mL load volume cartridge with a 4mm effective membrane diameter was used. It is important to note this is the high density membrane cartridge with a reported sorbent mass of 4.2 mg silica. Polyimide capillary columns were purchased from Polymicro inc. and in each respective setup 75 μm id was used.

2.2.2 Apparatus

Two different apparatuses were built in the presented work. Common to both setups are the following. An Omnichrome 325nm He/Cd laser with line filter was used as the excitation source. Laser power was in the 20 mW range with power reading being taken daily with +/- 5% daily power deviation. In both setups F/1 lenses were used to focus and collect fluorescent signals. A cyan filter (Edmund optics) in conjunction with a baffle was used to filter background scatter. Fluorescence signal was focused using an F/1 plano-convex lens on to a RCA-1-P28 photomultiplier tube linked to a Pacific Precision Instruments photometer (Concord, California). Output voltage from the photometer was converted at 1Hz by a PMD-1208LS data acquisition

board with TracerDaq strip software (Measurement Computing; Middleboro, MA). The PMD-1208LS was configured in single-ended format using differential channels allowing for 12-bit resolution.

The hydrodynamic loading 90° LIF (HD-90°-LIF) setup is depicted in Figure 11. This setup was used to measure analyte recovery from the HPEDC when performing single component analysis. For multi-component analysis using the HPEDC a confocal LIF detection setup was built. The same optics were used in the confocal setup except the use of a two mirrors ,with the function of directing the fluorescent signal towards PMT, and a single F/1 lens was used in focusing the laser line and collection of fluorescent signal.

2.2.3 Methods

The HPEDCs were loaded with appropriate sample volumes 1ml at a time and centrifuged at 2000rpm using a Fischer Centrific model #228. The cartridge itself was placed in a BVD vial and capped. The vial was 10ml in volume, however only 5ml were loaded at a time so the solvent would not come in contact with the HPEDC. The eluting solvent was also passed through the HPEDC by centrifugation. As is noted later 50μL of extraction solvent was found to be optimal. Prior to extraction the loaded HPEDC would be transferred to a new BVD vial and eluting solvent would be pipetted into the cartridge then centrifuged. The 50μL methanol containing extracted analyte would be transferred to a modified vial that had been cut and sized for a volume of roughly 100μL. This sample holder as it were was simply raised to the capillary inlet until the inlet itself was barley submerged in the solvent. Capillary action drove the liquid as high as a window that had been created by burning the polyimide coating from the capillary.

As can be seen in Figure 11 the LIF detection was in a 90° collection alignment and fluorescent signals were recorded.

With regards to the 90° LIF setup the capillary was rinsed with a series of methanol solutions until the initial baseline reading was reached. This process avoided any carry over of contamination between recorded measurements and in most cases a single rinse is all that was needed. When electrophoretic separations were performed the columns were initially conditioned in the subsequent manner: 1M NaOH rinse for 10 minutes; 1mM NaOH rinse for 10 minutes; DI water rinse for 5 minutes, mobile phase rinse for 10 minutes. Between separation runs columns were flushed with 1mM NaOH for 10 minutes, DI water for 10 minutes, and buffer solution for five minutes. Prior to separation runs the system was allowed to equilibrate for 10 minutes at 5kV before the separation voltage of 11.4 kV was applied. The separation voltage was applied for 5 minutes before injection. Injections were made hydrostatically. The sample vial was simply placed 10mm above the inlet vial. The inlet of the capillary was placed in the sample vial for 10 seconds and immediately returned to the inlet vial. The separation voltage was then applied. Initial separations modeled on a Hewlett Packard-3D-Capillary Electrophoresis (HP-3D-CE) instrument used a background solution consisting of 25mM sodium dodecyl sulfate, 12.5mM borate anion, 10% methanol. Other experiments used the same background solution with the addition of 10 mM β-Cyclodextrin where noted.

2.3 Results and Discussion

2.3.1 Characterization of High Performance Extraction Disc Cartridge

The HPEDCs were initially characterized by the analysis of extracts using the experimental setup depicted in Figure 11. Different organic solvents as well as varying extraction volumes of said solvents were tested for optimum analyte recovery with greater than 90% recovery of a 100 fold concentration as a suitable benchmark. Steps involving sample clean-up by way of extra rinse steps of the HPEDC sorbent disc were not incorporated in lieu of speed and cost. When dealing with a clean sample matrix 'clean up' steps are unnecessary[84] and gauging the HPEDC pre-filter's ability to provide such clean-up was sought. The compounds selected in this study showed sufficient fluorescence when excited by the He/Cd laser at 325nm. Calibrations were performed to determine the feasibility of testing in the desired ppb concentration range while maintaining signal to noise ratios of at least 3 to 1 with selected solvents. The selected compounds were spiked into deionized water at nanomolar concentrations and subjected to a 100 fold concentration followed by elution with a varying volume and composition of eluting solvent. A 50 µL extraction volume of HPLC grade methanol was determined to give more than adequate results except when eluting the most nonpolar analyte in the study dibenzo[a,h]anthracene (DBA).

In table 1 the results of 100 fold concentration of analytes in deionized water with methanol extraction are reported.

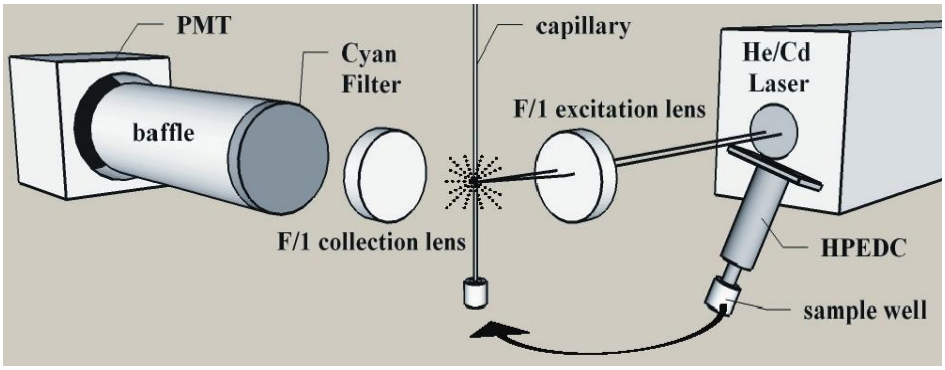


Figure 11. For 100 fold concentration enrichments 5 mL of spiked water is passed through the HPEDC followed by 50 µL organic solvent for subsequent extraction. The extracts were then analyzed using a platform utilizing hydrodynamic loading of solution with LIF Detection in a 90° geometry as shown.

Table 1. HPEDC Studies. All data was collected from the analysis of extracts using setup in Figure 11.

Analyte	Desired Con'n Factor	Achieved Con'n Factor AVG
pyrene	100	106
fluoranthene	100	103
Dibenzo[a,h] anthracene	100	86
aflatoxin G-2	100	105
aflatoxin B-1	100	92
camptothecin	100	98
naproxen	100	105
aflatoxin G-2	100	102
aflatoxin G-2	500	480
aflatoxin G-2	1000	822
aflatoxin G-2*	100	98
aflatoxin G-2**	100	103
aflatoxin G-2^	100	107
aflatoxin G-2^	500	289
aflatoxin G-2^^	100	24
aflatoxin G-2^^	500	194
Dibenzo[a,h] anthracene	100	see below

Table 1. Continued

	Achieved Con'n Factor	
Extraction Solvent	**AVG**	**RSD %**
MeOH	80	4.3
ACN	69	3.5
EtOH	96	1.0

a) All analytes were initially at ppb concentration levels prior to SPE.
b) **1:1 Bisphenol A(M):aflatoxin G-2 (M) **1:10000 Bisphenol A(M):aflatoxin G-2 (M).* Bisphenol A showed no response to the He/Cd 325nm laser line.
c) *real sample matrix: ^ Second Creek, Knoxville TN ^^ Tennessee River, Knoxville TN*.

The achieved concentration factor is simply the average of duplicate analyses. Slight variation in load volumes and elution volume contribute to the range of concentration factors as well as evaporation of methanol in the aliquot during the hydrodynamic loading LIF analysis. Kiss et al.[85] reported many factors related to SPE of PAH compounds from aqueous media among them the variability in analyte recovery when the sorbent bed was not relieved of water. The cartridge sorbent disc was not dried and trace amounts of water from initial loading steps could also have contributed to variability in analyte recovery.

Aflatoxin G-2 was chosen to further characterize the HPEDC in terms of capacity, load volume efficiency, and to gauge the HPEDC's performance when interrogating real sample matrices. 3M's HPEDCs come in varying sizes with regards to differing load volumes and associated sorbent masses. To characterize load volume efficiency concentrating factors of 100, 500, and 1000 with respective load volumes, 5, 25, and 50 ml, were analyzed. As seen before the 100 fold concentration step was successful as well as the 500 fold concentrating step with 102% and 96% average recoveries. The 1000 fold concentration step yielded an 82.2% average recovery which is less than ideal. To account for this lower recovery, capacity or the small unretained analyte concentration present in the 1 ml loading steps, which would not manifest itself except in large load volume profiles, was investigated. 3M states the sorbent mass of the high density cartridge is roughly 4 mg silica. Capacity can be estimated at 1-10% of the sorbent mass in most cases. Therefore it was determined the 1000 fold concentration step from nanolmolar range should not approach a capacity defined limit on loaded mass from a DI water matrix. To test this, as denoted by the * and ** in table 1, the aflatoxin G-2 was again concentrated a hundred fold in the presence of Bisphenol-A a common plasticizer and suspected

EDC. Bisphenol A was chosen because it showed no fluorescence response to the 325nm excitation line. Initially the aflatoxin G-2 and Bisphenol A were at 1:1 molar concentrations denoted as * in table 1. The recovered aflatoxin G-2/Bisphenol A aliquot showed the same response as the same concentration methanol standard aflatoxin G-2. The same 100 fold concentration step was performed again except the molar ratio of aflatoxin G-2 to Bisphenol A was changed to 1:10000. Again the 50 μL methanol extract showed the same response as the aflatoxin G-2 methanol standard.

This experiment did not represent the upper limit of the estimated capacity at 1% the sorbent mass. However it does show the ability of the sorbent phase to retain analyte in terms of mass is not what determines or rather contributes to inefficiency of 1000 fold concentration steps from the nanomolar range. Rather the inefficiency lies in the fact that although the retention factor is large in SPE, during loading there does exist a given equilibrium based on a variety of factors including analyte affinity for the stationary phase, composition of elution solvent, temperature, etc. Consequently a small percentage of analyte mass is unretained in each loading step. Once the stationary phase begins retaining analyte mass through the 1ml loading steps the stationary phase is also changing in the number and position of available adsorption sites. For this reasons less than ideal recovery percentages may be expected for large load volume profiles.

2.3.2 Real Sample Matrices Analysis using hydrodynamic-LIF analysis platform

Aflatoxin G-2 was spiked into water collected from both the Tennessee River and a tributary of the river, Second Creek, concentrated, and extracted from the HPEDC using methanol. Adequate recovery percentages with respect to 100 and 500 fold concentration steps

were obtained in previously mentioned work. Therefore these concentrating factors or rather respective recovery percentages were compared with extractions from real sample matrices. As seen in table 1 the Second Creek 100 fold concentration step was successful with 107% recovery while the 500 fold concentration yielded a 58% recovery. The concentration of aflatoxin G-2 in the Tennessee River samples was poor with 24% recovery for the 100 fold concentration and 39% recovery for the 500 fold concentration.

Poor recovery of analyte from a real sample matrix could be the result of a capacity limitation and/or the presence of a suppressive matrix effect. The former was investigated by the analysis of several different extractions from Second Creek water spiked with aflatoxin G-2 over several orders of magnitude concentration levels. A calibration plot was built (data not shown) that reflected a linear fit of calibration points with higher signal levels than prepared aflatoxin G-2 methanol standards. The real sample background levels were indeed higher but data from calibration standards also indicated a decrease in sensitivity due to a sample matrix effect.

To address whether this was an analyte dependent effect, Pyrene was spiked into the Second Creek Samples and subjected to the same regiment. Figure 12 shows both a linear fit of Pyrene methanol standards and a calibration fit based on methanol extracts from the spiked Second Creek samples. Easily seen is the fact the slope of the calibration fits are different and therefore ascertaining the HPEDC ability to concentrate target analytes from real sample matrices is precluded by the limiting matrix effect when comparing baseline subtracted signals of methanol standards and extracts. The ability to gauge the effectiveness of the HPEDC ability to concentrate and to clean-up polar interferences called for a separation capable of fractionating the interfering species from the target analytes.

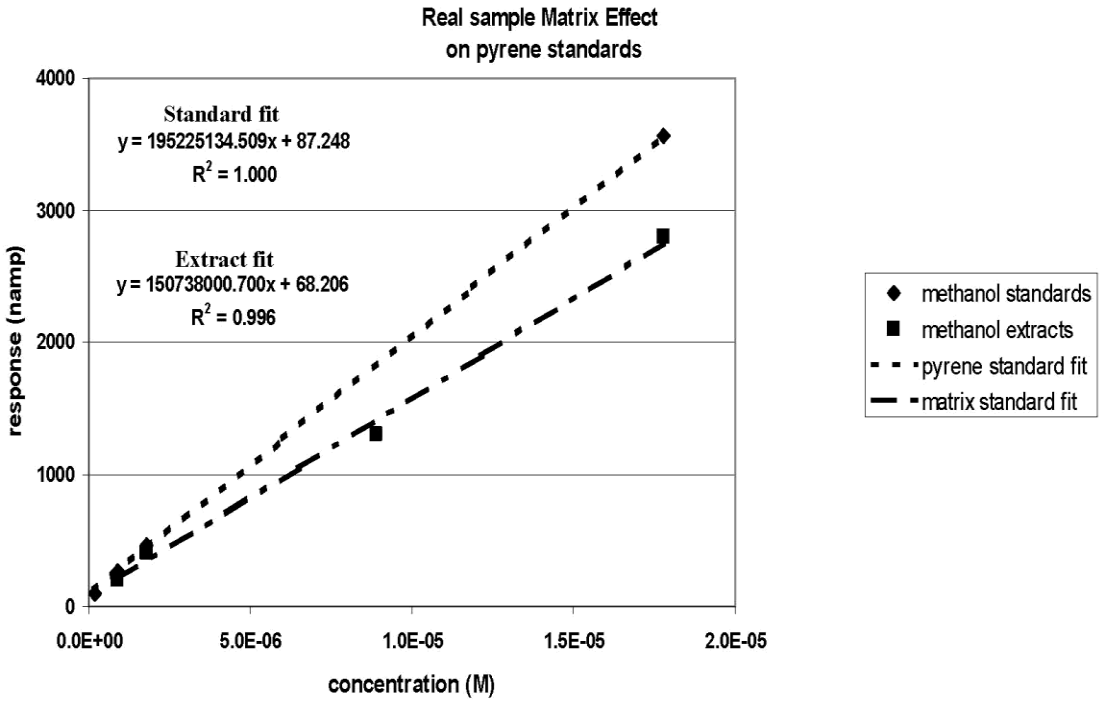


Figure 12. The above calibration data shows both a linear fit of Pyrene methanol standards and a calibration fit based on methanol extracts from spiked Second Creek samples over the same concentration range as the standards.

2.3.3 Reproducibility and Solvent Study

The conjugated five member ring structure of DBA (Figure 10) is retained by the C-18 sorbent phase more strongly requiring the need for a different extraction solvent. Table 1 shows both an elution solvent study and reproducibility data related to the loading and extraction of DBA using the HPEDC. The use of methanol as the eluting solvent shows an 80% recovery of DBA. It was anticipated acetonitrile would show better recovery, however, the recovery percentage was less than that of methanol with the acetonitrile recovery at 69%. Ethanol was the best solvent to elute the DBA with a 96% average recovery from the HPEDC. The RSD% is also presented in table 1 (n=5 for each solvent). The range of data related to the extraction solvent study is as follows: methanol 75-84, acetonitrile 66-71, and ethanol 94-97.

The fact methanol shows better recovery than the acetonitrile could be the result of a wetting issue and/or solvent polarity. Beyond wetting the C18 phase differently the solvents also interact with DBA differently. Sun et al.[86] actually reported the SPE recovery percentage of 16 PAH compounds was higher when using methanol over acetonitrile, with acetone being the best for their reported procedure. Polarity of given solvents can be reported as a dielectric constant, ε, with acetonitrile having a value of 38, methanol 33, and ethanol 25 at room temperature.[87] In the elution of DBA the molecule interacts with the solvent through van der Waals forces. Any type of hydrogen bonding interaction would not be present or substantial in the elution of such a non-polar analyte from the C18 disk. Methanol interacts with DBA more strongly than acetonitrile resulting in poor recovery percentages. The use of ethanol as the extracting solvent is the best choice for more non-polar species.

2.3.4 Analyte Mixture Separation Using CD-Modified/MEKC Platform

Initial separations modeled on a Hewlett Packard-3D-Capillary Electrophoresis (HP-3D-CE) instrument showed a buffer solution consisting of 25mM sodium dodecyl sulfate, 12.5mM borate anion, 10% methanol, could easily separate the pharmaceutical and aflatoxin compounds efficiently. It was unclear however whether adequate separation was taking place with the PAH compounds. A confocal LIF detection setup was built in house for the separation and detection of the selected compounds in the sub-ppm range. After optimization of the confocal detection setup separations were carried out using the same buffer system used in the HP-3D-CE work. All compounds save the PAH compounds eluted in under eight minutes and were resolved. The PAH compounds eluted with the micelles at 'tm'. Adding cyclodextrin to the buffer solution changed the selectivity of the system and reduced the excessive capacity factors so that the PAH compounds could be separated. The elution order of the three PAH compounds were determined to be pyrene, fluoranthene, and DBA. Pyrene and fluoranthene have the same molecular weight with differing chemical structure. Apparently the structure of Pyrene allows for better interaction with the hydrophobic β-CD cavity. As can be seen in Figure 13 the resolution between fluoranthene and DBA is roughly 1. Using a capillary with a slightly longer effective length would allow for more complete resolution under the reported experimental conditions. When changing the selectivity of the original buffering system with the addition of β-CD, naproxen and aflatoxin G-2 eluted at the same time. For this reason G-2 was not used in the extraction and separation study of analyte mixtures. Using the buffer system without CD would indeed separate these two compounds. It was decided the CD modified MEKC system would be studied.

Standards of the six component mixture were made in the mobile phase solution, injected, and separated. Peak height was used when producing calibration curves and for reporting reproducibility statistics. As seen in table 2 best fit lines and related correlation values are reported using four data points for each analyte. Reproducibility data is reported in table 2 based on 5 sequential injections of a standard mix. RSD percentages below 10% are reported and variability in peak height is most likely due to manual hydrodynamic loading of the capillary. The calibration data shows satisfactory correlation values. The DBA calibration was performed with only three data points. The highest concentration data point showed poor peak shape related to a solubility issue.

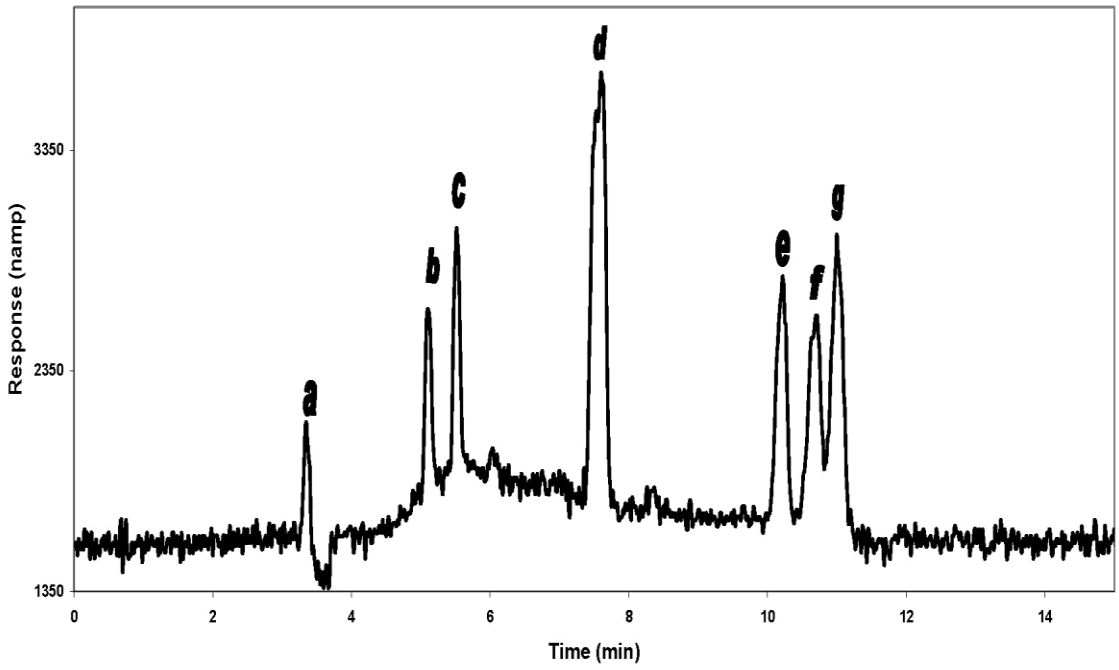


Figure 13. Separation of Second Creek Sample spiked with analytes at ppb concentration range prior to SPE. 75-fold concentration achieved Conditions: Applied Voltage-*220V/cm* Mobile Phase composition- *25mM SDS 10mM β-CD 12.5mM borate 10% MeOH* Effective Length-*24cm* Analytes: a-*methanol RI change* b-*camptothecin* c-*Naproxen* d-*aflatoxin B-1* e-*pyrene* f-*fluoranthene* g-*Dibenzo[a,h]anthracene.*

Table 2. Calibration data related to a real water sample and DI water sample.

peak #	Reproducibility Data		Calibration Data	
	AVG height	***RSD %***	***equation for the line***	R^2
1	5390.00	3.22	y = 5.00E+10x + 1096.50	1.00
2	5055.00	3.31	y = 3.00E+09x - 125.80	1.00
3	5428.00	4.94	y = 1.00E+10x + 516.70	1.00
4	5349.00	6.03	y = 2.00E+10x + 674.70	1.00
5	5123.00	5.39	y = 7.00E+09x + 519.20	1.00
6	7177.00	6.04	y = 1.00E+10x + 1043.60	1.00
1^	2475.40	2.98	y = 2.00E+10x – 1774.00	0.94
2^	2315.60	7.75	y = 5.00E+08x - 743.30	0.83
3^	*	*	y = 6.00E+09x + 3499.40	0.83
4^	2104.20	3.51	y = 3.00E+09x + 2595.70	0.72
5^	2095.60	3.09	y = 1.00E+09x + 2480.80	0.71
6^	2107.60	3.39	y = 4.00E+09x + 3980.40	0.67

a) ^ represents data recorded from six component analyte mixtures loaded and extracted from spiked Second Creek water subsequent to CD-MEKC-LIF analysis.

b) * designates aflatoxin B-1 which saturated the detector during calibration analysis.

c) N=4 for both the standard and real sample calibrations.

2.3.5 HPEDC Extract Analysis of Mixtures using CD-Modified/MEKC/LIF

Load volumes ten times the elution volume can be loaded across the HPEDC sorbent phase with greater than 95% retention with most analytes. A 75-fold enrichment of a real sample spiked with a six component analyte mixture was achieved. Efficient separations were achieved with plate counts ranging from 10^3 to 10^4 on a shortened 24 cm fused silica capillary. Using a He/Cd laser operating at 325 nm all analytes were easily detected in the ppb range except naproxen. Detection of Naproxen using a different lasing line would have provided greater sensitivity, however sufficient fluorescence is achieved with the 325nm excitation line that shows the usefulness of the developed techniques in the desired concentration range.

Six component mixtures were spiked into Second Creek water and concentrated onto the HPEDC sorbent phase.. A 15 mL volume of spiked Second Creek water was loaded onto the HPEDC followed by 50 μL methanol extraction. The 50 μL aliquot was then diluted with 150μL run buffer solution without methanol. This solution was then hydrodynamic injected into the capillary prior to separation and detection. Calibration plots and reproducibility statistics were as described above. As seen in table 2, peak #3, aflatoxin B-1, is not recorded because of detector saturation in the signal range used for this portion of the study. As seen with the serial injected standards RSD% values related to the five extracts are below 10%. The calibration data has an interesting trend. Peak #3 is included in this portion of the study where the concentration was adjusted as to range all peaks during a single separation run. Peak #1, Camptothecin, produced an adequate calibration plot with a correlation value of 0.944. Peaks 2 through 6 show a downward trend of correlation values with peak #6, DBA, being 0.6703. When examining the individual calibration plots (data not shown) the later eluting peaks show a consistent leveling

effect when concentrating into the upper ppm range. The injected extraction solution is different than the injected standard solution in composition. Where the standard consists of 10% methanol the extraction solution consists of 33% methanol. Apparently the discontinuity between the injected sample plug and running buffer creates adverse conditions at the higher concentrations of the calibration leading to non-linearity. However, working in the ppb concentration range and lower allows for better calibration data.

2.4 Conclusions

Several device and method optimization strategies involving the use of High Performance Extraction Disk Cartridges were realized with simple load and extraction schemes. Polar matrix interferences and undetectable concentration levels are eliminated by way of the reversed phase extraction technique that is vital to obtaining detectable concentrations of the target environmental contaminates in the parts per billion range. The presented CD-MEKC separation method allowed for adequate separation and detection of multi-component extracts. Analytes spiked into real sample matrices at ppb levels were easily separated and detected. Moreover, the presented techniques are useful in the analysis of aqueous samples whether that be effluent waste water, potable water, or other aqueous samples. The methods presented here could easily be extended to microfluidic platforms with little to no optimization for on-site testing.

Chapter 3

Direct Sampling and Analysis from Solid Phase Extraction Cards using an Automated Liquid Extraction Surface Analysis Nanoelectrospray Mass Spectrometry System

3.1 Introduction

Direct sampling and ionization of materials present on surfaces under ambient conditions is an expanding area of research and application in mass spectrometry (MS).[5,88,89,90,91,92,93,94,95,96] These ambient ionization methods for surface sampling have been defined by some to include only those techniques that require no or minimal sample pre-treatment. However, sample preparation knowledge would dictate,[97] and several recent ambient surface sampling/ionization studies have clearly illustrated[98,99,100,101,102] that, under certain analysis scenarios, the use of sample preparation methods prior to analysis greatly improve the analytical figures of merit. One such sample preparation scheme that has been used with these techniques is solid phase extraction (SPE).[103] For example, Takáts' group[47] showed that a SPE-based sample preparation scheme improved the overall detection level for desorption electrospray ionization (DESI) by up to six orders of magnitude. More relevant to the current report, Wachs and Henion[12] demonstrated the automated quantitative analysis of analytes captured with a conventional 96-well format SPE plate using a continuous flow surface sampling probe.

Direct liquid extraction based surface sampling probes, such as those originally used by Wachs and Henion[12] and similar or alternative versions used by us and others,[8,13,104,15,16,105,106,107] are well-suited to take advantage of sample concentration and cleanup by SPE. These liquid

extraction probes use a simple, well-controlled, solid-liquid extraction mechanism to extract or reconstitute materials from a surface. The resulting extract is transported to a liquid introduction ionization source like electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), and the constituent species in the extract are ionized and subsequently mass analyzed. The droplet sampling mode that has been implemented with both continuous flow[108] and autonomous pipette[32,34] versions of these liquid extraction surface sampling probes is expected to be particularly useful for the analysis of SPE surfaces. In this sampling mode, the sample end of a probe is placed about 0.2 mm above the surface, a small volume droplet (e.g., 1-2 µL or less) is dispensed to contact the surface, while remaining attached to the probe. After an appropriate extraction time the droplet is aspirated back into the probe and the solution is directly analyzed[32,34] or further processed using, for example, HPLC.[38]

The autonomous pipette droplet sampling mode of operation has recently been implemented as the Liquid Extraction Surface Analysis (LESA) mode on the commercially available TriVersa NanoMate® chip-based infusion nanoelectrospray ionization (nanoESI) system.[109,110] Herein, we demonstrate the use of the TriVersa NanoMate® (and Nanomate 100®) in LESA mode for the read-out of a custom, planar SPE card utilizing a monolithic reversed-phase capture phase in a 96-well microtiter plate-like format. Characterization of the SPE capture card and the optimization of LESA extraction parameters, including extraction/nanoESI solvent composition, solvent volume, and extraction times, are discussed. Quantitative and linear response for the system is demonstrated using the drug propranolol with propranolol-d7 as an internal standard. The analytical utility of this analysis approach is further demonstrated by

the cleanup of a NaCl containing sample of the peptide Angiotensin II and the capture and identification of herbicides in a multicomponent herbicide mixture at ppb concentration levels.

3.2Experimental

3.2.1 Materials

LC-MS grade acetonitrile, methanol and water with and without 0.1% formic acid (v/v) (Chromosolv Sigma Aldrich, St Louis, MS, USA) were used for all extraction/nanoESI experiments and for SPE card well conditioning. HPLC grade water and methanol (J.T. Baker, Phillipsburg, NJ, USA) were used for making standard solutions. Angiotensin II, ammonium acetate and sodium chloride were obtained from Sigma Aldrich. Propranolol hydrochloride (Acros Organics, Morris Plain, NJ, USA) and 99.2% pure propranolol-d7 (TLC PharmaChem., Inc., Concord, Ontario, Canada) were obtained commercially and used without further purification. The herbicides alachlor [2-Chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide], triallate [S-(2,3,3-trichloroprop-2-enyl) N,N-di(propan-2-yl)] and ramrod [2-Chloro-N-isopropylacetanilide] were obtained from PolyScience Corporation (Niles, Illinois, USA). All stock standard solutions were made in methanol at millimolar concentrations then diluted with methanol or water to appropriate concentrations prior to analysis. μFocus MALDI plates were purchased from Hudson Surface Technology (Fort Lee, NJ, USA).

3.2.2 Multi-Well Solid Phase Extraction Card

The SPE cards used in this work were made in planar 96 well microtiter plate format. The 1 and 2 mm diameter SPE capture wells were made from a C-8 chromatographic media immobilized in a hydrophobic monolithic polymer. 2 mm diameter wells were used in the

linearity and reproducibility studies performed with propranolol. 1 mm diameter wells were used in the peptide and herbicide analyses. The card wells were conditioned, loaded, and washed per conventional SPE protocols using a modified Millipore vacuum manifold. Photographs of the SPE cards and the manifold device as well as a description of its assembly and use are detailed in Figure 14.

Figure 14. A) A SPE Card is shown with two Teflon gaskets. The SPE card wells consist of C-8 chromatographic media in a hydrophobic monolithic polymer. The SPE card is sandwiched between the gaskets such that each well is isolated from one another. B) The SPE card sandwiched between the gaskets is shown in the middle while a steal casing, both a bottom and top section, is shown on either side. Again the SPE card with Teflon gaskets was sandwiched in between these metal holders and tightened with both clips and 4 bolts with locking nuts.

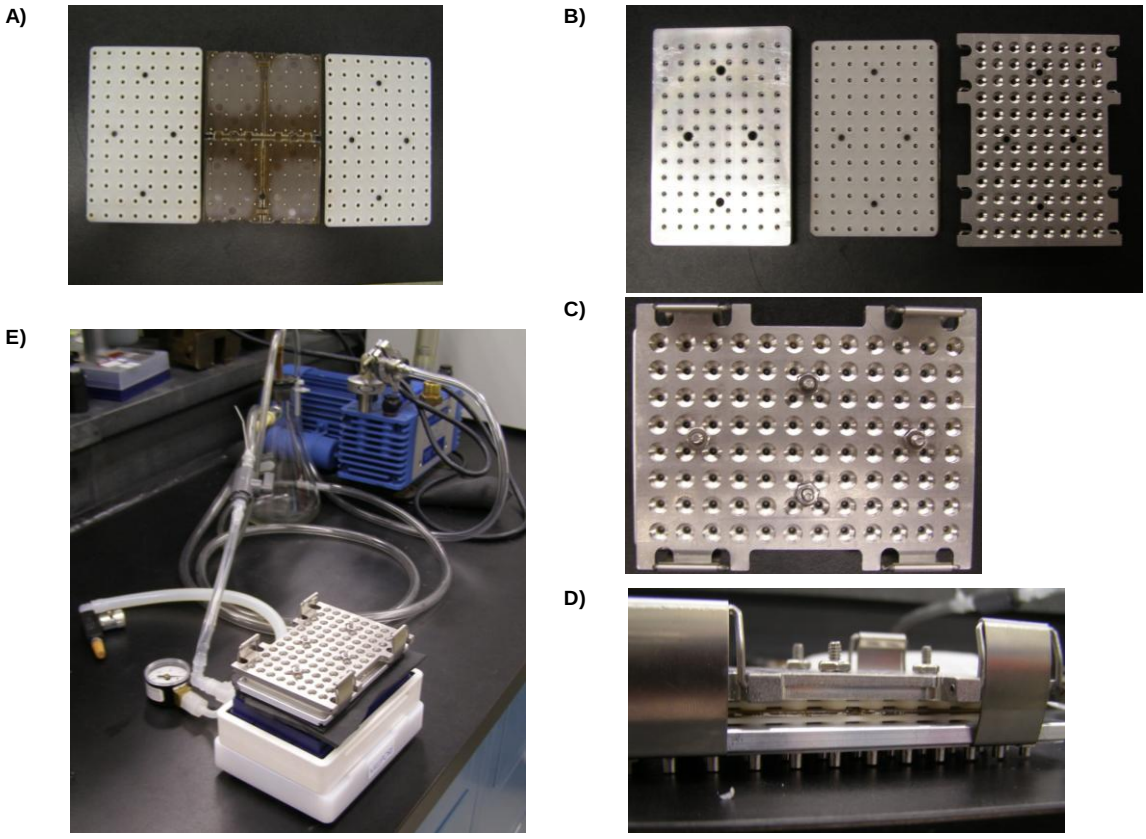


Figure 14. C) A top view image of the entire holder with SPE card. D) Cross section view of entire SPE card holder that shows each layer of the device. The top steel holder was manufactured with wells that could hold up to 200 µL of solvent at a time. E) The SPE card holder is positioned on a Millipore vacuum manifold. This was used to aspirate solvents through the SPE card wells. A rubber gasket between the SPE card holder and manifold allowed sufficient vacuum pressure on the 96 wells to pull solvent through to a waste reservoir. The vacuum pull was measured at 20 in Hg. Immediately before loading, the extraction phase was conditioned with 50 µL LC/MS grade methanol, followed by 100 µL LC/MS grade water. The extraction phase was then loaded with HPLC grade water with analyte(s) spiked in at indicated concentration levels. In some experiments an additional rinse of HPLC grade water was used.

3.2.3 Automated Liquid Extraction Surface Analysis

All SPE cards were analyzed using either a NanoMate 100® (Advion BioSciences, Inc. Ithaca, NY, USA) coupled to a 4000 QTRAP® hybrid triple quadrupole/linear ion trap mass spectrometer (MDS SCIEX, Concord, Ontario, Canada) or a TriVersa NanoMate® (Advion BioSciences) system coupled to a LTQ XL linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA). A nanoESI voltage of 1.61 kV and gas pressure of 0.6 psi was applied in all experiments. Customized robotic arm (mandrel) movements and liquid handling for surface analysis was set up in the LESA panel of the ChipSoftManager control software for the NanoMate 100®/TriVersa NanoMate®. For all experiments, the mandrel was programmed to acquire a pipette tip, aspirate 1.7 or 2.0 µL, (1.7 µL for 1 mm dia. SPE card wells and 2.0 µL for 2 mm dia. SPE card wells) of extraction/nanoESI solvent from a solvent reservoir, and move to within 0.2 – 0.4 mm above selected SPE card wells. For the 2 mm diameter SPE card wells, 1.3 µL of solvent was dispensed, and for 1 mm diameter wells 0.7 µL was dispensed. The SPE wells were extracted for 5 to 40 seconds as noted. The mandrel was programmed to lower the tip to 0.2 mm below the dispense height and aspirate the extraction solvent back into the tip. The aspirated solvent volume was set as 0.4 µL more than the dispensed volume to ensure maximum extract pickup. The pipette tip was then programmed to engage with the nanoESI chip. Analyte detection in selected reaction monitoring (SRM) mode on the 4000 QTRAP® was optimized by direct infusion of 1 µM analyte standards. Figure 15 shows the structures and the monitored precursor ions for the compounds of interest.

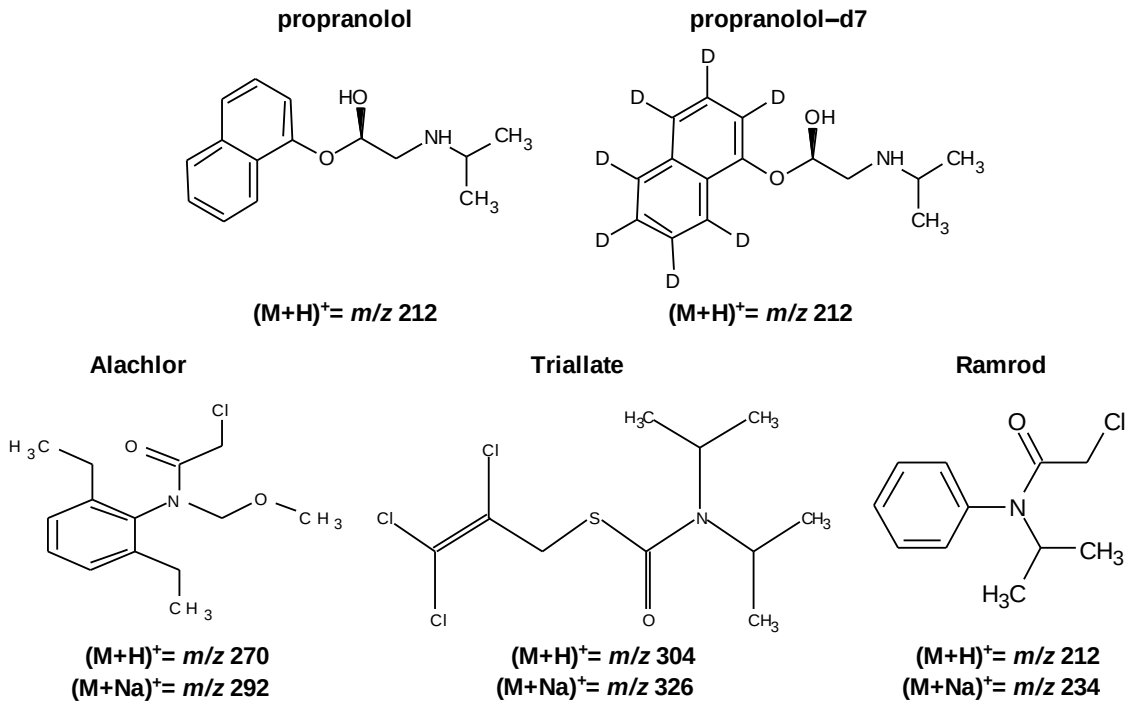


Figure 15. Structure and mass-to-charge ratio observed for the compounds investigated.

