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The application of weak-anion exchange chromatography for the analysis of organic zwitterions
using LC/MS/MS

by

Michael Bishop

Under the Direction of Gabor Patonay

ABSTRACT

A rapid and accurate quantitative method was developed and validated for the analysis of four urinary organic acids with nitrogen containing functional groups, formiminoglutamic acid (FIGLU), pyroglutamic acid (PYRGLU), 5-hydroxyindoleacetic acid (5-HIAA), and 2-methylhippuric acid (2-METHIP) by liquid chromatography tandem mass spectrometry (LC/MS/MS). The chromatography was developed using a weak anion-exchange amino column that provided mixed-mode retention of the analytes. The elution gradient relied on changes in mobile phase pH over a concave gradient, without the use of counter-ions or concentrated salt buffers. A simple sample preparation was used, only requiring the dilution of urine prior to instrumental analysis. The method was validated based on linearity ($r^2 \geq 0.995$), accuracy (85–115%), precision (C.V. < 12%), sample preparation stability ($\leq 5\%$, 72h), and established patient ranges. The method was found to be both efficient and accurate for the analysis of urinary zwitterionic organic acids.

INDEX WORDS: Weak anion exchange, Formiminoglutamic acid, Pyroglutamic acid, 5-Hydroxyindoleacetic acid, 2-Methylhippuric acid, LC/MS/MS, Organic acids

THE APPLICATION OF WEAK-ANION EXCHANGE CHROMATOGRAPHY FOR THE
ANALYSIS OF ORGANIC ZWITTERIONS USING LC/MS/MS

by

MICHAEL BISHOP

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2006

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Michael Jason Bishop
2006

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by

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List of abbreviations

AcN	Acetonitrile
MeOH	Methanol
FIGLU	Formiminoglutamic acid
PYRGLU	Pyroglutamic acid
5-HIAA	5-Hydroxyindole-3-acetic acid
2-METHIP	2-Methylhippuric acid
GABA	Gamma hydroxybutric acid
CNS	Central nervous system
MHA	Methylhippuric acid
GC	Gas chromatography
LC	Liquid chromatography
MS	Mass spectroscopy
EI	Electron impact
CI	Chemical Ionization
APCI	Atmospheric pressure chemical ionization
ESI	Electrospray ionization
MRM	Multiple reaction monitoring

Chapter 1: Introduction

1.1 Background

Metabolic disorders, and related disease, are quickly emerging as the most prevalent cause of preventable death in the United States [1]. These findings are not unique to the U.S., as many industrialized nations experience an ever-growing population afflicted with varying forms of metabolic disorder. This is a result of increased exposure to toxins coupled with poor diet and exercise, facilitated by current customs in developed countries [2,3]. A direct consequence of these factors is the prevalence of obesity, which has been related to many metabolic disorders [4]. Growing knowledge of these disorders, along with an increased use of testing in conjunction with technological advancement has lead to early detection and treatment of many metabolic diseases [5]. However, delayed detection and treatment has significant drawbacks that can alter the long-term health of patients with disease [6]. There is a need for rapid and accurate testing to facilitate early detection of metabolic disease. In an attempt to lower cost and improve availability, many tests for metabolic diseases have been developed that provide non-invasive sample collection that requires little or no supervision by a healthcare professional [5,7].

The measurement of urinary formiminoglutamic acid (FIGLU), L-pyroglutamate (PYRGLU), 5-Hydroxyindole-3-acetic acid (5-HIAA), and 2-methylhippurate (2-METHIP) has been reported in the assessment of specific metabolic disorders and toxicity. The quantification of FIGLU and PYRGLU has been related to the status of specific metabolic pathways. The functional state of folate metabolism and the evaluation of formiminotransferase deficiencies have been related to FIGLU excretion [8,9]. The measurement of PYRGLU has been used in the assessment of glycine insufficiencies and the diagnosis of 5-oxoprolinuria [10,11]. The major form of metabolized serotonin, 5-HIAA has been reported as a marker for the content and

turnover of gastrointestinal serotonin [12,13]. It has also been related to carcoid syndrome as well as a number of neurological disorders [14-17]. The measurement of urinary 2-METHIP has been associated with a specific form of toxic exposure, arising from contact with substances containing xylene and toluene [18-20].

1.1.1 Biological Significance of FIGLU

Urinary excretion of FIGLU has been related to various disease states associated with histidine, folate, and vitamin B₁₂ metabolism [8,9,21,22]. These diseases states are all related to the biological pathway required to fully metabolize histidine [9]. This metabolic pathway is a series of enzyme-catalyzed reactions that require the presence of both folate and vitamin B₁₂ as cofactors. The enzymatic pathway is composed of five different enzymes functioning in series to fully degrade histidine into constituent by-products that include glycine and NH₄⁺ (Fig. 1.1). The products formed by each enzyme are substrates for the next enzyme forming a sequential cascade that is controlled by the rate of formation of each enzyme in sequence. The second to last enzyme in this cascade, glutamate formiminotransferase, requires the coenzyme tetrahydrofolate for the degradation of FIGLU into glutamic acid and 5-formimino-tetrahydrofolate. This enzymatic reaction is responsible for the transfer of the formimino-functional group to the substrate tetrahydrofolate to form 5-formiminotetrahydrofolate. At this point in the pathway, any interruption of enzymatic activity either from the absence of tetrahydrofolate or by improper functioning of the enzyme would result in the build-up of FIGLU [9]. Over abundance of FIGLU stores within the body then presents in elevated levels of urinary excretion. The measured concentration of FIGLU in urine can then be used to identify diseases and disorders that are related to the metabolic catabolism of histidine [9, 21, 22].

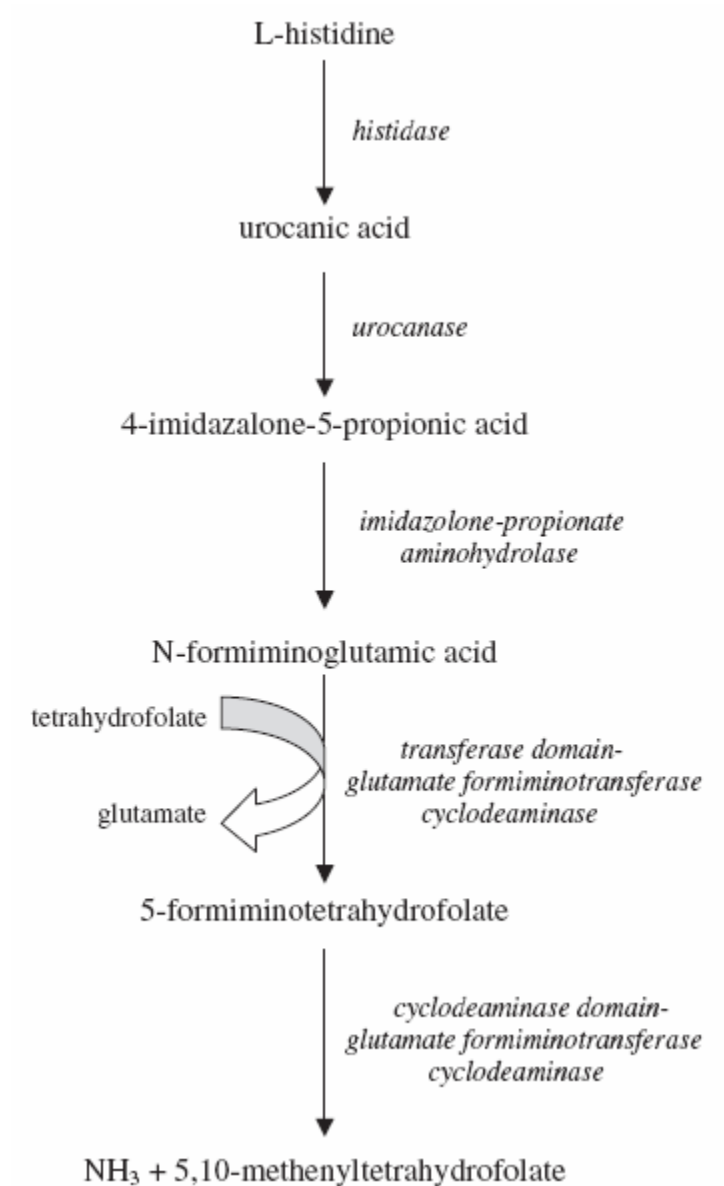


Figure 1.1: The metabolic pathway of histidine catabolism [9]