Two SRM transitions for propranolol (*m/z* 260.1 → 183.1, 116.0) and propranolol-d7 (*m/z* 267.1 → 189.1, 116.0) were monitored using a 50 ms dwell time and a collision energy (CE) of 27 eV. Using the LTQ, full scan (*m/z* 100-1000) and MS^n data were recorded with automatic gain control (ACG) on. For MS^n experiments using the LTQ, a normalized collision energy of 35% was used with a *m/z* 1.5 isolation width for each experiment.

3.3 Results and Discussion

3.3.1 Solid Phase Extraction Card.

The 96 well formatted card used for SPE in this work was originally developed as a capture cartridge slide for peptides and proteins separated and enriched using gel electrophoresis.[111] Hydrophobic monolith media were formed in 1 or 2 mm diameter through-holes in a 1 mm thick microtiter plate size polymer slide for the retention of target analytes. These capture cartridge slides had not been designed, tested, or otherwise optimized for SPE. The use of monoliths in SPE is not common, but they have been used successfully for similar applications.[112] To use these particular cards for SPE, they were positioned on a conventional SPE plate vacuum manifold that allowed up to 200 µL of solution to be loaded in the wells at one time in a serial or parallel format.

3.3.2 Extraction Optimization and Quantitation of Linearity and Reproducibility

Figure 16 shows a cartoon depiction of the LESA process.

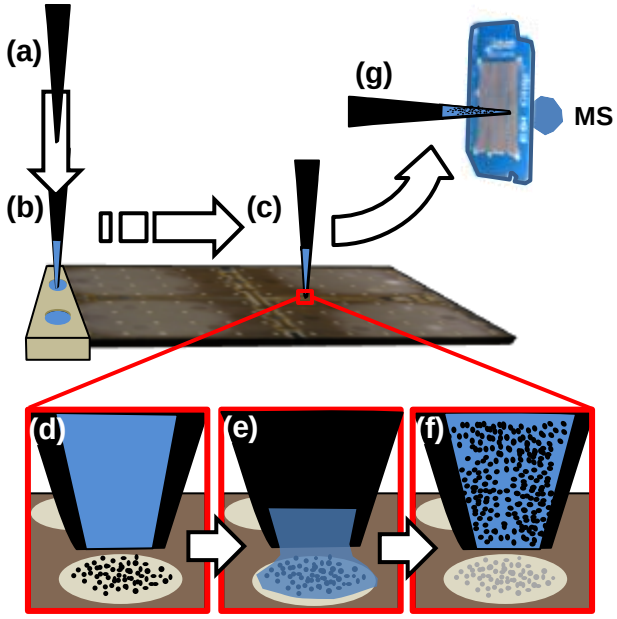


Figure 16. Scheme illustrating individual steps of LESA process. (a) TriVersa NanoMate robot mandrel picks up pipette tip. (b) Mandrel moves tip to extraction solvent reservoir and aspirates extraction solvent into tip. (c) Mandrel moves tip to position above one of the 96 wells on the SPE card. (d) Tip is moved to within approximately 0.2 mm above the well spot. (e) Extraction solvent (0.7 – 1.3 µL) is dispensed and a liquid microjunction is formed between the tip and the SPE well. (f) After a set time (1 - 40 s) the extraction solvent is aspirated back into the tip. (g) Mandrel moves tip to microchip interface and extract is directly electrosprayed.

Propranolol extraction was used to optimize the LESA technique for sampling 2 mm diameter SPE card wells. Wells were conditioned with methanol before loading 100 ng of propranolol in water. For this analyte, a 20 s extraction time produced a higher mass spectral signal for propranolol (SRM, *m/z* 260 → 116) than either 5 or 10 s, but longer extraction times did not dramatically increase response. Composition of the extracting/nanoESI solution had an impact on both extraction efficiency and ionization efficiency, and likely varies for different compounds of interest. For propranolol, methanol/water and acetonitrile/water combinations with formic acid were investigated. An extraction solution of 80/20/0.1 methanol/water/formic acid (v/v/v) gave the highest analyte response. The use of higher concentrations of organic solvent made it more difficult to maintain a liquid microjunction with the SPE well.

The propranolol SRM signal abundance data plotted in Figure 17 was obtained from six consecutive 20 s extractions and analyses from the same well that had been loaded with 100 ng of propranolol. New pipette tips and nanoESI nozzles were used for each individual extraction/analysis eliminating potential sample carryover. The first extraction resulted in the highest propranolol signal, but the extraction was not 100% efficient. A second extraction resulting in a signal just 30% as intense as the first with negligible signal observed after the fourth extraction.

A calibration was performed using extractions from SPE card wells which had been conditioned before being loaded with 100 µL aliquots of solutions of varying concentrations of propranolol (1 – 1000 ng/ml) and 500 ng/mL propranolol-d7 as an internal standard. The SPE card wells were sampled using 80/20/0.1 methanol/water/formic acid (v/v/v) for 20 s. The mass spectral response ratio for the drug and internal standard (SRM: propranolol, *m/z* 260 → 116,

propranolol-d7, *m/z* 267 → 116) plotted versus the loaded mass of propranolol is shown in Figure 18.

This calibration plot shows the signal response ratio to be reproducible and linear up to 10 ng loaded. The response ratio begins to show a less than linear response at 100 ng loaded. The RSD values for the replicate analyses ranged from 0.22% to 6.8%. Using the linear part of the calibration curve, from 0.1 – 10 ng propranolol loaded, a statistical analysis of the data estimated the lower limit of detection (LOD) and lower limit of quantitation (LLOQ) as 0.10 ng and 0.35 ng loaded, respectively.[113] Without an internal standard, signal from the SPE well sampling experiments also showed linear responses to changes in propranolol concentration, but RSD percentages were greater than $\geq$30% in the best cases.

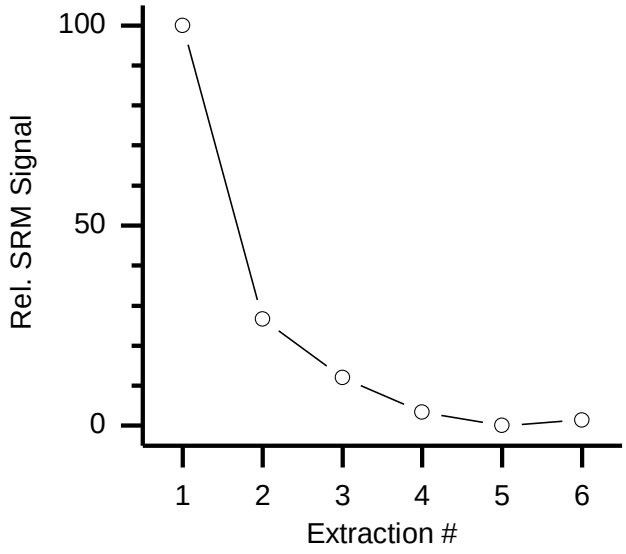


Figure 17. The relative SRM signal level for propranolol (*m/z* 260 → 116, CE 27 eV) was recorded from each of six consecutive extractions of a single 2 mm diameter SPE well card that had been conditioned and loaded with 100 ng propranolol. 2.0 µL of extraction solvent composed of 80/20/0.1 methanol/water/formic acid (v/v/v) was aspirated into a tip. The tip was directed to an SPE card well where 1.3 µL of that solution was dispensed. After 20 s the extraction solvent was re-aspirated into the tip and subjected to nanoESI for two minutes using a NanoMate 100 coupled to a 4000 QTRAP.

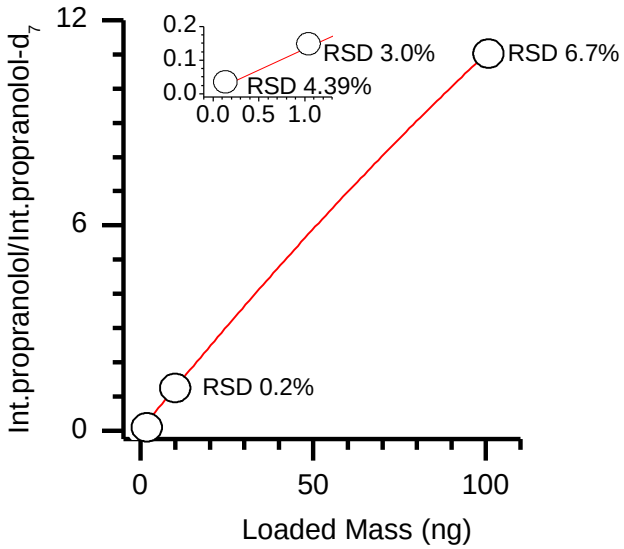


Figure 18. Average of the ratio of the integrated SRM signal of propranolol (1 – 1000 ng/ml) and that of propranolol-d7 (500 ng/mL) (Avg. Intensity$_{propranolol}$/Avg. Intensity$_{propranolo-d7}$) as a function of the loaded mass of propranolol ($c_{propranolol}$) in the solution loaded (100 µL) onto the SPE card spot analyzed. The data from an average of 3 replicates at each sample concentration were analyzed using a 2nd order polynomial regression with no weighting and fit the model of Avg.Intensity$_{propranolol}$/Avg. Intensity$_{propranolo-d7}$= 0.0126cpropranolol – 1.55x10^{-6}($c_{propranolol}$)^2. %RSD of the Avg. Intensity$_{propranolol}$/Avg.Intensity$_{propranolo-d7}$ for the replicates at each $c_{propranolol}$ is shown in the graph. A NanoMate 100 in LESA mode coupled to a 4000qtrap was used to analyze the SPE card extracts.

3.3.3 Combined Capture and Extraction Efficiency

To estimate a combined SPE/LESA capture/extraction efficiency, signal level comparisons were made between direct infusions, extractions from a stainless steel MALDI plate, and extractions from a capture card using propranolol as a test analyte. Detection was again performed using SRM on a triple quadrupole. A calibration was performed via direct infusion using propranolol solution standards (1 - 1000 ng/mL) in 80/20/0.1 methanol/water/formic acid (v/v/v). An equation for instrumental response was generated based on the plots of these direct infusion signal levels. This equation was used to determine expected signal values for 100% extraction efficiency from the SPE wells for the entire loaded mass of propranolol from each well into the given extraction volume.

To determine a "best case scenario" LESA extraction efficiency, a commercially available μFocus MALDI plate was prepared with multiple replicate spots of between 1 – 1000 pg of propranolol by depositing 1 μL solution volumes of various propranolol concentrations (80/20/0.1 methanol/water/formic acid (v/v/v)). The analyte spots in this case were on a nonporous surface and significantly smaller in size than the liquid microjunction providing the possibility that all material could be contacted by the solvent, reconstituted and extracted from the surface. For this analysis, 0.7 μL of solvent from a total of 1.7 μL in the pipette tip was dispensed onto a spot and after 10 s aspirated back into the tip. Figure 19 (a) shows the averaged data (n=5), linear regression, and %RSD values for each analysis.

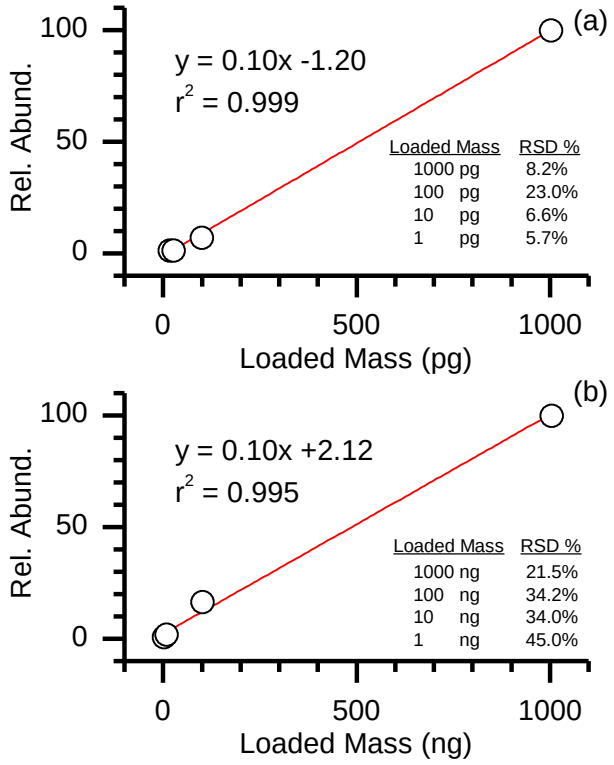


Figure 19. (a) µFocus MALDI plates were spotted with 1 µL volumes of propranolol in 80/20/0.1methanol/water/formic acid (v/v/v) and left to dry such that 1 – 1000 picograms was deposited. The plate was then read out using LESA. 1.7 µL of 80/20/0.1 methanol/water/formic acid (v/v/v) was aspirated into the pipette tip, 0.7 µL was deposited on the µFocus MALDI spot and allowed to dwell for ten seconds before the solvent was then aspirated into the pipette tip and analyzed using a 4000 QTRAP mass spectrometer in SRM mode. The fitted line is shown with no weighting and corresponding RSD percentages, n=5, for each analysis. The LOD for this analysis is a conservative ~0.4 ng of propranolol. (b) SPE card wells were loaded with 1 – 1000 ng propranolol. These wells were then read out using LESA using a 1.3 µL extraction solvent volume (2.0 µL initial aspiration

) and 40 second dwell time. The fitted line is shown with no weighting, and corresponding RSD percentages, n=5, for each analysis.

Statistical analysis of data[113] estimated the LOD for this analysis as 0.4 ng of propranolol spotted. The LOD for this analysis was higher than the propranolol analysis with the SPE card using an internal standard. This is a direct result of higher RSD values, which ranged from 5.7% – 23%, when using the raw propranolol signals only. The estimate capture/extraction efficiency (capture is assumed to be 100% in this case) determined from these data ranged from 61% – 78% (Table 3).

To estimate the combined capture/extraction efficiency from the SPE card wells, multiple replicate wells were conditioned and loaded with 1 – 1000 ng propranolol. Wells were read out using the same LESA conditions as for the μFocus MALDI plate analysis except extraction volumes of 1.3 μL (2.0 μL initial aspiration) were used due to the larger sampling area of the 2 mm diameter SPE card well. Figure 19 (b) shows the signal, and a calibration for the loaded masses. These data, as well as the corresponding data from direct infusions, are reported in Table 3. With the SPE card, the combined capture/extraction efficiency, into 2 μL, ranged from 0.64% to 0.80%. The highest efficiency was seen in the case of the lowest loaded mass (i.e., 1 ng) and the lowest efficiency in the case of highest mass loading (i.e., 100 ng load). These efficiencies can also be compared to a 10% extraction efficiency determined for the LESA analysis of 10 ng of propranolol deposited and dried on the SPE card surface, rather than loaded onto a conditioned well using the vacuum manifold. While 10% efficiency is lower than the efficiencies seen with the MALDI plate, it is over an order of magnitude higher than that of the conventionally loaded sample, indicating ineffective analyte capture or potentially ineffective extraction form the entirety of the SPE well monolith volume.

Table 3 Estimated combined Capture/Extraction Efficiencies.

Surface	Loaded Mass (ng)	LESA Response (cps)*	Expected Value** (cps)	Capture/Extraction Efficiency (%)
MALDI Plate	0.1	8.74E+04	1.41E+05	62.0
MALDI Plate	0.01	8.48E+03	1.39E+04	61.0
MALDI Plate	0.001	1.07E+03	1.38E+03	77.5
SPE Card	1	8.77E+03	1.14E+06	0.8
SPE Card	10	7.40E+04	1.15E+07	0.6
SPE Card	100	6.20E+05	1.15E+08	0.5

* Recorded using NanoMate 100® coupled to 4000 QTRAP® mass spectrometer in SRM mode.

** Based on 100% extraction efficiency into 1.7 µL volume for MALDI plate and 2.0 µL volume for SPE card.

The extraction media was designed for the capture and release of intact proteins and may not be as suitable for small molecule applications. The effectiveness of LESA to sample the entirety of the SPE card well depth would also dictate recovery percentages. Regardless, the ability of LESA to sample the conventionally loaded SPE card wells using only 1 – 2 µL of solvent allowed analysis of low nanogram masses of propranolol loaded.

3.3.4 Application to Peptides

Angiotensin II was chosen as a model analyte to demonstrate the use of these SPE well cards for sample cleanup. Separate 1 µL aliquots of a 5 µM angiotensin II solution containing 5 mM NaCl was spotted on two different 1 mm diameter SPE wells. One of the wells was immediately analyzed, while the second well was washed with 100 µL water to remove the salt from the sample before analysis. In each case, 80/20/0.1 methanol/water/formic acid (v/vv) was used for extraction and nanoESI. The full scan mass spectrum in Figure 20 (a) obtained from the unwashed well showed little discernable signal for angiotensin II. In the full scan mass spectrum from the washed sample, shown in Figure 20 (b), signals from both doubly and triply charged angiotensin II were clearly observed at roughly 10 times the intensity of the same peaks in Figure 20 (a). These results demonstrate the sample cleanup utility of LESA from an SPE phase.

3.3.5 Application to Herbicides

The EPA and other regulatory agencies across the world mandate 0.1 - 1.0 ppb detection levels for most environmental contaminants.[114,115] To demonstrate qualitative detection of low level environmental contaminants, we examined an herbicide mixture of ramrod, triallate and

alachlor prepared at the part per billion level in water. A comparison between direct nanoESI infusion and LESA from a SPE well was made. A 200 µL mixture of the herbicides at 2 ppb each was loaded onto a 1 mm diameter SPE card well. For these experiments, neither conditioning nor rinse steps after loading were performed. Full scan, MS/MS and MS^3 product ion spectra focusing on the protonated molecule of each of the herbicides were recorded for a LESA experiment.

Figure 21 (a) shows the full scan mass spectrum from the direct infusion of a 200 ppb reference solution of the herbicide mixture in 80/20/0.1 (v/v/v) methanol/water/ammonium acetate via nanoESI. Each herbicide was observed both as protonated and sodiated molecules. Figures 21 (b) and 21 (c) are full scan spectra of a 2 ppb dilution of this reference solution loaded and extracted from an SPE card and directly infused, respectively. In both (b) and (c) the peaks of interest, identified in (a), are masked by other ions. Figures 21 (d)-f show the MS^3 spectrum for the fragmentation of alachlor (*m/z* 270 → *m/z* 238 → ○) from the 200 ppb direct infusion, 2 ppb SPE card extract, and 2 ppb direct infusion. MS/MS product ion spectrum targeting the protonated alachlor (data not shown) was substantially different from that of the reference spectra indicating isobaric overlap of one or more other compounds extracted from the capture monolith. However, the MS^3 product ion spectrum of the alachlor protonated molecule (*m/z* 270 → *m/z* 238 → ○) shown in Figure 21 (e) matches well with that from the standard infusion spectrum Figure 20 (d). Figure 22 shows that the MS^3 spectra of the other two herbicides extracted from the capture card wells also match the MS^3 spectra from infusion of herbicide standard mixture.

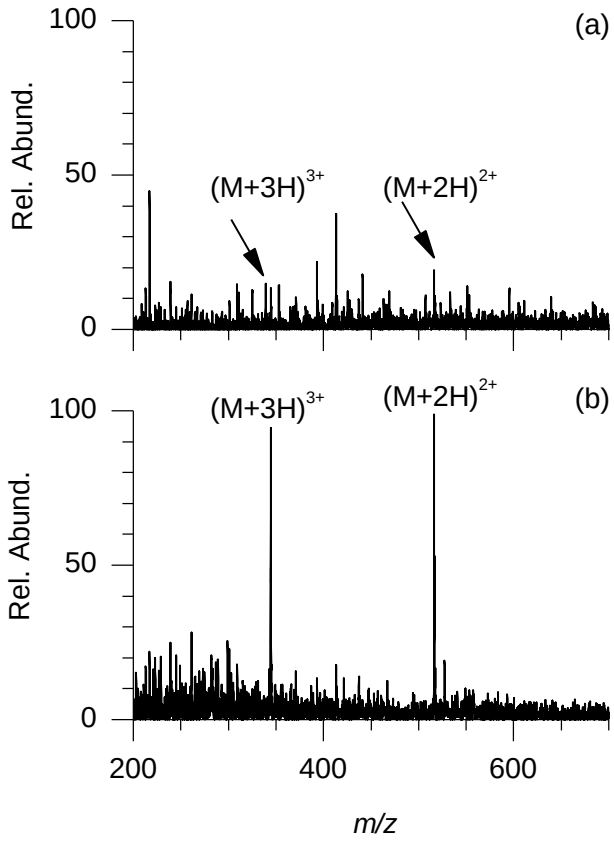


Figure 20. Angiotensin II from a solution (1 µL of 5 µM (5.9 ng)) containing 5 mM NaCl was spotted on separate 1 mm diameter SPE card wells. The full scan mass spectra shown were obtained by (a) directly analyzing from one of the wells, and by (b) analyzing the well after washing it with 100 µL of water to the remove salt. Ion signals in spectra (a) and (b) were normalized to the $(M+2H)^{2+}$ ion signal level observed in the spectrum in panel (b). A TriVersa NanoMate coupled with a Thermo LTQ-XL directly nano electrosprayed the LESA extracts. The wells were extracted for 40 seconds. A 1.7µL aliquot of 80/20/0.1 methanol/water/formic acid was used for extraction/nanoESI.

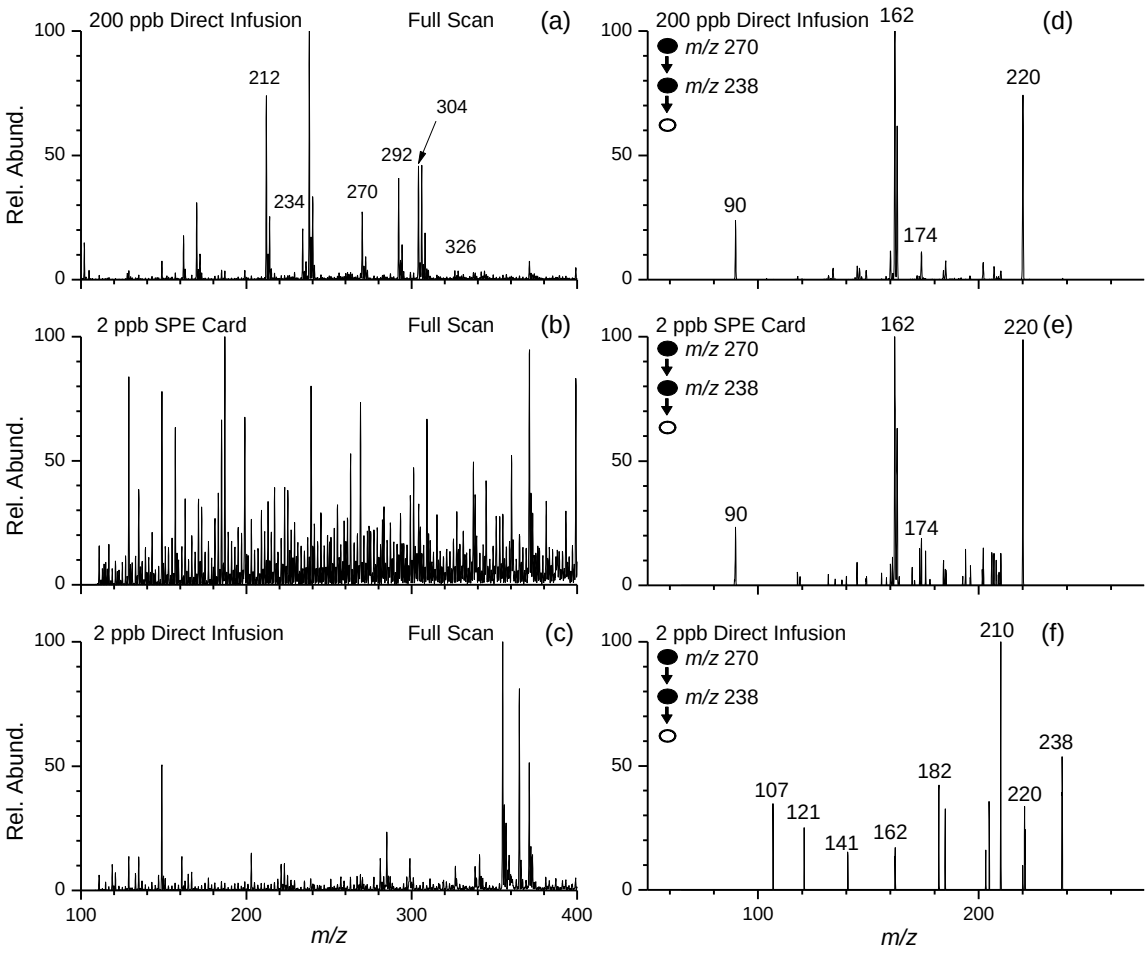


Figure 21. The analysis of a herbicide mixture at 2 ppb using the SPE card read out of 1 mm diameter wells with LESA is shown. For comparison, full scan mass spectra are shown from the herbicide mixture (a) directly infused at 200 ppb, (b) from the extraction off an SPE card loaded with 200 µL of a 2 ppb solution, and (c) directly infused at 2 ppb. MS^3 product ion spectra are shown of alachlor (*m/z* 270 → m/z 238 → ○) from the (a) 200 ppb direct infusion, (e) 2 ppb SPE card extract, and (f) direct infusion of 2 ppb mixture. Extraction/nanoESI solution was composed of 80/20/0.1 (v/v/v) methanol/water/ammonium acetate. In each case, a TriVersa NanoMate coupled with a Thermo LTQ-XL directly sprayed for two minutes. For the LESA experiment, an extraction time of 40 s was used.

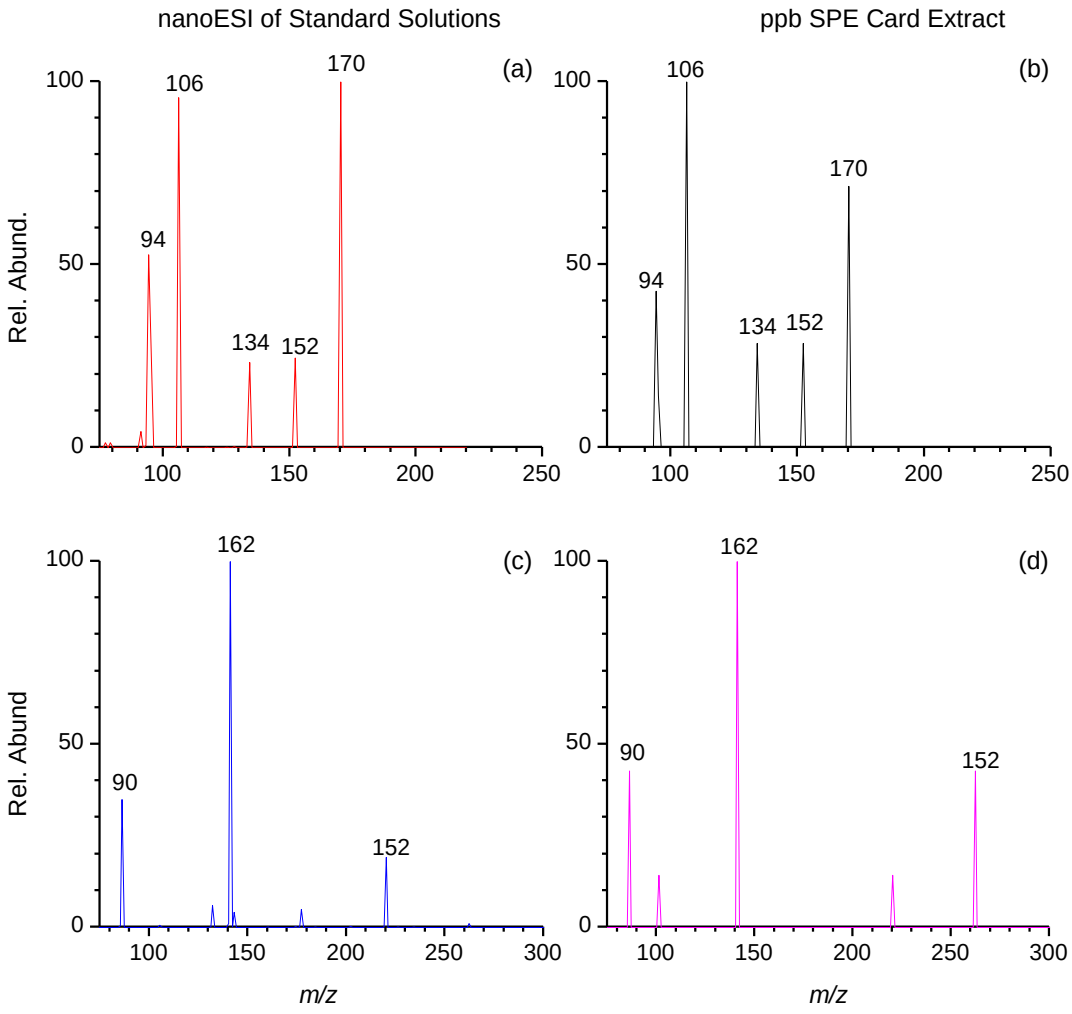


Figure 22. A 200 µL herbicide mixture at 2 ppb concentration level was concentrated onto a 1 mm extraction well (0.4 ng). Neither conditioning nor rinse steps after loading were performed in this analysis. Extraction/nanoESI solution was composed of 80/20/.1 v/v/v methanol/water/ammonium acetate. A TriVersa coupled with a Thermo LTQ directly nano electrosprayed the extract for two minutes. Multiple full MS scans, MS/MS scans, and MS^3 scans were recorded. (a) MS^3 spectrum of Ramrod standard (b) MS^3 spectrum for ppb Ramrod extract (c) MS^3 spectrum of Triallate standard. (d) MS^3 spectrum for ppb Alachlor extract. MS^3 fingerprint allows absolute qualitative determination of compounds in aqueous samples. This technique allows sufficient time (minutes) to perform multiple MS experiments with a single extract.

The MS^3 fingerprint allows absolute qualitative determination of such compounds in aqueous samples. In Figure 21 (f), the MS^3 product ion spectrum from direct infusion of 2 ppb alachlor does not match the 200 ppb product ion spectrum seen in Figure 21 (d), due to the concentration of the solution being below the detection threshold. Note also that the extraction/nanoESI solution used in this case was composed of 80/20/.1 v/v/v methanol/water/ammonium acetate. We found that compared to the same solvents with formic acid added, as used in all the other experiments above, the ammonium acetate diminished the formation of sodium adduct ions of the herbicides, $(M+Na)^+$, simplifying the spectra and increasing the signal levels observed for the herbicide protonated molecules, $(M+H)^+$. Improving the concentrating effect of the SPE card would allow orders of magnitude improvement over the ppb detection level reported here. Of important note is the fact that single LESA extracts could be infused for minutes allowing ample time for these multiple MS experiments.

3.4 Conclusions

The recently released LESA mode on the TriVersa® NanoMate®, an automated nanoESI system, was applied to the analysis of analytes from custom SPE card wells. Using this strategy, analytes were prepared for analysis by binding and concentrating them on planer SPE cards, washed to remove polar contaminating species, and eluted from the surface in an ESI-friendly solvent. The eluent was analyzed by direct infusion nanoESI mass spectrometry. This approach to sample preparation has the advantage of being easily performed in a parallel fashion, enabling high throughput. Another key advantage is that the surfaces exposed to the sample, along with the sampling pipette tip and ESI emitter, are made to be used for a single sample. This approach

eliminates carryover effects that hinder, and in many cases prevent, trace analysis of organic compounds.

We showed that this method of analysis to be useful for both quantitative and qualitative assays. Linear and reproducible calibration data was obtained for propranolol using a deuterated internal standard. A 10-fold concentration and cleanup of angiotensin II at micromolar concentration in a concentrated salt solution was demonstrated. A multicomponent herbicide mixture at ppb concentration levels was analyzed using MS^3 spectra for identification in the presence of interferences. The analysis method presented here turns the traditional elution step from an SPE plate into a direct extractive analysis, minimizing sample dilution and providing extended time, if required, for complex mass spectral detection and characterization experiments. Future studies will focus on tailoring an SPE card for LESA analysis such that greater concentration factors will be realized.

Hydrophobic Treatment Enabling Analysis of Wettable Surfaces using a Liquid Microjunction Surface Sampling Probe/Electrospray Ionization-Mass Spectrometry System

4.1 Introduction

Sampling and ionizing materials present on surfaces under ambient conditions is an expanding area of research and application in mass spectrometry (MS).[5,116,117,118,119,120,121] Direct liquid extraction based surface sampling probes are one way to perform atmospheric pressure surface sampling and ionization.[5] One particular probe of this type, a continuous flow liquid microjunction surface sampling probe (LMJ-SSP),[8,9,12,13,14,15,16,17,18,19,105] reconstitutes or extracts an analyte from a surface by means of a wall-less liquid microjunction between the sampling end of the probe and the surface. The liquid extraction solvent is brought to the surface through the annular space between two coaxial tubes at the sampling end of the probe and is then carried on thru the inner tube to the ionization source through a self-aspirating electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) emitter. This LMJ-SSP approach to surface sampling/ionization can be applied to all species that can be dissolved and conducted into the probe and subsequently ionized by the respective ionization method being used.

The LMJ-SSP has two general modes of operation, viz., a discrete spot sampling mode and a scanning (or imaging) mode, each of which can be used either manually or as an automated procedure.[17,32] The discrete sampling mode allows the operator to sample selected single spots from a surface for analysis by forming a liquid microjunction separately at each of those points. The imaging mode allows continuous sampling of material from a surface by maintaining a

liquid microjunction with the surface as the sample is moved in both x and y coordinates relative to the stationary probe. Analytical applications of this continuous flow LMJ-SSP in these two different modes of operation have involved the sampling and analysis of dried drugs or proteins or solutions thereof from wells on microtiter plates, drugs captured in solid-phase extraction cards,[12] a variety of dyes, inks, or pharmaceuticals on paper or separated on hydrophobic reversed-phase (C8 and C18) thin-layer chromatography plates,[13,14,15,16] exogenous compounds from thin tissue sections,[19] and surface deposited and affinity captured proteins.[18]

The requirement to form a liquid microjunction with the surface can limit the effectiveness of the coaxial tube LMJ-SSP when sampling from particular types of wettable, absorbent surfaces. This is particularly problematic in the scanning mode which requires a sustained, well controlled, liquid microjunction for an extended period of time (often many min). An important example of this limit is development lane scanning during the analysis of wettable and absorbant high performance thin layer chromatography (HPTLC) plates. Not only can the solvent be lost into these surfaces, but delivery of the solvent at the point of sampling tends to develop analytes out from the vicinity of the probe before they can be sampled. As such, the application of the LMJ-SSP for the analysis of HPTLC plates has been limited to date to hydrophobic reversed-phase (RP) C8 and C18 plates.[13,14,15,16,20] Herein, we describe a simple, inexpensive surface treatment method, implemented post plate development, that enables the LMJ-SSP to effectively sample from previously wettable HPTLC phases. Proper aerosol application of one or more silicone based products to the developed plates is performed to create a hydrophobic surface that enables liquid microjunction formation, allows efficient extraction of the analytes from the plate, and does not contribute significant chemical background in the mass

spectra. The surface treatment process is described and explained for different plate phases and the effectiveness of the treatment is illustrated by the LMJ-SSP/ESI-MS analysis of alkaloids from Goldenseal (*Hydrastis canadensis*) root on a normal phase silica gel 60 F_{254S} plate and peptides from protein tryptic digests separated on a ProtoChrom® HPTLC Silica gel 60 F_{254S} plate and a ProtoChrom® HPTLC cellulose sheet.

4.2 Experimental

4.2.1 Materials and Reagents

Preparation of the standards and TLC plate development involving berberine chloride (Sigma Aldrich, St. Louis, MO) and Goldenseal root (Botanical Liasions, Boulder, CO) used HPLC grade ethyl acetete and methanol from Fisher Scientific (Fair Lawn, NJ), 99%+ formic acid from Acros (Morris Planes, NJ) and HPLC grade water from Spectrum (Gardena, CA). HPTLC glass 20 x 10 cm Silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany) were used for the separation of these samples. Ammonium bicarbonate, trypsin, LiChrosolv® methanol and HPLC-grade water (Merck KGaA) were used for tryptic digest TLC. LC-MS grade Chromosolv® solvents acetonitrile and water both with 0.1% formic acid (v/v) were obtained from Sigma Aldrich for use with the LMJ-SSP/ESI-MS analyses. Proteins bovine cytochrome c., equine myoglobin, beta-casein from bovine milk, bovine serum albumin and lysozyme from chicken egg white were obtained from Sigma Aldrich (Fluka, Buchs, Switzerland). ProteoChrom® HPTLC Cellulose sheets and ProteoChrom® HPTLC Silica gel 60 F_{254S} plates used for separations of the tryptically digested proteins were acquired from Merck KGaA. Trio Magic Carfa Silicone Oil aerosol spray was purchased from CAMAG (Muttenz,

Switzerland). KIWI Camp Dry Heavy Duty Water Repellent spray was purchased over the counter locally.

4.2.2 Tryptic Digest

Tryptic digestion of model proteins was performed using ProteoExtract™ All-in-One Trypsin Digestion Kit. The protocol for digestion of proteins in solution was followed with an initial concentration of 2 mg ml^{-1} in the first step. Tryptic digests were performed by dissolving each model protein in 25 mM ammonium bicarbonate buffer. The final protein concentration based on ProteoExtract™ All-in-One Trypsin Digestion Kit's protocol is 2 μg μL^{-1}. The concentration of tryptic peptides recovered from the digestion was not determined using a BCA or UV/VIS spectroscopy analysis such that the final protein concentration as stated is at best a high estimation. Trypsin was added to the protein buffer mixture such that the trypsin:protein ratio was 1:100. The mixture was incubated for 15 h at 37 °C.

4.2.3 Thin-layer Chromatography

Berberine and Goldenseal. A 1.0 mg mL^{-1} berberine standard was prepared in methanol. This solution was serially diluted in methanol to make standards of 0.1 – 0.001 mg mL^{-1}. 1 μL of these solutions was applied as 6 mm bands on a HPTLC Silica Gel 60 F_{254S} plate and developed in ethyl acetate, water, formic acid (80/10/10 v/v/v). To prepare a Goldenseal root standard solution, 0.25 g of powdered sample was added to 5 mL of a methanol/water (80:20 v/v) solution and sonicated for 30 min. The solution was filtered twice with filter paper each time washing the filter with 2 mL of methanol. The filtrate and washings were combined and brought to a final volume of 20 mL with methanol. These standard solutions were applied in volumes from 0.5 to

10 μL to a HPTLC glass 20 x 10 cm Silica gel 60 F_{254S} plate as 8 mm long bands using a ATS 4 fully automated sample applicator (CAMAG, Muttenz, Switzerland). The samples were applied 20 mm from the bottom of the HPTLC plate and the distance between bands was 11.4 mm. Plates were developed vertically in a twin trough chamber with solvent saturated atmosphere using ethyl acetate, water, formic acid (80/10/10 v/v/v). The plates were air dried and images acquired with a TLC visualizer (CAMAG). The plates were then treated by spraying a coating of Carfa Magic Silicone oil onto the surface after which the plate was left to dry in the vertical position for 12 hours prior to mass spectrometric analysis (drying time can be accelerated by drying a treated plate in a fume hood as is noted later).

Tryptic Digests. The tryptic protein digest separation was performed on ProteoChrom® HPTLC Cellulose sheets or ProteoChrom® HPTLC Silica gel 60 F_{254S} plates. Sample application was done using either an ATS 4 fully automated sample applicator or a Linomat V semiautomated sample applicator (CAMAG). A total sample volume of 7 μL was applied as 6-mm bands at a dosage speed of 50 nL s^{-1} equivalent to about 14 μg of the original protein per band. The samples were applied 10 mm from the bottom of the HPTLC plate and the distance between bands was 15 mm. The development of the HPTLC plates was carried out in a normal flat-bottomed chamber using 2-butanol/pyridine/ammonia/water (39:34:10:26, v/v/v/v) for the ProteoChrom® Silica gel 60 F_{254S} plates and 2-butanol/pyridine/acetic acid/ water (30:20:6:24, v/v/v/v) for the ProteoChrom® HPTLC Cellulose sheets. The migration distance on the ProteoChrom® Silica gel 60 F_{254S} plate and the ProteoChrom® HPTLC Cellulose sheet used for the analysis was 50 mm achieved in 45–60 min.

Hydrophobic coating procedures for the silica gel plates and cellulose plates differed. In both cases the plates were placed in a fume hood, laying flat, and spray coated with the Trio Magic Carfa Silicone Oil. Plates were coated evenly until the silicone layer visibly oversaturated the stationary phase. A kimwipe tissue was hand pressed down onto the HPTLC plate using a glass plate for 5 s to remove the excess silicone. This blotting procedure was then performed again using a new kimwipe. At this point silica gel 60 HPTLC plates were allowed to dry in open air for 7-10 hrs. In the case of the HPTLC cellulose plates, after the second kimwipe blotting, the plates were sprayed for 2 s with a light coat of KIWI Camp Dry Heavy Duty Water Repellent. Immediately following this application excess silicone resting on top of the cellulose stationary phase was removed using the double blotting procedure described above. The cellulose plates were allowed to dry in a fume hood for 7-10 hrs after coating.

4.2.4 LMJ-SSP/ESI-MS

An LTQ XL linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) with Xcalibur software version 2.0 was used in this work. The particular LMJ-SSP probe system used was exactly the same as that which has been described previously[8,13] with only the mounting system modified to accommodate the particular mass spectrometer. The self aspirating LMJ-SSP probe was built using a stainless steel tee, a 10 cm long inner sampling/emitter capillary with a 254 µm o.d. and a 127 µm i.d., an outer tube on the sampling end with 635 µm o.d. and 327 µm i.d., and a nebulizer tube on the spray side. Photographs of the current setup can be found in Chapter 4 Supplemental Information (SI) 1. The nebulizing gas flow was used to adjust the probe aspiration rate to be in balance with the pumped flow of eluting/spray solvent (10 µL min^{-1} using a 1-mL syringe attached to a syringe pump) into the probe. An approximately 27 cm

long section of PEEK tubing (127 µm inner diameter and 1/16 inch outer diameter) with an upstream ground point was used to supply the elution solvent to the probe/emitter. The ESI voltage applied to the probe and the capillary and tube lens voltages were independently optimized for the Goldenseal alkaloids and peptides by infusing micromolar level standards of each through the probe.

An MS2000 robotic x, y, z platform (Applied Scientific Instrumentation, Inc., Eugene, OR, USA) was used to hold and maneuver the TLC plates in a perpendicular position relative to the stationary LMJ-SSP for analysis. As described elsewhere,13 the original microscope slide holder supplied with the stage was replaced with a home-built TLC plate holder made from rigid, nonconductive polymer. The MS2000 platform could be controlled manually by use of a joystick in the x and y-directions and by use of a jog wheel for z-direction control for initial alignment and LMJ formation. A camera used to observe the liquid microjunction during operation was equipped with an Optem 70 XL zoom lens (Thales Optem Inc., Fairport, NY, USA). All TLC plate lane scans were enabled by using HandsFree TLC/MS, software written in-house to control the ASI 2000 stage. Before scanning a lane, a LMJ was created at a position along the development lane below the spotting point by manual adjustment of the jog wheel and joystick via the ASI 2000 control system. After making the LMJ, the mass spectrometer data acquisition process was initiated simultaneous with the beginning of the lane scan (100 µm s^{-1}). When the scan and data collection processes were finished, the LMJ was broken by moving the stage away from the probe in the z-direction.

Full scan mass spectra were acquired with surface scans of all the Goldenseal related plates. During surface scans of the tryptic digest separations lanes, automatic gain control was

used with MS/MS product ion mass spectra acquired in data dependent mode with the three most abundant peaks within each full scan mass spectrum subjected to dissociation. The normalized collision energy was set to 35% and three microscans were acquired for each spectrum over a product ion range of 200-2000 Da. Dynamic exclusion was set to three so MS/MS would be performed on a certain *m/z* peak a maximum of three times. To identify the peptides observed, the MS/MS spectra were extracted from raw data files and converted to MS2 file format[122]. The MS2 files were searched using the DBDigger[123] proteomics database search program which used the MASPIC[124] scoring scheme and the DTASelect[125] algorithm for filtering the MS2 files. The DTA Select algorithm used a ΔCN of at least 0.08 and cross correlation (X_{corr}) scores of 20 (+1), 25 (+2) and 40 (+3).

4.3 Results and Discussion

4.3.1 Surface Treatment and Analysis Concept

To expand the use of the LMJ-SSP probe to include analysis of wettable or absorbent surfaces, such as normal phase HPTLC plates, simple, inexpensive surface treatment methods were explored. We found that proper aerosol application and curing of one or more silicone-based products could create a hydrophobic surface that enabled liquid microjunction formation, allowed extraction of the analyte, but did not contribute significant chemical background in the mass spectra. It is worth noting that the modification of planar chromatographic surfaces with nonpolar, hydrophobic materials like paraffin and silicone oil has a rich history as a means to create, hydrophobic reversed-phase TLC plates.[126] These procedures were developed before the wide availability of bonded phases.[127] Although the focus of the results and discussion here is on

HPTLC, these same treatment and analysis methods were successfully applied to other types of wettable or absorbent surface like frosted glass slides, kimwipes, and dried blood spot paper (Figures 23 and 24). The surface treatment process developed and the subsequent surface sampling procedure used here are illustrated schematically in Figure 25. In this case, shown for a developed HPTLC plate, the analyte of interest was dispersed in a band on the surface of the stationary phase particles. By aerosol application, the complete phase, including the analyte, was impregnated with a hydrophobic silicone material. Depending on the type of surface, one or more silicone products were applied in proper order and amount to achieve the desired surface hydrophobicity and analyte extractability (see **Experimental** section). During the analysis, the extraction solvent from the LMJ-SSP penetrated the hydrophobic coating, wetted the stationary phase at the sampling point, and dissolved the analyte that was then aspirated into the probe, electrosprayed, and detected in the mass spectrometer.

After sampling of the treated surfaces, whether in spot sampling or scanning mode, the sampled region had a different visual appearance than the rest of the plate. In offline studies it was found that neither silicone product showed significant solubility in methanol, acetonitrile, or methanol/water or acetonitrile/water mixtures. Rather the silicone material was immiscible with these solvents being dispersed therein as microdroplets or forming a colloidal or emulsion like phase. Thus, during the LMJ-SSP analysis of the surface it is possible that some immiscible silicone material may be removed in the extraction solvent as microdroplets or pushed out from the immediate vicinity of the probe by the extraction solvent. It is also possible that the solvent simply penetrates into the silicone layer, possible by a swelling mechanism known to occur from the interaction of organic solvents and silicone polymer networks[128].

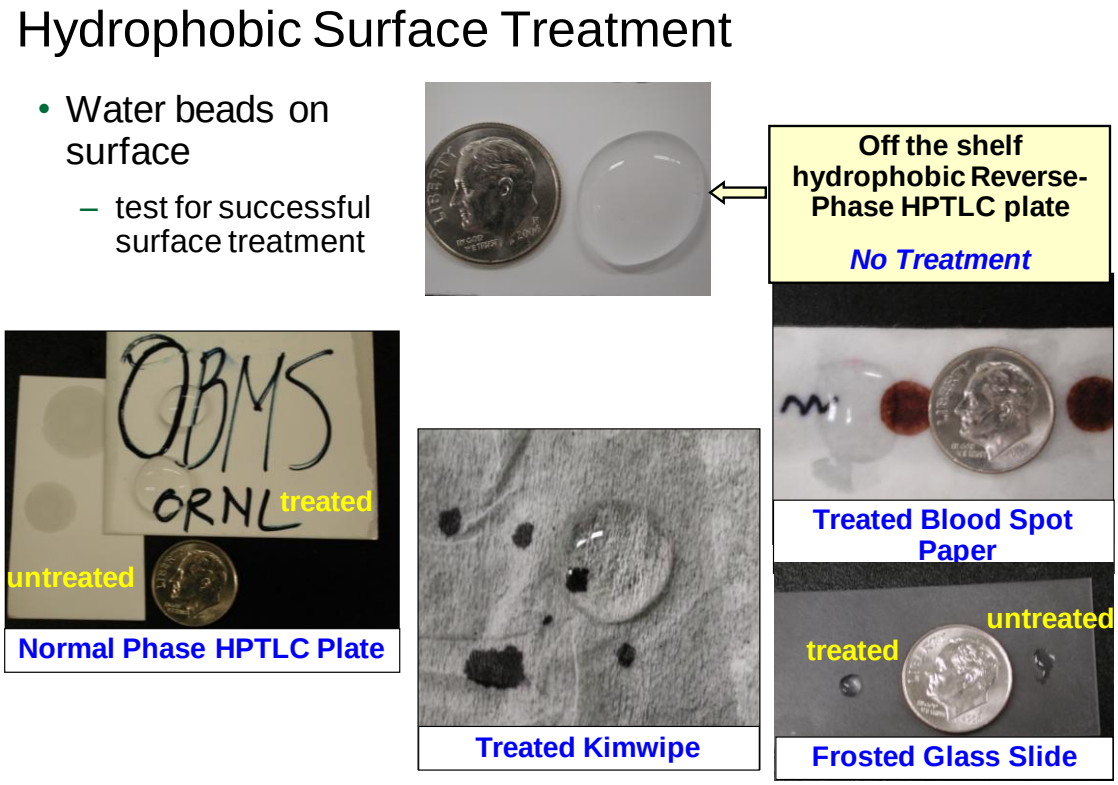


Figure 23. Hydrophobic surface treatment examples.

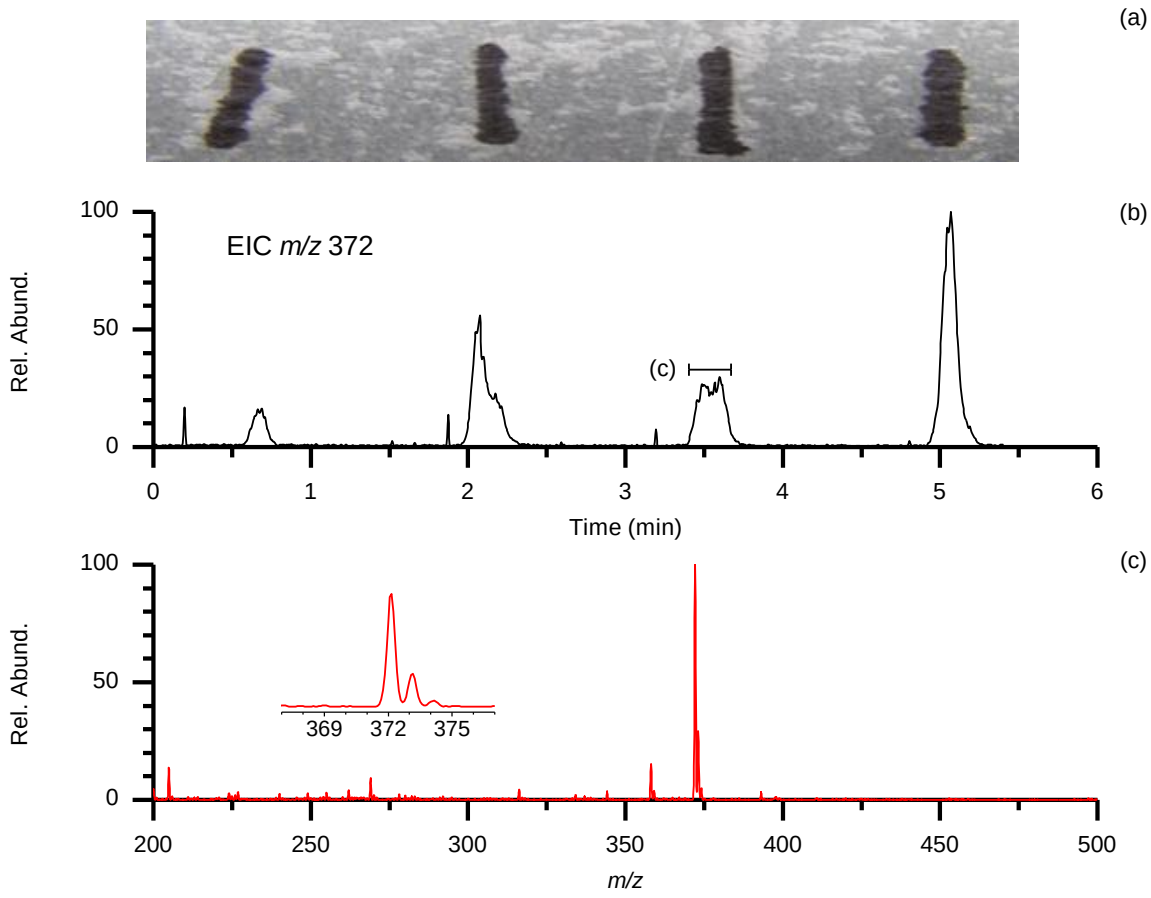


Figure 24. LMJ-SSP spot sample of ink on treated kimwipe. Black sharpie marker on kimwipes was spot sampled in 4 different marker lines. The extraction solvent was composed of 60/40 methanol/water (v/v). A Thermo LTQ Mass spectrometer was used for detection in full scan mode *m/z* 150 – 2000.

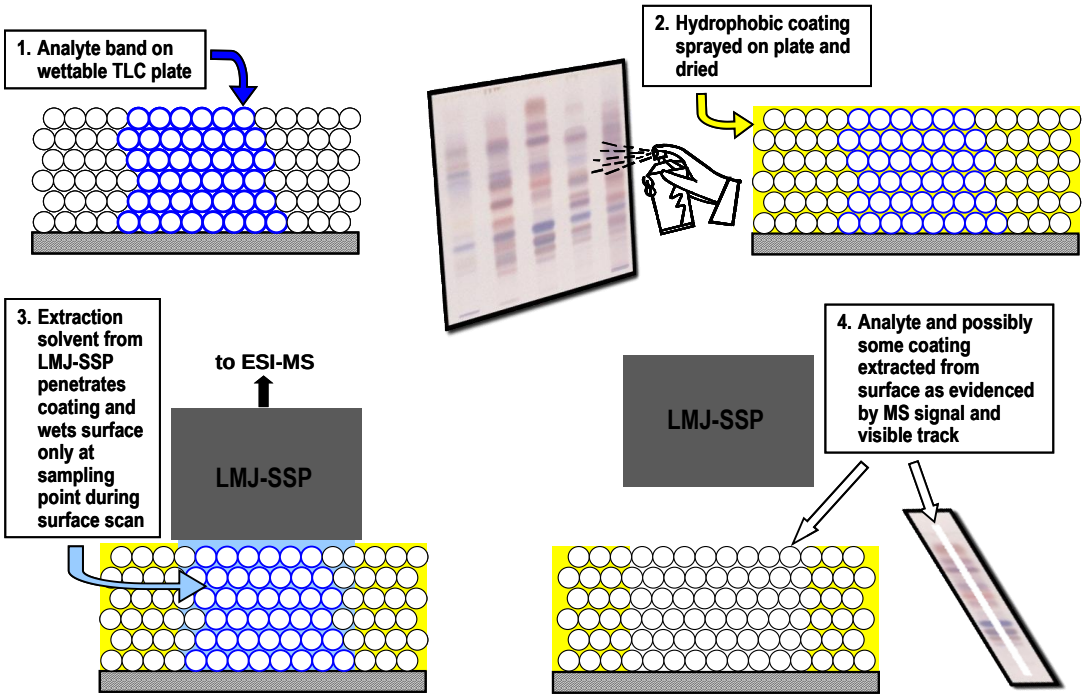


Figure 25. Surface treatment process developed and the subsequent surface sampling procedure schematically illustrated for a wettable HPTLC plate.

In any case, no significant mass spectral signal was observed when sampling from an appropriately cured treated surface. Silicone polymer ion signals were sometimes observed if the curing procedures were not followed as shown in Figure 26. Large polymer chains without sufficient charging if present would be outside the *m/z* range of the mass analyzer. However, the successful analyses illustrated by the data presented below indicate that little if any signal suppression from the coating took place.

4.3.2 Goldenseal Alkaloids on Silica Gel 60 HPTLC Plates

Authentic Goldenseal extracts contain four primary alkaloids, viz., berberine, tetrahydroberberine, hydrastine, and often hydrastinine, a degradation product of hydrastine.[129] Other alkaloids are known to also be present, but at much lower amounts. Figure 27 (a) shows the image of the Goldenseal extract development lane acquired using 366 nm UV light. (a) UV (366 nm) image of Goldenseal root extract developed on a HPTLC glass backed Silica gel 60 F254s plate using 80/10/10 ethyl acetate/water/formic acid (v/v/v). The extracted ion current profiles for the major and several minor Goldenseal alkaloids are plotted in panels (b) *m/z* 190, hydrastinine (observed as *m/z* consistent with water loss during sampling/ionization),[130] (c) *m/z* 384, hydrastine, (d) *m/z* 338, jatrorrhizine, (e) *m/z* 352, berberastine, (f) *m/z* 336, berberine, (g) *m/z* 370, canadaline and (h) *m/z* 340, tetrahydroberberine as indicated. The signal intensity in each panel was normalized to the signal from the most intense alkaloid berberine (*m/z* 336 in panel (f), rel. abund. 100 = 2.03×10^7 cnts). The development lane was scanned relative to the LMJ-SSP from low to high R_f at 100 μm s^{-1} using an extraction/ESI solvent composed of 40/60 methanol/water (v/v) flowing at 10 μL min^{-1}.

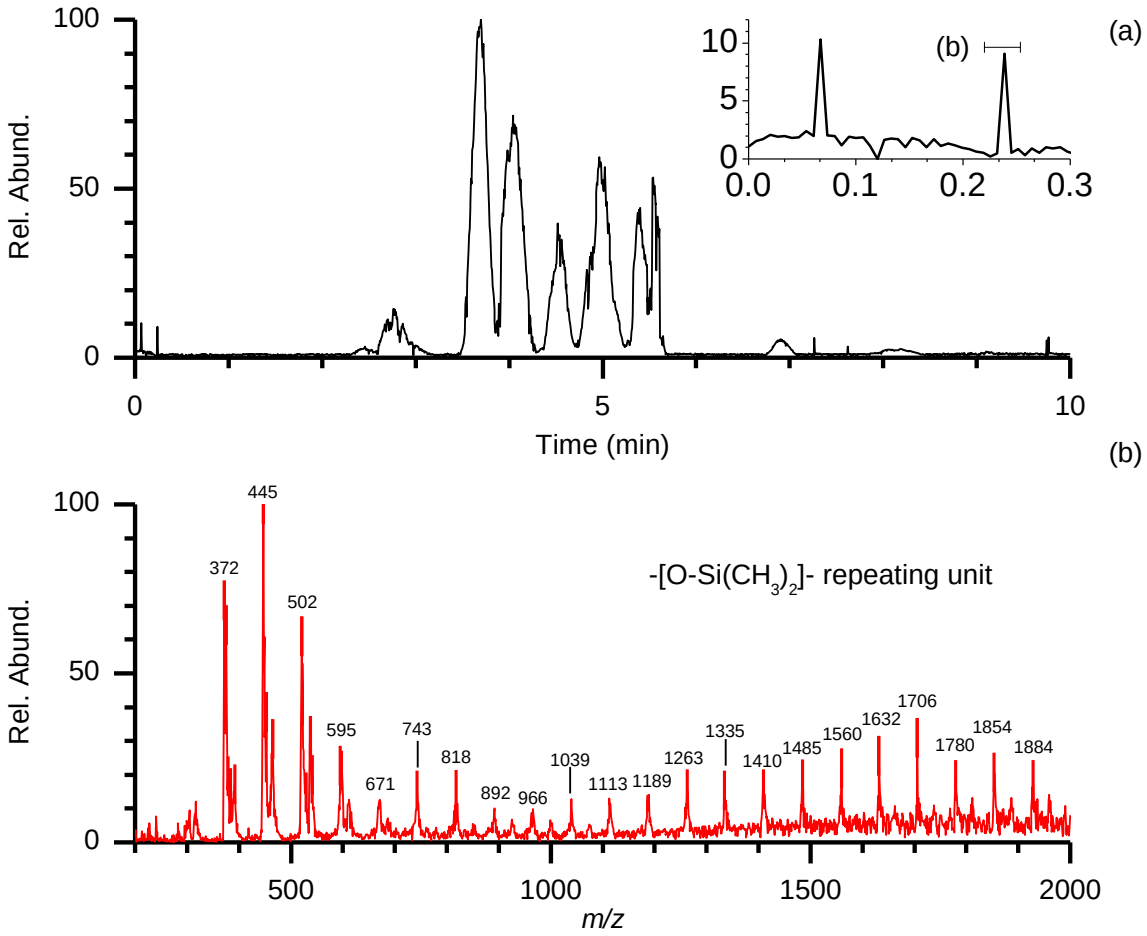


Figure 26. (a) Base peak chromatogram of LMJ-SSP silica gel 60 HPTLC lane scan of Cytochrome C tryptic digest. A 100 µms-1 scan rate was used with an extraction solvent of 60/40 H20/ACN 0.1% formic acid. (b) Representative spectra of silicone polymer signal that is observed when treated surfaces are not allowed sufficient time to dry.

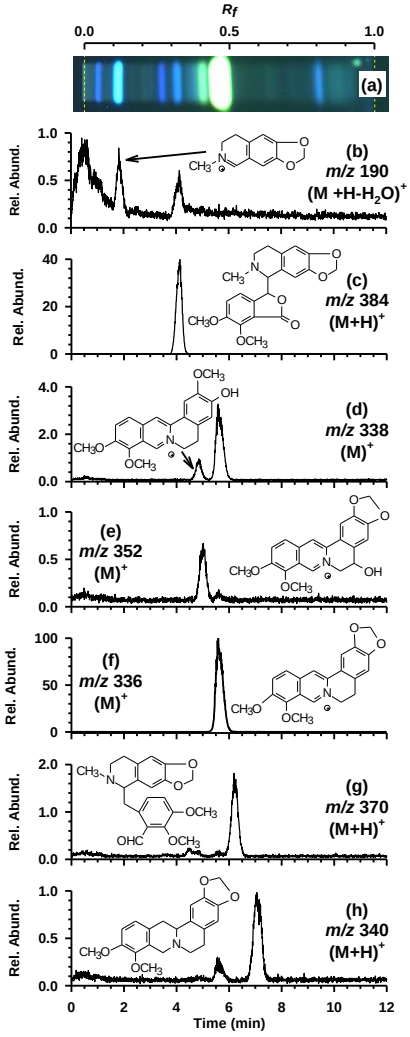


Figure 27. (a) UV (366 nm) image of Goldenseal root extract developed on a HPTLC glass backed Silica gel 60 F254s plate using 80/10/10 ethyl acetate/water/formic acid (v/v/v). The extracted ion current profiles for the major and several minor Goldenseal alkaloids are plotted in panels (b) *m/z* 190, hydrastinine (observed as *m/z* consistent with water loss during sampling/ionization),[130] (c) *m/z* 384, hydrastine, (d) *m/z* 338, jatrorrhizine, (e) *m/z* 352, berberastine, (f) *m/z* 336, berberine, (g) *m/z* 370, canadaline and (h) *m/z* 340, tetrahydroberberine as indicated. The signal intensity in each panel was normalized to the signal from the most intense alkaloid berberine (*m/z* 336 in panel (f), rel. abund. 100 = 2.03 x 10^7 cnts). The development lane was scanned relative to the LMJ-SSP from low to high R_f at 100 µm s^{-1} using an extraction/ESI solvent composed of 40/60 methanol/water (v/v) flowing at 10 µL min^{-1}.

Figures 27(b) – 27(h) show the extracted ion chronograms for the two major alkaloids and several minor abundant alkaloids expected in an authentic Goldenseal extract obtained during a surface sampling lane scan with the LMJ-SSP/ESI-MS system. The most intense signals extracted from full scan (*m/z* 100 - 1000) mass spectral data were observed for *m/z* 384 (Retention factor (R_f)= 0.32) and *m/z* 336 (R_f = 0.46, rel. abund. 100 = 2.03 x 10^7 cnts) which are assigned as hydrastine and berberine, respectively. Hydrastinine, which decomposes during ESI-MS analysis,[130] was observed at *m/z* 190 (R_f = 0.12), berberastine at *m/z* 352 (R_f = 0.41), canadaline at *m/z* 370 (R_f = 0.51) and tetrahydroberberine at *m/z* 340 (R_f = 0.60). Other discernable peaks were observed above a signal level arbitrarily set at 1 x 10^5 cnts, including *m/z* 365 (R_f = 0.02), *m/z* 503 (R_f = 0.35), *m/z* 368 (R_f = 0.46), and *m/z* 342 (R_f = 0.48). These species were not definitively identified. Peaks corresponding to primary alkaloids of adulterants or admixtures commonly substituted for Goldenseal, such as coptisine (*m/z* 320), were not detected. However, the *m/z* 338 peak at R_f = 0.39 would be consistent with the presence of jatorrhizine, but this alkaloid is not a reported component of Goldenseal.[129] The more abundant *m/z* 338 peak (R_f = 0.46) is the (M+2) isotope peak from berberine. Some bands obvious in the image of the plate (e.g., bands at R_f = 0.27 and 0.80) did not give detectable mass spectral response under the conditions used. The species in these bands may not be easily extracted using the current extraction solvent composition, may not be effectively ionized in positive ion mode ESI or may be outside of the *m/z* range scanned. There is no direct evidence to indicate that the lack of signal from these bands was caused in any way by the silicone treatment.

To test detection levels, a berberine reference standard was applied at six different concentrations in triplicate, the plate was developed, silicone treated, and read out using the

LMJ-SSP/ESI-MS. The plate was scanned at fixed R_f across the replicates rather than up each development lane (Figure 28 (a)). Additional bands other then the characteristic light green fluorescent zone of berberine at R_f 0.48 were visible on the plate indicating the reference standard was not pure. A representative mass spectrum is shown in Figure 28(b) for one of the 10 ng berberine band scans which shows berberine ion as the base peak in the spectrum. The calibration ion curve in Figure 28(c) is based on the integrated peak area values of the extracted ion current for *m/z* 336. The data was best fitted with a second order polynomial due to the 'roll over' at the 1000 ng band levels attributed to detector saturation. The readout of the 100, 10 and 1 ng bands had acceptable reproducibility with RSD values of 20.5%, 13.2% and 15.0%. The low nanogram detection levels observed here are similar to those levels reported for similar small molecules using the LMJ-SSP to analyze hydrophobic reversed-phase TLC plates15 and in the case of another ambient surface sampling methods, desorption electrospray ionization (DESI)-MS, when analyzing this same compound from a normal phase plate.[130]

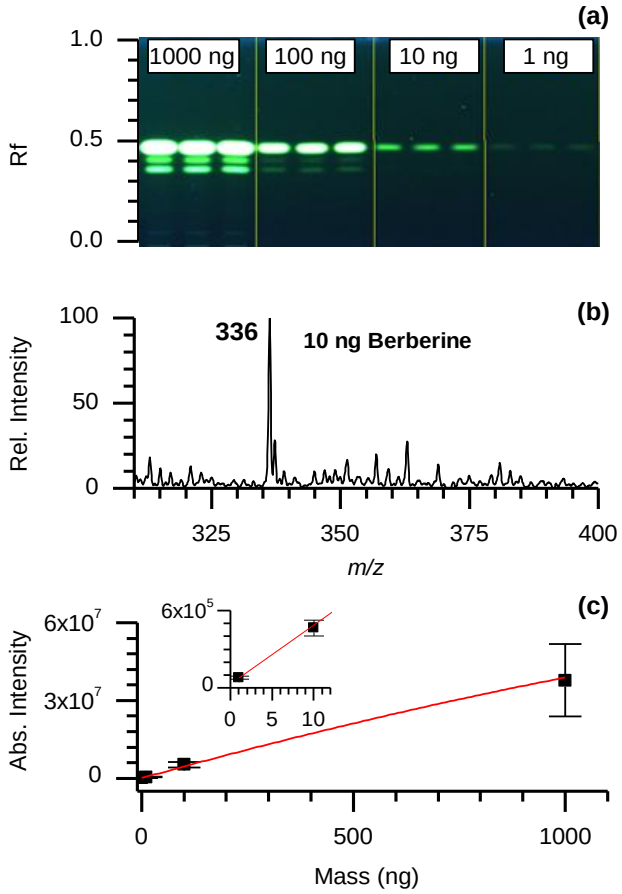


Figure 28. (a) UV (366 nm) image of berberine standard developed on a HPTLC glass backed silica gel 60 F_{254S} plate using 80/10/10 ethyl acetate/water/formic acid (v/v/v). The development lane was scanned relative to the LMJ-SSP in a direction perpendicular to the development direction across the berberine bands at R_f 0.48 at 100 µm s^{-1} using an extraction/ESI solvent composed of 40/60 acetonitrile/water/formic acid (v/v/v) flowing at 10 µL min^{-1}. (b) Full scan mass spectrum from one of the 10 ng band replicates. (c) Calibration curve based on integrated peak areas from the *m/z* 336 extracted ion current.

4.3.3 Tryptic Peptides on ProtoChrom® HPTLC Silica gel 60 Plates and Cellulose Sheets

The separation of peptides from the tryptic digestion of proteins was performed on both ProteoChrom® HPTLC Silica gel 60 Plates and Cellulose Sheets. After development and drying, the plates were treated with the aerosol silicone sprays as described in the **Experimental** section. The different nature of the two stationary phases required a different coating procedure for each plate type to achieve the desired surface properties for LMJ-SSP analysis. Each development lane was scanned separately in data dependent MS/MS mode. To illustrate the quality of the read out and the mass spectral signal achieved, the extracted ion current profiles for all peptides identified by LMJ-SSP/ESI-MS/MS of a BSA tryptic digest separated on a ProteoChrom® HPTLC Silica gel 60 F_{254S} Plate are shown in Figure 29. The photo in Figure 29 (a) is that of a ninhydrin stained plate of the separated digest similar to the plate actually analyzed. Averaged full scan mass spectra over the indicated regions along the scanned development lane are presented Figures 29(c), 29(d) and 29(e). The identity of the peptides ions observed in these spectra is indicated by the number annotation which can be correlated with the complete tabulation of identified peptides for this protein in Table 4. Low R_f bands were not identified and may be the result of irreversible binding of peptides onto the ProteoChrom® HPTLC Silica gel 60 F_{254S} stationary phase. Chapter 4 SI 2 shows analyte bands that were not identified by MS/MS but could possibly represent some of the unidentified tryptic peptides. Of course, the peptide identifications were based on data base matching of the product ion spectra of the peptide precursor ions not on peptide mass alone.

The protein sequence coverage achieved in these experiments are reported in Table 5 for all five proteins with both ProteoChrom® HPTLC plate types.

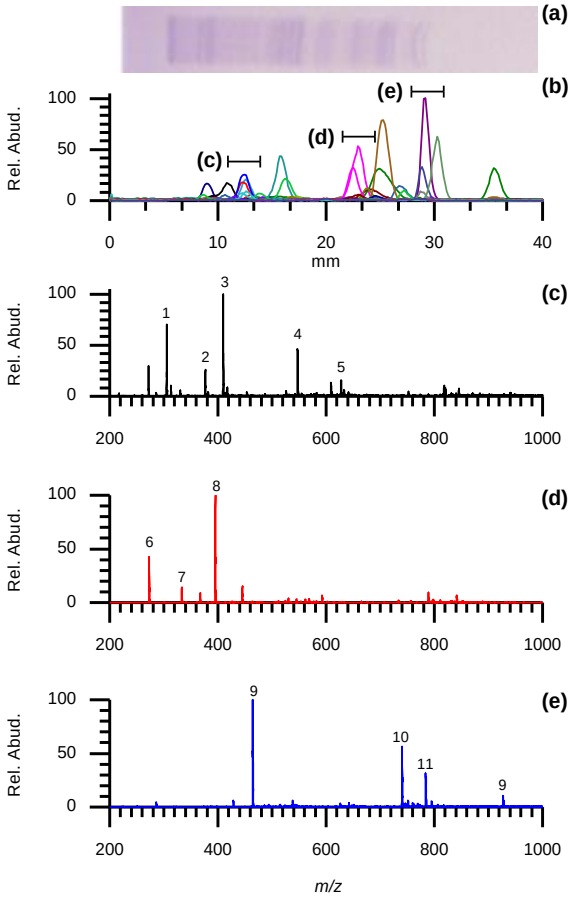


Figure 29. (a) Image of BSA tryptic peptides developed on ProteoChrom® HPTLC Silica gel 60 F_{254S} using 2-butanol/pyridine/ammonia/water (39:34:10:26, v/v/v/v) and stained using ninhydrin. (b) Extracted ion current profiles for all peptide precursor ions that were identified by the LMJ-SSP/ESI-MS/MS scan of the development lane in an unstained duplicate lane to that shown in (a). Representive full scan, averaged mass spectra over the areas indicated in panel (b) are shown in panels (c) (d) (e) respectively. Ion signals labeled numerically 1-11 that were identified as tryptic peptides using MS/MS spectra are reported with numbered superscripts in Table 2 with all other identified peptides. The development lane was scanned relative to the LMJ-SSP from low to high R_f at 100 µm s^{-1} using an extraction/ESI solvent composed of 40/60 acetonitrile/water/formic acid (v/v/v) flowing at 10 µL min^{-1}.

Table 4. MS/MS identified peptides from the LMJ-SSP/ESI-MS/MS analysis of BSA tryptic digests separated on a ProteoChrom HPTLC Silica gel 60 F254s plate.

Peptide	Charge State			R_f
	1	2	3	
SEIAHR		*		0.29
FKDLGEEHFK		*	*	0.24
DLGEEHFK		*		0.21
GLVLIAFSQYLQQC"PFDEHVK			*	0.48
LVNELTEFAK	*	*		0.42
VASLR[6]		*		0.45
DDSPDLPK		*		0.19
LKPDPNTLC"DEFK			*	0.22
FW+G:KYLYEIAR		*		0.48
YLYEIAR[9]	*	*		0.53
GAC"LLPK		*		0.48
VLTSSAR		*		0.42
FGER	*			0.42
AW+SVAR		*		0.48
AEFVEVTK	*	*		0.29
LVTDLTK[8]	*	*		0.47
EC"C"HGDLLEC"ADDR			*	0.22
ADLAK	*	*		0.29
YIC"DNQDTISSK		*		0.21
SHC"IAEVEK			*	0.22
DAIPENLPPLTADFAEDK		*		0.22
NYQEAK[2]		*		0.23
DAFLGSFLYEYSR[11]		*		0.52
RHPEYAVSVLLR			*	0.24
HPEYAVSVLLR		*	*	0.49
DDPHAC"YSTVFDK			*	0.22
HLVDEPQNLIK		*	*	0.30
QNC"DQFEK		*		0.21
LGEYGFQNALIVR[10]		*	*	0.55
KVPQVSTPTLVEVSR[4]			*	0.23
VPQVSTPTLVEVSR		*		0.48
M*PC"TEDYLSLILNR		*		0.49
TPVSEK	*	*		0.22
C"C"TESLVNR		*		0.34

Table 4. Continued

peptide		Charge state		R_f
	1	2	3	
RPC"FSALTPDETYVPK[5]			*	0.23
AFDEK[1]	*	*		0.22
LFTFHADIC"TLPDTEK			*	0.36
ATEEQLK[3]		*		0.21
TVM*ENFVAFVDK		*		0.41
LVVSTQTALA.-		*		0.65
IETMR[7]		*		0.19

Table 5. Protein sequence coverages obtained by LMJ-SSP/ESI-MS/MS analysis of tryptic digests separated on ProteoChrom HPTLC Silica gel 60 F_{254S} plates and ProteoChrom HPTLC Cellulose sheets.