Folic acid is a vitamin that must be consumed in mammalian diets or from microorganisms in the intestinal tracts to ensure the proper function of various biological pathways, the most important of which leads to the formation of thymine [23]. However, folic acid is not biologically active and must first be converted to tetrahydrofolate, a key coenzyme for many important enzymatic reactions. Tetrahydrofolate transports activated methyl groups, or

one-carbon units to enzymes for the formation of numerous biological compounds. The one-carbon unit responsible for the coenzyme's activity is bonded to nitrogen atoms within the compound. This formation can exist in several oxidation states; the most reduced form carries a single methyl group, while the most oxidized forms carry a formyl, formimino, or methylenyl group [23]. The different oxidation states of tetrahydrofolate can be formed interchangeably, making the coenzyme a versatile element of various biosynthetic reactions. The presence of tetrahydrofolate is needed for enzymatic pathways that are responsible for producing essential components of normal mammalian biology. These essential compounds include methionine and thymine, important compounds needed for protein synthesis and DNA production [23]. The enzymatic reaction responsible for the degradation of FIGLU to 5-formimino-tetrahydrofolic acid and glutamic acid requires tetrahydrofolate as a coenzyme. The absence of tetrahydrofolate within the body then presents as an elevated level of urinary FIGLU, possibly indicating a folate deficiency [24].

Vitamin B₁₂ is another biological compound that is essential for the normal function of mammalian biochemistry. And like folate, vitamin B₁₂ cannot be produced by most mammals and must be consumed. The deficiencies of vitamin B₁₂ can result in serious health effects related to liver and nerve cell function [23]. Previous examinations of vitamin B₁₂ deficiencies were related to an increase in the levels of urinary FIGLU [22]. The cause of this observation was not well understood at the time of their findings. Later studies, would relate vitamin B₁₂ deficiencies to the histidine catabolism cascade which normally results in the formation of glutamic acid [24]. Corresponding to these finding, it was observed that the absence of vitamin B₁₂ within the body results in a build up of methylated tetrahydrofolate mentioned previously. Methylated tetrahydrofolate is normally used in biological processes as a methyl donor. The

enzyme responsible for this reaction requires vitamin B₁₂ as a cofactor. Deficiencies in vitamin B₁₂ will not allow the proper conversion of methylated tetrahydrofolate back to tetrahydrofolate. This results in an increase of methylated tetrahydrofolate, depleting the stores of tetrahydrofolate. In the absence of tetrahydrofolate, histidine catabolism is restricted at the step of formimino- group transfer to tetrahydrofolate. This results in an increase of urinary FIGLU excretion [24].

1.1.2 Biological Significance of PYRGLU

Urinary pyroglutamic acid is another important biomarker which represents a measure of the physiological glycaemic index [10, 11, 25, 26, 27]. Glycine is a neutral amino acid that acts as an inhibitory neurotransmitter, similar in function to gamma hydroxybutyric acid (GABA). Unlike GABA, the distribution of glycine throughout the central nervous system is more localized and functions in more specific neurological signaling. Nearly half of all inhibitory synapses found in the human spinal cord rely on the inhibitory effects of glycine [28]. Glycine is synthesized from serine by an enzymatic pathway that utilizes serine hydroxymethyltransferase, a mitochondrial iso-form, and requires tetrahydrofolate as a cofactor. As mentioned above, tetrahydrofolate is also the cofactor required for the catabolism of histidine that results in the degradation of FIGLU. Following the conversion of serine to glycine, vesicular inhibitory amino acid transporters load glycine into neuronal vesicles for future release into synapses [28]. The process of vesicle loading is similar for all inhibitory amino acids, including GABA. The effects of glycine in the synaptic cleft are modulated by the action of glycine transporters, within the plasma membrane of the presynaptic cell, which act to rapidly remove the amino acid from the synaptic junction. A related disease state which involves glycine results from the malformation

of transport enzymes as a result of genetic abnormalities. The resulting accumulation within the synaptic junction can cause lethargy, seizures, and mental retardation [28].

Often considered to be a non-essential amino acid, the nutritional importance of glycine has often been overlooked [10, 25, 27]. As a non-essential amino acid, current research suggests that the body is capable of producing enough of the compound for proper biochemical functioning. However, as research continues to explore the biological availability of such compounds, compromising situations have been observed that may cause normal metabolic systems to fail [10, 27]. Some metabolic disturbances can result in the depletion of ample supplies of endogenous biological compounds. Under these circumstances, non-essential components of metabolism may become too low, requiring supplementation from outside sources to maintain proper bodily functions [26]. Glycine functions as a coenzyme in a metabolic pathway responsible for the production of glutathione (Fig. 1.2). Glycine insufficiency disrupts this pathway which causes the formation of PYRGLU. The measurement

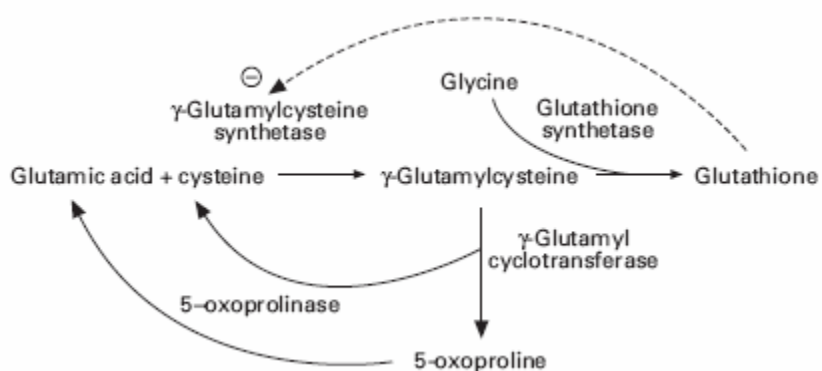


Figure 1.2: Illustration of glycine metabolism [27]

of urinary levels of PYRGLU provides a clinical means of determining glycine stores within the body [25, 26, 27]. Therefore the analytical assessment of urinary PYRGLU is essential for determining the need for exogenous supplementation to restore natural metabolic functioning.

1.1.3 Biological Significance of 5-HIAA

Proper neurological function requires specific components of intercellular neuronal signaling. The major compounds responsible for the signaling between neuronal cells are neurotransmitters. Serotonin is considerably one of the most important neurotransmitters utilized within the mammalian brain. Serotonin is produced within the body from the essential amino acid tryptophan by the action of bacterium within the gut and through cellular anabolism within the central nervous system [28]. Given the extent of serotonin production within these cells, an adequate metabolic pathway is required to degrade the compound in order to maintain the appropriate concentrations within the cell. Serotonin is mainly metabolized by the action of the enzyme, monoamine oxidase-A. As seen in Fig. 1.3, one of the most prevalent metabolites formed in the degradation of serotonin is 5-hydroxyindole-3-acetic acid [12, 29]. Metabolized serotonin, in the form of 5-HIAA, is then excreted in urine. The measurement of urinary 5-HIAA can therefore be used as an accurate approximation for serotonin content within the body [12, 13]. As mentioned, serotonin functions in neurological cell signaling, for which the cellular content must be adequately maintained. Therefore, the assessment of serotonin content within the body, through the measurement of urinary 5-HIAA is a helpful tool in the diagnosis of related neurological disease [14, 15, 16]. Other diseases, such as carcinoid syndrome, are related to abnormal serotonin production within the body [17]. Carcinoid syndrome is a result of the formation of carcinoid tumors, which are tumors of hormonal cells found throughout the body.