Protein	% Sequence Coverage Obtained with each HPTLC Plate Type	
	ProteoChrom® HPTLC Cellulose	ProteoChrom® HPTLC Silica gel 60
bovine serum albumin	40.5	60.5
beta casein	22.3	29.5
cyctochrome *c*	90.4	89.4
myoglobin	98.0	85.6
lysozyme	75.2	88.4

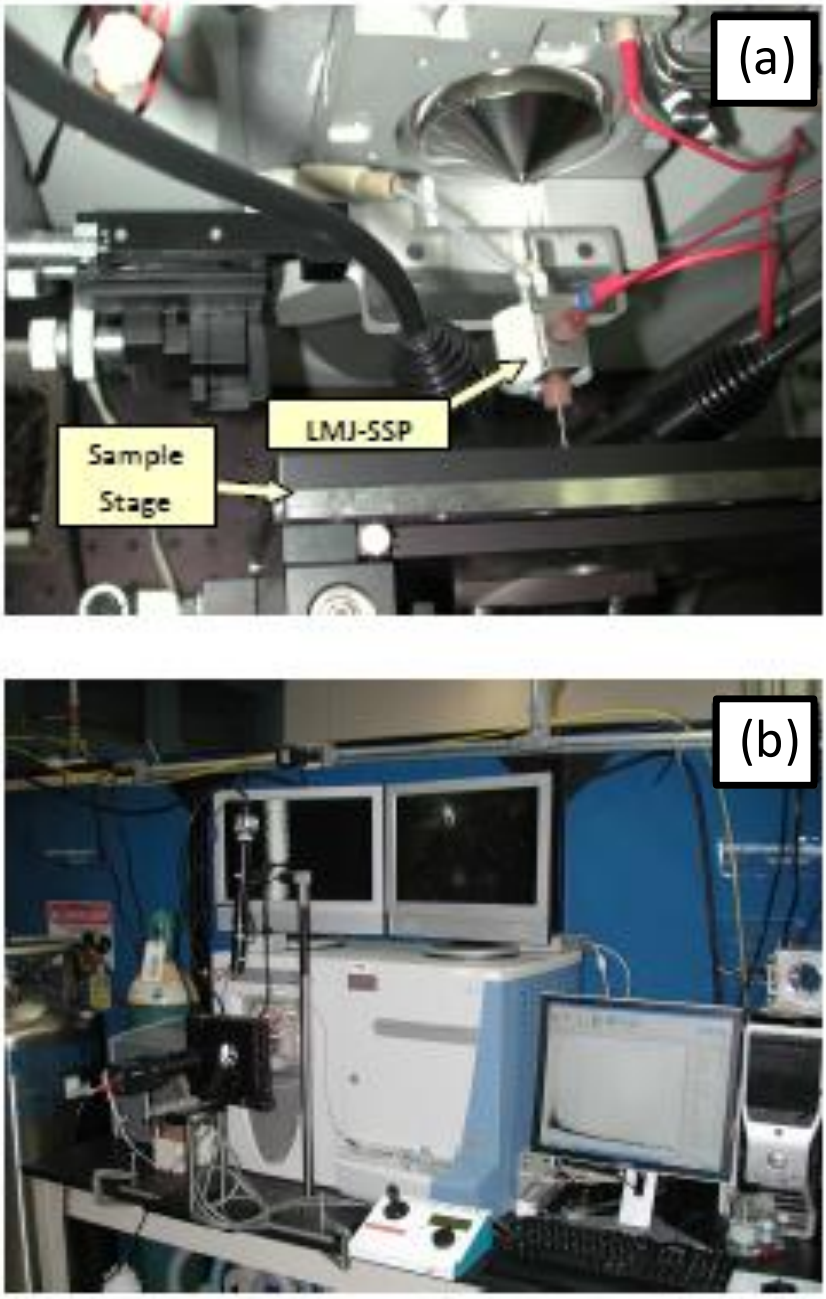
Amino acids sequences for each tryptic protein analyzed, indicating identified peptides, can be found in Chapter 4 SI 3. The sequence coverage from each plate type was similar for all the protein digests examined except for bovine serum albumin (BSA). The BSA analysis on the ProteoChrom® HPTLC cellulose sheet resulted in 40.5% sequence coverage whereas a substantially higher coverage, 60.5%, was achieved with the ProteoChrom® HPTLC Silica gel 60 F_{254S} plate. Twenty-nine BSA tryptic peptides were identified through their respective MS/MS spectra in a single cellulose lane scan (see chapter 4 SI 4), while forty-one BSA tryptic peptides were identified in a single BSA lane scan on the ProteoChrom® HPTLC Silica gel 60 F_{254S} plate. The higher sequence coverage, as it relates to the BSA digest, can be attributed to the greater separation efficiency afforded of peptides on the ProteoChrom® HPTLC Silica gel 60 F_{254S} media versus ProteoChrom cellulose under the reported development conditions. Beta casein gave the lowest sequence coverage of the examined proteins on both ProteoChrom® HPTLC Silica gel 60 F_{254S} and cellulose HPTLC plates. When the base peak chromatograms from beta casein lane scans on both HPTLC plate types was examined closely it revealed many significant ion signals, corresponding to the visualized bands, were not being identified as beta casein tryptic peptides. Further investigation revealed the possibility that the digested beta casein contained phosphorylated serine residues which precluded their identification using DBdigger.[123] Collision induced dissociation (CID) can be used to identify the presence of phosphorylation; however, because of H_3PO_4 (98 Da) and/or HPO_3 (80 Da) losses the fragmentation spectra are complex. The use of electron capture dissociation (ECD), for example, would provide more sequence data and unequivocal determination of phosphorylated peptides.[131]

In any case, the protein sequence coverage we achieved here was better in all cases than the coverage we have reported for the same protein digests using either the LMJ-SSP and hydrophobic reversed-phase HPTLC plates[20] or DESI-MS and these same normal phase (untreated) plates.[132] The higher sequence coverage reported may be due in part to the use of a newer more sensitive ion trap in the present studies. However, the quality of the data still attests to the effectiveness of the surface treatment and the subsequent LMJ-SSP readout of these surfaces that otherwise could not have been analyzed by this type of sampling probe.

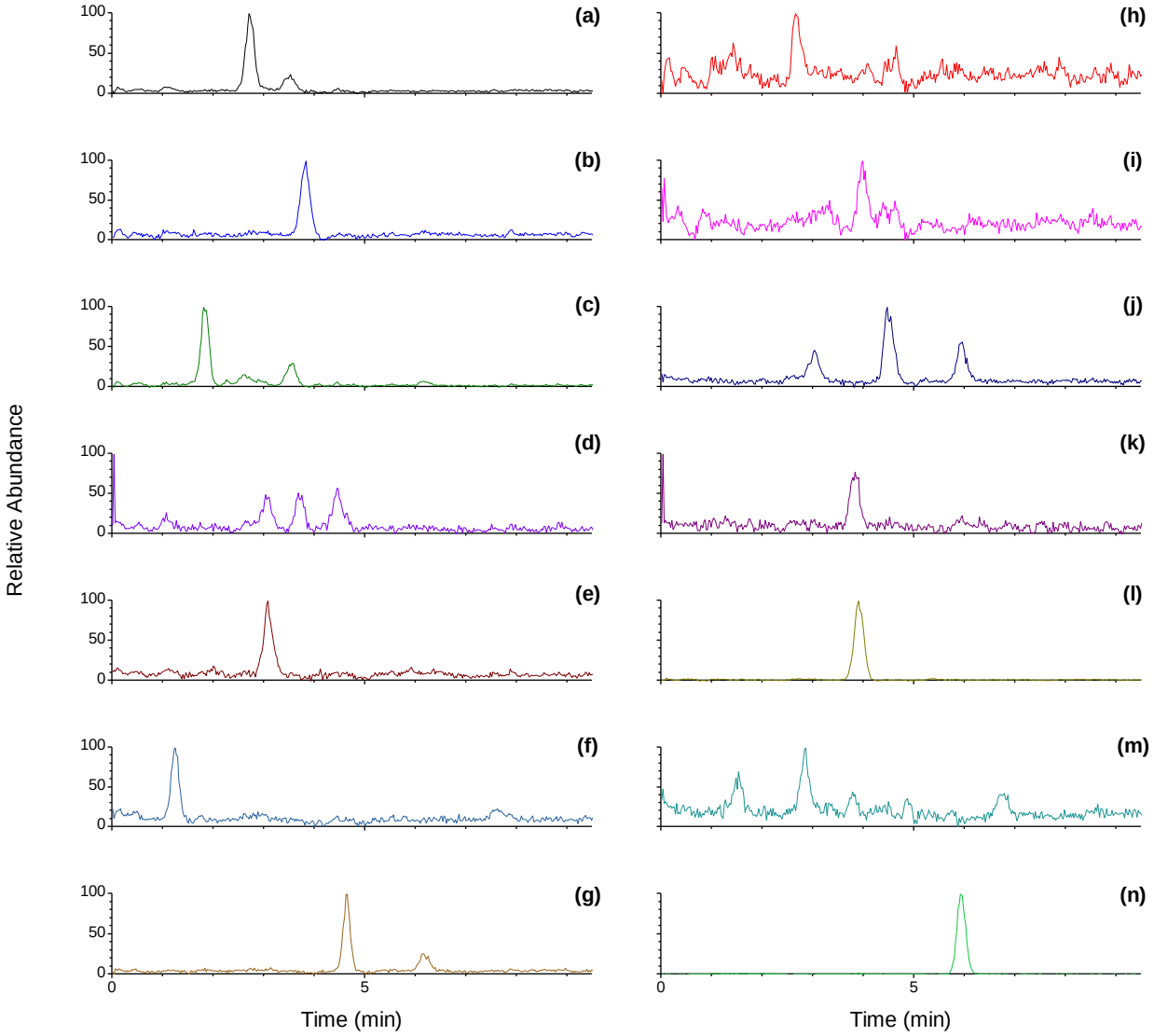
4.4 Conclusions

We demonstrated in this paper that a simple, inexpensive surface treatment process using commercially available silicone aerosol sprays could be used to create the proper surface characteristics for effective LMJ-SSP analysis of wettable surfaces that have been previously inaccessible to this surface sampling approach. The treatment process and subsequent analysis presented here emphasized wettable HPTLC phases, using examples of separated small molecule natural products and peptides from protein tryptic digests on three different stationary phase types. As we also mentioned, providing data in the Supplemental Section, other wettable surfaces like dried blood spot paper and kimwipes can be made amenable to analysis with this treatment. Thus, this simple surface treatment process significantly expands the analytical surfaces that can be analyzed with the LMJ-SSP, and therefore, also expands the analytical utility of this liquid extraction based surface sampling approach.

Chapter 4 Supplemental



Chp 4 SI 1. Photographs showing the LMJ-SSP setup on LTQ mass spectrometer. (a) Close up view of LMJ-SSP and atmospheric pressure interface region of mass spectrometer. (b) Wide angle view of complete instrumental setup.



Chp 4 SI 2. Extracted ion chromatograms of some BSA tryptic peptide signals not identified by MS/MS. In some chromatograms multiple peaks are seen as a result of isobaric signals from other peptides or redundant signals from other tryptic peptides in different charge states. Because the SSP only samples a small portion of the development lane MS parameters can be adjusted to search deeper into full scans or signals identified as real chromatographic bands can be targeted to elucidate or confirm identification. (a) LK (b) LR (c) LAK/ALK (d) QIK (e) SLGK (f) ADEK (g) FW+GK (h) EK (i) FK/MK (j) VTK (k) FPK (l) VTR (m) LSQK (n) WVTFISLLLLFSSAYSR.

Chp 4 SI 3.
Peptide ID Information
Amino acids in black font and underlined was identified by MS/MS database searching. Amino acids in red font only were not identified.

BSA Digest
Proteochrome HPTLC cellulose plate
MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPF
DEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQ
EPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPEL
LYYANKYNGVFQECCQAEDKGACLLPKIETMREKVLTSSARQRLRCASIQKFGERALK
AWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDT
ISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSF
LYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIK
QNCDQFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPC
TEDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTF
HADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEAC
FAVEGPKLVVSTQTALA
Sequence Coverage 40.5%

Proteochrome HPTLC silica gel 60 plate
MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPF
DEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQ
EPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPEL
LYYANKYNGVFQECCQAEDKGACLLPKIETMREKVLTSSARQRLRCASIQKFGERALK
AWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDT
ISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSF
LYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIK
QNCDQFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPC
TEDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTF
HADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEAC
FAVEGPKLVVSTQTAL
Sequence Coverage 60.5%

Lysosyme Digest
Proteochrome HPTLC cellulose plate
KVFGRCELAAAMKRHGLDNYRGYSLGNWVCAAKFESNFNTQA
TNRNTDGSTDYGILQINSRWWCNDGRTPGSRNLCNIPCSALLSS
DITASVNCAKKIVSDGNGMNAWVAWRNRCKGTDVQAWIRGCRL
Sequence Coverage 75.2%

Proteochrome HPTLC silica gel 60 plate
KVFGRCELAAAMKRHGLDNYRGYSLGNWVCAAKFESNFNTQA
TNRNTDGSTDYGILQINSRWWCNDGRTPGSRNLCNIPCSALLSS

DITASVNCAKKIVSDGNGMNAWVAWRNRCKGTDVQAWIRGCRL
Sequence Coverage 88.4%

Myoglobin Digest
Proteochrome HPTLC cellulose plate
GLSDGEWQQV LNVWGKVEAD IAGHGQEVLI RLFTGHPETL
EKFDKFKHLKTEAEMKASEDLKKHGTVVLT ALGGILKKKG HHEAELKPLA
QSHATKHKIP IKYLEFISDA IIHVLHSKHPGDFGADAQGA MTKALELFRN
DIAAKYKELG FQG
Sequence Coverage 98.0%

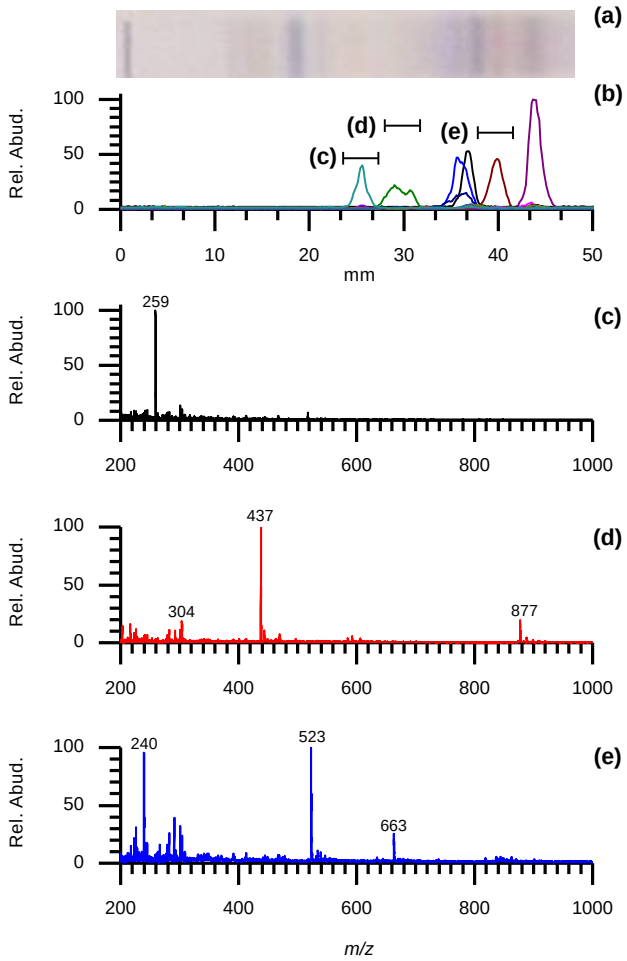
Proteochrome HPTLC silica gel 60 plate
GLSDGEWQQV LNVWGKVEAD IAGHGQEVLI RLFTGHPETL
EKFDKFKHLKTEAEMKASEDLKKHGTVVLT ALGGILKKKG HHEAELKPLA
QSHATKHKIP IKYLEFISDA IIHVLHSKHPGDFGADAQGA MTKALELFRN
DIAAKYKELG FQG
Sequence Coverage 85.6%

Beta Casein Digest
Proteochrome HPTLC cellulose plate
MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQD
KIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKE
MPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQS
KVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV
Sequence Coverage 22.3%

Proteochrome HPTLC silica gel 60 plate
MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQD
KIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKE
MPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQS
KVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV
Sequence Coverage 29.5%

Bovine Cytochrome C Digest
Proteochrome HPTLC cellulose plate
GDVEKGKKIF VQKCAQCHTV EKGGKHKTGP NLHGLFGRKT GQAPGFSYTD
ANKNKGITWGEETLMEYLEN PKKYIPGTKM IFAGIKKKGE REDLIAYLKK ATNE
Sequence Coverage 90.4%

Proteochrome HPTLC silica gel 60 plate
GDVEKGKKIF VQKCAQCHTV EKGGKHKTGP NLHGLFGRKT GQAPGFSYTD
ANKNKGITWGEETLMEYLEN PKKYIPGTKM IFAGIKKKGE REDLIAYLKK ATNE
Sequence Coverage 89.4%



Chp 4 SI 4. (a) Tryptic digest of lysosyme separated on ProteoChrom® HPTLC Cellulose sheets and stained using ProteoChrom® Color Peptide Stain. (b) Extracted ion current profiles for all peptides identified by LMJ-SSP/ESI-MS/MS scan of the development lane. A 100 µms-1 scan rate was used with an extraction solvent of 60/40/0.1 H_2O/ACN/formic acid (v/v/v). (c) (d) (e) represent full scan mass spectra averaged over indicated distances along scanned development lane.

Direct Analysis of Reversed-Phase HPTLC Separated Peptides from Protein Tryptic Digests using a Surface Sampling Probe/ESI-MS System.

5.1 Introduction

Protein identification is commonly done via "bottom up"[133,134] approaches that use HPLC-MS/MS to identify and characterize enzymatically digested proteins. Peptide ions are commonly generated from protein digests by either matrix assisted laser desorption ionization (MALDI)[135,136] or electrospray[137] ionization (ESI). However use of ESI is often preferred because ESI produces multiply charged peptides that typically provide more informative MS/MS data than singly charged peptides[138,139]. While HPLC-MS methods are unlikely to be completely supplanted by HPTLC for peptide separations, there are some drawbacks of being time consuming and involving the use of copious amounts of solvents as well as inhibiting the repeat analysis of a particular protein digestion. Thin-layer separation methods advantages over HPLC methods include the ability to separate several samples in parallel and to archive a separation on a TLC plate and offer the ability to analyze a separation several times. Low detection levels and molecular identification capabilities[140,141,142,143] make mass spectrometry the analytical method of choice for the readout of analytes separated on TLC plates. MALDI-MS has recently been shown to be a very successful analysis method for TLC plates[144, 145, 146, 147, 148], and recent efforts have been made to use electrospray based ionization methods for TLC plate analysis.[149]

To date, desorption electrospray ionization (DESI)[7] has probably shown the most promise in the ionization of biomolecules, namely proteins and peptides[150, 151, 152, 153, 154, 155, 156]. Recently, DESI has been used to analyze tryptic digests of five model proteins, which were separated on

one dimensional[157] and two dimensional[158] normal phase (NP) HPTLC plates, with a high degree of success. Although DESI has been quite successful in the analysis of peptides separated on NP HPTLC plates in our laboratory at ORNL, it has not been successful in the analysis of hydrophobic RP HPTLC plates because the hydrophobic plates are destroyed during DESI analysis when using the preferred DESI solvent water. While it may be possible to find a solvent system that allows DESI analysis of hydrophobic HPTLC plates our efforts in this regard so far have been futile.

A method for hydrophobic RP TLC plate analysis involves the use of a liquid microjunction surface sampling probe (LMJ-SSP), which forms a wall-less liquid microjunction with the surface of the TLC plate. This LMJ-SSP, based on a design first described by Wachs and Henion[159], consists of a pair of coaxial tubes with space between the inner and outer capillaries. Liquid flows down this annular space to the surface to be sampled, extracts the analyte from the surface, and then draws up the solution in the inner tube from which it is sprayed into the mass spectrometer via an electrospray emitter source. Earlier demonstrations of the analytical utility of this LMJ-SSP include the sampling and analysis of dried drugs or proteins or solutions thereof from wells on microtiter plates[9], drugs captured in solid-phase extraction cards[12], a variety of dyes, inks, or pharmaceuticals on paper or separated on hydrophobic reversed-phase (C8 and C18) thin-layer chromatography plates[8, 13, 14, 15, 16] , exogenous compounds from thin tissue sections,[19, 160] and surface-deposited and affinity-captured proteins.[18]

In this work, the LMJ-SSP is used to directly analyze peptides from tryptic protein digests separated on hydrophobic RP-C8 and C18 HPTLC plates. The results are compared and contrasted with a previous work using DESI to analyze the same tryptic proteins digests from NP

HPTLC plates. Although the separations on the RP HPTLC plates had lower band resolution when compared with separations performed on NP plates, the overall protein sequence coverage obtained from the RP plates via LMJ-SSP/ES-MS/MS analysis were similar to that obtained from DESI-MS/MS analysis of NP HPTLC plates. Proteins cytochome c. and myoglobin gave the highest sequence coverage, and RP-C8 plates separated the tryptic peptides slightly better and gave slightly higher sequence coverage than the RP-C18 plates. The LMJ-SSP system provides a way to obtain mass spectrometric readout of peptides separated on hydrophobic RP HPTLC plates. LMJ-SSP and DESI offer comparable results from the readout of RP and NP TLC plates and these techniques are complementary because DESI's use of water as the primary solvent destroys the hydrophobic TLC plates and the LMJ-SSP cannot be used on NP plates because a liquid microjunction cannot be formed on a NP plate.

5.2 Experimental

5.2.1 Materials and Reagents

Ammonium bicarbonate, trypsin, HPLC-grade methanol and HPLC-grade water were used for TLC and were obtained from Merck KGaA (Darmstadt, Germany). LC-MS grade Chromosolv® solvents acetonitrile and water both with 0.1% formic acid were obtained from Sigma Aldrich (St. Louis, MS) for use with the LMJ-SSP. Proteins bovine cytochrome c., equine myoglobin, beta-casein from bovine milk, bovine serum albumin and lysozyme from chicken egg white were obtained from Sigma Aldrich (Fluka, Buchs, Switzerland). Hydrophobic HPTLC RP-18 and HPTLC RP-8 plates for separation of the tryptically digested proteins were acquired from Merck KGaA (Darmstadt, Germany).

5.2.2 Tryptic Digestion

Digestions of the five model proteins with trypsin were performed by dissolving each protein in 25 mM ammonium bicarbonate to a final protein concentration of 2 µg/µL. Trypsin was added to the protein buffer mixture so the trypsin:protein ratio was 1:100 and the mixture was incubated for 15 h at 37° C.

5.2.3 Thin-layer Chromatography

Application of the samples was done using an ATS 4 fully automated sample applicator from CAMAG (Muttenz, Switzerland). A sample volume of 7-µL was applied in a 6-mm band at a dosage speed of 45-nL/s to give a total of 14-µg of protein per band. Protein concentrations per band were as follows: cytochrome c. (1132 pmol), myoglobin (824 pmol), lysozyme (979 pmol), beta casein (560 pmol), and bovine serum albumin (210 pmol). All samples were applied 10 mm from the bottom of the HPTLC plate with the bands spaced 15 mm apart. Development of HPTLC plates was performed in a flat bottomed chamber using 70% methanol and 30% water solution with 0.1 M ammonium acetate to develop the RP plates. Migration distances for both sets of plates were 50-mm, which was achieved in 45-60 minutes. The RP plates were stained with 0.2% ninhydrin using a DS20 automated TLC sprayer (Sarstedt (DESAGA), Nurnbrecht, Germany) and were coated with fluorescamin using a normal TLC sprayer was and lastly were heated for 2 min at 120° C.

5.2.4 Mass Spectrometry

Two mass spectrometers were used in this work, an LCQ-DECA (Thermo Electron, San Jose, CA) and a 4000 QTRAP (MDS SCIEX, Concord, Ontario, Canada). The LCQ-DECA was

chosen because it is the same instrument used to analyze tryptic digested proteins separated on NP TLC plates via DESI and data dependent MS/MS, which provided the NP TLC protein sequence coverages listed in this work. In this way, the results from DESI analysis of separated protein digests on NP TLC plates and the results from LMJ-SSP analysis of protein digests separated on hydrophobic RP plates could be more directly compared. The 4000 QTRAP, however, provided better full scan mass spectra than the LCQ, so the data from the 4000 QTRAP were used to construct the ion profiles for the peptides identified from each protein digest. Figure 30 shows the LMJ-SSP-ESI-MS setup. A detailed description of each instrument setup with the LMJ-SSP has been described in detail elsewhere.[8] While, the surface sampling probes used with the 4000 QTRAP and the LCQ-DECA have the same internal components they have different outer casings. The LMJ-SSP on the 4000 QTRAP was built from a MicroIonSpray II emitter (MDS SCIEX) and was attached to the 4000 QTRAP via a modified nanospray source. The LMJ-SSP on the LCQ-DECA was designed in a similar fashion to that described by Wachs and Henion, as described previously, and was attached to the instrument via a modified LCQ-DECA nanospray ion source (Thermo Electron). Both LMJ-SSP probes had the following dimensions: a inner sprayer/emitter capillaries with a 254-µm outer diameter and a 127-µm inner diameter, and the sampling end of the probe had an outer capillary with a 635-µm outer diameter and a 327-µm inner diameter. The only difference in the two LMJ-SSP probes was the length of their respective capillaries such that the probe on the LCQ-DECA was 10 cm long and the probe on the 4000 QTRAP was 8 cm long.

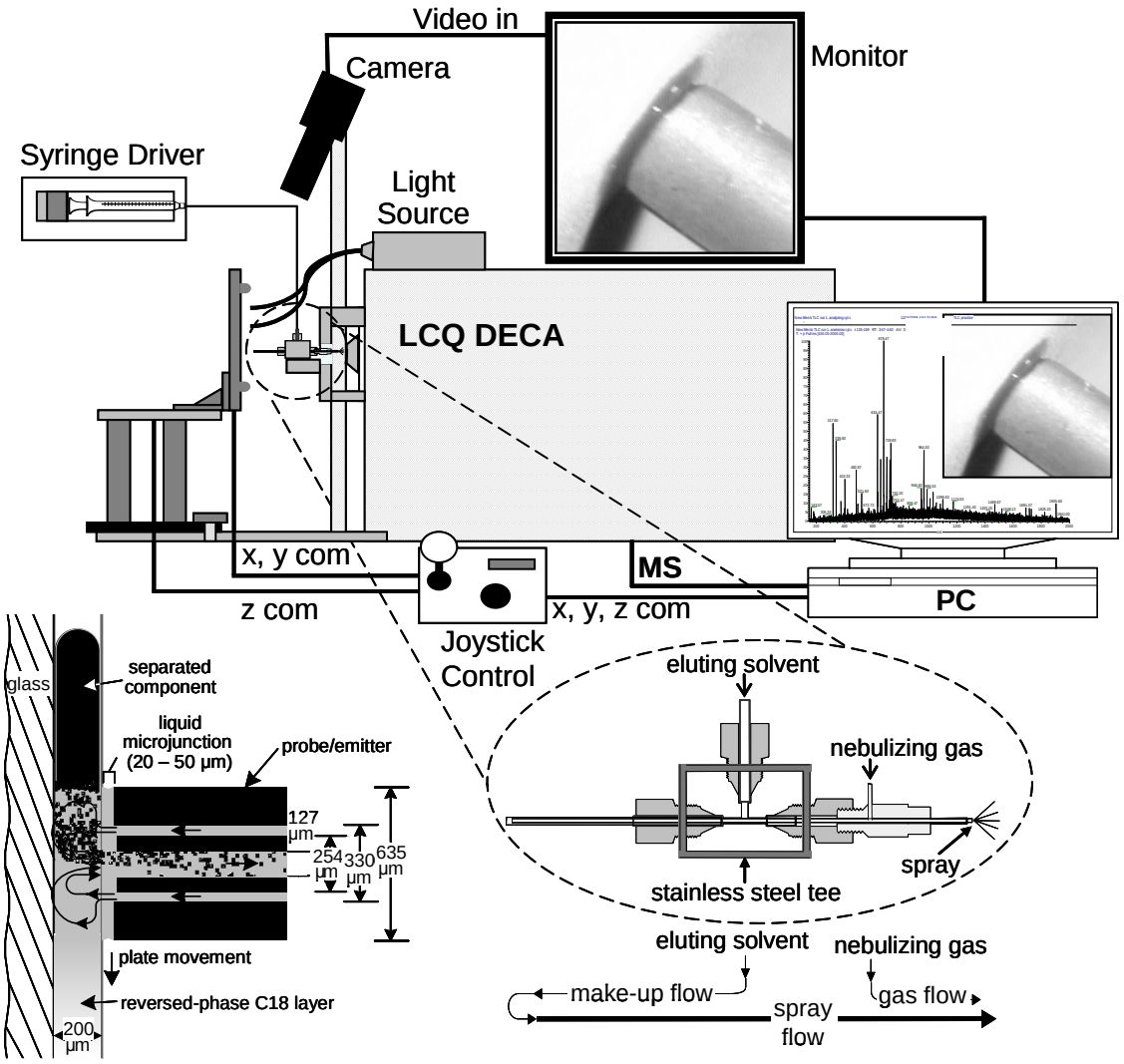


Figure 30. Schematic of liquid microjunction surface sampling probe ESI-MS/MS setup (not to scale) on the LCQ-DECA for analysis of HPTLC plates. The left side of the probe (magnified section) was responsible for making the liquid microjunction on the TLC plate and the solvent flow and electrospray process can be understood by viewing the diagram below the probe.

Operation of the LCQ Deca 3D ion trap was performed using Xcalibur software version 1.3 and the typical instrument parameters consisted of an ESI voltage of 4 kV, a capillary voltage of 7 V, a tube lens voltage of -35 V and a capillary temperature of 200° C. Automatic gain control was used for all measurements and tandem mass spectra were acquired by operating the instrument in data dependent mode so that the three most abundant peaks within each full scan mass spectrum would be subjected to tandem mass spectrometry and with dynamic exclusion set to three so MS/MS would be performed on a certain peak a maximum of three times. The normalized collision energy was set to 35% and three microscans were acquired for each spectrum over a product ion range of 200-2000 daltons. An example of the setup of the LMJ-SSP with the LCQ-DECA system can be seen in Figure 30. The 4000 QTRAP was controlled by Analyst software version 1.4.2. Typical operating conditions consisted of setting the ESI voltage to 4.5 kV, the curtain gas at 20 instrumental units and the declustering potential to 100 V. In both instruments the nebulizing gas used for probe aspiration was adjusted to the necessary level for formation of a liquid microjuction.

An elution solvent composition of 70/30 (v/v) water/acetonitrile with 0.1% formic acid by volume was used to extract the analyte peptides from the TLC plates and was pumped into the probe emitter at a rate of 10-µL/min using a 1-mL or 2-mL syringe attached to a Harvard Syringe pump. An approximately 27-cm long section of Peek tubing (127-µm inner diameter and 1/16 inch outer diameter) was used to supply the elution solvent to the probe/emitter.

An MS2000 robotic x, y, z platform (Applied Scientific Instrumentation, Inc., Eugene, OR, USA) was used to hold and maneuver the TLC plates relative to the stationary LMJ-SSP for analysis. The original microscope slide holder supplied with the stage was replaced with a

home-built TLC plate holder made from rigid, nonconductive polymer. This TLC plate holder held the TLC plate in a 100 x 100 x 1 mm milled out groove via finger-tightened plastic screws with semi-circular heads. The TLC plate was held in the vertical position and perpendicular to the LMJ-SSP. The MS2000 platform was controlled by use of a joystick in the x and y-directions and by use of a jog wheel for z-direction control. Sampling operations on both the LCQ and the 4000 QTRAP were monitored in the horizontal and vertical planes by two Panasonic GP-KR222 closed-circuit cameras (Panansonic Matsushita Electric Corporation of America, Secaucus, NJ, USA). The camera used to observe the liquid microjuntion during operation was equipped with a Optem 70 XL zoom lenses (Thales Optem Inc., Fairport, NY, USA).

All the TLC plate lane scans were enabled by using software[161] written in-house to control the ASI 2000 stage. Before scanning a lane, a liquid microjunction was created at a position along the development lane below the spotting point by manual adjustment of the jog wheel and joystick via the ASI 2000 control system by the instrument user. After making the liquid microjunction, the mass spectrometer data acquisition process was initiated simultaneous with TLC plate positioning software. The HPTLC plate was typically moved 60-mm at a scan speed of 45-µm/s or 27-µm/s and a typical scan time for an individual TLC lane was 22 min and 35 min, respectively. Both the instrument data acquisition time and TLC lane scan time were set so that the instrument would acquire data from the distance below the spotting point to beyond the solvent front. When the scan and data collection processes were finished, the liquid microjunction was broken by moving the stage away from the probe in the z-direction and then repositioning the stage for the next lane scan using the jog wheel and joystick.

Identification of the peptides observed during the TLC lane scans was performed by extracting the MS/MS spectra from raw data files and converting them to MS2 file format[122]. The MS2 files were searched using the DBDigger[123] proteomics database search program which used the MASPIC[124] scoring scheme and the DTASelect[125] algorithm for filtering the MS2 files. The DTASelect algorithm used a ΔCN of at least 0.08 and cross correlation (X_{corr}) scores of 20 (+1), 25 (+2) and 40 (+3). Surface positions of the identified peptides reveal the location where the MS/MS spectrum of the peptide was obtained**.**

5.3 Results and Discussion

5.3.1 Overview of results

The majority of the tryptic peptides from the five protein digests (cytochrome c., myoglobin, beta casein, lysozyme and bovine serum albumin), which were separated on both RP-C8 and RP-C18 TLC plates, were located in the middle R_f region of the TLC plates. The presence of most of the peptide signals in the middle of the TLC plate indicates that the separation quality of the RP TLC plates is sub-optimal. An example of these separations can be observed from Figure 31 which shows the separations of all five protein digests on both RP-C8 and RP-C18 TLC plates.

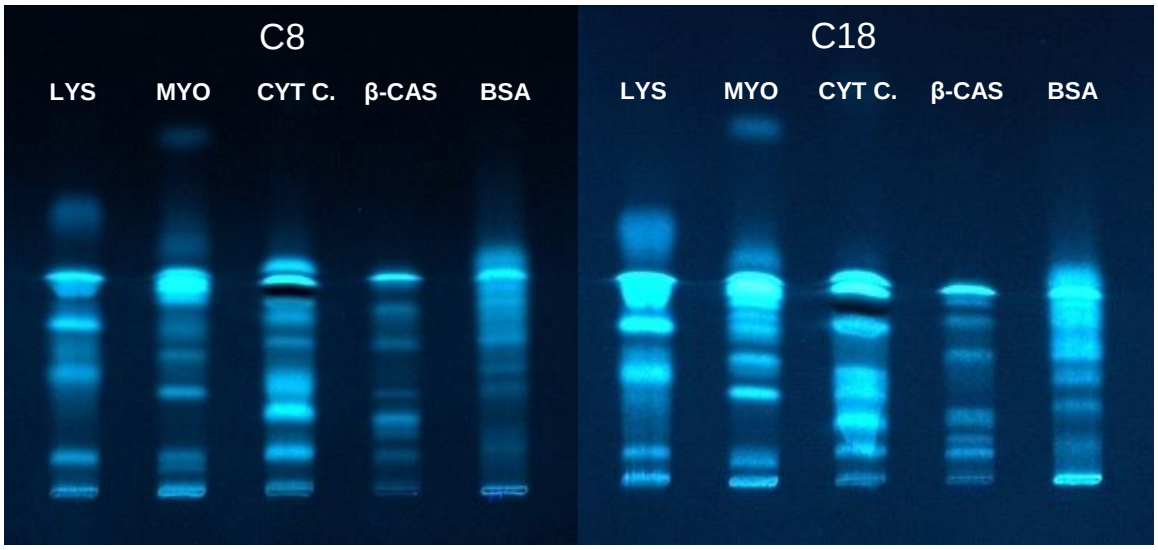


Figure 31. Shows reversed phase C8 (left) and C18 (right) HPTLC plates that were ninhydrin stained. The C8 plates on left have tighter, more resolved bands and the peptides also traveled further up the C8 plates than they did the C18 plates.

From looking at the stained plates, the bands on the C18 plate are brighter and broader, whereas the bands on the C8 plates are narrower and more separated as the peptides traveled further up the C8 plate. Higher quality peptide separations were achieved on the RP-C8 plate possibly because the C8 plate is less hydrophobic than the C18 plate and the peptides would interact more with the C18 stationary phase, leading to less efficient transport up the C18 TLC plate. A visual comparison of peptide separations on hydrophobic RP plates to those on NP and cellulose plates reveals that the NP plates separate peptides better than the RP plates but that the cellulose plates gave the best overall separations. Additionally, the peptide separations performed on the RP and NP plates do have one thing in common; the peptide layout order is similar on both types of plates. This similarity is odd considering that RP plates should, theoretically, separate peptides in the opposite order as NP plates. Examination of the peptides from cytochrome c. and myoglobin by use of the ProtParameters tool within the ExPASy proteomics server reveal no apparent trend in their peptide separations. Additionally, the peptides do not appear to be separated on the basis of hydrophobicity, molecular weight, charge state or by the number of acidic/basic sites.

The sequence coverage for the five proteins analyzed on both RP and NP plates is shown in Table 6 and the coverage from the RP-C8 plate is comparable to that achieved from analysis of the NP plate by DESI-MS. One difference in analysis on the RP versus NP plates is that the NP plates were scanned at a 100-μm/s velocity whereas the RP plates were scanned at a 45-μm/s or 27-μm/s velocity. The RP plates were scanned at a slower velocity to help compensate for the lower quality separations of the RP plate by giving the mass spectrometer more time to analyze every peptide.

Table 6. Sequence coverages obtained for five protein tryptic digests analyzed by LMJ-SSP-MS/MS for the RP-C8 and RP-C18 HPTLC plates and by DESI-MS/MS for the NP HPTLC plates (NP sequences coverages obtained from reference157).

**** BSA 210 pmol**

Protein	RP-C8 HPTLC	RP-C18 HPTLC	Silica gel[26] HPTLC
BSA (66 kDa, 21 pmol)	5.3%	8.6%	14.3%
Beta Casein (25 kDa, 560 pmol)	12.1%	12.1%	17.4%
Cytochrome C. (12 kDa, 1132 pmol)	62.5%	59.6%	59.6%
Myoglobin (17 kDa, 824 pmol)	58.2%	54.2%	66.0%
Lysozyme (14 kDa, 979 pmol)	45.7%	34.1%	27.1%

Scanning at a 27-μm/s gave the highest sequence coverage, but the sequence coverage was typically only 3-5% higher than that obtained from the 45-μm/s velocity.All the sequence coverage percentages listed in Table 6 are from the 27-μm/s velocity unless there was no difference between the 27-μm/s and 45-μm/s velocities as was the case with beta-casein and BSA.

Proteins cytochrome c. and myoglobin give much higher sequence coverage on the RP plates than the other three proteins lysozyme, beta casein and bovine serum albumin (BSA). When looking at the sequence coverage for the five proteins, it is important to consider that the proteins were not all applied in equal amounts. Not surprisingly, BSA (210 pmol) and beta-casein (560 pmol) were applied in the lowest amounts and gave much lower sequence coverage than cytochrome c. (1132 pmol), lysozyme (979 pmol) and myoglobin (824 pmol), which were applied in much higher amounts. It should also be noted that the lane scans of the protein digests performed for this work were approximately 0.5 mm wide and the band widths in each TLC lane were 6 mm wide, so the LMJ-SSP was only picking up material from 8% of the total amount of sample. This observation suggests that the LMJ-SSP analysis technique is potentially more sensitive than the data presented here suggest.

Further investigation of the full scan mass spectra of all five proteins reveals tryptic peptides that were not identified by the MS/MS data dependent scans on the LCQ-DECA but that are consistent with the expected peptide masses for tryptic digests of these proteins. The highest number of these peptides, whose identifications were not confirmed by MS/MS data, was found for BSA (eleven new peptides). New unconfirmed peptides were also discovered for cytochrome c. (three new peptides), myoglobin (five new peptides), lysozyme (two new

peptides) and beta casein (only one new peptide). The use of more sensitive instrumentation would likely enable identification of several of these missed peptides, increasing the sequence coverage obtained from RP plates. Close examination of the full scan mass spectra of all the protein digests analyzed in this work shows the presence of sodium adducts on almost every major peptide ion, which is consistent with observations made during the study of NP plates by DESI-MS. The sodium adducts observed during use of the LMJ-SSP were commonly about 15-30% of the intensity of the protonated parent ion. Sodium adducts decrease the overall ion signal available from protonated versions of a peptide, which in turn decreases the overall quality of the MS/MS data acquired from a peptide leading to lower sequence coverages.

Several peptides in three of the proteins investigated here (beta casein, cytochrome c. and lysozyme) show evidence of peptide modifications such as oxidation and N-terminal acetylation that would inhibit confident identification of these peptides if these modifications were not included in the database searching parameters. The modifications, which are indicated by a symbol placed after the modified amino acid, are represented by the symbols * and + which indicate single oxidation and double oxidation and : which indicates N-terminal acetylation. Examples include the peptides M*IFAGIK, GITW+GEETLM*EYLENPKK and N-terminally acetylated GDVEK in cytochrome c., the HPGDFGADAQGAM*TK peptide from myoglobin, the EM*PFPK peptide in beta-casein, and the IVSDGNGM*NAW+VAW+R and GTDVQAW+IR peptides in lysozyme.

Every stained band on a TLC lane correlated with a peptide mass, however, this assumption is now in question because bands exist on TLC plate that do not correlate with any observed peptide m/z value. The relatively abundant N-terminally acetylated peptide G:DVEK

from the cytochrome c. digest, for example, cannot be easily correlated with a particular band even though it has the highest ion abundance at the end of the lane. N-terminal acetylation of this peptide prevents it from reacting with the ninhydrin[162] and appearing as a brightly stained band. Although the amine group on the lysine side chain would react with the ninhydrin stain, it reacts with ~33% less efficiency than the N-terminus amine, decreasing the probability of this peptide being visible via ninhydrin staining.

5.3.2 Cytochrome c. detailed analysis

Analysis of cytochrome c. digests separated on RP-C8 and C18 HPTLC plates via mass spectrometry gives sequence coverage of 62.5% and 59.6%, respectively. These results agree with previous observations made from visual inspections of the stained C8 and C18 TLC plates which showed that C8 plates provide slightly better separations than C18 plates. The sequence coverage of the C8 plate is a few percent higher because the peptide IFVQKCAQCHTVEK is identified on the C8 plate but not the C18 plate. Although the peptides KATNE and KTGQAPFSYTDANK were identified in the C18 plate but not the C8, peptides with similar sequences were identified in the C8 plate effectively providing the same sequence information as the two former peptides.

Approximate locations of each peptide identified from the cytochrome c. tryptic digest on the RP-C8 and C18 TLC plates can be observed by referring to extracted ion profiles of the cytochrome c. peptides given in Figures 32 and 33, which show that most of the peptides identified by MS/MS are located in a relatively narrow ~10-mm distance on both the C8 and C18 plate. One peptide which was always observed in high intensities on both the C8 and C18 plates but which never contributed to the sequence coverage was GITWGEETLMEYLENPK. Both the

modified and unmodified versions of this peptide were identified from the LCQ full MS scan data at m/z 1005 and m/z 1030 but adequate fragmentation information was not obtained for confident identification, although doing so would have increased sequence coverage by ~10 percent. The modified version of this peptide contained a double oxidation on tryptophan and a single oxidation on methionine, and the relative intensity ratio of modified to unmodified appeared to be proportional to the length of time the TLC plate was exposed to air.

Details regarding the sequence coverage obtained for cytochrome c. analysis on RP-C8 and C18 plates as well as on NP plates and on cellulose plates can be viewed in Table 7. This table lists the observed peptides on the left hand column followed by their molecular weight and charge state in the next two columns. In the rest of the columns, stars in each box indicate whether a peptide was observed in the full scan mass spectra for that type TLC plate and whether MS/MS data was obtained for that peptide. Although the NP-HPTLC plates provide higher resolution separations, as previously discussed, comparable sequence coverage is obtained by the LMJ-SSP-ES-MS/MS analysis of hydrophobic RP plates (see Table 7). It is possible that the extraction efficiency of the LMJ-SSP probe compensates for the poorer separation of the RP plates. These results suggest that the use of the LMJ-SSP for analysis of hydrophobic RP HPTLC plates makes it a complementary method to DESI which is better suited for analysis of NP HPTLC plates. Further examination of Table 7 will show that the majority of the peptides were identified on both types of RP plates as well as on the NP and cellulose plates. Another similarity is that the peptide GITWGEETLMEYLENPK was not correctly identified on any of the three types of plates.

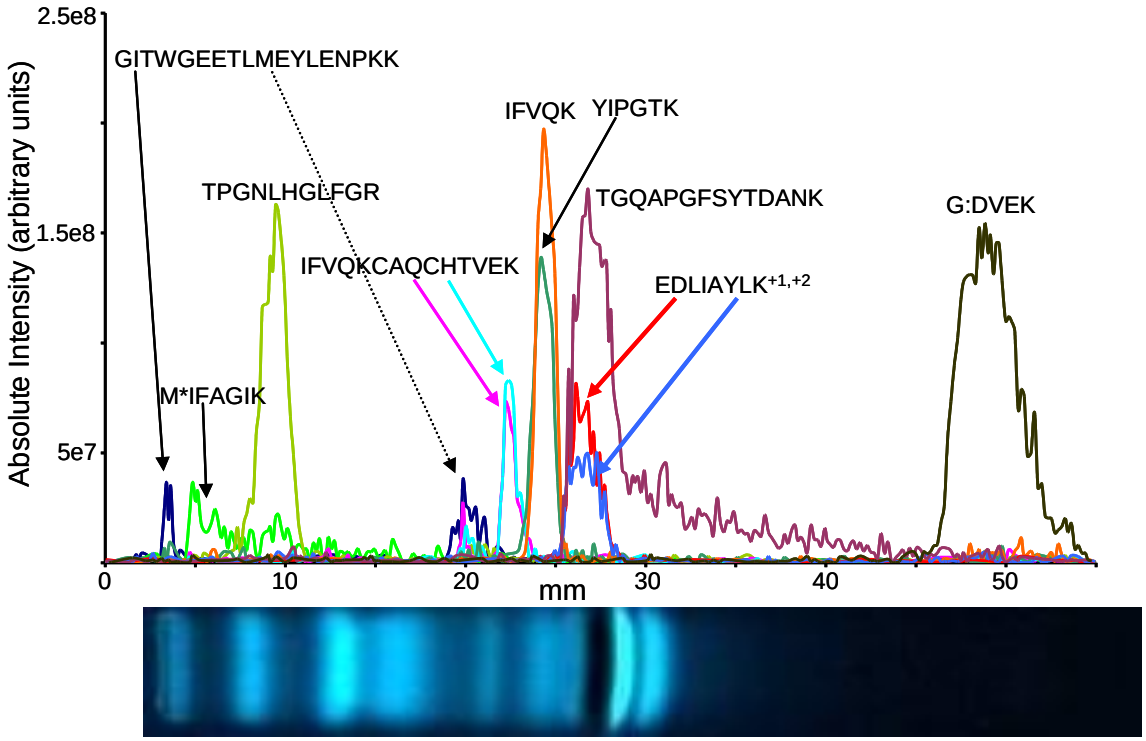


Figure 32. Extracted ion profiles for all peptides identified by LMJ-SSP-MS\MS of a cytochrome c. tryptic digest separated on a RP-C8 TLC plate. Most of the peptide signal is found in the middle of the TLC plate, demonstrating that better TLC plate separations may result in higher sequence coverage.

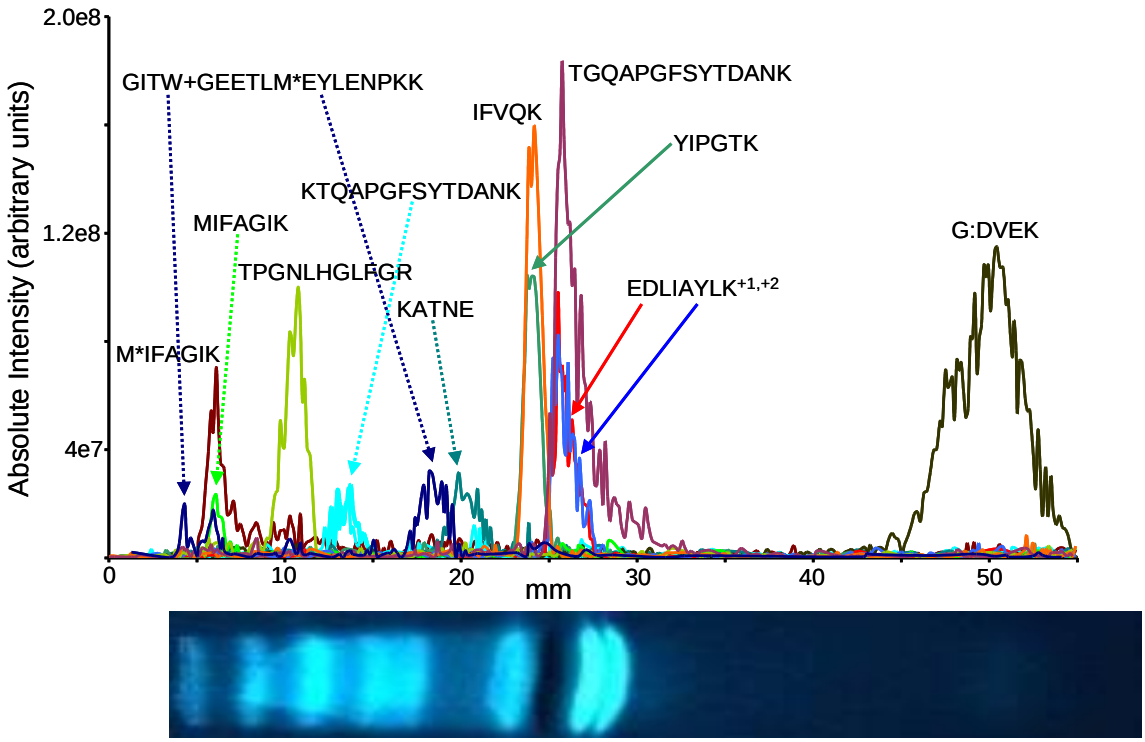


Figure 33. Extracted ion profiles for all peptides identified by LMJ-SSP-MS\MS of a cytochrome c. tryptic digest separated on a RP-C18 TLC plate. The RP-C18 HPTLC plates provided lower quality separations than the RP-C8 HPTLC plates, which resulted in lower sequence coverage being obtained from the C18 plates.

Table 7. Cytochrome c. tryptic digest peptides observed from MS and MS/MS analysis of four types of TLC plates.

			LMJ-SSP\ESI					DESI[26]			
			RP-C18		RP-C8			Silica gel		Cellulose	
Peptide	MW	z	MS	MS/MS	MS	MS/MS	z	MS	MS/MS	MS	MS/MS
GGK or NK	260	1					1	*			
GER	360	1					1	*			
KGER	489	1	*		*		1	*		*	*
KATNE	562	1	*	*	*		1	*	*	*	*
G:DEVK	589	1	*	*	*	*					
IFVQK	634	1	*	*	*	*	1	*	*	*	*
YIPGTK	678	1	*	*	*	*	1,2	*	*	*	*
KIFVQK	762	1					1,2			*	*
MIFAGIK	779	1,2	*	*			1,2	*	*	*	*
M*IFAGIK	795	1,2	*	*	*	*					
KYIPGTK	806	1	*				1,2			*	*
EDLIAYLK	964	1,2	*	*	*	*	1	*	*	*	*
TGPNLHGLFGR	1168	2	*	*	*	*	1,2	*		*	*
GEREDLIAYLK	1307	2					2	*	*		
MIFAGIKK	1378	1,2					1,2			*	
KGEREDLIAYLK	1435	2					1	*	*	*	*
TGQAPGFSYTDANK	1457	2	*	*	*	*	1,2	*	*		
KTGQAPGFSYTDANK	1585	2,3	*	*			2	*		*	*
IFVQKCAQCHTVEK	1634	2,3	*		*	*	2	*		*	*
G:ITW:G:EETLMEYLENPKK	2060	2,3	*		*						
GITWGEETLMEYLENPKK	2138	2,3	*		*		2,3			*	
Sequence Coverage				59.6%		62.5%			58.0%		72.0%

Small peptides, under 500 molecular weight, were also difficult to identify as well as peptides with low R_f values and high numbers of acidic or basic sites. As mentioned earlier, the majority of the peptides on both the NP and RP plates appear at similar development distances (R_f values). However, three peptides (MIFAGIK, TPGNLHGFLGR and TGQAPGFSYTDANK) have quite different R_f values on RP versus NP HPTLC plates. The former two peptides appear at lower R_f values or shorter development distances in the RP plate (5 mm versus 31 mm for MIFAGIK and 9.5 mm versus 26 mm for TGPNLHGLFGR) than the NP plate. The peptide TGQAPGFSYTDANK appears at a higher R_f value in the RP plate (26.5 mm versus 17.5 mm) and may have a longer development distance on the RP plate than the previous two peptides because of its lower hydrophobicity.

5.3.3 Myoglobin detailed analysis

The analysis of tryptic digested myoglobin on RP-C8 and RP-C18 plates leads to myoglobin sequence coverages of 58% and 54%, respectively. As with cytochrome c., myoglobin also gave higher sequence coverage from the RP-C8 TLC plate than the RP-C18 plate and the peptides were separated slightly better and traveled further up the C8 plate than the C18 plate. Extracted ion profiles of peptides observed from the myoglobin C8 and C18 HPTLC lanes are shown in Figures 34 and 35.

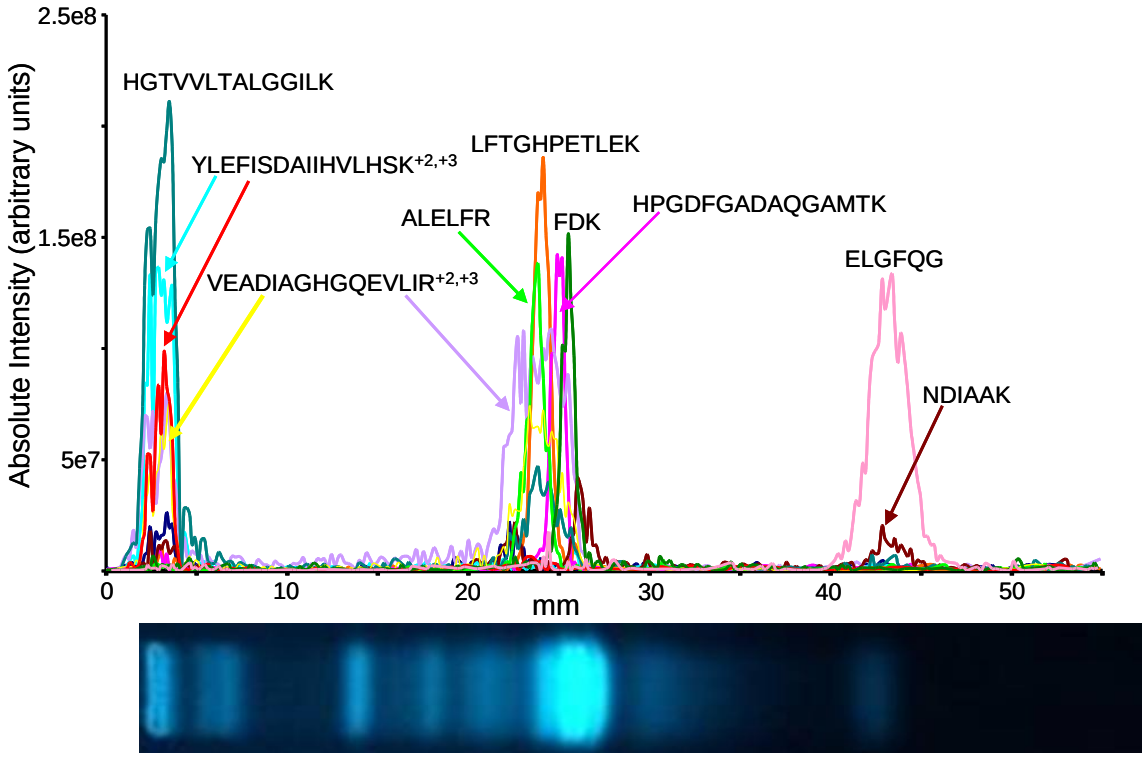


Figure 34. The extracted ion profiles for myoglobin peptides identified from a RP-C8 plate via LMJ-SSP-MS analysis. The peptide separations are better on the RP-C8 plate than on the RP_C18 plate (see Figure 35). Note that the NDIAAK peptide traveled further up the C8 plate than the C18 plate.

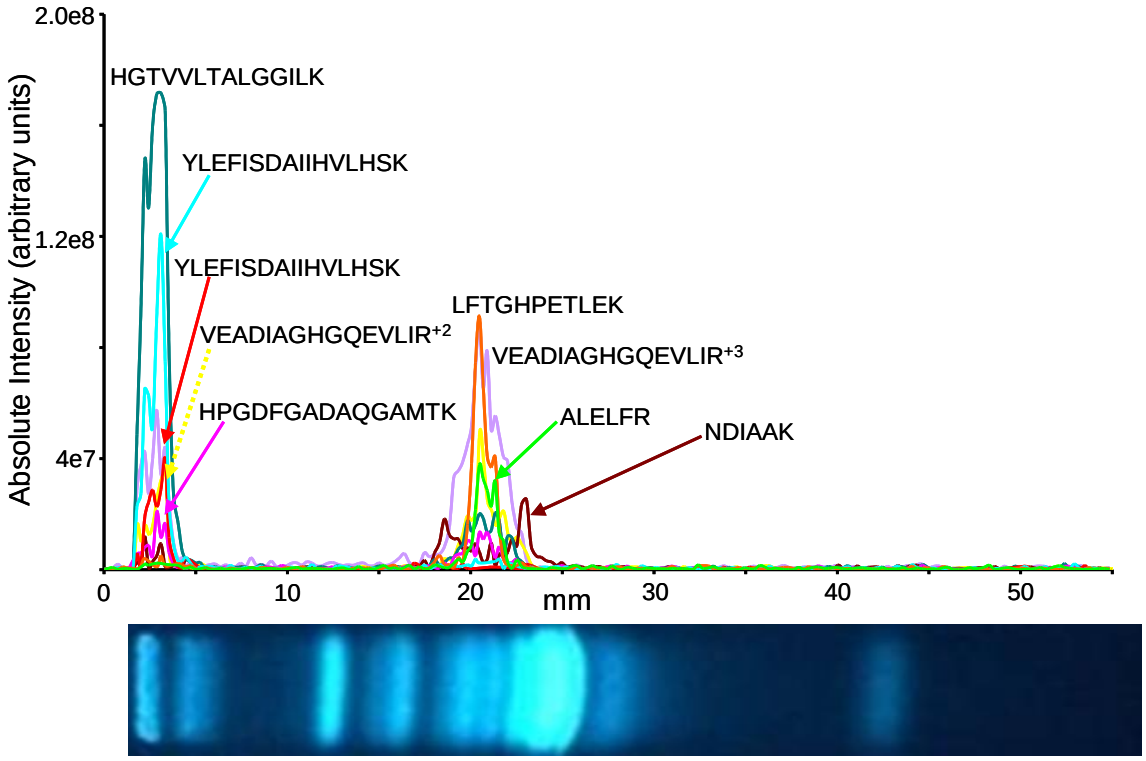


Figure 35. Extracted ion profiles for myoglobin peptides identified from LMJ-SSP-MS on a RP-C18 TLC plate. The peptides are not very well separated here and some of the peptides do not appear to move from the spotting point.

The slightly higher sequence coverage associated with C8 plate can be contributed to the identification of the ELGFQG peptide being identified on the C8 plate but not on the C18 plate. This peptide, found at 43 mm on the C8 plate, accounts for the majority of the ion signal at that position but is only identified by MS/MS once on the C8 plate despite its high abundance. A close look at the C18 myogobin data shows that the ELGFQG peptide is considered for fragmentation but does not fragment very efficiently, as reported in the literature[163], thus making its MS/MS scores too low for confident identification by DBDigger. Another myoglobin peptide that was very visible in the full scan data but not identified by MS/MS was the peptide FDK at 409 m/z. The FDK peptide appears at approximately 26 mm (see Figure 35) on the C8 myoglobin plate and has a high intensity and a similar position relative to other peptides identified by MS/MS. One possible explanation as to why the FDK peptide was not identified by MS/MS is its relatively small size, which may mean an insufficient number of fragment ions less than below 500 molecular weight were not easily identified by MS/MS from RP, NP or cellulose TLC plates, indicating that these smaller peptides may not fragment efficiently in the LCQ.

Another interesting observation from comparing the C8 and C18 TLC plates is that the NDIAAK peptide traveled approximately 20 mm further up the C8 plate than it did on the C18 plate, whereas all the other peptides that are observed on both the C8 and C18 plates differ in distance traveled by only about 5 mm on average, compare Figures 35 and 36. It appears that the NDIAAK peptide did not travel as far up the C18 plate because it may have interacted more strongly with the stationary phase on the C18 plate.

Further examination of the myoglobin data shows that several of the peptides identified from both the C8 and C18 TLC plates were present at the spotting point as well as about halfway up the developed TLC plate (~27 mm). The presence of identified peptides at the spotting point is indicative of the solvent not adequately moving the peptides up the plate during development. While the presence of some peptides at the spotting point is also observed to a lesser degree in cytochrome c. and BSA, this phenomenon is not readily noticeable in the development of the TLC plates containing lysozyme and beta casein and this phenomenon may be related to peptide composition.

A comparison of the myoglobin separation on RP versus NP HPTLC plates shows that four additional peptides were identified on the NP plate giving it a ~4% higher sequence coverage than the RP-C8 plate. Detailed information regarding this comparison can be obtained from Table 8. The first two columns in Table 8 give the peptide sequence and molecular weight, while the following columns provide m/z for each peptide and the asterisk reveal whether the peptide was identified by in the full scan MS or by MS/MS for each separation type. While most of the myoglobin peptides appeared at similar R_f values on the RP and NP plates, three peptides, however, vastly change their positions. The peptide YLEFISDAIIHVLHSK appears at 28-mm on the NP plate and 4 mm on the RP plate, traveling much further up the NP plate possibly because of the peptide's high hydrophobicity. The other two peptides ASEDLKK and NDIAAK traveled shorter distances on the NP plate at 8 mm on NP versus 22.5 mm on RP for ASEDLKK and 12 mm on NP versus 42.5 mm on RP for NDIAAK. It is plausible that the two former peptides interacted more strongly with the hydrophilic NP plate, resulting in lower R_f values.

Table 8. Lists all myoglobin tryptic digest peptides observed from MS and MS/MS analysis of four types of TLC plates. LMJ-SSP-MS/MS was used to analyze the RP (C8 and C18) TLC plates and DESI-ES-MS/MS was used to analyze both the NP and cellulose TLC plates. The RP and NP plates provided similar sequence coverage; however, as with cytochrome c. the cellulose TLC plate provided the highest overall sequence coverage.

			LMJ-SSP/ESI					DESI[26]			
			RP-C18		RP-C8			Silica gel		Cellulose	
Peptide	MW	z	MS	MS/MS	MS	MS/MS	z	MS	MS/MS	MS	MS/MS
YK	309.4						1			*	
HLK	396.5						1			*	*
FDK	408.5	1	*		*		1	*		*	
IPIK	469.6						1	*		*	*
NDIAAK	630.7	1	*	*	*	*	1	*	*	*	*
ELGFQG	649.7	1	*		*	*	1	*			
FDKFK	683.8						1			*	*
TEAEMK	707.8	1					1	*	*		
HKIPIK	734.9						1,2			*	*
ALELFR	747.9	1	*	*	*	*	1	*	*	*	*
ASEDLKK	789.9	1,2	*		*		1,2	*	*	*	*
YKELGFQG	941.1	1,2	*		*		1,2	*	*		
LFTGHPETLEK	1271.4	2	*	*	*	*	2	*	*	*	*
TEAEMKASEDLK	1351.5						2	*			
FKHLKTEAEMK	1361.6						2	*			
TEAEMKASEDLK	1479.0	2,3	*		*						
HGTVVLTALGGILK	1378.7	2	*	*	*	*	1,2	*		*	*
HPGDFGADAQGAMTK	1502.6	2	*	*			2	*	*		
HPGDFGADAQGAM*TK	1519.2	2	*		*	*	2	*	*		
VEADIAGHGQEVLIR	1606.8	2,3	*	*	*	*	1,2	*	*	*	
LFTGHPETLEKFDK	1661.9						1,2	*	*	*	*
GLSDGEWQQVLNVWGK	1816.0	2	*		*						
GHHEAELKPLAQSHATK	1855.0	3	*		*						
YLEFISDAIIHVLHSK	1885.2	2,3	*	*	*	*	1,2	*	*	*	*
KGHHEAELKPLAQSHATK	1982.2						2,3			*	*
Sequence Coverage				54.2%		58.2%			62.0%		68.0%

5.3.4 Detailed Analysis of BSA, Beta-Casein and Lysozyme

The TLC plate separations of the other three proteins (lysozyme, beta-casein and BSA) investigated in this study provide similar results to the more in depth discussions involving myoglobin and cytochrome c presented above. The RP C8 and C18 TLC plate separations of beta-casein, lysozyme and BSA protein digests also show that C8 separations are slightly better than C18 separations and have slightly higher sequence coverage. In addition, the NP plates provide better separations of these three protein digests than do RP plates, but the sequence coverages obtained from the RP plates are still comparable to those derived from the NP plates. Some interesting observations were made during analysis of the beta casein, lysozyme and BSA separations. Although the separation of BSA peptides appeared adequate on the RP plates, the large size (66 kDa) and relatively low amount of BSA (210 pmol) spotted on the TLC plates are responsible its low sequence coverage, which was less than 10% in both RP-C8 and C18 TLC plates. Like BSA, the larger size of beta-casein deposited (560 pmol) on the TLC plate contributed to the low seqence coverage obtained from this protein, which was 12.1% for both the C8 and C18 plates. The relatively narrow separation range of the beta-casein peptides on the HPTLC plate which was 7.5-mm for the C8 plate and 5-mm for the C18 plate also played a role in the low sequence coverage afforded beta-casein. Lysozyme had the third highest sequence coverage of the peptides tested (34% and 46% for C18 and C8, respectively). These coverages are attributable to the relatively good separations afforded lysozyme on both C8 and C18 plates as well as to its smaller size of 14 kDa, which is 2000 daltons smaller than myoglobin. The only explanation as to why lysozyme didn't provide higher sequence coverage than myoglobin is that it was less well separated on the HPTLC plates. Other factors that influence the sequence

coverage attainable from a tryptic protein digests using this surface sampling analysis method would include efficiency of the tryptic digestion, average size of the tryptic peptides produced from a protein, the ionization efficiency of the peptides produced, and the separation efficiency of the peptides on the TLC plate.

5.4 Conclusions

A liquid microjunction surface sampling probe which also acts as an electrospray ionization source has been used to extract and ionize tryptic peptides from hydrophobic reversed phase HPTLC plates for analysis via tandem mass spectrometry. Sequence coverages obtained from analysis of five model protein digests on a RP-C8 TLC plate using this method were 62.5% for cytochrome c., 58.2% for myoglobin, 45.7% for lysozyme, 12% for beta-casein and 8.5% for bovine serum albumin. Analysis of both RP-C8 and RP-C18 HPTLC plates was performed with the C8 plates providing slightly better separations and slightly higher sequence coverage on average than the C18 plates. The hydrophobicity of the C18 HPTLC plates may be responsible for the lower quality peptide separations on the C18 plates. Sequence coverages of the five proteins analyzed via LMJ-SSP-MS/MS are similar to those obtained via DESI-MS/MS of the same five protein digests separated on NP TLC plates, despite the fact that the NP TLC plates provided better peptide separations. Successful analysis of hydrophobic RP plates with the LMJ-SSP adds to the list of thin layer media that can be readout by ambient ionization techniques. The LMJ-SSP's ability to analyze hydrophobic RP TLC plates makes it a complementary ambient ionization method to DESI which is better suited to the analysis of NP TLC plates. Further advances in the quality of peptide separations on NP and RP TLC plates will likely

improve the protein sequence coverage obtainable by these ambient ionization/surface sampling techniques.

Chapter 6

Spatial LC-MS: HPTLC- Surface Sampling Analysis of Intact Proteins

6.1 Introduction

The direct readout of intact proteins spatially separated on an analytical surface, such as planar chromatographic media or gels, is attractive. [164,165] Direct readout would ultimately increase the throughput of intact protein analyses that are fractionated on gels or planar chromatographic media. As of late intact proteins separated on gel slabs must be blotted on to membranes, cleaned of detergents and buffers, and extracted off the blotting membrane prior to analysis. The use of column based LC-MS for intact protein analysis does not entail as much sample preparation and is fact a higher throughput technique that couples with MS. The development of intact proteins on commercially available high performance thin layer chromatography (HPTLC) plates has little history[166,167,168] and is inherently difficult due to the complex nature of intact proteins. The use of spatial chromatography for intact protein analysis may prove useful as a result of less sample preparation, when compared to a gel based analysis. Spatial chromatography also allows for the analysis of bands or spots that are not transient but static. Because separations on spatial chromatographic media can be readout after the separation many advantages related to the use of multiple detection schemes for analyte characterization/identification are applicable. In many intact protein analyses using multiple analytical technologies (including fluorescent tagging of proteins for LIF detection, NMR,

MALDI-MS, ESI-MS, and so on) are already used. Here we present a chromatographic system able to fractionate and efficiently move intact proteins of molecular weight >5000 Da.

The use of atmospheric pressure surface sampling/ionization techniques for the coupling of spatial separations with mass spectrometry has gained much attention.[169,170,171] Atmospheric Surface Sampling/Ionization Mass Spectrometry has experienced much growth and attention the last ten years. [172,173,174,175,176,177,178,179] Direct liquid extraction based surface sampling probes can be used to directly analyze material at or just below a surface of interest, but also have the ability to further process extracted material in analytical workflows. This ability to further process samples of extracted material can serve to increase the amount of attainable chemical information, improve sensitivity, and enhance selectivity, among other attributes.

A sealing surface sampling probe (S-SSP) introduced by Luftmann[180], is an example of a direct liquid extraction based surface sampling probe. With this probe, analyte is extracted from a surface by sealing the probe to the surface using a knife edge on the probe that cuts into the surface. By principle, this sealing mechanism does not readily allow analysis of analytes simply deposited on the surface of hard, inflexible, nonporous materials like metal, glass, or various plastics sheets. For this reason, this Luftmann-type SSSP has been mainly used for the analysis of mixtures and extracts (pharmaceuticals, components of plant extracts, etc) separated on normal phase TLC plates. More importantly, a modified version of the Luftmann-type S-SSP became commercially available recently by the name of "TLC-MS interface". [181] Soon after its release, this interface was used for analysis of dried blood spots on paper and small animal whole-body thin tissue sections on adhesive tape. Recently the TLC-MS interface was used for the successful readout of single peptides of angiotensin-converting enzyme inhibitors.[182] The

readout of intact protein separations on spatial chromatographic media using atmospheric pressure surface sampling/ionization techniques is needed. Here we show the successful development of intact proteins on ProteoChrom® Silica gel 60 F254s plates with S-SSP-MS readout. Development and S-SSP-MS readout were optimized using a standard intact protein, myoglobin. The development of five different proteins and their direct MS readout is also presented. This approach to intact protein analysis should not be considered an alternative to global proteomics studies or workflows as of yet. This work does represent the first attempt to intact protein analysis usually reserved for column based LC-ESI-MS or matrix assisted laser desorption ionization methods.

6.2 Experimental

6.2.1 Materials and Reagents

LC-MS grade Chromosolv® solvents acetonitrile (ACN) and water both with 0.1% formic acid (v/v) were obtained from Sigma Aldrich (St. Louis, MO) for use with the S-SSP/ESI-MS analyses. ProteoChrom® HPTLC Silica gel 60 F254s plates used for separations of proteins were acquired from Merck KGaA. Bovine cytochrome c., equine myoglobin, and beta-casein from bovine milk were obtained from Sigma Aldrich (Fluka, Buchs, Switzerland). Glucagon from hog pancreas (>70% HPLC) and ubiquitin from red cow blood bodies were obtained from Fluka Analytical. 1-Butanol ACS reagent, ≥99.4%, and Pyridine Chromasolv® Plus, for HPLC, ≥99.9% , Ammonium hydroxide 28% NH_3 Sigma Aldrich (St. Louis, MO), and LC-MS grade Chromasolv® water Sigma Aldrich (Allentown, PA) were used to make HPTLC development solutions which were prepared fresh daily.

6.2.2 Thin-layer Chromatography

The development of the HPTLC plates was carried out in a normal flat-bottomed chamber using 1-butanol/pyridine/NH_3/water 39/20/10/31 (v/v/v/v) for the ProteoChrom® Silica gel 60 F254s plates. The chamber was not saturated prior to development and development solvents were prepared daily. The migration distance on the ProteoChrom® Silica gel 60 F254s plate used for the analysis was 50 mm achieved in 60 min. A total sample volume of 3-7 μL was applied as a 6-mm band at a dosage speed of 50 nL s^{-1} using a Linomat 5. The Linomat 5 is a sample applicator system using a spray on technique. The Linomat 5 applies bands of sample versus manual spotting. Table 9 lists protein standards and their concentrations. Glucagon and ubiquitin at 0.35 and 1.35 mg ml^{-1} standards were in water. Myoglobin, cyctochrome c, and β-casein, each at 4.0 mg ml^{-1} standards were in 85/15 water/ACN (v/v). Plates were dried for ten minutes prior to development. Staining after development and sufficient drying time was done with a 0.25% ninhydrin solution followed by treatment with heat, 2 minutes at 110°C.

Table 9. Single intact protein solution and application conditions for figure 38.

Track	Sample	Concentration	Application volume (µL)	Loaded Mass (µg)	Loaded Mass/mm
1	Glucagon	0.35mg/ml	7	2.45	0.41 µg/mm
2	Myoglobin	4mg/ml	3	12	2.0 µg/mm
3	Cytochrome C	4mg/ml	3	12	2.0 µg/mm
4	ß-Casein	4mg/ml	4	16	2.67 µg/mm
5	Ubiquitin	1.35mg/ml	7	9.45	1.58 µg/mm

1) A Linomat 5 was used to spot 6 mm bands on the plate

6.2.3 Sealing Surface Sampling Probe Analysis

The inlet of the S-SSP (TLC-MS interface, CAMAG, Muttenz,Switzerland) was coupled to an Agilent 1100 High Performance Liquid Chromatography (HPLC) system and to a Thermo LTQ-XL mass spectrometer. Figure 36 shows an image of the Camag TLC-MS interface as well as a chart showing the modes of operation as a spot sampling technique. Sampled material can be subjected to direct infusion, flow injection analysis, or inject on to a chromatographic column. The gas pressure applied via a piston to the plunger of the SSSP interface was about 5 bar. The flow rate of the extraction solvent was 50 µL/min in all cases. When sampling the 6 port injection valve would be switched to extraction position and left open for the duration of the experiment. After sampling the extraction head would be cleared several times with the high pressure air flow. An insulin standard at 1 µM concentration water/ACN/formic acid 50/50/.1 (v/v/v) was directly infused and used to tune MS instrument parameters. The LTQ-XL mass spectrometer was operated in positive ion ESI mode, Automatic Gain Control on, the spray voltage was set at 5 kV, 8.0 arbitrary sheath gas flow rate, capillary voltage 1.03, and capillary temperature 200.0 °C. Full MS scans were recorded with a 3 microscan count average scanning *m/z* 200.0-2000.0.

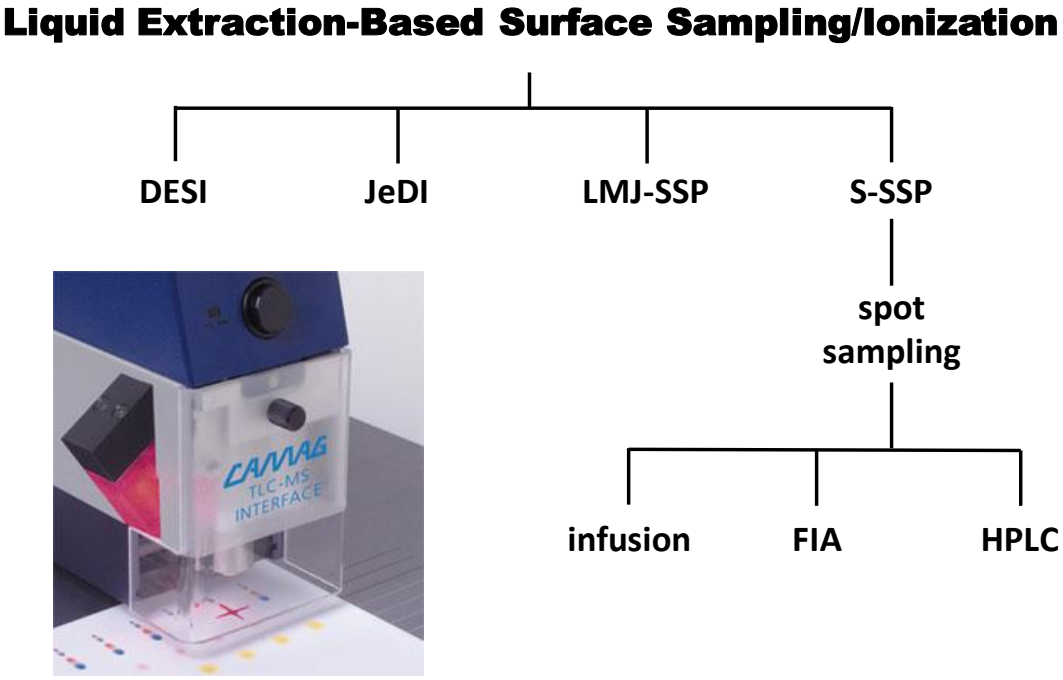


Figure 36. Sealing-Surface Sampling Probe Modes of Operation

6.3 Results and Discussion

Development of myoglobin on a HPTLC plate was performed. 3 µL of a myoglobin solution at 4.0 mg ml^{-1} was spotted as a 6 mm band on a HPTLC plate such that 12 µg of protein was deposited. The band was positioned 8 mm from the bottom of the plate. The HPTLC plate was developed using 1-butanol/pyridine/NH3/water 39/20/10/31 (v/v/v/v) in an unsaturated glass twin trough TLC tank. The composition of the development solution is critical to intact protein movement from the spotting point. Using the same solvents with little variation in percent composition will strongly influence the selectivity of the chromatographic system. After development one band could be identified visually. The visible band was later determined to be the disassociated heme. The myoglobin band position was identified by post development staining of one of the development lanes with ninhydrin. The heme band and intact protein band were directly analyzed using a S-SSP (Camag TLC-MS interface). The extraction/ESI solution was composed of 70/30/.1 water/ACN/formic acid (v/v/v) and was delivered at 50 µL min^{-1} using an Agilent 1100 LC pump. The 6 port injection valve on the TLC-MS interface was left in the 'inject' position for the entirety of the sampling. The entire dead volume post extraction and excluding the inlet frit is ~27 µL. Figure 37 (a) shows the averaged MS spectra from minute 2 to 8. Figure 37 (b)-(f) show the extracted ion chromatograms (EICs) for different charge states. The data suggests there may have been a gradient like extraction or the presence of suppressing species preventing the formation of higher charge state intact protein ions. Higher charge states were initially suppressed but as the extraction time continued the ion packet shifted to higher charge state formation. This experiment demonstrated a 16.7 kDa protein could be moved as a

band on planar chromatographic media and the band could be analyzed using liquid extraction based surface sampling mass spectrometric techniques.

Single protein solutions were spotted as 6 mm bands on a ProteoChrom HPTLC Silica gel 60 F(254S) plates 8 mm from the bottom of the plate. Details of applied absolute masses applied are found in table 9. Glucagon, myoglobin, cytochrome c, beta-casein, and ubiquitin were used. Plates was developed using 1-butanol/pyridine/NH_3/water 39/20/10/31 (v/v/v/v) under the same conditions mentioned above. After development and drying one plate was stained with ninhydrin to visualize protein bands. Figure 38 shows an image of the stained plate. The glucagon band is faintly visible at an R_f of ~0.6. The heme of the myoglobin sample is at an R_f of ~0.6 and the intact myoglobin moved to R_f 0.5. In track 3 cytochrome c moved to R_f of 0.15, beta casein R_f 0.4 and ubiquitin did not move. Glucagon and ubiquitin are roughly the same molecular weight and glucagon moved the farthest while ubiquitin did not such that the mechanism of separation or movement relates more to structure and other chemical properties independent of molecular weight. Table 10 lists ten proteins that were studied using this development system including whether they moved on the plate or not.