These hormonal cells, which have the potential to develop into cancerous tumors, respond to the release of neurotransmitters from adjacent neuronal cells. Upon stimulation, these hormonal cells release various hormones into the body as a part of an intricate signaling cascade that is important for a number of cellular functions. Generally, the hormones released differ widely

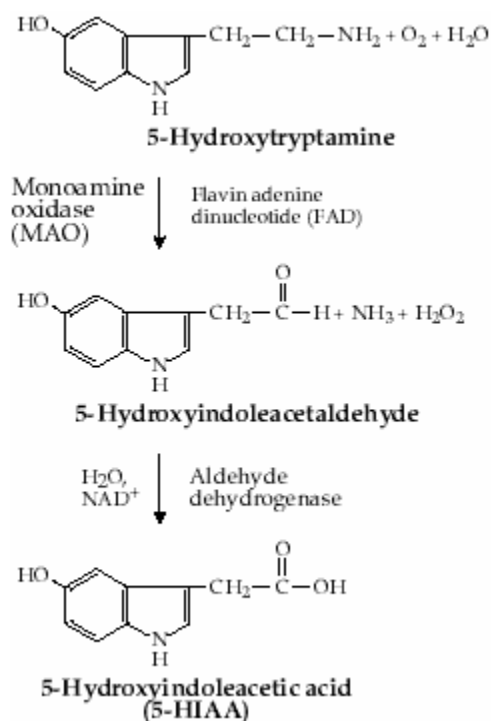


Figure 1.3: Serotonin (5-HT) metabolism into 5-HIAA [29]

depending upon the location and function of these cells within the body. An overgrowth of these cells can cause tumor formation and abnormal excretion of hormones. The associated syndrome is observed in nearly 10% of all carcinoid tumors patients. As a result of increasing hormone

levels, including serotonin, carcinoid syndrome can be characterized by the elevation of urinary 5-HIAA [17]. Other dysfunctions and diseases are thought to be related to the serotonin content within the body as well. These conditions include neurological abnormalities such as schizophrenia, depression, migraine, and autism [14]. Although further research is needed to confirm the role of serotonin in these conditions, the development of a non-invasive method to determine serotonin levels will certainly aid in these discoveries. Therefore, the measurement of urinary 5-HIAA in the form of a non-invasive and efficient analytical method is certainly needed to extend research in this area.

1.1.4 Biological Significance of 2-METHIP

Given the extent of increased industrialization around the world, the amount of environmental toxins produced is on the rise. These pollutants can often have dramatic effects on both the surrounding populous and environment [30]. The toxins produced by various manufacturing processes can be detrimental resulting in numerous adverse effects. The greatest toxic exposure is often observed within the factories and plants themselves. As workers consistently come in contact with contaminated environments during the course of a workweek, the levels of toxins within their own bodies rise [19]. The result of individual exposure to these toxins can vary from a mild to severe reaction. The symptoms of such exposure are not always obvious, and accurate measurement of the levels of these toxins within the body is essential to the proper treatment. The need for efficient testing to confirm or disprove toxin exposure can be great.

Xylene is one such compound that is commonly found in both the industrialized environment and within industries themselves [19, 30]. Xylene is used for a number of industrial

applications that include the manufacturing of paints, inks, dyes, adhesives, pharmaceuticals, and detergents [18, 19, 20]. Xylene is used as an organic solvent in the industrial process involved in the production of these products, and xylene can also be found within the products themselves. Xylene exists in three different forms, which are all structural isomers of a substituted aromatic ring. The isomers are denoted based upon the relative position of methyl groups on the substituted aromatic ring; these isomers are identified as o-, p-, and m-xylenes. Of the three isomers, m-xylene is considered to be the most abundant in the natural and industrial environment, while o-xylene is thought to be the least [31]. However, the abundance of these isomers is largely dependent upon the industrial application of the compound.

The chemical properties of xylene, as a non-polar, low-molecular weight aromatic compound, are what make it useful as an organic solvent. These characteristics allow for various organic molecules to be dissolved within the solvent, which aid in a number of areas within the manufacturing process. Xylene is also found in many petroleum-based fuels as an additive to help preserve machinery and moving parts. As a result, xylene is released into the surrounding environment by the emissions of these machines, such as automobiles, jet planes, and other vehicles that utilize petroleum fuels [30]. Given the ubiquitous use of xylene, it is not uncommon to observe toxic levels within areas where industry and mechanized transportation are prevalent. Large metropolises common to industrialized nations are examples of such areas. Large cities are also characterized by a large mobile population, which increases the risk of toxic exposures. The liver metabolizes xylene once it enters into the body following contact or exposure [32]. Xylene is metabolized into 2-, 3-, and 4-methylhippuric acids (MHA), each corresponding to the structural isomer of xylene absorbed (Fig. 1.4).

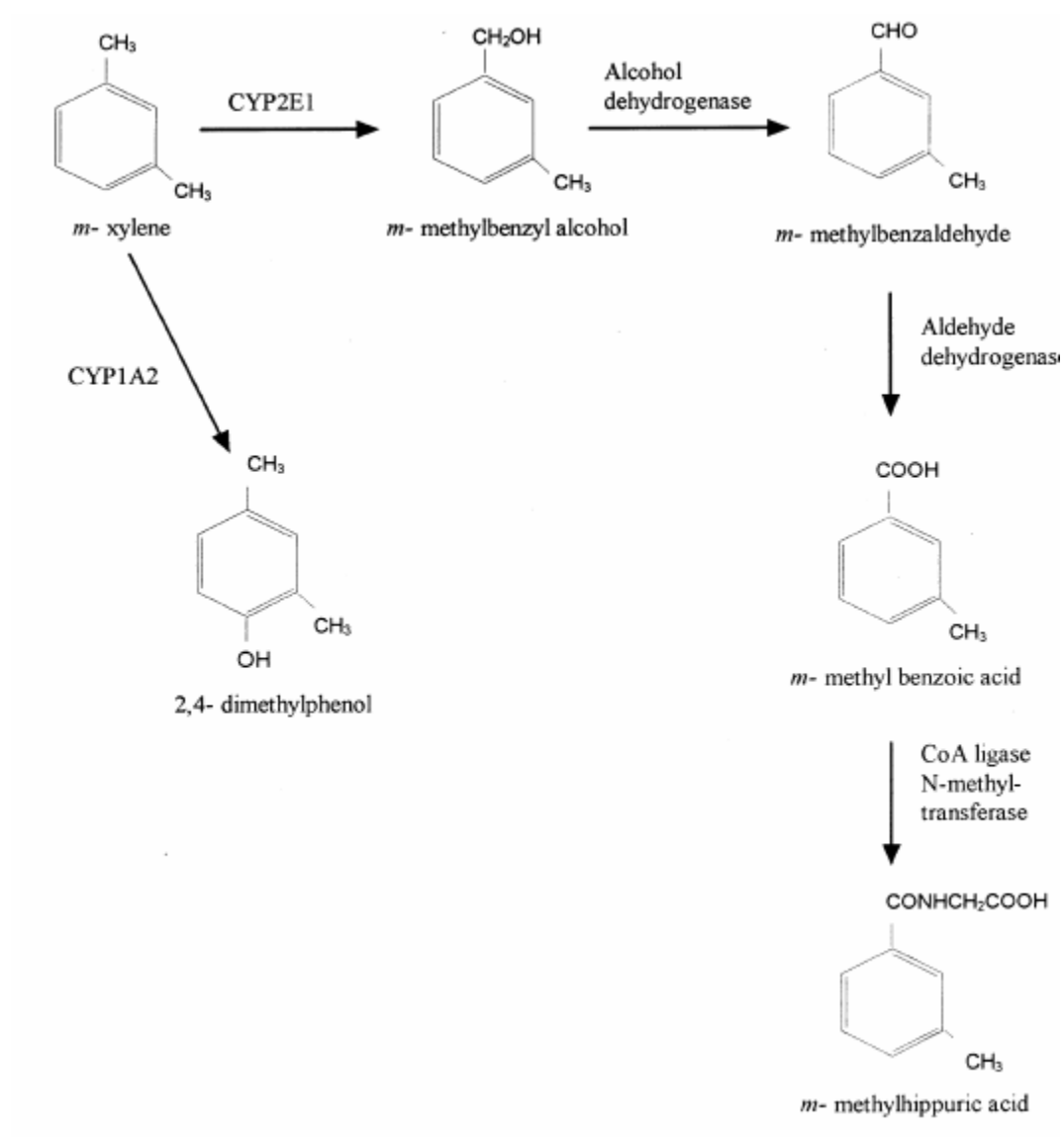


Figure 1.4: Illustration of *m*-xylene metabolism to *m*-methylhippuric acid [32]