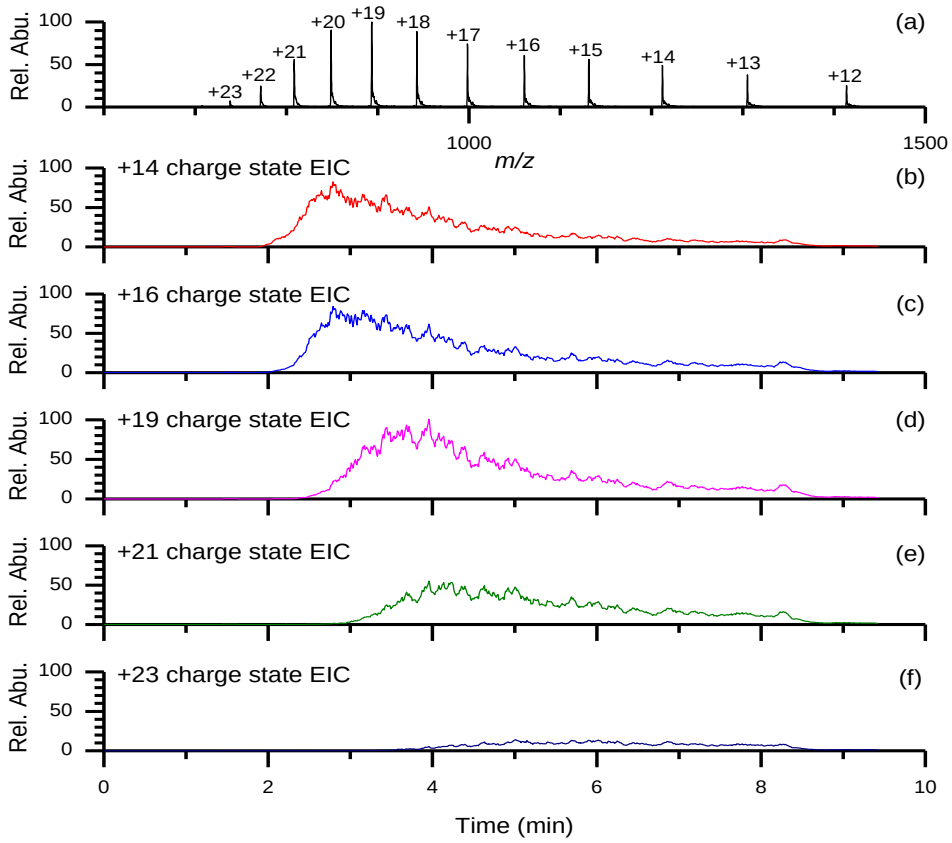


Figure 37. 3 µL of a myoglobin solution, 5 mg ml-1, was spotted as a 6 mm bands on a ProteoChrom HPTLC Silica gel 60 F(254S) plate and developed using 1-butanol/pyridine/NH3/water 39/20/10/31 (v/v/v/v). The myoglobin band position was identified (supplemental picture 1) by using ninhydrin on one of the developed bands. A S-SSP (Camag TLC-MS interface) was used to sample the band. The extraction/ESI solution composed of 70/30/.1 water/ACN/formic acid (v/v/v) was delivered at 50 µL min-1 using an Agilent 1100 LC pump. The 6 port injection valve was left in the 'inject' position for the entirety of the sampling. A 55 cm section of peek tubing at 0.127 mm ID connected to the zero dead volume S.S. union followed by 30 cm of 0.05 mm ID fused silica capillary into the LTQ-XL ESI source constituted the dead volume after extraction of ~ 7.5 µL excluding the extraction valve. The entire dead volume post extraction and excluding the inlet frit is ~27 µL. (a) shows the averaged MS spectra from minute 2 – 8. (b)-(f)

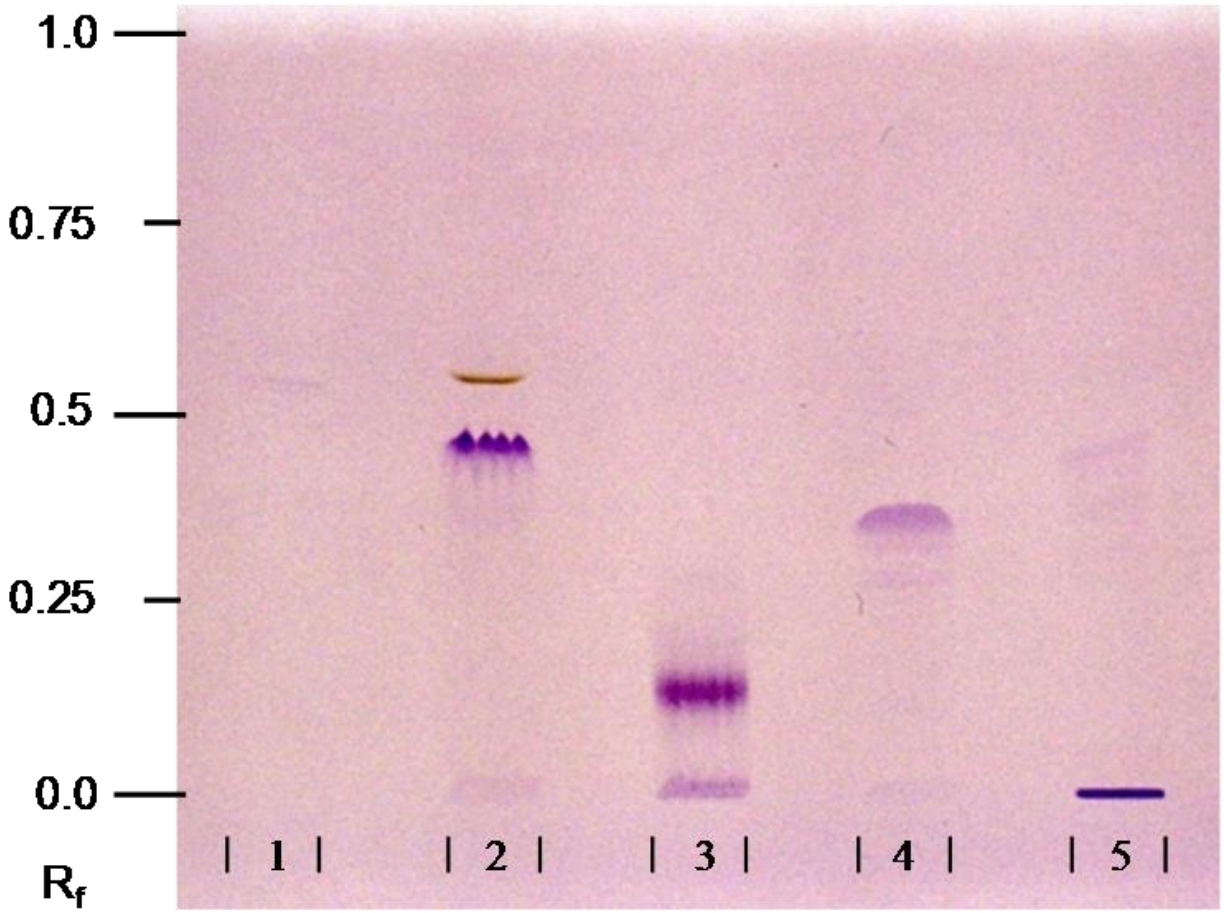


Figure 38. Single Protein solutions were spotted as a 6 mm bands on a ProteoChrom HPTLC Silica gel 60 F(254S) plate and developed using 1-butanol/pyridine/NH3/water 39/20/10/31 (v/v/v/v). Track 1: glucagon, track 2: Myoglobin, track 3: cytochrome c, track 4: beta-casein, and track 5: ubiquitin. Plate development in an unsaturated twin trough development tank took ~55 minutes.

A HPTLC plate with the five proteins shown in Figure 38 that was not stained was readout using the Camag TLC-MS interface. The same experimental conditions were used as previously stated. Figure 39 (a)-(d) shows the averaged MS spectra over a one minute period when protein signals were initially detected. From track 1 the glucagon signal of both a protonated and sodiated multiply charged species is seen in Figure 39 (a). The myoglobin and cytochrome c signals, Figure 39 (b) and (c), are well defined and no sodium adducts are detected. Ubiquitin, Figure 39 (d), was detected. Beta casein was not detected. The pI of beta casein is ~4.6 and the pH of a 1.0% formic acid solution is ~2.2. So the extraction solution would not have readily dissolved beta casein because of its high molecular weight and weak positive charge (almost neutral) at pH 2.2. When standards of beta casein were directly infused using the extraction solution poor intensity and S/N ratios were observed.

Table 10. Intact protein development progress using ProteoChrom HPTLC Silica gel 60 F(254S) plate and developed using 1-butanol/pyridine/NH3/water 39/20/10/31 (v/v/v/v).

Protein	MW (kDa)	pI	Moved	Detected
Glucagon	3.5	6.2	*	*
Myoglobin	16.7	7.5	*	*
Cytochrome C	12.0	10.2	*	*
ß-Casein	24	4.6	*	
α-Casein	23.6	4.6	*	
Ubiquitin	8.5	6.79		*
BSA	68.0	4.8		
Insulin	5.8	5.4	*	*
Hemoglobin	17	6.5-7.5	*	*
Lysosyme	14.7	10.9		*

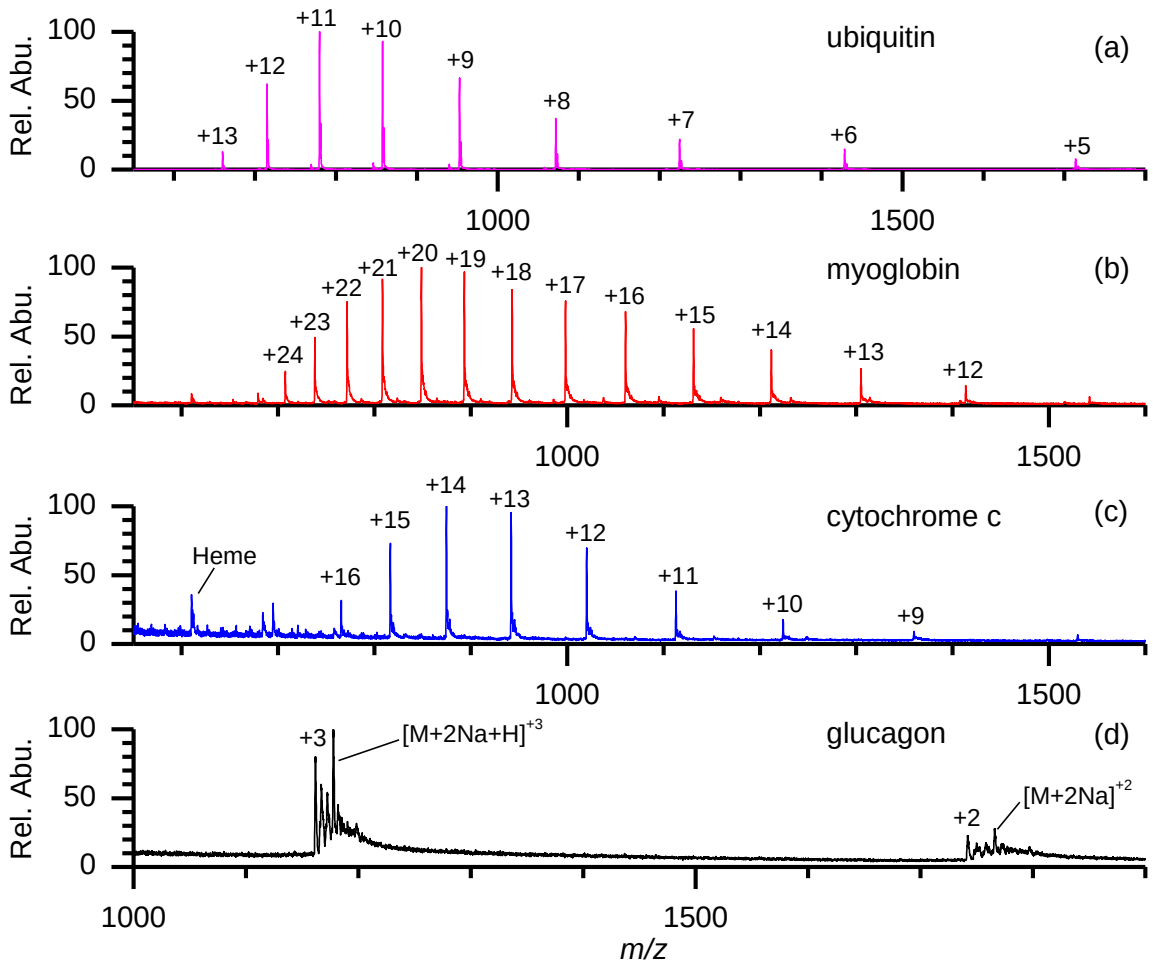


Figure 39. A S-SSP (Camag TLC-MS interface) coupled with an LTQ-XL MS was used to sample the band. The extraction/ESI solution composed of 70/30/.1 water/ACN/formic acid (v/v/v) was delivered at 50 µL min-1 using an Agilent 1100 LC pump. The 6 port injection valve was left in the 'inject' position for the entirety of the sampling. (a)-(d), corresponding to Tracks 1-3, 5 of figure 3, shows the averaged MS spectra over a one minute period when protein signals were initially detected.

6.4 Conclusions

The direct readout of intact proteins spatially separated on an analytical surface, ProteoChrom® Silica gel 60 F254s plates, was presented. The development of intact proteins on commercially available HPTLC plates followed by atmospheric pressure surface sampling/ionization readout may provide an alternative to traditional column based LC-MS analysis. The advantage of reading out static chromatographic bands and having the time to perform different MS experiments as well as gas phase reactions on target analytes should be exploited. Future experiments will involve the readout of complex mixtures using the presented HPTLC development conditions. Further study and optimization of the chromatographic conditions, including different stationary phases and development solutions, is also planned.

Chapter 7

Total Ion Mapping Scan Function for Direct Infusion and Spatial LC-MS: Chromatographic Peak Parking Study

7.1 Introduction

The analysis of complex samples using planar chromatographic media where components are spatially separated and analyzed has not been introduced in the literature to our knowledge. The efficient coupling of planar chromatography, a branch of liquid chromatography, with mass spectrometry has been called the next technology holy grail.[183] We prefer to call this field *spatial* Liquid Chromatography/Mass Spectrometry (LC-MS). Spatial chromatography takes place in space rather than time. The main advantage of this approach being the construction of two or more separation dimensions becomes more convenient.[184] The time for subsequent separations (two or more dimensions) happens in parallel such that the exponential increase in serial fractionation analysis time in multi-dimensional column or time based chromatography is also not a detrimental factor.[185] There are several advantages to utilizing spatial chromatography coupled with mass spectrometry. Some advantages include the static analysis of fractionated samples such that analysis times and number and type of MS experiments are not dictated by column based chromatographic peak times. These advantages arise from a classic advantage of spatial chromatography which is 'peak parking'. This manuscript represents the first complex sample analysis, a bacterial proteome, attempted using spatial LC-MS.

Atmospheric Pressure Surface Sampling/Ionization Mass Spectrometry has experienced much growth.[186,187,188,189,190,191,192,193] Direct liquid extraction based surface sampling probes can be used to analyze material at or just below a surface of interest, but also have the ability to further process extracted material in analytical workflows.[172] This ability to further process samples of extracted material can serve to increase the attainable chemical information, improve sensitivity, or enhance selectivity. One particular probe of this type, a continuous flow liquid microjunction surface sampling probe (continuous flow-LMJ-SSP),[194,195,196,197,198,199,200,201,202,203] reconstitutes or extracts an analyte from a surface by means of a wall-less liquid microjunction between the sampling end of the probe and the surface. The continuous flow-LMJ-SSP can utilize electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources while spot sampling or scanning surfaces of interest. Analytical applications of this continuous flow-LMJ-SSP have involved the sampling and analysis of dried drugs or proteins or solutions thereof from wells on microtiter plates, drugs captured in solid-phase extraction cards,[12] a variety of dyes, inks, or pharmaceuticals on paper or separated on hydrophobic reversed-phase (C8 and C18) thin-layer chromatography plates,[194,13,14,15,16] exogenous compounds from thin tissue sections,[19] and surface deposited and affinity captured proteins.[18]

A droplet sampling mode has been implemented with both a continuous flow[204] and autonomous pipette[205,206] versions of these LMJ-SSPs. In this sampling mode, the sample end of a probe is placed about 0.2 mm above the surface, a small volume droplet (e.g., 1-2 µL or less) is dispensed to contact the surface, while remaining attached to the probe. After an appropriate extraction time the droplet is aspirated back into the probe and the solution is directly analyzed[108,205] or further processed using, for example, HPLC.[206] The autonomous pipette droplet

sampling mode of operation has recently been implemented as the Liquid Extraction Surface Analysis (LESA) mode on the commercially available TriVersa NanoMate® chip-based infusion nanoelectrospray ionization (nanoESI) system.[205,207,208]

The use of atmospheric pressure surface sampling/ionization techniques for the coupling of planar separations with mass spectrometry has gained much attention.[209,210,211] Herein, we demonstrate the use of the TriVersa NanoMate® (and Nanomate 100®) in LESA mode and a continuous flow-LMJ-SSP-ESI-MS system for the read-out of spatial fractionations of complex peptide mixtures in a work flow we call spatial LC-MS. The advantage of having analyte peaks or bands 'parked' allows for total ion mapping MS scans when using LESA. The ability of an mass spectrometer to analyze direct infusions of complex samples in total ion mapping mode was studied to ascertain the gain or loss of chemical information when separation power was much less than in traditional column based LC-MS workflows. We used one-dimensional development of planar chromatographic media for its simplicity and speed when compared to most one and two dimensional column chromatography methods. We show the successful planar readout of a tryptically digested seven protein mixture on ProteoChrom Silica Gel 60 F_{254S} HPTLC plates. Using these approaches we were able to identify greater than 117 peptides in a single lane scan with all proteins being indentified from unique peptide signatures. Preliminary results using the same procedure on a more complex sample, an *E. coli* proteome digestion, resulted in the identification of 2,385 peptides from 909 different proteins in a single lane scan.

7.2 Experimental

7.2.1 Materials and Reagents

LC-MS grade Chromosolv® solvents acetonitrile and water both with 0.1% formic acid (v/v) were obtained from Sigma Aldrich (Milwaukee, WI) for use with the S-SSP/ESI-MS analyses. HPTLC Silica gel 60 F254s plates (Merck KGaA, Darmstadt, Germany) used for separations were developed using 1-Butanol ACS reagent, ≥99.4%, Pyridine Chomosolv® Plus, for HPLC, ≥99.9%, Ammonium hydroxide 28% NH_3, and LC-MS grade Chromosolv® water (Sigma Aldrich, St. Louis, MO). Development solutions were prepared fresh daily. Modified sequencing grade trypsin from Promega (Madison, WI) was used for all protein digestion reactions.

7.2.2 Tryptic Digest

Modified sequencing grade trypsin was used for all protein digestion reactions. An equal molar 1ml solution, based on 1 mg ml^{-1} myoglobin, of seven proteins was made and placed in a 15 ml centrifuge tube. The solution was diluted with 6 M guanidine and reduced with dithiothreitol (DTT) at 5 mM for 1 hour at 60°C. After cooling to room temperature the sample was diluted to 1 M guanidine using 50 mM Tris, 10 mM $CaCl_2$ at pH 7.5. The Promega trypsin was added at a concentration of 10 µg mg^{-1} of protein. The tubes were put in a 37°C incubator on a rotator for 12 hours. Another aliquot of trypsin (10 µg mg^{-1} of protein) was added for an additional hour followed by addition of 10 mM DTT for further reduction. Samples were then cleaned-up for analysis using +C18 Sep-Paks (Waters). A 10 ml syringe was used to pre-condition the Sep-Pak with acetonitrile/formic acid 100/0.1(v/v)followed by an additional rinse,

at twice the volume, with water/formic acid 100/0.1(v/v). The digested protein mix was then passed through the cartridge two times and washed with an additional portion of water/formic acid 100/0.1 (v/v). Tryptic peptides were then eluted with 1 ml of acetonitrile/formic acid 100/0.1 (v/v). Samples were dried using a Savant Speed Vac system without removal of all solvent and reconstituted in water/formic acid 100/0.1 (v/v) to a final volume of 1 mL. For *E. coli* and *Rhodopseudomonas palustris* (Rpal) samples, 1 mg of cell material was placed in a 15 ml centrifuge tube. 50 µL of 6 M guanidine/ 10 mM DTT in 50 mM Tris/10 mM $CaCl_2$ pH7.5 was added to the tube and sealed. The solution was vortexed every ten minutes for an hour and then incubated at 37°C for 12 hours. Tryptic digestion was performed as stated above with 5 µg trypsin followed by another aliquot of 5 µg trypsin for 24 hours. Vials were centrifuged and supernatant collected and cleaned with Sep-Pak protocol as stated above. Final solution volume was 500 µL in water/formic acid 100/0.1 (v/v).

7.2.3 Thin-layer Chromatography

The development of the HPTLC plates was carried out in a normal flat-bottomed chamber using 2-butanol/pyridine/ammonia/water 39/34/10/26 (v/v/v/v) for the HPTLC plates. The migration distance on the plates used for the analysis was 55-60 mm achieved in 55–60 min. A total sample volume of 20 µL was applied as a 5-mm band at a dosage speed of 50 nL s^{-1} Plates had to be made hydrophobic prior to LMJ-SSP analysis so that a liquid microjunction could be formed on the surface of the HPTLC plate. Developed plates were placed in a fume hood, lying flat, and spray coated with the Trio Magic Carfa Silicone Oil. Plates were coated evenly until the silicone layer visibly oversaturated the stationary phase. A kimwipe tissue was hand pressed down onto the HPTLC plate using a glass plate for 5 s to remove the excess

silicone. This blotting procedure was then performed again using a new kimwipe. At this point silica gel 60 HPTLC plates were allowed to dry in open air for 7-10 hrs.

7.2.4 HPLC/ESI-MS

The HPLC separations were conducted on an Agilent 1100 Series Capillary LC System. Separation was achieved on an Acclaim PepMap 100 C18 column (1 mm x 150 mm; 3 µm particle size; Dionex) at ambient temperature. The mobile phase consisted of water (0.1% formic acid) (A) and acetonitrile/ water/formic acid (80:20:0.1 v/v/v) (B). The mobile phase was applied in a gradient elution starting at 0 min with 96% A: 4% B, and changed over the next 30 min to 80%A: 20%B, and then held at 80% A : 20% B for 20 min. Each run was followed by a 20- min equilibration period. The flow rate was 30 µl/min and the injection volume was 1 µl. The LC system, with a PALs autosampler, was coupled to an LTQ-XL mass spectrometer (Thermo Electron, San Jose, CA) with Xcalibur software version 2.0. MS/MS spectra were acquired in data dependent mode with dynamic exclusion and automatic gain control (AGC) on. The five most abundant peaks in every full scan mass spectra were subjected to MS/MS analysis at a normalized collision energy of 35%. Three microscans were acquired per spectrum.

7.2.5 LMJ-SSP/ESI-MS

The continuous flow-LMJ-SSP probe system used was identical to that which has been described previously[8,13] with only the mounting system modified to accommodate the particular mass spectrometer. The self aspirating LMJ-SSP probe was built using a stainless steel tee, a 10 cm long inner sampling/emitter capillary with a 254 µm o.d and a 127 µm i.d., an outer tube on the sampling end with 635 µm o.d. and 327 µm i.d., and a nebulizer tube on the spray side. The

nebulizing gas flow was used to adjust the probe aspiration rate to be in balance with the pumped flow of eluting/spray solvent (10 µL min^{-1} using a 1-mL syringe attached to a syringe pump) into the probe. An approximately 27 cm long section of PEEK tubing (127 µm inner diameter and 1/16 inch outer diameter) with an upstream ground point was used to supply the elution solvent to the probe/emitter. The ESI voltage applied to the probe and the capillary and tube lens voltages were independently optimized.

An MS2000 robotic x, y, z platform (Applied Scientific Instrumentation, Inc., Eugene, OR, USA) was used to hold and maneuver the TLC plates in a perpendicular position relative to the stationary LMJ-SSP for analysis. As described elsewhere,13 the original microscope slide holder supplied with the stage was replaced with a home-built TLC plate holder made from rigid, nonconductive polymer. The MS2000 platform could be controlled manually by use of a joystick in the x and y-directions and by use of a jog wheel for z-direction control for initial alignment and LMJ formation. A camera used to observe the liquid microjunction during operation was equipped with an Optem 70 XL zoom lens (Thales Optem Inc., Fairport, NY, USA). All TLC plate lane scans were enabled by using HandsFree TLC/MS, software written in-house to control the ASI 2000 stage. Before scanning a lane, a LMJ was created at a position along the development lane below the spotting point by manual adjustment of the jog wheel and joystick via the ASI 2000 control system. After making the LMJ, the mass spectrometer data acquisition process was initiated simultaneous with the beginning of the lane scan (100 µm s^{-1}). When the scan and data collection processes were finished, the LMJ was broken by moving the stage away from the probe in the z-direction.

An LTQ-XL mass spectrometer (Thermo Electron, San Jose, CA) with Xcalibur software (version 2.0) was used with the LMJ-SSP. During surface scans of the tryptic digest separation lanes, automatic gain control was used with MS/MS product ion mass spectra acquired in data dependent mode with the three most abundant peaks within each full scan mass spectrum subjected to dissociation. The normalized collision energy was set to 35% and three microscans were acquired for each spectrum over a product ion range of *m/z* 200-2000. Dynamic exclusion was set to three so MS/MS would be performed on a certain *m/z* peak a maximum of two times.

7.2.6 Automated Liquid Extraction Surface Analysis

A TriVersa NanoMate® (Advion BioSciences) system coupled to a LTQ-XL linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA) was operated in Liquid Extraction surface Analysis (LESA) mode for HPTLC plate readout. HPTLC plates were scanned using LESA points software that allowed the positioning of extraction points at predefined spacing along the HPTLC development lane. A nanoESI voltage of 1.70 kV and gas pressure of 1.0 psi was applied in all experiments. Customized robotic arm (mandrel) movements and liquid handling for surface analysis was set up in the LESA panel of the ChipSoftManager control software for the TriVersa NanoMate®. For all experiments, the mandrel was programmed to acquire a pipette tip, aspirate 2.0 µL of extraction/nanoESI solvent from a solvent reservoir, and move to within 0.2 – 0.4 mm above the HPTLC plates. 0.7 µL of solvent was dispensed and ~1mm diameter areas were extracted for 3 seconds. The mandrel was programmed to lower the tip to 0.2 mm below the dispense height and aspirate the extraction solvent back into the tip. The aspirated solvent volume was set as 0.4 µL more than the dispensed volume to ensure maximum extract pickup. The pipette tip was then programmed to engage with the nanoESI chip. The

LTQ-XL was operated in total ion mapping mode. A mass range of *m/z* 350-1500 was scanned through at *m/z* 1.0 steps. The normalized collision energy was set to 35% and three microscans were acquired for each spectrum over a constant product ion range of 100-2000 Da. MS/MS data was searched and peptides identified using the same procedure as stated above.

7.2.7 Database Searching

To identify the peptides observed, the MS/MS spectra were extracted from raw data files and converted to MS2 file format[212]. The MS2 files were searched using the DBDigger[213] proteomics database search program which used the MASPIC[214] scoring scheme and the DTASelect[215] algorithm for filtering the MS2 files. The DTA Select algorithm used a ΔCN of at least 0.08 and cross correlation (X_{corr}) scores were set such that a false discovery rate of less than 1% was realized. Databases of *E. coli*, Rpal, and the seven protein mix were created in house using SwissProt. Because no full scans were performed and only MS/MS data was collected at predefined *m/z* values every MS/MS spectra was treated as a +1, +2, or +3.

7.3 Results and Discussion

A LMJ-SSP is one of four types of liquid extraction based surface sampling techniques as seen in Figure 40. Other liquid extraction based surface sampling techniques include DESI, jet desorption ionization (JeDI), and a sealing –surface sampling probe (S-SSP). LMJ-SSPs employ an open-air, wall-less liquid microjunction (LMJ) between the sampling end of a solvent delivery apparatus and a surface. Material coming in contact with a liquid microjunction may be extracted into the liquid phase based on inherent chemical properties of the material.

7.3.1 Column based LC-MS and Direct Infusion Analysis of Seven Protein Digest

A seven protein equal molar mix was subjected to tryptic digestion (see experimental). To ensure the quality of the digestion and to set a baseline for other analysis techniques to be used, a traditional column based LC-MS system was used to analyze the digestion. Column liquid chromatography was performed using an Acclaim PepMap 100 C18 column (1 mm x 150 mm x 3 µm particle size) in gradient elution mode. An LTQ-XL mass spectrometer in data dependent mode was used as the detector performing full and MS/MS scans of eluting analytes. MS/MS spectra were searched using DBDigger proteomics database searching software to identify peptides. Identified peptides were correlated with respective proteins. Protein sequence coverages were calculated and are reported in Table 11.

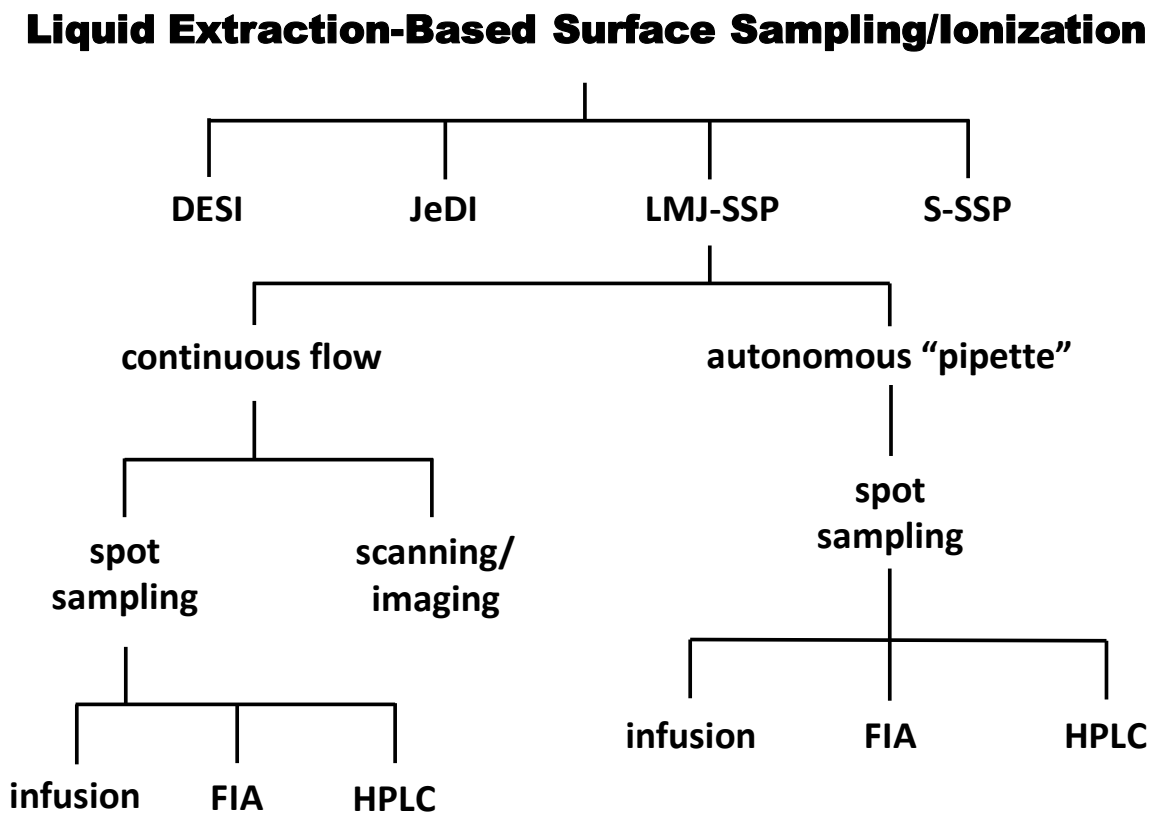


Figure 40. Liquid microjunction surface sampling probe modes of operation.

Table 11. 7 protein tryptic digest analysis using data dependent scan function with LC-MS and ion mapping scan function with direct infusion chip based nanoESI.

	1D LC-MS.	Direct Infusion Ion Mapping Eq. M^	Direct Infusion Ion Mapping^^
	10 µL	1 µL	1 µL
HORSE HEART CYTOCHROME C	77.1	77.1	76.2
BOVIN CARBONIC ANHYDRASE II	56.4	43.6	46.3
BOVIN HEMOGLOBIN BETA CHAIN	82.8	77.9	69.0
BOVIN HEMOGLOBIN ALPHA CHAIN	51.1	69.5	80.1
HORSE LIVER CHAIN A ALCOHOL DEHYDROGENASE	20.5	0	0
HORSE MYOGLOBIN	100	100	63.4
CHICK Lysozyme C	93.8	91.5	95.3
RABBIT GLYCERALDEHYDE-3-PHOSPHATEDEHYDROGENASE	57.4	53.5	50.8
Proteins IDs	7	6	6
peptide IDs	233	136	124

^ An equal molar mix
^^ 7 Protein mix with dynamic range (0.5 mg/protein)
1) Modifications- : G (+42.0106), M (+15.9949), W (+31.9898)

A hemoglobin sample that had both alpha and beta chain species was treated as one protein but searched for separately. All 7 proteins were identified with sequence coverages ranging from 20.5 – 100.0%. 233 unique peptide IDs were used in the identification of the 7 proteins and were used to calculate the respective sequence coverages.

The use of total ion mapping MS scan functions in complex sample analysis has not been studied. The use of the TriVersa NanoMate for the analysis of a BSA tryptic digestion in a static direct infusion nanoESI mode has been reported using peptide mapping.[216] The equal molar 7 protein mix was directly analyzed using a TriVersa NanoMate in direct infusion mode with 1 µL of the standard digest solution. The LTQ-XL was operated such that the mass spectrometer would scan *m/z* 350-1500 one Da at a time. 1150 tandem mass spectra were collected in just under 30 minutes. The MS/MS data was searched using the same parameters as stated above. Six of the seven proteins were identified with sequence coverages ranging from 43.6 – 100.0% (excluding the unidentified Horse Liver Chain A alcohol Dehydrogenase) as reported in Table 11. 136 unique peptide IDs were used in the identification of the proteins. Figure 41 shows the total ion current chromatogram for the total ion mapping experiment. The x axis is plotted in terms of time; however the defined scan range of *m/z* 350-1500 could have been plotted as well. Figure 41 (b) - (d) shows higher molecular weight peptides that were identified with high confidence and were not suppressed by lower molecular weight peptides that may have greater proton affinity. The same protein mix only with greater dynamic range, i.e. different molarities based on 0.5 mg ml^{-1} of each protein, was subjected to the same analysis procedure.

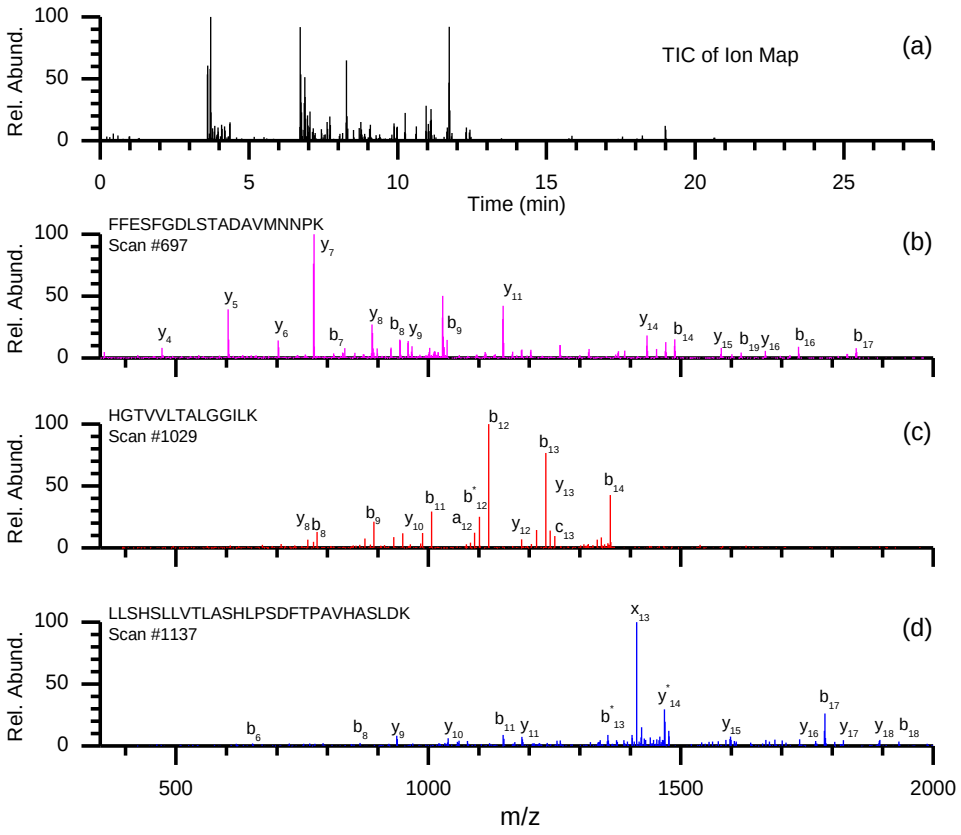


Figure 41. (a) The Total Ion Current (TIC) chromatogram of an ion mapping experiment is shown. A tryptically digested seven protein mix was directly infused using a TriVersa NanoMate in direct infusion mode using 1.7 kV nanoESI spray voltage and a 1.0 (arb.) gas backing pressure. For detection an LTQ-XL was operated in positive ion mode, CID isolation width was set at 1.5, CE at 35%, Activation Q 0.250. Ion mapping settings were set so the parent mass step was 1.0 m/z through m/z 350.0 – 1500.0 and product mass range was a static m/z 100.0 – 2000.0. The entire experiment ran for 29.38 minutes or 0.0255 min m/z -1 . (b) Scan 697 shows the isolation and fragmentation of m/z 1046 a doubly charged peptide identified as R.FFESFGDLSTADAVMNNPK.V (82.0 score) . (c) Scan 1029 shows the isolation and fragmentation of m/z 1379 a singly charged peptide identified as K.HGTVVLTALGGILK.K (24.0 score). (d) Scan 1137 shows the isolation and fragmentation of m/z 1486 a doubly charged peptide identified as K.LLSHSLLVTLASHLPSDFTPAVHASLDK.F (35.0 score).