Unlike benzene, which is chemically similar, xylene exposure has not been found to cause significant damage to the liver. However, the toxic effects of other substances acquired through exposure coincidentally with xylene have been shown to increase toxicity. The major negative biological effects of xylene exposure are on the central nervous system (CNS), although other biological systems can be affected including pulmonary, cardiovascular, and gastrointestinal organs [31]. Xylene effects on the CNS are significant because they can occur as a result of both short and long term exposure. The CNS symptoms that accompany xylene exposure vary based upon the amount of exposure. Low to moderate exposure can result in nausea, headache, dizziness, and vomiting. Higher exposures result in more severe symptoms such as lack of muscle coordination, confusion, and loss of balance. The most extreme cases of xylene exposure can result in the loss of consciousness. Given the wide array of symptoms that can be attributed to xylene exposure, an efficient method to detect the level of exposure is needed. The measurement of urinary levels of MHAs provides an efficient and non-invasive means to evaluate xylene toxicity and exposure.

1.2 Measurement of Biomarkers: LC/MS/MS, systems, and methodology

The most common analytical technique used for the evaluation of low molecular weight biological organic acids has been gas chromatography/mass spectrometry (GC/MS) [33]. However, in recent years the introduction of liquid chromatography/tandem mass spectrometry (LC/MS/MS) has allowed for more rapid analytical techniques to be developed [34]. The decrease in time needed for analysis is attributed to the high degree of selectivity offered by LC/MS/MS instrumentation. However, many published reports for the rapid analysis of small compounds are flawed by improper use of the mass spectrometer or by their lack of adequate

chromatography [35]. In some cases, the selectivity of small molecules is not sufficient enough to offer total accuracy in the absence of chromatographic separation [36]. The chromatographic separation developed must provide enough retention to separate compounds from interferences and avoid matrix effects seen by co-elution within the column void volume [35-37]. The observed interferences and matrix effects are directly related to the interface used to couple hyphenated analytical techniques such as HPLC and MS analysis.

1.2.1 LC/MS/MS interfaces

The analysis of molecules by mass spectrometry generally requires that the compounds be introduced into the MS system in a gas phase ionized state. The principle of MS requires that the compounds of interest be ionized producing an overall charged state. This charged state is then related to the mass of the compound by a mass over charge ratio, or m/z [38]. This ratio of charge to mass is one of the single most important aspects of MS analysis. Chemical species are separated using MS according to the individual m/z for each compound. Traditionally, this process involves the use of magnetic fields generated by electromagnets to control the velocity of ions moving through a vacuum [38]. However, modern MS systems utilize quadrupole technology that can perform equally well yet requires much less space than previous systems. With this technology, charged compounds are selectively monitored or filtered using an alternating electronic signal that produces electromagnetic fields. These fields only allow for specific charged masses to pass while all other ions are removed from the stable ion path [39]. This technology has allowed for bench-top models of MS detectors to be made readily available to consumers commercially. However, interfacing to these detectors has been a challenge for many years. Initially MS systems were coupled to GC systems that allowed for the introduction

of gas phase molecules into the MS. The molecules upon entering the MS are charged using a number of chemical mechanisms, the most common being electron impact (EI) [39]. The process of EI ionization produces a number of charged fragments that can be used to accurately identify chemical compounds. The principle problem with GC/MS techniques is that it requires the compounds of interest to be volatile and thermally stable. For non-volatile compounds, extensive sample preparation was required for derivatization forming volatile products. These preparations normally involve the use of strong chemical reagents that may cause chemical decomposition of the analyte and are not always effective for all compounds. Liquid chromatography had been used as an alternate method of separation for compounds not compatible with GC. However, limited detection methods were available to interface with LC systems, the most common being absorption spectroscopy. Given that the LC separations relied on liquid phases, the prospect of producing volatile charged chemical species was not obvious. However, research into the area of interfacing LC systems to MS detectors was soon explored [40].

One early system used to interface LC with MS instrumentation was the moving belt interface. This interface consisted of a Kapton ribbon on which liquid sample was deposited. The belt moved along different areas of vacuum with decreasing pressure approaching the MS analyzer aperture. Along with decreasing pressure the temperature varied as well, increasing inversely with pressure. Near the aperture for the MS analyzer the liquid volume is almost entirely volatilized with mobile phase and analytes making up the gaseous phase. The gas phase is then charged by either EI or chemical ionization (CI). The entire process results in the production of charged gas phase molecules that are drawn into the MS for analysis. The belt is then cleaned and returned to the initial point where the liquid was first placed on the belt. This

technique was one of the first attempts to interface LC with MS detection, however the entire process was very inefficient.

More efficient methods for delivering LC sample analytes to MS detectors began with the technique of direct liquid interface. This technique utilized a small opening within a diaphragm that partitioned between two areas of pressure to achieve a jet of liquid. The entire process took place in a reduced pressure chamber that allowed for the formation of tiny droplets of the liquid phase. The chamber is connected to a CI source that caused the formation of charged molecules. Solvent and solvent additives are commonly used with this technique to help in the desolvation process required to form gaseous ions. This process was limited by a maximum allowable flow rate of 100 $\mu\text{L}/\text{min}$ and by the frequent clogging of the 4- μm -I.D. diaphragms used in this interface technique. Although this process represents a more efficient means of ionization than the previous method of the moving-belt interface, more efficient means of volatilization would be developed.

Thermospray ionization was developed as the next improvement in LC/MS interface technology. The thermospray interface creates gaseous charged molecules from the solvent phase by heating the liquid as it passes into an area of low pressure. The solvent molecules are volatilized by the increasing temperature and the low area of pressure and are directed into the MS analyzer by a deflector plate. Ionization of gaseous molecules can be achieved by CI or promoted through the use of particular mobile phases and mobile phase additives. The entire process is more efficient than both the moving-belt and direct liquid introduction interface. Thermospray was at one point the most common LC/MS interface used commercially, however more sensitive techniques have all but replaced these interfaces.

Yet another type of LC/MS interface that is more commonly used with magnetic sector instruments is continuous-flow or dynamic fast-atom bombardment (CF-FAB) interface. This interface functions by eluting the liquid phase through a fused silica capillary onto a stainless-steel frit or gold-plated FAB target where an equilibrium between a uniform liquid film and gaseous formation is achieved. The film is then bombarded with fast atoms or ions generating ionic species within the gas phase. These ions are drawn into the MS for analysis. Given the relative simplicity of this technique, especially with reference to magnetic sector analysis, the interface continues to be widely used.

The above-mentioned interfaces for LC/MS systems illustrate the variety of methods available for the ionization of chemical compounds from liquid mediums. Some techniques have

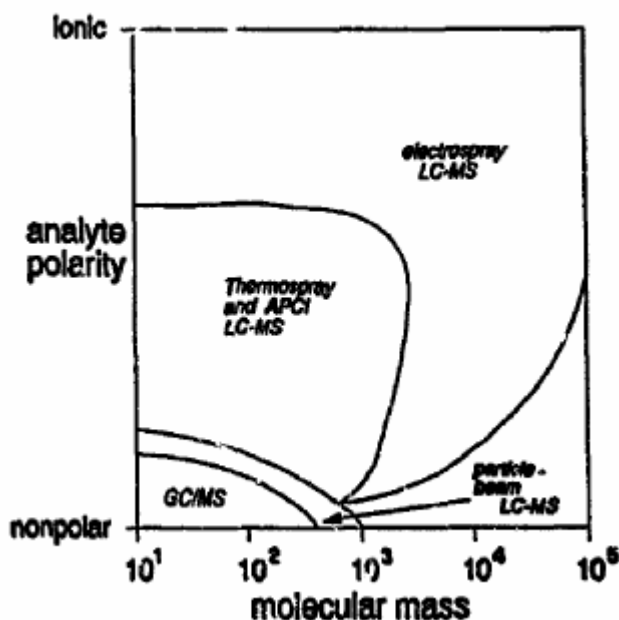


Figure 1.5: Representation of the most suitable analytical instrument for the measurement of specific compounds based upon analyte polarity and molecular mass [41]

proven useful for many years and will continue in to do so in the future, while others have served more as a step in the right direction for newer techniques. All LC/MS interfaces have both positive and negative aspects given a unique set of conditions that must be followed for the best operation (Fig. 1.5). Currently the most common interface between LC and MS that provides the greatest overall performance are atmospheric ionization techniques that include APCI and ESI [41]. These techniques allow for greater sensitivity while also providing a wide range of operating perimeters that are essential for robust method development using LC techniques.

1.2.2 Atmospheric pressure ionization: APcI and ESI

Atmospheric pressure ionization is a technique used as an interface in LC/MS where the liquid sample volume is volatilized into a chamber at pressures near atmospheric pressure. These systems normally function by removing the liquid solvent by means of adjusting temperature and pressure. In most cases the sample enters a capillary which reduces the liquid volume before it is volatilized as a spray consisting of mobile phase and the surrounding desolvation gas. The droplets of liquid that form out of the spray continue to disassociate into smaller and smaller droplets. The first interface into the MS analyzer usually positioned near the forming spray draws in the droplets by potential charge and vacuum. Passing through the first interface into the MS analyzer, the small cluster of particles continue through areas of decreasing pressure and in some cases mass filters, commonly referred to as a hexapole. This process further desolvates the droplets and filters particles that did not fully ionize or volatilize properly. The resulting ions then pass through the quadrupole mass analyzer. This is a brief description of how an atmospheric pressure interface accomplishes the task of introducing liquid components into the MS system in a charged gaseous state (Fig. 1.6).

Another form of atmospheric ionization, atmospheric chemical ionization (APCI) is very similar to the interface just described however the ionization process is aided by the introduction of energy through the use of a corona pin. The corona pin is a metallic pin that is connected to a potential in the interface. The pin comes out of the interface to form a small tapered point that is positioned in front of the MS aperture below the API probe. As the chamber is filled with

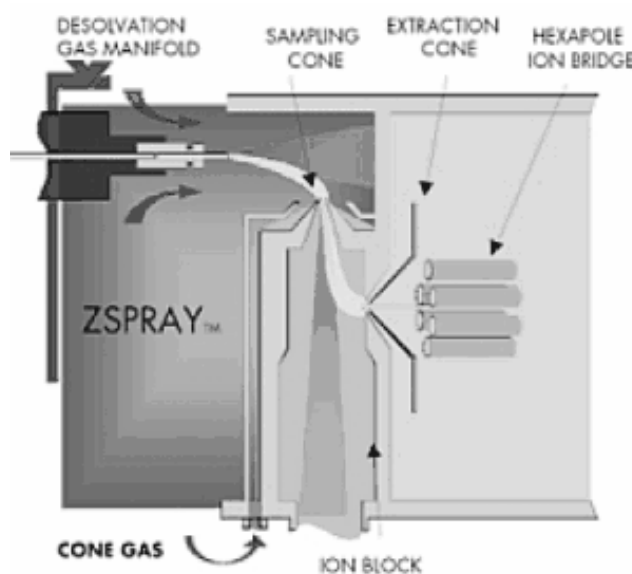


Figure 1.6: Illustration of API interface for a Waters QuattroMicro, (Waters corp.)

nitrogen or any other inert gas the molecules begin to react with high voltage supplied by the corona pin to form charged radical chemical species. The same effect results in the charging of both solvent and analyte particles as they form out of API probe. The resulting gas phase environment is then composed of a number of charged particles. This process allows for charge transfer between gaseous molecules and analyte molecules that can be very beneficial in cases where the analytes are neutral in solution (Fig. 1.7). This process makes APCI a very useful technique for the analysis of neutral compounds by LC/MS [42].

Electrospray ionization is another API technique, however, ESI is better suited for analysis of compounds that are ionic in solution [42, 43]. The ESI interface consists of a stainless steel capillary that carries an electric potential which sprays liquid medium into the interface. The charge carried by the capillary is transferred to the mobile phase liquid which upon elution from the tip creates what is known as a Taylor cone [43]. The Taylor cone is a charged aqueous formation where the electric potential is distributed along the surface of the droplet. The potential in effect forces charged species within the mobile phase to the outside of the droplet. The potential in effect forces charged species within the mobile phase to the outside of the droplet.

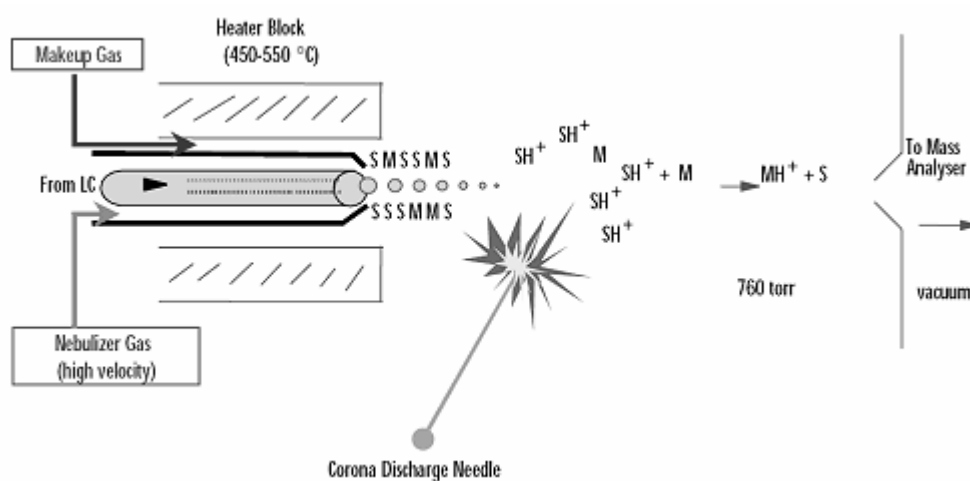


Figure 1.7: Illustration of charge formation produced by an APcI probe source, (Waters corp.)

As the charges build, coulombic interactions increase until the surface tension of the droplet can no longer contain the droplet. Budding droplets result, and as more and more droplets form the dry heated gas in the interface chamber causes evaporation of the liquid phase. As this process continues, charged compounds enter the gas phase. The MS aperture is positioned near the probe tip and also carries a potential charge which draws ions into the analyzer (Fig. 1.8).

ESI interfaces are well suited for the ionization of charged chemical species within solution, however due to the nature of the Taylor cone formation it is possible to have a number of interferences [44]. Overall ESI represents one of the most common ionization techniques used today for LC/MS systems [45].