Six of the seven proteins were again identified with sequence coverages ranging from 46.3 – 95.3% (excluding the unidentified Horse Liver Chain A alcohol Dehydrogenase) as reported in Table 11. 124 unique peptide IDs were used in the identification of the proteins. The ability to identify well over a hundred different peptides in a single infusion experiment using 1 µL of solution is attractive. The use of static infusion total ion mapping MS scan functionality can be coupled with column based LC through fraction collection, or can be used in the readout of static analyte bands spatially separated on planar chromatographic media.

7.3.2 Spatial LC-MS Analysis of Seven Protein Digest

The TriVersa NanoMate® chip-based infusion nanoelectrospray ionization (nanoESI) system has recently been equipped with a liquid extraction surface analysis (LESA) mode.[217,218,219] This mode of surface sampling can be applied to planar chromatographic media and was, until recently, only possible on reversed phase C8 or C18 glass backed HPTLC plates. Wettable or hydrophilic surfaces, such as normal phase HPTLC silica gel plates, can now be readout using liquid microjunction techniques by way of a simple post development procedure with a silicone aerosol spray.[220] HPTLC plates were spotted with 20 µL of the equal molar 7 protein digest sample. The band was placed 8 mm from the bottom of the plate at a length of 5 mm. Plates were developed, dried and treated with aerosol silicone spray. Some plates were not treated and were derivatized with ninhydrin for visualization of peptide bands. A derivatized or stained 7 protein digest development lane is shown in Figure 42 (a) and (b). Figure 42 (a) represents a ninhydrin stained development lane while Figure 42 (b) is the same sample prior to derivatization under a UV lamp. The fluorescent indicator in the HPTLC plate allows visualization of some bands.

Plates that were treated were allowed sufficient drying time prior to being readout using both the LESA and continuous flow-LMJ-SSP techniques.

The continuous flow-LMJ-SSP-ESI-MS analysis of the seven protein digest was performed in data dependent mode. Figure 43 (b) shows the base peak chromatogram of a single lane scan at 100 µm s^{-1}. Tandem mass spectra collected were analyzed using database searching software which identified six of the seven proteins. Seventy unique peptides were identified in this ten minute analysis using only ~1/5 of the sample band. The protein sequence coverages ranged from 7.3 – 79.0%. As noted in previous work[20] sodium adduct formation is prevalent when examining full scan data. Figure 44 shows the full scan MS spectrum of a tryptic peptide that shows an intense signal of singly protonated species and many different sodium adducts. This in fact prevented the identification of this peptide in the single continuous flow-LMJ-SSP lane scan. Examples of sodiated peptide tandem mass spectra are shown in figure 44 (b) and (c) where the MS/MS spectra lack enough fragment peaks to confidently identify the sodiated peptide species. This was not a consequence of the sample, development solution, or silicone treatment. Rather the HPTLC plate itself can have a significant concentration of metal embedded in the stationary phase that is not removed in the solvent development process. The production of a higher quality or MS quality HPTLC plate is needed. The sensitivity of the peptide analysis can be improved if metals such as sodium are removed in the manufacturing process.

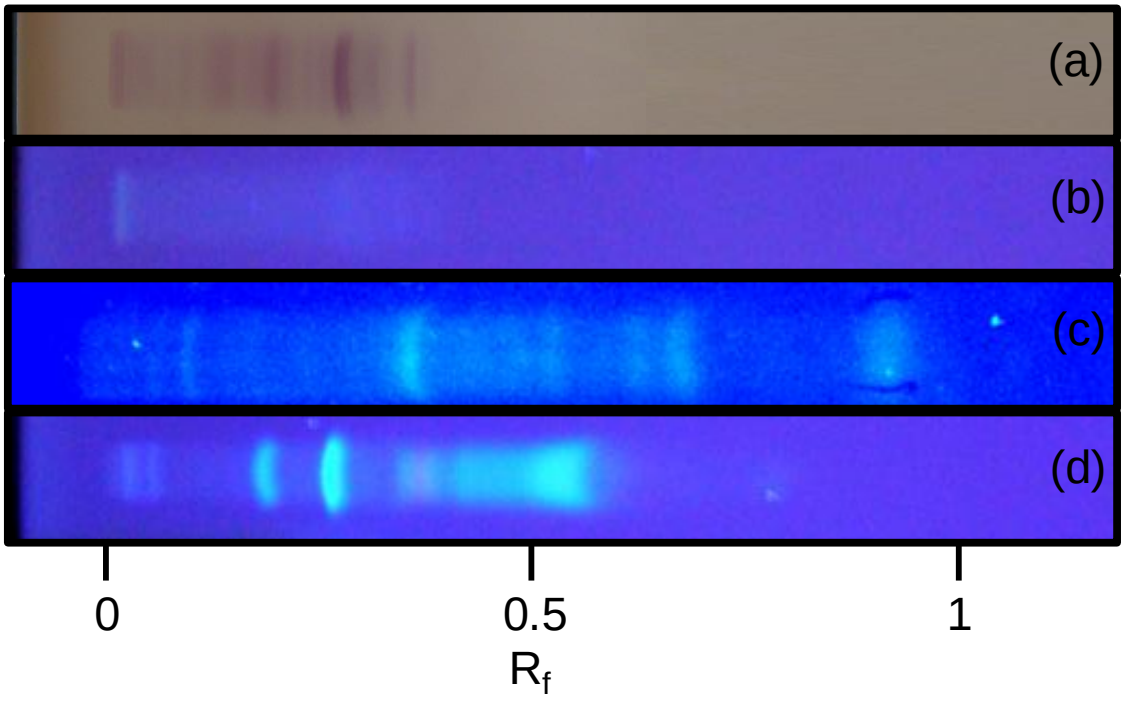


Figure 42. Tryptic peptides developed on ProteoChrom HPTLC Silica gel 60 F(254S) plates using 2-butanol/pyridine/ammonia/ water (39:34:10:26, v/v/v/v) are shown. In each development bands were spotted 8.0 mm from bottom of the plate and developed to ~ 60 mm. Development time ranged from 55 – 60 minutes in a twin trough development tank saturated for 20 minutes prior to plate immersion in development solvent. (a) Image (white light) of 7 protein digest stained with ninhydrin (20 µL applied as 5 mm band) (b) Image (under black light blue lamp) of 7 protein digest used for HPTLC-MS LESA Ion Mapping experiments (20 µL applied as 5 mm band). (c) Image (under black light blue lamp) of Escherichia coli digest used for HPTLC-MS LESA Ion Mapping experiments (100 µL applied as 4 mm band). (d) Image (under black light blue lamp) of Rhodopseudomonas palustris digest (80 µL applied as 4 mm band).

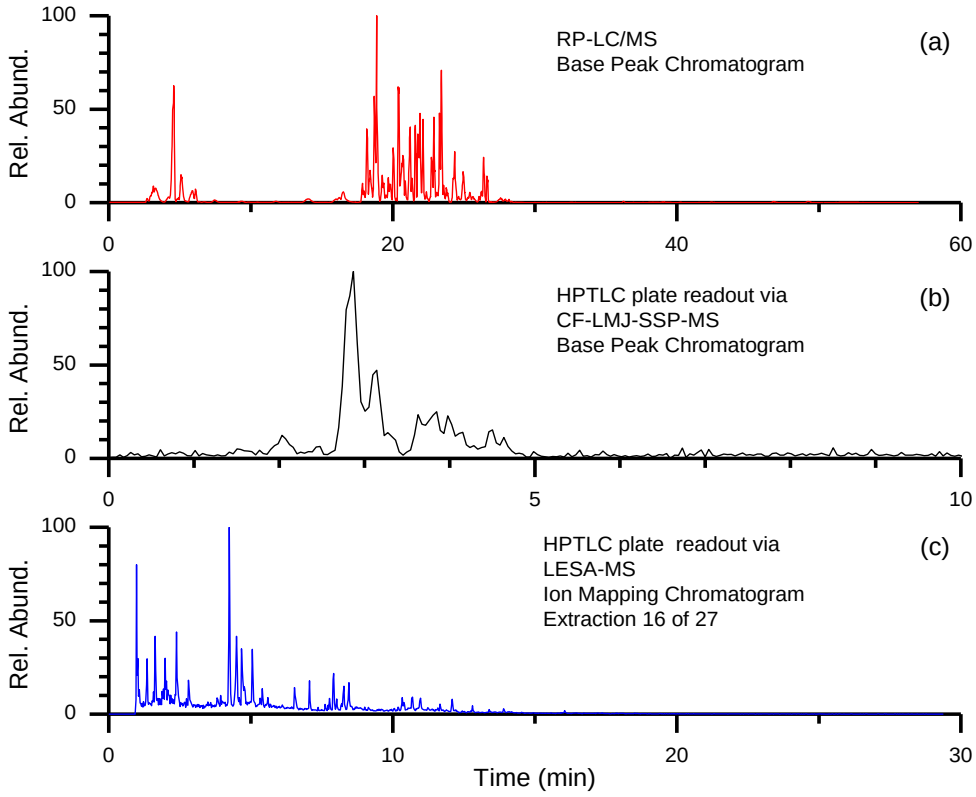


Figure 42. A tryptically digested seven protein mix was analyzed using three different LC-MS methods using an LTQ-XL operated in positive ion mode. CID isolation width was set at 1.5, CE at 35%, Activation Q 0.250 (a) A n Acclaim® PepMap100 C18 column, at 1.0 mm id and 15 cm length, was operated using a 45 min gradient at a 50 µL min-1 flow rate. (b) A continuous flow LMJ-SSP was used to read out a single HPTLC development lane at 100 µm min-1 using 70/30/0.1 water/ACN/formic acid (v/v/v). (c) The Total Ion Current (TIC) chromatogram of an ion mapping experiment in shown. The HPTLC read out using a TriVersa NanoMate in LESA mode using 1.7 kV nanoESI spray voltage and a 1.0 (arb.) gas backing pressure. Ion mapping settings were set so the parent mass step was 1.0 m/z through m/z 350.0 – 1500.0 and product mass range was m/z 100.0 – 2000.0. One extract was analyzed for 29.38 minutes or 0.0255 min m/z -1. In this experiment 27 extractions were performed at 2 mm step sizes for a total of 54 mm of development lane.

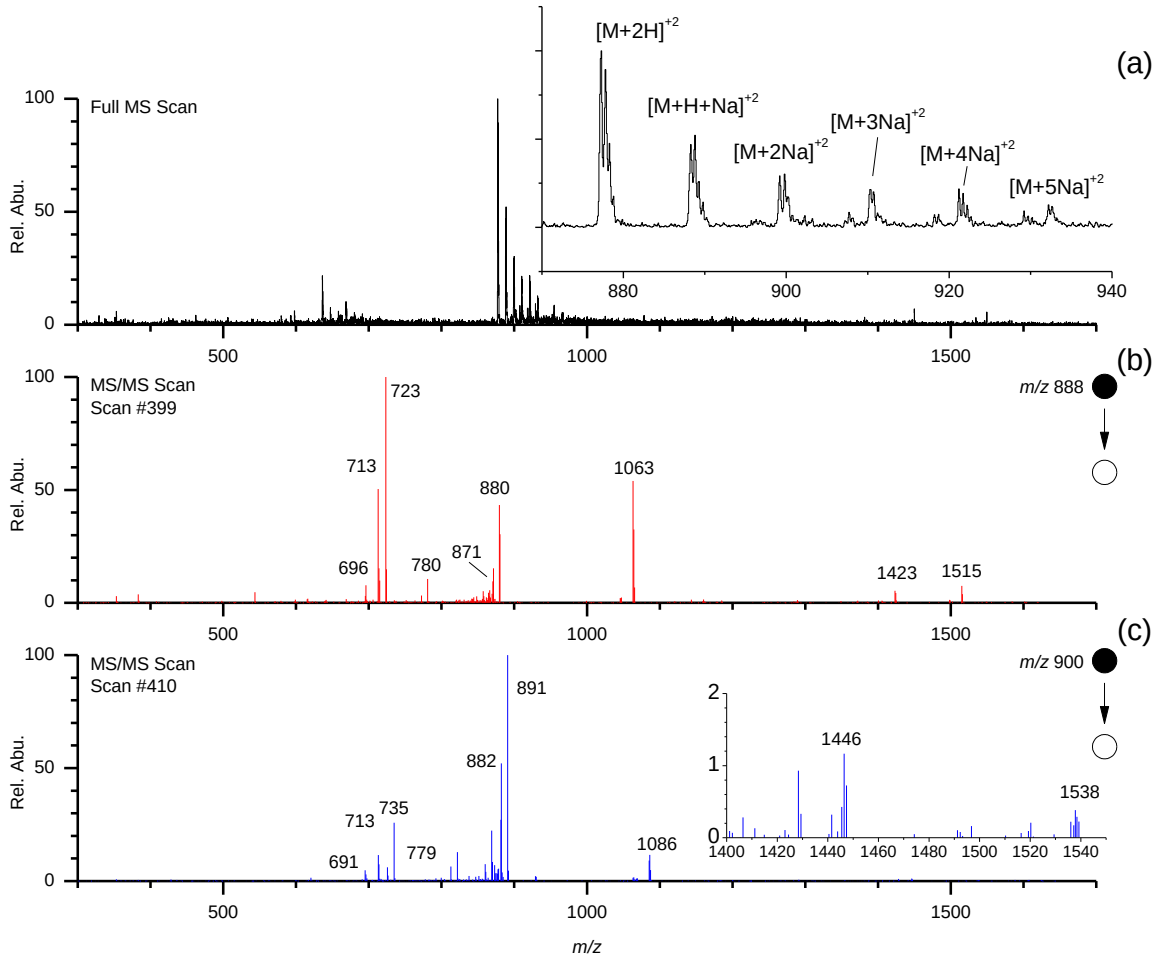


Figure 44. A continuous flow LMJ-SSP was used to readout a seven protein tryptic digest, spotted as a 5 mm band, from a HPTLC development lane at 100 µm min^{-1} using 70/30/0.1 water/ACN/formic acid (v/v/v). The presence of sodium in the plate leads to the formation of multiple adducts species which are selected for MS/MS in data dependent mode. (a) In the full scan ms spectrum the base peak is an intense doubly charged peptide species that was not identified because of its particular *m/z* value being put on the exclusion list 20 seconds before it appeared in the full scan. Both (b) and (c) show the selected MS/MS spectra of the sodiated species that, even though intense, are much less information rich than the singly protonated species due the metal species, in this case sodium, taking the charge upon fragmentation.

The LESA analysis of a seven protein digest fractionated on a HPTLC plate was performed with the LTQ-XL in total ion mapping mode. Using LESA points software a development lane image was produced. Using this image, a line of extraction points, from the spotting position to the development front with 2 mm spacing, could be defined to sample from. The HPTLC plate was then placed in the TriVersa NanoMate sample holder and automatically readout. Twenty-seven extractions were performed at 2 mm resolution. Extracts were statically infused into the MS for less than thirty minutes. No full scan spectra were collected. The TIC of extraction 16 is shown in figure 43 (c). 54 mm of the 60 mm development lane were scanned using the LESA-MS method. Tandem mass spectra were collected under the same conditions as the direct infusion ion mapping experiments mentioned above. The entire readout took ~13.5 hours. Table 12 shows seven of seven proteins were identified from 117 unique peptide identifications. Protein sequence coverages ranged from 7.5 – 81.0%.

Table 12. 7 protein tryptic digest developed on ProteoChrom HPTLC Silica gel 60 F(254S) plates and readout using data dependent scan function with continuous flow-LMJ-SSP-MS and ion mapping scan function LESA chip based nanoESI.

	continuous flow – LMJ-SSP	LESA Ion Mapping
	5mmBand 20 µL ap	5mmBand 20 µL ap
HORSE HEART CYTOCHROME C	79	81
BOVIN CARBONIC ANHYDRASE II	7.3	22.3
BOVIN HEMOGLOBIN BETA CHAIN	44.1	55.2
BOVIN HEMOGLOBIN ALPHA CHAIN	43.3	43.3
HORSE LIVER CHAIN A ALCOHOL DEHYDROGENASE	0	7.5
HORSE MYOGLOBIN	76.5	52.9
CHICK Lysozyme C	73.9	53.5
RABBIT GLYCERALDEHYDE-3-PHOSPHATEDEHYDROGENASE	33	31.8
Proteins IDs	6	7
peptide IDs	70	117

1) 7 protein tryptic digest equal molar mix

2) Modifications- G (+42.0106), M (+15.9949), W (+31.9898)

7.3.3 Spatial LC-MS of a Proteome

The use of LESA for the readout of spatially separated HPTLC bands is certainly not as fast as the continuous flow-LMJ-SSP analysis nor the traditional HPLC-MS analysis. The use of LESA in the readout of static R_f position perpendicular to the development direction, however would not sacrifice throughput. Methods, such as multidimensional column based LC-MS, are applicable to LESA-HPTLC analysis time frames. Multi dimensional protein identification technology (MudPIT) work flows are often entail 24 hours analysis times for the analysis of entire proteomes. To gauge the effectiveness of LESA-MS against workflows or techniques with comparable analysis time the preparation and development of a tryptic digest of both E. coli and Rpal bacterium on HPTLC plates was done. Figure 42 (c) and (d) show developed bands under a UV light prior to aerosol silicone treatment. The visualized bands indicate the entire development lane is being utilized. After silicone treatment and sufficient dry time the E coli development lane was readout using LESA-MS. The LESA MS analysis was performed in total ion mapping mode. 49 mm of the development lane was profiled with a readout resolution of 1 mm. The entire readout took 23.5 hours. Collected tandem mass spectra were subjected to database searching software using an Ecoli database. As seen in Table 13 909 non redundant proteins were identified. 2,385 unique peptides were identified with a false discovery rate of 0.7%.[221]

Table 13. E. coli digest developed on ProteoChrom HPTLC Silica gel 60 F(254S) plates and readout using data dependent scan function with continuous flow-LMJ-SSP-MS and ion mapping scan function LESA chip based nanoESI.

LESA Ion Mapping^	Proteins	Peptide IDs	Copies	FDR%
Redundant	924	2815	3530	
Nonredundant	909	2385	3085	0.7
continuous flow-LMJ-SSP^^				
Redundant	89	313	443	
Nonredundant	85	296	426	0.3

1) 100 µL of E. coli trypsin digest was spotted as a 4 mm band for each analysis

2) Modifications- G (+42.0106), M (+15.9949), W (+31.9898), Acetylation of N terminus

^ LESA experiment consisted of 49 extraction of HPTLC development lane using 70/30/.1 Water/ACN/formic acid (v/v/v) as extraction/nanoESI solvent

^^ continuous flow-LMJ-SSP was scanned at 50 µm s^{-1} through HPTLC development lane using 70/30/.1 Water/ACN/formic acid (v/v/v) extraction/ESI solvent

A continuous flow-LMJ-SSP-ESI-MS system was also used to analyze the E coli tryptic digestion developed on a separate development lane. The probe was scanned at 50 µm s^{-1}. The MS was operated in data dependent mode. Data base searching software identified 85 proteins from 296 unique peptide hits from the twenty minute MS experiment. The FDR was determined to be 0.3%.

7.4 Conclusions

The analysis of complex samples using planar chromatographic media where components are spatially separated and analyzed has been demonstrated. There are several advantages to utilizing such techniques including what was demonstrated here: static analysis of fractionated samples such that analysis times and number and type of MS experiments are not dictated by column based chromatographic peak times or transient signals. The analysis of complex mixtures today and most certainly tomorrow will require and demand novel approaches such as the coupling of spatial LC with mass spectrometry.

Chapter 8

Conclusions and Recommendations

The studies presented in this dissertation showed the use of LMJ-SSP technology coupled with mass spectrometry for the readout of different analytical surfaces including: reversed phase and normal phase high performance thin layer chromatography plates and solid phase extraction cards. These studies indicate more research is needed in this developing field, however, the future of LMJ-SSP based technology is promising. Most notably the use of spatial LC-MS as a analytical technique for complex sample analysis may prove to be the technology 'holy grail' of the future. Spatial LC-MS is made possible through the use of atmospheric pressure surface sampling methods for mass spectrometry.

Atmospheric pressure surface sampling/ionization techniques for mass spectrometry are not presently supplanting proven analytical techniques. One reason may be the adoption of new analytical technologies over proven methods must not come at the cost of reproducibility and/or sensitivity. In many cases the direct analysis of samples that are complex in nature will not allow for sensitive and/or reproducible measurements without sample preparation prior to analysis. The use of LMJ-SSP-MS technology as a robust means of sample analysis is not time proven as of yet; however, the future holds much promise as this is the only atmospheric pressure surface sampling/ionization technique where the fundamental operation involves 'sampling' followed by sample processing. If progress in coming years is not made towards alleviating or addressing signal suppression inherent in many direct analysis API based techniques then the future of many techniques, including DESI, LDI and MALDI, may not be one where these techniques are widely used or adopted by industry or academia. It is probably

worth noting these aforementioned techniques, including LMJ-SSP technology, would benefit greatly from progress in ionization efficiency and ion mobility based chromatography prior to MS analysis. However more traditional techniques most notably column based LC-MS, direct infusion, and MS based flow injection analysis would realize similar gains in sensitivity and/or selectivity over current benchmarks, thus making any technological advantage null. Therefore extraction efficiency and throughput will become significant areas of surface sampling techniques coupled with mass spectrometry as new techniques that can overcome matrix effects are developed.

To embrace sample preparation as an inherent part of atmospheric pressure surface sampling/ionization methods is needed. The ability to attain spatially resolved chemical information by way of efficient sampling and analysis is a promising field of analytical chemistry. Future work in the area of high resolution readout LMJ-SSP is needed. LMJ-SSP probes with readout resolutions less than 50 µm could be easily built by a moderately skilled worker. Readout resolution of less than a micron using LMJ-SSP probes is also feasible, however more obstacles including efficient sampling, analyte transport, capillary clogging, flush times, and MS detector sensitivity become more apparent and make this prospect less trivial (not impossible though).

Future studies involving current and future atmospheric pressure surface sampling/ionization methods should always be compared with traditional analysis method. When performing any LMJ-SSP-MS based experiments a simple liquid extraction followed by flow injection analysis should always be performed if the targeted analyte or material is not being directed to an analytical surface in a logical workflow or if sensitivity and reproducibility are

compromised. The advantages of speed and simplicity are for not if the data is incorrect or questionable.

The synergy between analytical surfaces and atmospheric pressure surface sampling/ionization methods will ultimately dictate the future of atmospheric pressure surface sampling/ionization methods. The work presented in this dissertation involving the custom SPE card readout using a TriVersa NanoMate in LESA mode is an example. The use or development of a SPE card that has a high sample load capacity and small load depth profile would be useful for the developed application. The development of such a SPE card would allow for significant concentration of analyte at a surface depth easily accessible to the LESA mode of analysis. The readout of planar chromatographic media holds the most promise for LMJ-SSP technology. Some may argue the analysis of mounted tissue sections may be more promising; however, the replacement (or a comparable alternative) of column based LC-MS with spatial LC-MS would have far reaching effects beyond biological based analyses. Analytical fields that are concerned with environmental, food security, quality control, and forensic analysis that use column based LC-MS would potentially benefit from a new approach. Much work, of course, still needs to be accomplished though. This work includes the development of MS quality planar chromatographic materials. Until now there has not been a need to demand higher quality planar chromatographic media. The ability to run separations reproducibly using either forced flow or capillary action based flow is also paramount. The adoption of more stringent spatial chromatographic development procedures is also called for so that results can be reproduced easily.

The future of LMJ-SSP technology is full of promise and the next few years will likely see more researchers adopt variations of LMJ-SSP technology for different analysis platforms. One could say the LMJ-SSP approach to surface analysis is akin to a 'Swiss army knife' in that it allows the user many choices or options in ionization technique, further sample fractionation or cleanup, changing of readout resolution, and so on. The ability of a sample introduction or surface sampling system to morph into a compatible interface for a given task is obviously attractive. LMJ-SSP technology coupled with mass spectrometry offers this ability.

LIST OF REFERENCES

[1] Benninghoven, A.; Hagenhoff, B.; Niehuis, E.; "Surface MS: probing real-world samples." *Ana. Chem.* **1993**, 65: 630A.

[2] Todd, P.J.; Schaaff, T.G.; Chaurand, P.; Caprioli, R.M.; "Organic ion imaging of biological tissue with secondary ion mass spectrometry snd matrix-assisted laser desorption/ionization." *JMS.* **2001,** 36: 355.

[3] Hillenkamp, F.; Karas, M.; MALDI MS. In A Practical Guide to Instrumentation, Methods and Applications, Hillenkamp F, Peter- Katalinic J (eds). Wiley-VCH Verlag GmbH and Co. KgaA: Weinheim, **2007**.

[4] Winograd, N. "The Magic of Cluster SIMS." *Anal. Chem.* **2005**, *77*, 142A-149A.

[5] Berkel, G. J. V.; Pasilis, S. P.; Ovchinnikova, O. " Established and emerging atmospheric pressure surface sampling/ionization techniques for mass spectrometry." **2008**, 1161-1180.

[6] Laiko, V. V.; Baldwin M.A.; Burlingame, A.L.; "Atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry" *Anal. Chem.* **2000,** 72, 652.

[7] Tak´ats Z.; Wiseman, J.M.; Gologan, B.; Cooks R.G.; "Mass spectrometry sampling under ambient conditions with desorption electrospray ionization." *Science* **2004**, 306: 471.

[8] Van Berkel G. J.; Sanchez, A. D.; Quirke, J. M. E. "Thin-Layer Chromatography and Electrospray Mass Spectrometry Coupled using a Surface Sampling Probe." *Anal. Chem.* **2002**, 74, 6216-6223.

[9] Wachs, T.; Henion, J. "Electrospray Device for Coupling Microscale Separations and Other Miniaturized Devices with Electrospray Mass Spectrometry." *Anal. Chem.* **2001**, 73, 632-638.

[10] Snyder, L.R. "Classification of the Solvent Properties of Common Liquids." Journal of Chromatography. **1974**. 7412, 92, 223-230.

[11] Ovchinnikova, O. S.; Kertesz, V.; Van Berkel, G. J. "Combining Laser Ablation/Liquid Phase Collection Surface Sampling and High-Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry." Analytical chemistry**. 2011**, 83, 1874-8.

[12] Wachs, T.; Henion, J. "A Device for Automated Direct Sampling and Quantitation from Solid-Phase Sorbent Extraction Cards by Electrospray Tandem Mass Spectrometry." *Anal. Chem.* **2003**, *75*, 1769-1775.

[13] Ford, M. J.; Van Berkel, G. J. "An Improved Thin-Layer Chromatography/Mass Spectrometry Coupling Using a Surface Sampling Probe Electrospray Ion Trap System." *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1303-1309.

[14] Ford, M. J.; Kertesz, V.; Van Berkel, G. J. "Thin Layer Chromatography/Electrospray Ionization Triple Quadrupole Linear Ion Trap Mass Spectrometry System: Analysis of Rhodamine Dyes Separated on Reversed-Phase C8 Plates." *J. Mass Spectrom.* **2005**, *40*, 866-875.

[15] Ford, M. J.; Deibel, M. A.; Tomkins, B. A.; Van Berkel, G. J. "Quantitative Thin-Layer Chromatography/Mass Spectrometry Analysis of Caffeine using a Surface Sampling Probe Electrospray Ionization Tandem Mass Spectrometry System." *Anal. Chem.* **2005**, *77*, 4385-4389.

[16] Asano, K. G.; Ford, M. J.; Tomkins, B. A.; Van Berkel, G. J. "Self-Aspirating Atmospheric Pressure Chemical Ionization Source for Direct Sampling of Analytes on Surfaces and in Liquid Solutions." *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2305-2312.

[17] Kertesz, V.; Ford, M. J.; Van Berkel, G. J. "Automation of a Surface Sampling Probe/Electrospray Mass Spectrometry System." *Anal. Chem.* **2005**, *77*, 7183-7189.

[18] Van Berkel, G. J.; Ford, M. J.; Doktycz, M. J.; Kennel, S. J. "Evaluation of a Surface Sampling Probe Electrospray Mass Spectrometry System for the Analysis of Surface Deposited and Affinity Captured Proteins." *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1144-1152.

[19] Van Berkel, G. J.; Kertesz, V.; Koeplinger, K. A.; Vavrek, M.; Kong, A. T. "Liquid Micro-Junction Surface Sampling Probe Electrospray Mass Spectrometry for Detection of Drugs and Metabolites in Thin Tissue Sections." *J. Mass Spectrom.* **2008**, *43*, 500-508.

[20] Kertesz, V.; Van Berkel, G. J.; Vavrek, M.; Koeplinger, K. A.; Schneider, B.B.; Covey, T. R. "Comparison of Drug Distribution Images from Whole-Body Thin Tissue Sections Obtained Using Desorption Electrospray Ionization Tandem Mass Spectrometry and Autoradiography." *Anal. Chem.* **2008**, *80*, 5168-5177.

[21] Alpmann, A.; Morlock, G. "Improved online coupling of planar chromatography with electrospray mass spectrometry: extraction of zones from glass plates." *Anal. Bioanal. Chem.* **2006**, 386, 1543–1551.

[22] Aranda, M.; Morlock, G. "Automated interface for hyphenation of planar chromatography with mass spectrometry." *Rapid Commun. Mass Spectrom.* **2007**, 21, 1297-1303.

[23] Luftmann, H.; Aranda, M.; Morlock, G. ” Automated interface for hyphenation of planar chromatography with mass spectrometry.” *Rapid Commun. Mass Spectrom.* **2007**, 23, 3772-3776.

[24] Morlock, G. E.; Jautz, U. J. ” Comparison of two different plunger geometries for HPTLC-MS coupling via an extractor-based interface.” *Planar Chromatogr.* **2008**, 21, 367-371.

[25] Dytkiewitz, E.; Morlock, G. E. J. AOAC Int. **2008**, 91, 1237–1243.

[26] Aranda, M.; Morlock, G. “Simultaneous determination of riboflavin, pyridoxine, nicotinamide, caffeine and taurine in energy drinks by planar chromatography-multiple detection with confirmation by electrospray ionization mass spectrometry.” *J. Chromatogr., A.* **2006**, 1131, 253–260.

[27] Morlock, G.; Ueda, Y. “New coupling of planar chromatography with direct analysis in real time mass spectrometry.” *J. Chromatogr., A.* **2007**, 1143, 243–251.

[28] Aranda, M.; Morlock, G. “New coupling of planar chromatography with direct analysis in real time mass spectrometry.” *J. Chromatogr. Sci.* **2007**, 45, 251–255.

[29] Jautz, U.; Morlock, G. “Efficacy of planar chromatography coupled to (tandem) mass spectrometry for employment in trace analysis.” *J. Chromatogr., A.* **2006**, 1128, 244–250.

[30] Morlock, G.; Schwack, W.”Determination of isopropylthioxanthone (ITX) in milk, yoghurt and fat by HPTLC-FLD, HPTLC-ESI/MS and HPTLC-DART/MS.” *Anal. Bioanal. Chem.* **2006**, 385, 586–595.

[31] Van Berkel, G. J.; Kertesz, V. “Electrochemically Initiated Tagging of Thiols Using an Electrospray Ionization-Based Liquid Microjunction Surface Sampling Probe Two-Electrode Cell.” *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1380-1386.

[32] Van Berkel, G. J.; Kertesz, V.; King, R. C. “High Throughput Mode Liquid Microjunction Surface Sampling Probe.” *Anal. Chem*. **2009**, 821, 7096-7101.

[33] Van Berkel, G. J.; Kertesz, V. “Application of a Liquid Extraction Based Sealing Surface Sampling Probe for Mass Spectrometric Analysis of Dried Blood Spots and Mouse Whole-Body Thin Tissue Sections.” *Anal Chem*., **2009**, 81, 9146-9152.

[34] Kertesz, V.; Van Berkel, G. J. "Fully Automated Liquid Extraction-Based Surface Sampling and Ionization Using a Chip-Based Robotic Nanoelectrospray Platform." *J. Mass Spectrom.* **2009**, 45, 252-260.

[35] Abu-Rabie, P.; Spooner, N. " Direct Quantitative Bioanalysis of Drugs in Dried Blood Spot Samples Using a Thin-Layer Chromatography Mass Spectrometer Interface." Anal. Chem. **2009**, 81, 10275-10284.

[36] Amstalden van Hove, E.R.; Smith, D.F.; Heeren, R. M. A. "A concise review of mass spectrometry imaging." *Journal of Chromatography A* **2010**, 1217, 25, 3946-3954.

[37] Edwards, R. L.; Creese, A. J.; Baumert, M.; Griffiths, P.; Bunch, J.; Cooper, H. J. "Hemoglobin variant analysis via direct surface sampling of dried blood spots coupled with high-resolution mass spectrometry." *Analytical chemistry*. **2011**, *83*, 2265-70.

[38] Kertez, V.; Van Berkel, G.J. "Liquid Microjunction Surface Sampling Coupled with High-Pressure Liquid Chromatography-Electrospray Ionization-Mass Spectrometry for the Analysis of Drugs and Metabolites in Whole-Body Thin Tissue Sections." *Analytical Chemistry*. **2010**, 82, 5917-5921.

[39] Luftmann, H. "A simple device for the extraction of TLC spots: direct coupling with an electrospray mass spectrometer." Anal. Bioanal. Chem. 2004, 378, 964–968.

[40] Prosek, M.; Milivojevic, L.; Krizman, M.; Fir, M. "On-line TLC-MS." *Journal of Planar Chromatography*. **2004,** 17, 420.

[41] Van Berkel, G. J.; Pasilis, S. P.; Ovchinnikova, O. "Established and Emerging Atmospheric Pressure Sampling/Ionization Techniques for Mass Spectrometry." *J. Mass. Spectrom*. **2008**, *43*, 1161-1180.

[42] Burton, W.G.; Nugent, K.D.; Slattery, T.K.; Summers, B.R.; Synder, L.R. "Separation of proteins by reversed-phase high-performance liquid chromatography: I. Optimizing the column." *J. Chromatogr. A*. **1988**, 443, 363-379.

[43] Chaurand, P.; Schriver, K. E.; Caprioli, R. M. "Instrument design and characterization for high resolution MALDI-MS imaging of tissue sections." *J. Mass Spectrom*. **2007**, 42, 476.

[44] Soparawalla, S.; Salazar, g.A.; Sokol, E.; Perry, R.H.; Cooks, G.R. "Trace detection of non-uniformly distributed analytes on surfaces using mass transfer and large-area desorption electrospray ionization (DESI) mass spectrometry." *Analyst*. **2010**, 135, 1953 -1960.

[45] Harris, G. a; Galhena, A. S.; Fernández, F. M." Ambient Sampling/Ionization Mass Spectrometry: Applications and Current Trends.." *Analytical chemistry*. **2011**.

[46] Chen, Y.; Guo, Z.; Wang, X.; Qiu, C. "Sample preparation." *J. Chromatogr. A*. **2008**, *1184*, 191-219.

[47] Dénes, J.; Katona, M.; Hosszú, A.; Czuczy, N.; Takáts, Z. "Analysis of Biological Fluids by Direct Combination of Solid Phase Extraction and Desorption Electrospray Ionization Mass Spectrometry." *Anal. Chem.* **2009**, *81*, 1669-1675.

[48] Chipuk, J. E.; Gelb, M. H.; Brodbelt, J. S. "Rapid and Selective Screening for Sulfhydryl Analytes in Plasma and Urine using Surface-Enhanced Transmission Mode Desorption Electrospray Ionization Mass Spectrometry." *Anal.Chem.* **2010**, *82*, 4130-4139

[49] Figueiredo, E. C.; Sanvido, G. B.; Arruda, M. A. Z.; Eberlin, M. N. "Molecularly Imprinted Polymers as Analyte Sequesters and Selective Surfaces for Easy Ambient Sonic-Spray Ionization." *Analyst* **2010**, *135*, 726-730.

[50] Kennedy, J. H.; Aurand, C.; Shirey, R.; Laughlin, B. C.; Wiseman, J. M. "Coupling Desorption Electrospray Ionization with Solid-Phase Microextraction for Screening and Quantitative Analysis of Drugs in Urine." *Anal. Chem.* **2010**, *82*, 7502–7508.

[51] Van Biesen G.; Wiseman, J. M.; Li, J.; Bottaro, C. S. "Desorption Electrospray Ionization-Mass Spectrometry for the Detection of Analytes Extracted by Thin-Film Molecularly Imprinted Polymers." *Analyst* **2010**, *135*, 2237-2240.

[52] Venn, R. F.; Merson, J.; Cole, S.; Macrae, P. "96-Well Solid-Phase Extraction: A Brief History of its Development." *J. Chromatogr. B* **2005**, *817*, 77-80.

[53] Wachs, T.; Henion, J. "A Device for Automated Direct Sampling and Quantitation from Solid-Phase Sorbant Extraction Cards by Electrospray Tandem Mass Spectroemtry." *Anal. Chem.* **2003**, *75*, 1769-1775.

[54] Morlock, G.; Schwack, W. "Coupling of planar chromatography to mass spectrometry." *Trends in Analytical Chemistry*. **2010**, *29*, 1157-1171.

[55] Cheng, S.-C.; Huang, M.-Z.; Shiea, J. "Thin Layer Chromatography/Mass Spectrometry." *Journal of Chromatography A*. **2011**.

[56] Pasilis, S. P.; Van Berkel, G. J. "Atmospheric pressure surface sampling/ionization techniques for direct coupling of planar separations with mass spectrometry." *Journal of chromatography. A.* **2010**, *1217*, 3955-65.

[57] Poole, C. F. "Planar chromatography at the turn of the century." *Journal of chromatography. A.* **1999**, *856*, 399-427.

[58] Vivo, G.; Wal, S. V. D.; Schoenmakers, P. J. "Comprehensive Study on the Optimization of Online Two-Dimensional Liquid Chromatographic Systems Considering Losses in Theoretical Peak Capacity in First- and Second-Dimensions : A Pareto-Optimality Approach" *Analysis.* **2008**, 3090-3100.

[59] Płocharz, P.; Klimek-Turek, A.; Dzido, T. H. "Pressurized planar electrochromatography, high-performance thin-layer chromatography and high-performance liquid chromatography-Comparison of performance." *Journal of chromatography. A.* **2010**.

[60] Tyihák, E.; Mincsovics, E." Forced-flow planar liquid chromatographic techniques (after twenty-two years)." *JPC - Journal of Planar Chromatography - Modern TLC.* **2010**, *23*, 382-395.

[61] Kuster, M.; De Alda, M.J.; Hernando, M.D.; Petrovic, M. "Analysis and occurrence of pharmaceuticals, estrogens, progestrons and polar petsicides in sewage treatment plant effluents, river water and drinking water in the Llobregat river basin (Barcelona, Spain)." Journal of Hydrology **2008**, 358, 112-123.

[62] Kudlak, B.; Namiesnik, J."Environmental Fate of Endocrine Disrupting Compounds-Analytical Problems and Challenges." *Crit. Rev. Anal. Chem.* **2008**, 38, 242-258.

[63] Iguchi, T.; Katsu, Y. " Commonality in signaling of endocrine disruption from snail to human." *Bioscience.* **2008**, 58, 1061-1067.

[64] Focazio, M. J.; Kolpin, D. W.; Barnes, K. K.; Furlong, E. T. "A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States - (II) Untreated drinking water sources." *Sci. Total Environ.* **2008**, 402, 201-216.