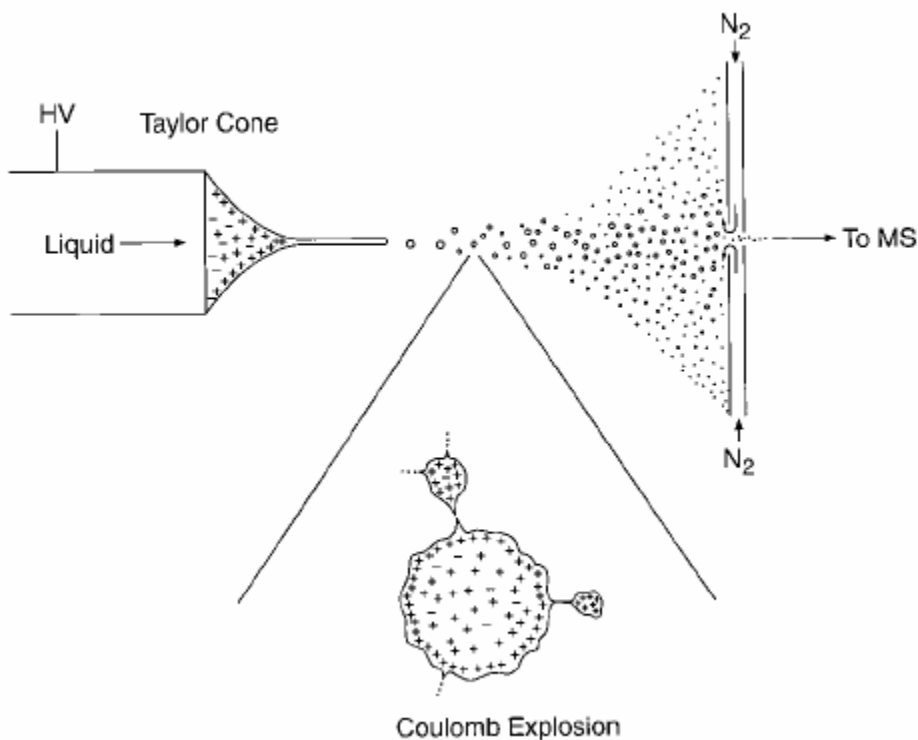


Figure 1.8: Illustration of Taylor cone formation by ESI [45]

1.2.3 LC/MS and LC/MS/MS applications:

Given that biological compounds are generally hydrophilic in nature, a number of HPLC techniques have been developed for biological analysis. However, with the advent of LC/MS systems, with various interfaces allowing for a number of applications, the analysis of biological

samples as been greatly improved. The term hyphenated technique is used to describe LC/MS systems because each instrument is capable of generating an analytical result, the combination of the two only helps to provide better understanding of the compounds being analyzed. The relative scope of applications that are made possible by LC/MS technology is seemingly limitless.

The more common biological applications of LC/MS systems have been in the area of protein identification and characterization [45]. The utility of MS analysis is that it can provide very specific chemical information based upon mass and fragmentation patterns. The process of analyzing proteins has shown to be useful in determining the make-up of amino acids and in the conformational structure based upon ionization [46, 47]. This process allows for accurate identification of a number of proteins and has been extremely useful for proteomic research.

LC/MS systems have also been widely used in the area of environmental chemistry [48, 49]. The evaluation of environmental samples for the existence of various contaminants is very useful for the preservation of the surrounding biological environments. The most common analytical techniques used in the evaluation environmental techniques have been HPLC and ICP-MS. Using current LC/MS technology, a number of new evaluations for these samples are available that provide great accuracy and efficient over older techniques. LC/MS systems have been used to determine impurities in waste waters and soil samples, as well as industrial processes to determine the level of waste products generated. LC/MS will likely continue to improve the analytical evaluations in this area in the future.

Clinical analysis, similar to biological analysis only more specialized in diagnostics, has also been helped greatly by the advent of LC/MS systems [50, 51]. A number of analytical techniques have been used in the evaluation of clinical samples including HPLC, GC/MS, and

ICP-MS. LC/MS and LC/MS/MS systems are greatly improving the way clinical samples are evaluated analytically by increasing through-put while maintaining, if not improving, accuracy and precision. Given that chromatography has the ability to separate any number of analytes effectively, the ability to selectively detect chemical species only adds to the capability of analytical analysis. The LC/MS systems allow for less sample preparation as a result, while also maintaining an acceptable level of accuracy. Runtimes and efficiency of sample analysis are also shorter owing to the fact that overlapping chromatographic peaks can now be spectrally identified and measured. These positive aspects of LC/MS and LC/MS/MS instrumentation have lead to the development of a number of clinical techniques that have greatly improved the analysis of relevant biomarkers and important biological compounds.

LC/MS systems have greatly improved the world of analytical chemistry by providing a number of new possibilities for the analysis of chemical compounds. The progress in this field is seemingly end-less although the cost of these instruments has certainly been a set back for research into this area. However, given the current progression of technology these instruments are becoming not only more accurate but also more affordable. In the future it is feasible that LC/MS and LC/MS/MS systems will be common place among all analytical laboratories, one day becoming a standard analytical technique.

1.3 Validating an analytical technique [52, 53]

All analytical methods should under go a stringent process of validation to ensure that measured results are always reliable. The process of validation involves the evaluation of well planned experiments proving that the technique is adequate, accurate, and precise. These protocols are prevalent in any analytical laboratory where the results maybe used for

environmental or diagnostic purposes. Currently there are no set specific guidelines for the validation of an analytical technique although certain agencies such as the Food and Drug Administration (FDA) or Environmental Protection Agency (EPA) require that certain evaluations be performed to determine the validity of the test or analytical results. Other accrediting establishments such as CLIA also require that certain criteria for testing be met in order to perform any analytical test for clinical use. Meeting these guidelines is normally concurrent with certification by certain state and local governments making it a requirement in order to legally report the analytical results. Therefore validating an analytical technique is of great importance if the developed technique is to be used effectively and for business purposes.

Validation procedures are not the same for every institution, although steps have been taken on an international level to help standardize the way everyone validates an analytical method. Certain evaluations are globally accepted as required for complete validation of an analytical technique:

- Linearity
- LOD and LOQ
- Evaluation of Matrix Effects
- Recovery or Accuracy experiments
- Precision
- Sample Stability

Each step is important in its own way in providing proof that the analytical method is viable and accurate. The steps listed above will be examined in more detail below.

Linearity is normally performed by preparing a set of calibration solutions of known concentration and measuring them using the specific analytical method. In the case of LC/MS systems, prepared standards should provide a linear response related to concentration. The matrix of the sample can have a dramatic effect on sensitivity when using LC/MS as an analytical technique. However, the use of internal standards is usually a suitable way of correcting for variations due to matrix. If these experiments produce results that are not consistent with what is expected then the analytical technique can not be validated.

The limit of detection (LOD) and limit of quantification (LOQ) are measurements of the accuracy of the calibration curve produced by the analytical method. There are several ways to calculate these values based upon the calibration curve itself. However, each calculation carries a certain degree of accuracy or inaccuracy. A generally accepted calculation of LOD and LOQ is given below:

$$\text{LOD} = \text{Std.Dev.}_{\text{slope}} \times 3 / \text{Slope}$$

$$\text{LOQ} = \text{Std.Dev.}_{\text{slope}} \times 10 / \text{Slope}$$

The importance of the measured LOD and LOQ is that it establishes a certain cut off for analytical measurement, ensuring that all reported values are accurate and precise to within 20 %RSD.

The evaluation of matrix effects is an important aspect of any analytical technique. LC/MS techniques are specifically susceptible to matrix effects that can lead to significant ion suppression and inaccuracies in reported results. There are a number of ways to assess matrix effects. A discussion of them all is not necessary; however, the evaluation of matrix effects is an

important part of method validation. One common evaluation is to measure the concentration of a standard in aqueous solution and then measured again in matrix. If the value changes significantly, more than 20%, then a large matrix effect exists in the assay for that compound. Large matrix effects must be rectified otherwise the analytical method does not meet the requirements of validation.

Experiments designed to assess the accuracy are obviously very important for the validation of any analytical technique. Spike recovery is an experiment routinely performed to determine the relative accuracy of an analytical technique. The process of establishing accuracy is usually carried out by spiking a standard of known concentration into a sample and measuring both a blank, and the spiked sample. If the analytical method accurately measures the correct spiked amount the method is then generally accepted. However, the number and concentration of the spike samples evaluated can vary. In general, two spikes of a concentration in the mid- and high-level of the calibration are used to establish accurate recovery. If the measured spike does not fall within 15% of the expected value the analytical method will not be considered for validation.

Precision is another important aspect of analytical method validation. The precision of an analysis relates to the ability to consistently report the sample measurement for a given sample or standard solution. Precision is normally assessed by evaluating a set of samples over an extended period of time, one week or possibly one month, while monitoring the measured value for precision. An acceptable value for a precise analytical method should vary no more than 20 %RSD from day-to-day, and no more than 10 %RSD within a single day. These requirements must be met for validation to be successful.

The stability of a sample for analytical measurement is also very important for any analytical method. If a certain methodology is established for the analytical measurement of a compound that is unstable then the time frame in which an accurate measurement can be made must be established. Methods for identifying sample stability vary. For clinical purposes the sample should only be stable long enough to provide an accurate measurement. This evaluation can take place by measuring a sample repeatedly over a period of time to determine sample stability. If during this time the measurements begin to vary greatly, more than 20 %RSD, then the noted time period is recorded as the time for which the sample is stable. If the sample is extremely unstable the analytical method may not be considered valid.

Chapter 2: Reversed phase separations of FIGLU, 2-METHIP, PYRGLU, and 5-HIAA

2.1 Introduction [39, 54]

Reversed phase chromatography is currently one of the most common techniques utilized in analytical separations by HPLC. The general mechanism of retention for reversed phase chromatography is based upon hydrophobic interactions. This category of chromatography generally consists of stationary phases composed of non-polar side chains of varying length and composition. The standard reversed phase analytical column is composed of alkyl carbon chains containing between eight and eighteen carbon atoms bound to a silica backbone by a silyl-ether linkage. However, more modern reversed phase columns contain various “polar” groups imbedded within the alkyl chain which add greater functionality and resilience to the column performance. Newer analytical columns are also composed of higher grade silica and are manufactured with greater precision that increases both durability and column life. The stability of the stationary phases was at one time very sensitive to certain chromatographic conditions which could cause folding or degradation leading to column failure. These issues have also been greatly improved by current advancements in analytical column development. Modern chromatographic techniques rely heavily on these advancements making use of reversed phase analytical columns for a wide range of separation methods.

There are disadvantages to the use of reversed phase analytical columns that are related to the mechanism of retention for these phases. Retention relies heavily on the transition of analytes into the non-polar or hydrophobic stationary phase. This retention is modulated to a large extent by the polarity of the mobile phase or combination of phases used in the separation method. These interactions greatly promote the retention of non-polar compounds. However,

the opposite is true for largely polar ionic or hydrophilic compounds. The lack of retention for hydrophilic compounds is a serious disadvantage for the use of reverse phase chromatography when target analytes are polar ionic. Or more commonly, when a group of compounds are being separated that vary greatly in ionic character. These issues have proved difficult for many chromatographers, who through experience have developed a number of solutions for this problem.

Optimization of reverse phase chromatography is essential for the analysis of multiple analytes that vary in chemical structure and related properties. Traditionally reversed phase chromatography requires the use of a non-polar stationary phase with mobile phases that contain both an aqueous and organic component. Evaluation of different stationary phases along with a number of mobile phase properties is essential for proper optimization of an analytical method. The aqueous phase can be buffered through a varying range of pH to promote greater retention of ionic compounds. The organic phase used for elution can also be varied to provide greater efficiency and sensitivity. Performing experiments that utilize these variables effectively can result in a suitable analytical method for the separation of a diverse mixture of analytes.

2.1.1 Instrumentation and materials

The chromatographic separations were performed on a Waters (Milford MA, USA) 2695 high-performance liquid chromatograph. Samples were analyzed on a Waters Quattro-micro tandem mass spectrometer equipped with an electrospray ionization source. All collected data was processed using MassLynx V4.0.

HPLC grade acetonitrile, methanol, and formic acid were purchased from VWR (VWR International, North America). Ammonium acetate, ammonium formate, and triethylamine were

obtained from Sigma (St. Louis, MO, USA). 1-Butanol and acetyl chloride were also purchased from Sigma (St. Louis MO, USA). The standards, 5-hydroxyindole-3-acetic acid and L-pyroglutamate were purchased from Sigma. 2-methylhippurate was purchased from Aldrich (St. Louis, MO, USA). Formiminoglutamic acid was obtained from PharmAgra Laboratories (Brevard, NC, USA). Internal standards, N-benzoylalanine (NBA) and 5-fluoroindole-3-acetic acid (5-FIAA) were obtained from Sigma. Glutamic acid, 2, 4, 4,-*d*3 (D3GLU), was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Separations were performed using two common analytical reversed phase columns composed of C-8 and C-18 stationary phases. A Waters (Milford, MA, USA) Sunfire column, 150 x 2.1 mm, 5 μ m, maintained at a constant temperature of 30 °C was used to evaluate analytical separations using a C-8 stationary phase. A Phenomenex (Torrance, CA, USA) Gemini column, 50 x 2.0 mm, 5 μ m maintained at a constant temperature of 30 °C was used to evaluate analytical separations using a C-18 stationary phase. Mobile phases used for each separation consisted of buffered aqueous (de-ionized H₂O) solutions containing ammonium formate or ammonium acetate adjusted with the corresponding acid suitable for the given pH evaluated. The organic phases used for each separation consisted of methanol or acetonitrile containing 0.02% of formic acid or acetic acid depending upon the aqueous buffer used. All chromatographic methods were evaluated at a constant flow rate of 0.300 mL/min.

All compounds were detected in electrospray positive ionization mode, with the desolvation gas set to 800 L/hr. To increase sensitivity the nebulizer gas was not used. Capillary voltage was maintained at 3.5 kV, with source and desolvation temperatures at 150 °C and 350 °C, respectively. Each mass transition was collected at unit mass resolution with a dwell time of 0.1 s. The cone and collision settings were established individually for each compound for

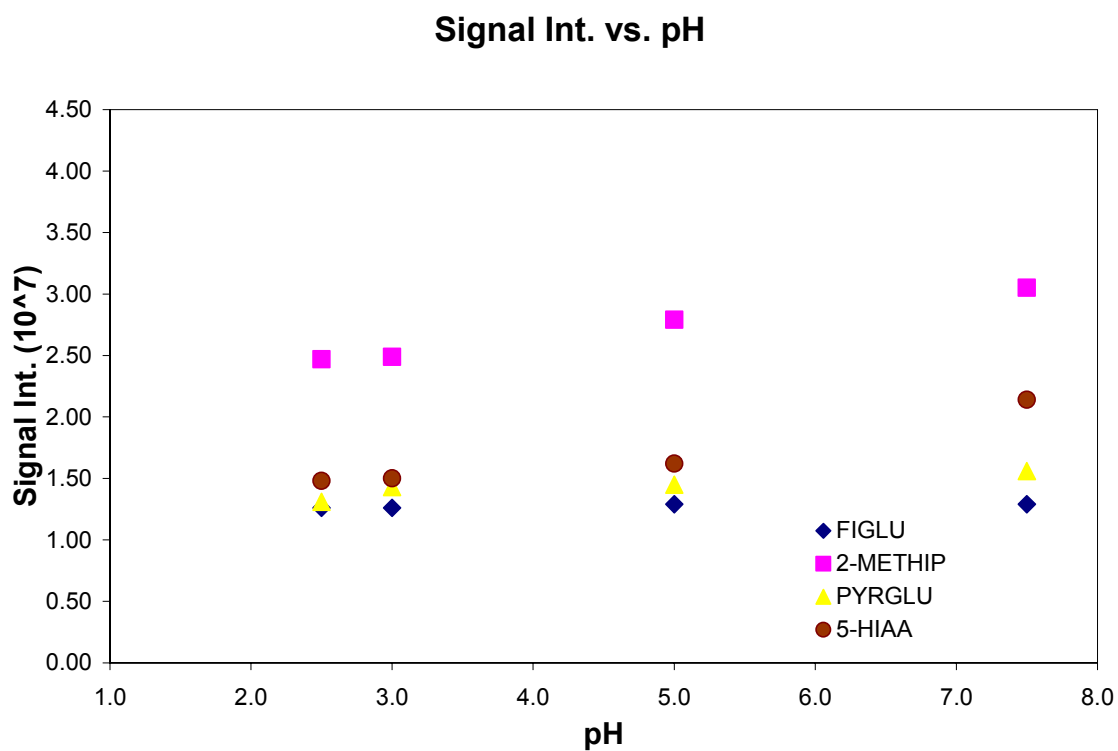
multiple reaction monitoring (MRM) detection. The conditions for detection of all analytes were obtained by direct infusion of a standard solution in line with the HPLC. Sensitivity for each analyte was evaluated at different pH values ranging from 2.5 to 7.5 using only aqueous buffer (Fig. 2.1). The MRM transitions and appropriate detection settings are presented in Table 2.1.