[65] Marumo, A.; Kumazawa, T.; Lee, X. P.; Hasegawa, C. "Utility of disk solid-phase extraction for whole blood samples: analysis of some tetracyclic antidepressants by gas chromatography with nitrogen-phosphorus detection." *Forensic Toxicology.* **2008**, 26, 13-18.

[66] Davi, L. M.; Baldi, M.; Penazzi, L.; Liboni, M. "Evaluation of the membrane approach to solid-phase extractions of pesticide residues in drinking water." *Pesticide Science.* **1992**, 35, 63-67.

[67] Rudaz, S.; Haerdi, W.; Veuthey, J. L." Evaluation of procedures for solid-phase extraction of [125I]-methadone from serum on to disks and cartridges." *Chromatographia.* **1997**, 44, 283-288.

[68] de Zeeuw, R. A.; Wijsbeek, J.; Franke, J. P. "SPEC disc solid-phase extraction for rapid broad-spectrum drug screening in urine." *Journal of Anal. Toxocology.* **2000**, 24, 97-101.

[69] Poole, C. F. "New trends in solid-phase extraction." *Trac-Trends in Analytical Chemistry.* **2003**, 22, 362-373.

[70] http://solutions.3m.com/wps/portal/3M/en_US/Empore/extraction/center/, technical-library/cartridge-faq/.

[71] Terabe, S.; Miyashita, Y.; Shibata, O.; Barnhart, E. R. et. al.."Separation of highly hydrophobic compounds by cyclodextrin-modified micellar electrokinetic chromatography." *Journal of Chroma. A.* **1990**, 1,23-31.

[72] Desiderio, C.; Fanali, S.; Sinibaldi, M.; Polcaro, C. "Cyclodextrin modified micellar electrokinetic chromatography for the chiral direct resolution of (+), (-)-trans-1,2-dihydrodiol metabolite of chrysene in vitro activated by rat liver microsome S9 fraction." *Electrophoresis.* **1995**, 16,784-788.

[73] Zhao, Y. F.; Luo, X. P.; Ming, Y. F.; Chen, L. R.; Li, Y. M." Rapid separation and determination of resibufogenin and cinobufagin in toad venom and Liushen tablet by β-cyclodextrin modified micellar electrokinetic chromatography." *Journal of Pharmaceutical and Biomedical Analysis*. **2006**, 41, 124-128.

[74] Gotti, R.; El-Hady, D. A.; Andrisano, V.; Bertucci, C. "Determination of the chiral and achiral related substances of methotrexate by cyclodextrin-modified micellar electrokinetic chromatography." *Electrophoresis.* **2004**, 25, 2830-2837.

[75] Rodriguez-Mozaz, S.; de Alda, M. J. L.; Barcelo, D." Advantages and limitations of on-line solid phase extraction coupled to liquid chromatography-mass spectrometry technologies versus biosensors for monitoring of emerging contaminants in water." *Journal of Chromatography A*. **2007**, 1152, 97-115.

[76] Lee, H. B.; Peart, T. E.; Svoboda, M. L." Determination of endocrine-disrupting phenols, acidic pharmaceuticals, and personal-care products in sewage by solid-phase extraction and gas chromatography-mass spectrometry." *Journal of Chromatography A.* **2005**, 1094, 122-129.

[77] Baugros, J. B.; Giroud, B.; Dessalces, G.; Grenier-Loustalot, M. F.; Cren-Olive, C. " Multiresidue analytical methods for the ultra-trace quantification of 33 priority substances present in the list of REACH in real water samples." *Analytica Chimica Acta*. **2008**, 607, 191-203.

[78] Zhang, J. H.; Wu, X. P.; Zhang, W.; Xu, L. J.; Chen, G. N. "A sweeping-micellar electrokinetic chromatography method for direct detection of some aromatic amines in water samples." *Electrophoresis* **2008**, 29, 796-802.

[79] Wakida, S.; Fujimoto, K.; Nagai, H.; Miyado, T.; et al. "On-chip micellar electrokinetic chromatographic separation of phenolic chemicals in waters." *Journal of Chromatography A.* **2006**, 1109,179-182.

[80] Regan, F.; Moran, A.; Fogarty, B.; Dempsey, E." Novel modes of capillary electrophoresis for the determination of endocrine disrupting chemicals." *Journal of Chromat. A.* **2003**, 141-152.

[81] Ravelo-Perez, L. M.; Hernandez-Borges, J.; Cifuentes, A.; Rodriguez-Delgado, M. A. "MEKC combined with SPE and sample stacking for multiple analysis of pesticides in water samples at the ng/L level." *Electrophoresis.* **2007**, *28*, 1805-1814.

[82] Regan, F.; Moran, A.; Fogarty, B.; Dempsey, E." Novel modes of capillary electrophoresis for the determination of endocrine disrupting chemicals." *Journal of Chromat. A.* **2003**, 141-152.

[83] Wu, D. P.; Qin, J. H.; Lin, B. C. "Electrophoretic separations on microfluidic chips." *Journal of Chromatography A*. **2008**, 1184, 542-559.

[84] Marce, R. M.; Borrull, F. "Solid-phase extraction of polycyclic aromatic compounds." *Journal of Chromatography A*. **2000**, 885, 273-290.

[85] Kiss, G.; VargaPuchony, Z.; Hlavay, J. "Determination of polycyclic aromatic hydrocarbons in precipitation using solid-phase extraction and column liquid chromatography." *Journal of Chromatography A.* **1996**, 725, 261-272.

[86] Sun, F. S., Littlejohn, D., Gibson, M. D. "Ultrasonication extraction and solid phase extraction clean-up for determination of US EPA 16 priority pollutant polycyclic aromatic hydrocarbons in soils by reversed-phase liquid chromatography with ultraviolet absorption detection." *Analytica Chimica Acta*. **1998**, 364(1-3), 1-11.

[87] Loudon, G. M. Organic Chemistry. Oxford University Press, New York **2002**.

[88] Harris, G. A.; Nyadong, L; Fernandez, F. "Recent Developments in Ambient Ionization Techniques for Analytical Mass Spectrometry." *Analyst.* **2008**, *133*, 1297-1301.

[89] Venter, A.; Nefliu, M.; Cooks, R. G. "Ambient Desorption Ionization Mass Spectrometry." *Trends Anal. Chem.* **2008**, *27*, 284-290.

[90] Ifa, D. R.; Jackson, A. U.; Paglia, G.; Cooks, R. G. "Forensic Applications of Ambient Ionization Mass Spectrometry." *Anal. Bioanal. Chem*. **2009**, *394*, 1995-2008.

[91] Chen, H.W.; Gamez, G.; Zenobi, R. "What Can We Learn from Ambient Ionization Techniques?" *J. Am. Soc. Mass. Spectrom.* **2009**, *20*, 1947-1963.

[92] Alberici, R. M.; Simas, R. C.; Sanvido, G. B.; Romao, W; Lalli, P. M.; Benassi, M.; Cunha, I. B. S.; Eberlin, M. N. "Ambient Mass Spectrometry: Bringing MS into the "Real World"." *Anal. Bioanal. Chem.* **2010**, *398*, 265-294.

[93] Ifa, D. R.; Wu, C.; Ouyang, Z.; Cooks, R. G. "Desorption Electrospray Ionization and Other Ambient Ionization Methods: Current Progress and Preview." *Analyst* **2010**, *135*, 669-681.

[94] Huang, M. Z.; Yuan, C. H.; Cheng, S. C.; Cho, Y. T.; Shiea, J. "Ambient Ionization Mass Spectrometry. " *Ann. Rev. Anal. Chem.* **2010**, *3*, 43-65.

[95] Pasilis, S. P.; Van Berkel, G. J. "Atmospheric Pressure Surface Sampling/Ionization Techniques for Direct Coupling of Planar Separations with Mass Spectrometry." *J. Chromatgr. A* **2010**, *1217*, 3955-3965.

[96] Weston, D. J. "Ambient Ionization Mass Spectrometry: Current Understanding of Mechanistic Theory; Analytical Performance and Application Areas." *Analyst* **2010**, *135*, 661-668.

[97] Chen, Y.; Guo, Z.; Wang, X.; Qiu, C. "Sample preparation." *J. Chromatogr. A.* **2008**, *1184*, 191-219.

[98] Dénes, J.; Katona, M.; Hosszú, A.; Czuczy, N.; Takáts, Z. "Analysis of Biological Fluids by Direct Combination of Solid Phase Extraction and Desorption Electrospray Ionization Mass Spectrometry." *Anal. Chem.* **2009**, *81*, 1669-1675.

[99] Chipuk, J. E.; Gelb, M. H.; Brodbelt, J. S. "Rapid and Selective Screening for Sulfhydryl Analytes in Plasma and Urine using Surface-Enhanced Transmission Mode Desorption Electrospray Ionization Mass Spectrometry." *Anal.Chem.* **2010**, *82*, 4130-4139

[100] Figueiredo, E. C.; Sanvido, G. B.; Arruda, M. A. Z.; Eberlin, M. N. "Molecularly Imprinted Polymers as Analyte Sequesters and Selective Surfaces for Easy Ambient Sonic-Spray Ionization." *Analyst* **2010**, *135*, 726-730.

[101] Kennedy, J. H.; Aurand, C.; Shirey, R.; Laughlin, B. C.; Wiseman, J. M. "Coupling Desorption Electrospray Ionization with Solid-Phase Microextraction for Screening and Quantitative Analysis of Drugs in Urine." *Anal. Chem.* **2010**, *82*, 7502–7508.

[102] Van Biesen G.; Wiseman, J. M.; Li, J.; Bottaro, C. S. "Desorption Electrospray Ionization-Mass Spectrometry for the Detection of Analytes Extracted by Thin-Film Molecularly Imprinted Polymers." *Analyst* **2010**, *135*, 2237-2240.

[103] Venn, R. F.; Merson, J.; Cole, S.; Macrae, P. "96-Well Solid-Phase Extraction: A Brief History of its Development." *J. Chromatogr. B* **2005**, *817*, 77-80.

[104] Kertesz, V.; Ford, M.J.; Van Berkel, G.J. "Automation of a surface sampling probe/electrospray mass spectrometry system." *Anal. Chem.* **2005,** *77*, 7183-7189.

[105] Emory, J.F.; Walworth, M.J.; Van Berkel, G,J; Schulz, M.; Minarik, S. "Direct Analysis of Reversed-Phase High-Performance Thin Layer Chromatography Separated Tryptic Protein Digests using a Liquid Microjunction Surface Sampling Probe /Electrospray Ionization Mass Spectrometry System." *Eur J Mass Spectrom*.**2010**, *16*, 21-33.

[106] Roach, P.J.; Laskin, J.; Laskin, A. "Nanospray Desorption Electrospray Ionization: An Ambient Method for Liquid-Extraction Surface Sampling in Mass Spectrometry." *Analyst* **2010**, *135*, 2233-2236.

[107] Roach, P.J.; Laskin, J.; Laskin, A.; "Molecular Characterization of Organic Aerosols Using Nanospray-Desorption / Electrospray Ionization-Mass Spectrometry". *Anal. Chem.* **2010**, *82*, 7970-7986.

[108] Van Berkel, G. J.; Kertesz, V.; King, R. C.; "High-Throughput Mode Liquid Microjunction Surface Sampling Probe." *Anal. Chem.* **2009**, *81*, 7096-7101.

[109] Marshall, P.; Toteu-Djomte, V.; Bareille, P.; Perry, H.; Brown, G.; Baumert, M.; Biggadike, K., "Correlation of Skin Blanching and Percutaneous Absorption for Glucocorticoid Receptor Agonists by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry Imaging and Liquid Extraction Surface Analysis with Nanoelectrospray Ionization Mass Spectrometry." *Anal. Chem.* **2010**, *82*, 7787–7794

[110] http://www.advion.com/biosystems/triversa-nanomate/LESA/

[111] Harkins, J. B.; Katz, B. B.; Pastor, S. J.; Osucha, P.; Hafeman, D. G.; Witkowski, C. E.; Norris, J. L. "Parallel electrophoretic depletion, fractionation, concentration, and desalting of 96 complex biological samples for mass spectrometry." *Analytical chemistry*. **2008**, 80, 2734-43.

[112] Svec, F. "Less Common Applications of Monoliths: Preconcentration and Solid-Phase Extraction." *J. Chromatogr. B* **2006**, *841*, 52-64.

[113] Miller J. C.; Miller J.N. Statistics for Analytical Chemistry, 2nd Edition Prentice Hall: Englewood Cliffs, NJ. **1992**.

[114] Greulich, K.; Alder, L. "Fast Multiresidue Screening of 300 Pesticides in Water for Human Consumption by LC-MS/MS." *Anal. Bioana. Chem.* **2008**, *391*, 183-197.

[115] Walworth, M. J.; Connatser, R. M.; Sepaniak, M. J. "Extraction, Separation, and Fluorometric Analysis of Selected Environmental Contaminants." *J. Sep.Sci.* **2009**, *32*, 2985-2992.

[116] Harris, G. A.; Nyadong, L; Fernandez, F. "Recent Developments in Ambient Ionization Techniques for Analytical Mass Spectrometry." *Analyst* **2008**, *133*, 1297-1301.

[117] Venter, A.; Nefliu, M.; Cooks, R. G. "Ambient Desorption Ionization Mass Spectrometry." *Trends Anal. Chem.* **2008**, *27*, 284-290.

[118] Chen, H.; Gamez, G.; Zenobi, R. "What can we Learn from Ambient Ionization Techniques?" *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 1947-1963.

[119] Weston, D. J. "Ambient Ionization Mass Spectrometry: Current Understating of the Mechanistic Theory; Analytical Performance and Applciation Areas." *Analyst* **2010**, *135*, 661-668.

[120] Ifa, D. R.; Wu, C.; Ouyang, Z.; Cooks, R. G. "Desorption Electrospray Ionization and Other Ambient Ionization Methods: Current Progress and Preview." *Analyst* **2010**, *135*, 669-681.

[121] Alberici, R. M.; Simas, R. C.; Sanvido, G. B.; Romão, W.; Lalli, P. M.; Benassi, M.; Cunha, I. B. S.; Eberlin, M. N. "Ambient Mass Spectrometry: Bringing MS into the Real World." *Anal. Bioanal. Chem.* **2010**, 398, 265-294.

[122] McDonald, W. H.; Tabb, D. L.; Sadygov, R. G.; MacCoss, M. J.; Venable, J.; Graumann, J.; Johnson, J. R.; Cociorva, D.; Yates, J. R. "MS1, MS2, and SQT – Three Unified, Compact, and Easily Parsed File Formats for the Storage of Shotgun Proteomic Spectra and Identifications." *Rapid Commun. Mass Spectrom.* **2004,** *18*, 2162-2168.

[123] Tabb, D. L.; Narasimhan, C.; Strader, B. L.; Hettich, R. L. "DBDigger: reorganized proteomic database identification that improves flexibility and speed." *Anal. Chem.* **2005**, *77*, 2464-2474.

[124] Narasimhan, C.; Tabb, D. L.; VerBerkmoes, N. C.; Thompson, M. R.; Hettich, R. L.; Uberbacher, E. C. "MASPIC: Intensity-Based Tandem Mass Spectrometry Scoring Scheme that Improves Peptide Identification at High Confidence." *Anal. Chem.* **2005** *77*, 7581-7593.

[125] Tabb, D. L.; McDonald, W. H.; Yates, J. R. "DTASelect and Contrast: Tools for Assembling and Comparing Protein Identifications from Shotgun Proteomics." *J. Proteome Res.* **2002**, *1*, 21-26.

[126] Gasparič, J. "Chromatography on Thin Layers Impregnated with Organic Stationary Phases." Chapter 3 in *Advances in Chromatography, Vol. 31*, Giddings, J. C; Grushka, E.; Brown, P. R., Eds., Marcel Dekker, New York, NY, **1992**, 153-252.

[127] Rabel, F. M. "Sorbants and Precoated Layers in Thin-Layer Chromatography." Chapter 4 in *Handbook of Thin Layer chromatography, Third Edition*, Sherma, J.; Fried, B., Eds., Marcel Dekker, New York, NY, **2003**, 99-133.

[128] Clarson, S. J. *Synthesis and Properties of Silicones and Silicone-Modified Materials.* American Chemical Society, Washington, D.C. **2003**.

[129] Upton, R., Ed., Goldenseal Root, *Hydrastis Canadensis*: Standards of Analysis, Quality Control, and Therapeutics. Santa Clara, CA, American Herbal Pharmacopoeia, 2001.

[130] Van Berkel, G.; Tomkins, B. A.; Kertesz, V. "Thin-Layer Chromatography/Desorption Electrospray Ionization Mass Spectrometry: Investigation of Goldenseal Alkaloids." *Anal. Chem*. **2007**, *79*, 2778-2789.

[131] Shi, S. D.; Hemling, M. E.; Carr, S. A.; Horn, D. M.; Lindh, I.; McLafferty, F. W. "Phosphopeptide/Phosphoprotein Mapping by Electron Capture Dissociation Mass Spectrometry." *Anal. Chem.* **2001**, *73*, 19-22.

[132] Pasilis, S. P.; Kertesz, V.; Van Berkel, G. J.; Schulz, M.; Schorcht, S. "Using HPTLC/DESI-MS for Peptide Identification in 1D Separations of Tryptic Peptide Protein Digests." *Anal. Bioanal. Chem.* **2008**, *391*, 317-324.

[133] Aebersold, R.; Goodlett, D.R. Mass spectrometry in proteomics. *Chem. Rev.* **2001**, *101*, 269-295.

[134] Hu, L.; Ye, M.; Jiang, X; Feng, S.; Zou, H. Advances in hyphenated analytical techniques for shotgun proteome and peptidome analysis-a review. *Anal. Chim. Acta* **2007**, *598*, 193–204.

[135] Karas, M.; Hillenkamp, F. "Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons." *Anal. Chem.* **1988**, 60, 2299.

[136] Hillenkamp, F.; Karas, M.; Beavis. R.C.; Chait, B.T. "Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers." *Anal. Chem.* **1991**, 63, 1193A.

[137] Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. "Electrospray ionization for mass spectrometry of large biomolecules." *Science* **1989**, *246*, 64-71.

[138] Dongre A.R.; Jones J.L.; Somogyi, A.; Wysocki, V.H. "Influence of peptide composition, gas-phase basicity, and chemical modification on fragmentation efficiency: Evidence for the mobile proton model." *J. Am. Soc. Mass Spectrom.* **1996**, 118, 64.

[139] He M.; Reid, G.E.; Shang, H.; Lee, G.U.; McLuckey, S.A. "Dissociation of multiple protein ion charge states following a single gas-phase purification and concentration procedure." *Anal. Chem.*, **2002**, 64, 4653.

[140] Busch, K.L. "Mass-spectrometric detectors for samples separated by planar electrophoresis." *J. Chromatogr. A* **1995**; 692: 275.

[141] Wilson, I.D.; Morden, W. "Advances and applications in the use of HPTLC-MS-MS." *J. Planar Chromatogr.* **1996**, 9, 84.

[142] Wilson, I.D. "The state of the art in thin-layer chromatography-mass spectrometry: a critical appraisal." *J. Chromatogr. A* **1999**, 856, 429.

[143] Gusev, A.I. "Interfacing matrix-assisted laser desorption/ionization mass spectrometry with column and planar separations." *Fresenius J. Anal. Chem.* **2000**, 366, 691.

[144] Gusev, A.I.; Proctor, A.; Rabinovich, Y.I.; Hercules, D.M. "Thin-Layer Chromatography combined with matrix-assisted laser-desorption ionization mass-spectrometry." *Anal. Chem.* **1995**, 67, 4565.

[145] Gusev, A.I.; Vasseur, O.J.; Proctor, A.; Sharkey, A.G.; Hercules, D.M. "Imaging of thin-layer chromatograms using matrix/assisted laser desorption ionization mass-spectrometry." *Anal. Chem.* **1995**, 67**,** 4565.

[146] Mehl, J.T.; Gusev, A.I.; Hercules, D.M. "Coupling protocol for thin layer chromatography matrix-assisted laser desorption ionization." *Chromatographia*. **1997**, 46, 358.

[147] Isbell, D.T.; Gusev, A.I.; Taranenko, N.I.; Chen, C.H.; Hercules, D.M."Analysis of nucleotides directly from TLC plates using MALDI-MS detection." *Fresenius J. Anal. Chem.* **1999**, 365, 625.

[148] Mehl, J.T.; Hercules, D.M. "Direct TLC-MALDI coupling using a hybrid plate." *Anal. Chem.* **2000**, 72, 68.

[149] *Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation and Applications*, Cole RB (ed). John Wiley: New York, **1997**.

[150] Takáts, Z.; Wiseman, J.M.; Cooks, R.G. "Ambient mass spectrometry using desorption electrospray ionization (DESI): instrumentation, mechanisms and applications in forensics, chemistry, and biology." J Mass Spectrom. **2005**, 40, 1261–1275.

[151] Myung, S.; Wiseman, J.M.; Valentine, S.J.; Takáts, Z.; Cooks, R.G.; Clemmer, D.E. "Coupling desorption electrospray ionization with ion mobility/mass spectromtery for analysis of protein structure: evidence for desorption of folded and denatured states." *J Phys Chem*. **2006.** 110, 4545–4551.

[152] Bereman, M.S.; Nyadong, L.; Fernandez, F.M.; Muddiman, D.C. "Direct high-resolution peptide and protein analysis by desorption electrspray ionization fourier transform ion cyclotron resonance mass spectrometry." *Rapid Commun Mass Spectrom*. **2006**, 20, 3409–3411.

[153] Shin, Y.S.; Drolet, B.; Mayer, R.; Dolence, K.; Basile, F. "Desorption electrospray ionization mass spectrometry of proteins." *Anal. Chem.* **2007**, 79, 3514–3518.

[154] Kaur-Atwal, G.; Weston, D.J.; Green, P.S.; Crosland, S.; Bonner, P.L.R.; Creaser, C.S. "Analysis of tryptic peptides using desorption electrospray ionization combined with ion mobility spectrometry/mass spectromtetry." *Rapid Commun. Mass Spectrom.* **2007**. 21, 1131–1138.

[155] Cotte-Rodriguez, I.; Mulligan, C.C.; Cooks, R.G. "Nonproximate detection of small and large molecules by desorption electrospray ionization and desorption atmospheric pressure chemical ionzation mass spectrometry: instrumentation and applications in forensics, chemistry, and biology." *Anal. Chem.* **2007**, 79, 7069–7077.

[156] Shin, Y.; Drolet, B.; Mayer, R.; Dolence, K.; Basile, F. "Desorption Electrospray Ionization-Mass Spectrometry of Proteins." *Anal. Chem.* **2007**, 79, 3514-3518.

[157] Pasilis, S.P.; Kertesz, V.; Van Berkel G.J.; Schulz, M.; Schorcht, S. "Using HPTLC/DESI-MS for peptide identification in 1D separations of tryptic protein digests." *Anal. Bioanal. Chem.* **2008**, 391, 317–324.

[158] Pasilis, S.P.; Kertesz, V.; Van Berkel, G.J.; Schulz, M.; Schorcht, S. "HPTLC/DESI-MS imaging of tryptic protein digests separated in two dimensions." *J. Mass Spectrom.* **2008**, 43, 1627–1635.

[159] Wachs, T.; Henion, J. "Electrospray device for coupling microscale separations and other miniaturized devices with electrospray mass spectrometry." *Anal. Chem.* **2001**, *73*, 632-638.

[160] Kertesz, V.; Van Berkel, G.J.; Vavrek, M.; Koeplinger, K.A.; Schneider, B.B.; Covey, T.R. "Comparison of Drug Distribution Images from Whole-Body Thin Tissue Sections Obtained

Using Desorption Electrospray Ionization Tandem Mass Spectrometry and Autoradiography." *Anal. Chem.* **2008**, 80, 5168.

[161] Van Berkel, G.J.; Kertesz, V. "Automated sampling and imaging of analytes separated on thin-layer chromatography plates using desorption electrospray ionizationmass spectrometry." *Analytical Chemistry.* **2006**, 78, 4938.

[163] Colinge, J.; Chiappe, D.; Lagache, S.; Moniatte, M.; Bougueleret, L. "Differential Proteomics via Probabilistic Peptide Identification Scores." Anal. Chem. **2005,** 77, 596-606.

[164] Bakry, R.; Bonn, G. K.; Mair, D.; Svec, F. "Monolithic porous polymer layer for the separation of peptides and proteins using thin-layer chromatography coupled with MALDI-TOF-MS." *Analytical chemistry*. **2007**, *79*, 486-93.

[165] Patton, W.; Panchagnula, V.; Rockney, E.; Krull, I. "Taking a Walk on the Wild Side with Planar Electrochromatography and Thin-Layer Electrophoresis: Of Peptides, Proteins, and Proteomics." *Journal of Liquid Chromatography & Related Technologies*. **2006**, *29*, 1177-1218.

[166] Luo, Q.; Andrade, J. D.; Caldwell, K. D. "Thin-layer ion-exchange chromatography of proteins." *Journal of chromatography. A*. **1998**, *816*, 97-105.

[167] Neth, T.; Greg, B. "PEPTIDES AND PROTEINS OF AMINO ACIDS , ; Z) F THE EFFECT OF pH AND ION-PAIR FQRMA- TION ON THE RETENTION OF PEPTIDES ON CHEMICALLY-BONDED." *North*. **1979**, *185*.

[168] Marutoiu, C.; Soran, M.L. Peptides and Proteins: TLC Analysis. Encyclopedia of Chromatography. **2009.** 1728.

[169] Morlock, G.; Schwack, W. "Coupling of planar chromatography to mass spectrometry." *Trends in Analytical Chemistry*. **2010**, *29*, 1157-1171.

[170] Cheng, S.-C.; Huang, M.-Z.; Shiea, J. "Thin Layer Chromatography/Mass Spectrometry." *Journal of Chromatography A*. **2011**.

[171] Pasilis, S. P.; Van Berkel, G. J. "Atmospheric pressure surface sampling/ionization techniques for direct coupling of planar separations with mass spectrometry." *Journal of chromatography. A*. **2010**, *1217*, 3955-65.

[172] Van Berkel, G. J.; Pasilis, S. P.; Ovchinnikova, O. *J. Mass Spectrom*. **2008**, *43*, 1161 - 1180.

[173] Harris, G. A.; Nyadong, L; Fernandez, F. "Recent Developments in Ambient Ionization Techniques for Analytical Mass Spectrometry." *Analyst* **2008**, *133*, 1297-1301.

[174] Venter, A.; Nefliu, M.; Cooks, R. G. "Ambient Desorption Ionization Mass Spectrometry." *Trends Anal. Chem.* **2008**, *27*, 284-290.

[175] Chen, H.; Gamez, G.; Zenobi, R. "What can we Learn from Ambient Ionization Techniques?" *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 1947-1963.

[176] Weston, D. J. "Ambient Ionization Mass Spectrometry: Current Understating of the Mechanistic Theory; Analytical Performance and Applciation Areas." *Analyst* **2010**, *135*, 661-668.

[177] Ifa, D. R.; Wu, C.; Ouyang, Z.; Cooks, R. G. "Desorption Electrospray Ionization and Other Ambient Ionization Methods: Current Progress and Preview." *Analyst* **2010**, *135*, 669-681.

[178] Alberici, R. M.; Simas, R. C.; Sanvido, G. B.; Romão, W.; Lalli, P. M.; Benassi, M.; Cunha, I. B. S.; Eberlin, M. N. "Ambient Mass Spectrometry: Bringing MS into the Real World." *Anal. Boanal. Chem.* **2010**, 398, 265-294.

[179] Harris, G. a; Galhena, A. S.; Fernández, F. M. "Ambient Sampling/Ionization Mass Spectrometry: Applications and Current Trends." *Analytical chemistry*. **2011**, DOI 10.1021/ac200918u.

[180] Luftmann, H. Anal. Bioanal. Chem. 2004, 378, 964–968.

[181] http://www.camag.de/ (accessed on January 28, 2010).

[182] Vovk, I.; Popovic, G.; Simonovska, B.; Albreht, A.; Agbaba, D. "Ultra-thin-layer chromatography mass spectrometry and thin-layer chromatography mass spectrometry of single peptides of angiotensin-converting enzyme inhibitors." *Journal of Chromatography A.* **2011**, 1218, 20, 3089-3094.

[183] Poole, C. F. "Planar chromatography at the turn of the century." *Journal of chromatography. A.* **1999**, *856*, 399-427.

[184] http://www.nwo.nl/projecten.nsf/vk2008/ned/2300137953

[185] Vivo, G.; Wal, S. V. D.; Schoenmakers, P. J. "Comprehensive Study on the Optimization of Online Two-Dimensional Liquid Chromatographic Systems Considering Losses in Theoretical Peak Capacity in First- and Second-Dimensions : A Pareto-Optimality Approach" *Analysis.* **2008**, 3090-3100.

[186] Van Berkel, G. J.; Pasilis, S. P.; Ovchinnikova, O. *J. Mass Spectrom.* **2008**, *43*, 1161 - 1180.

[187] Harris, G. A.; Nyadong, L; Fernandez, F. "Recent Developments in Ambient Ionization Techniques for Analytical Mass Spectrometry." *Analyst* **2008**, *133*, 1297-1301.

[188] Venter, A.; Nefliu, M.; Cooks, R. G. "Ambient Desorption Ionization Mass Spectrometry." *Trends Anal. Chem.* **2008**, *27*, 284-290.

[189] Chen, H.; Gamez, G.; Zenobi, R. "What can we Learn from Ambient Ionization Techniques?" *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 1947-1963.

[190] Weston, D. J. "Ambient Ionization Mass Spectrometry: Current Understating of the Mechanistic Theory; Analytical Performance and Applciation Areas." *Analyst* **2010**, *135*, 661-668.

[191] Ifa, D. R.; Wu, C.; Ouyang, Z.; Cooks, R. G. "Desorption Electrospray Ionization and Other Ambient Ionization Methods: Current Progress and Preview." *Analyst* **2010**, *135*, 669-681.

[192] Alberici, R. M.; Simas, R. C.; Sanvido, G. B.; Romão, W.; Lalli, P. M.; Benassi, M.; Cunha, I. B. S.; Eberlin, M. N. "Ambient Mass Spectrometry: Bringing MS into the Real World." *Anal. Boanal. Chem.* **2010**, 398, 265-294.

[193] Harris, G. a; Galhena, A. S.; Fernández, F. M. "Ambient Sampling/Ionization Mass Spectrometry: Applications and Current Trends." *Analytical chemistry*. **2011**, DOI 10.1021/ac200918u.

[194] Van Berkel G. J.; Sanchez, A. D.; Quirke, J. M. E. "Thin-Layer Chromatography and Electrospray Mass Spectrometry Coupled using a Surface Sampling Probe." *Anal. Chem.* **2002**, *74*, 6216-6223.

[195] Wachs, T.; Henion, J. "A Device for Automated Direct Sampling and Quantitation from Solid-Phase Sorbent Extraction Cards by Electrospray Tandem Mass Spectrometry." *Anal. Chem.* **2003**, *75*, 1769-1775.

[196] Ford, M. J.; Van Berkel, G. J. "An Improved Thin-Layer Chromatography/Mass Spectrometry Coupling Using a Surface Sampling Probe Electrospray Ion Trap System." *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1303-1309.

[197] Ford, M. J.; Kertesz, V.; Van Berkel, G. J. "Thin Layer Chromatography/Electrospray Ionization Triple Quadrupole Linear Ion Trap Mass Spectrometry System: Analysis of Rhodamine Dyes Separated on Reversed-Phase C8 Plates." *J. Mass Spectrom.* **2005**, *40*, 866-875.

[198] Ford, M. J.; Deibel, M. A.; Tomkins, B. A.; Van Berkel, G. J. "Quantitative Thin-Layer Chromatography/Mass Spectrometry Analysis of Caffeine using a Surface Sampling Probe Electrospray Ionization Tandem Mass Spectrometry System." *Anal. Chem.* **2005**, *77*, 4385-4389.

[199] Asano, K. G.; Ford, M. J.; Tomkins, B. A.; Van Berkel, G. J. "Self-Aspirating Atmospheric Pressure Chemical Ionization Source for Direct Sampling of Analytes on Surfaces and in Liquid Solutions." *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2305-2312.

[200] Kertesz, V.; Ford, M. J.; Van Berkel, G. J. "Automation of a Surface Sampling Probe/Electrospray Mass Spectrometry System." *Anal. Chem.* **2005**, *77*, 7183-7189.

[201] Van Berkel, G. J.; Ford, M. J.; Doktycz, M. J.; Kennel, S. J. "Evaluation of a Surface Sampling Probe Electrospray Mass Spectrometry System for the Analysis of Surface Deposited and Affinity Captured Proteins." *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1144-1152.

[202] Van Berkel, G. J.; Kertesz, V.; Koeplinger, K. A.; Vavrek, M.; Kong, A. T. "Liquid Micro-Junction Surface Sampling Probe Electrospray Mass Spectrometry for Detection of Drugs and Metabolites in Thin Tissue Sections." *J. Mass Spectrom.* **2008**, *43*, 500-508.

[203] Emory, J. F.; Walworth, M. J.; Van Berkel. G. J.; Schulz, M.; Minarik, S. "Direct Analysis of Reversed-Phase High Performance Thin Layer Chromatography Separated Tryptic Protein Digests Using a Liquid Microjunction Surface Sampling Probe/Electrospray Ionization Mass Spectrometry System." *Eur. J. Mass Spectrom.* **2010**, *16*, 21-33.

[204] Van Berkel, G. J.; Kertesz, V.; King, R. C.; "High-Throughput Mode Liquid Microjunction Surface Sampling Probe." *Anal. Chem.* **2009**, *81*, 7096-7101.

[205] Kertesz, V.; Van Berkel, G. J. "Fully Automated Liquid Extraction-Based Surface Sampling and Ionization using a Chip-Based Robotic Nanoelectrospray Platform." *J. Mass Spectrom.* **2009**, *45*, 252-260.

[206] Kertesz, V.; Van Berkel, G. J. "Liquid Microjunction Surface Sampling Coupled with High-Pressure Liquid Chromatography-Electrospray Ionization-Mass Spectrometry for Analysis of Drugs and Metabolites in Whole-Body Thin Tissue Sections." *Anal. Chem.* **2010**, *82*, 5917-5921.

[207] Marshall, P.; Toteu-Djomte, V.; Bareille, P.; Perry, H.; Brown, G.; Baumert, M.; Biggadike, K., "Correlation of Skin Blanching and Percutaneous Absorption for Glucocorticoid Receptor Agonists by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry Imaging and

Liquid Extraction Surface Analysis with Nanoelectrospray Ionization Mass Spectrometry." *Anal. Chem.* **2010**, *82*, 7787–7794

[208] http://www.advion.com/biosystems/triversa-nanomate/LESA/

[209] Morlock, G.; Schwack, W. "Coupling of planar chromatography to mass spectrometry." *Trends in Analytical Chemistry*. **2010**, *29*, 1157-1171.

[210] Cheng, S.-C.; Huang, M.-Z.; Shiea, J. "Thin Layer Chromatography/Mass Spectrometry." *Journal of Chromatography A*. **2011**.

[211] Pasilis, S. P.; Van Berkel, G. J. "Atmospheric pressure surface sampling/ionization techniques for direct coupling of planar separations with mass spectrometry." *Journal of chromatography. A*. **2010**, *1217*, 3955-65.

[212] McDonald, W. H.; Tabb, D. L.; Sadygov, R. G.; MacCoss, M. J.; Venable, J.; Graumann, J.; Johnson, J. R.; Cociorva, D.; Yates, J. R. "MS1, MS2, and SQT – Three Unified, Compact, and Easily Parsed File Formats for the Storage of Shotgun Proteomic Spectra and Identifications." *Rapid Commun. Mass Spectrom.* **2004,** *18*, 2162-2168.

[213] Tabb, D. L.; Narasimhan, C.; Strader, B. L.; Hettich, R. L. "DBDigger: reorganized proteomic database identification that improves flexibility and speed." *Anal. Chem.* **2005**, *77*, 2464-2474.

[214] Narasimhan, C.; Tabb, D. L.; VerBerkmoes, N. C.; Thompson, M. R.; Hettich, R. L.; Uberbacher, E. C. "MASPIC: Intensity-Based Tandem Mass Spectrometry Scoring Scheme that Improves Peptide Identification at High Confidence." *Anal. Chem.* **2005** *77*, 7581-7593.

[215] Tabb, D. L.; McDonald, W. H.; Yates, J. R. "DTASelect and Contrast: Tools for Assembling and Comparing Protein Identifications from Shotgun Proteomics." *J. Proteome Res.* **2002**, *1*, 21-26.

[216] Lu, A.; Waanders, L. F.; Almeida, R.; Li, G.; Allen, M.; Cox, J.; Olsen, J. V.; Bonaldi, T.; Mann, M. "Nanoelectrospray peptide mapping revisited: Composite survey spectra allow high dynamic range protein characterization without LCMS on an orbitrap mass spectrometer." *International Journal of Mass Spectrometry*. **2007**, *268*, 158-167.

[217] Harris, G. a; Galhena, A. S.; Fernandez, F. M. "Ambient Sampling/Ionization Mass Spectrometry: Applications and Current Trends." *Analytical chemistry*. **2011**, DOI: 10.1021/ac200918u.

[218] Marshall, P.; Toteu-Djomte, V.; Bareille, P.; Perry, H.; Brown, G.; Baumert, M.; Biggadike, K., "Correlation of Skin Blanching and Percutaneous Absorption for Glucocorticoid Receptor Agonists by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry Imaging and Liquid Extraction Surface Analysis with Nanoelectrospray Ionization Mass Spectrometry." *Anal. Chem.* **2010**, *82*, 7787–7794

[219] http://www.advion.com/biosystems/triversa-nanomate/LESA/

[220] Walworth, M. J.; Stankovich, J. J.; Van Berkel, G. J.; Schulz, M.; Minarik, S.; Nichols, J.; Reich, E. "Hydrophobic treatment enabling analysis of wettable surfaces using a liquid microjunction surface sampling probe/electrospray ionization-mass spectrometry system." *Analytical chemistry*. **2011**, *83*, 591-7.

[221] Peng, J.; Elias, J. E.; Thoreen, C. C.; Licklider, L. J.; Gygi, S. P. "Evaluation of Multidimensional Chromatography Coupled with Tandem Mass Spectrometry (LC/LC−MS/MS) for Large-Scale Protein Analysis: The Yeast Proteome." *Journal of Proteome Research*. **2003**, *2*, 43-50.

Vita

Matthew John Walworth was born in Fort Wayne, Indiana in the year nineteen hundred and seventy eight. He began his higher education at East Tennessee State University in the college of music. He became disillusioned with higher education after three years and lived in seclusion for several years. Part of this time was spent in a small mining town in the Colorado Rocky Mountains. In 2004 Matthew enrolled at the University of Tennessee and graduated in 2007 with a B.S. degree in Chemistry. He then joined the University of Tennessee Chemistry Department as a graduate student under the guidance of Dr. Michael J. Sepaniak. In 2009 Matthew began working under Dr. Gary J. Van Berkel at Oak Ridge National Lab with the Organic and Biological Mass Spectrometry group. In August 2011 he will fulfill his obligations to the graduate school and receive his Ph. D. in Chemistry.