Standard stock solutions were prepared for each analyte as follows: Working stock solution A (StkA) was made by the addition of 0.024 g of FIGLU to a 100 mL volumetric flask and brought to volume with de-ionized water. Working stock solution B (StkB) was made by the addition of 0.019 g of 2-METHIP to a 100 mL volumetric flask and brought to volume with de-ionized water. Working stock solution C (StkC) was made by the addition of 0.013 g of PYRGLU to a 100 mL volumetric flask and brought to volume with de-ionized water. Working stock solution D (StkD) was made by the addition of 0.019 g of 5-HIAA to a 100 mL volumetric flask and brought to volume with de-ionized water. The internal standard solution was prepared by the addition of 0.001g of NBA, 0.001 g of D3GLU, and 0.002 g of FIAA to a 500 mL volumetric flask and brought to volume with de-ionized water.

The high level calibration solution was prepared by the addition of equal parts of StkA, StkB, StkC, and StkD to a 100 mL volumetric flask. Calibration standards were prepared by serial dilution of the high level calibration solution for the desired calibration range. Five calibration standards, including a blank, were prepared for each analyte as follows: FIGLU, 0.00, 0.242, 0.942, 3.77, 15.1, 60.4 mg/L; PYRGLU, 0.00, 0.129, 0.503, 2.01, 8.07, 32.3 mg/L; 2-METHIP, 0.00, 0.193, 0.753, 3.01, 12.1, 48.3 mg/L; 5-HIAA, 0.00, 0.191, 0.746, 2.98, 11.9, 47.8 mg/L.

Table 2.1: MRM transitions and detection settings for all analytes and internal standards

Analytes and I.S.	Parent ion [M+H]	Product ion	Cone potential (V)	Collision energy (eV)
FIGLU	175	82.9	20	20
PYRGLU	130	83.9	25	15
5-HIAA	192	146	20	15
2-METHIP	194	119	15	10
D3GLU	151	86.8	15	15
NBA	194.1	104.9	20	15
5-FIAA	194	148	20	15

**Figure 2.1:** Plot of signal intensity versus pH for FIGLU, 2-METHIP, PYRGLU, and 5-HIAA

2.1.2 Method validation

Each experimental reversed phase analytical method was evaluated based upon linearity, accuracy, and observed matrix effects. Linearity was evaluated using a five point calibration curve. Accuracy was established by measuring spike recoveries for all analytes in a pooled urine sample when spiked with mid-level and high-level calibrators. The urine samples were spiked with no more than 10% of initial urine volume and calculated based on the average of three successive measurements for each level.

The linearity of the calibration curve was evaluated by linear regression, including the intercept ($y=mx+b$), weighted by $1/x$. Linear curves were comprised of six calibration levels, run in duplicate and quantified from a standard curve to evaluate precision and accuracy. All calculations were performed using EP Evaluator 6 software, (RHOADS, Kennett Square, PA, USA). The LOD ($S/N = 3$) and LOQ ($S/N = 10$) were determined using the regression approach based upon the linear regression of calibration from the established linear range [58]. The sensitivity of the present method was determined from these measurements.

Accuracy was evaluated by spike recovery from pooled urine samples. This baseline urine level was spiked with two levels of calibrators (mid-level and high-level). Both spiked samples were prepared by the addition of 10% v/v of the specific calibrate to the baseline urine. Mid-level solutions were spiked using calibrator level 3 (FIGLU 3.77 mg/L, PYRGLU 2.01 mg/L, 2-METHIP 3.01 mg/L, 5-HIAA 2.98 mg/L). High-level spikes were spiked using calibrator level 5 (FIGLU 60.4 mg/L, PYRGLU 32.3 mg/L, 2-METHIP 48.3 mg/L, 5-HIAA 47.8 mg/L). The baseline samples were prepared in a similar way i.e. made with 10% de-ionized

water in the place of calibrate. The baseline, mid-level, and high-level samples were run in duplicate and quantified using a standard curve.

The effects of sample matrix on calibration were measured using a simple matrix matching experiment. A pooled urine sample was prepared using intra-laboratory samples. To prepare calibration standards in matrix, a volume of pooled urine (500 μL) was added to 16 x 100 mm glass tubes and blown to dryness under a steady stream of nitrogen in a water bath maintained at 50 °C. The dried urine was reconstituted in 500 μL of calibration solutions, absent of matrix. The matrix matched calibration solutions were measured using the same method described in this report and compared with measured calibration solutions at the corresponding calibration levels.

2.2 Traditional buffered reversed phase separations

Using the high level calibration standard, reversed phase chromatographic conditions were observed for both C-8 and C-18 stationary phases with a buffered aqueous mobile phase A (pH 2.5, 5.0, and 7.5) and organic mobile phase B (MeOH and AcN). Multiple gradients (Waters gradients #2 – 9, Figure 2.2) were used to develop a working chromatographic method from the above experimental conditions. Each method was evaluated based upon separation efficiency, analyte intensity, and peak shape. The analytical method with the best overall chromatographic properties was then considered for validation. Experimental samples used to develop working chromatographic conditions were prepared by the addition of 200 μL of high

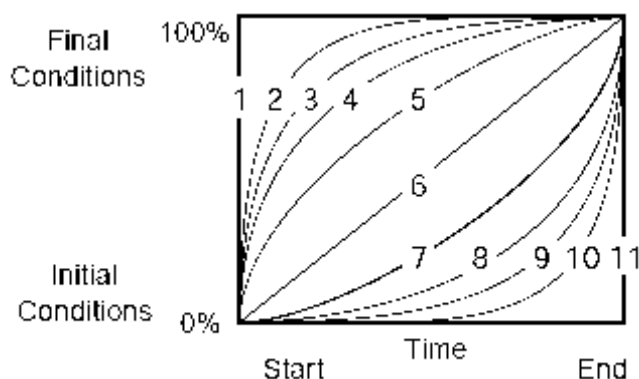


Figure 2.2: Waters gradient table illustrating number assignments for different pre-programmed elution profiles (Waters, corp.)

calibrator (level 5) to a vial containing 600 μL of the appropriate MPA. Experimental samples used for validation were prepared by the addition of 200 μL of sample to a vial containing 600 μL of internal standard solution. The internal standard solution was prepared using a solution containing mobile phase to match the initial on-column chromatographic conditions. Each chromatographic run was performed using a 25 μL sample injection. The experimental results are discussed below.

2.2.1 Buffered separations using a C-8 reversed phase column at a pH of 2.5, 5.0, and 7.5

The separation of all four analytes was observed using a C-8 column at a buffered pH of 2.5, 5.0, and 7.5. These conditions were tested by various gradient elution profiles with both AcN and MeOH individually evaluated as the organic phases. The use of AcN as the organic modifier at a buffered pH of 2.5 had no significant effect on the elution of FIGLU and PYRGLU for all gradient elution profiles used. The elution profile greatly affected the retention of 5-HIAA and 2-METHIP, with a linear gradient providing the best peak shape and intensity for both compounds at this pH (Fig. 2.3A). The use of AcN as the organic modifier at a buffered pH of

5.0 again had little effect on the elution of FIGLU and PYRGLU for nearly all gradient elution profiles used. PYRGLU was retained less at this pH and the peak shape was negatively affected. The retention of 5-HIAA at a pH of 5.0 was also unaffected by the use of different gradients, however a better overall peak shape was observed. At this pH, the retention of 2-METHIP increased slightly, and the peak shape only improved slightly (Fig. 2.3B). Increasing the pH provided no positive effects on the elution of FIGLU and PYRGLU for all elution profiles used. The retention of both compounds was greatly diminished, both eluting near the void. 5-HIAA was dramatically less retained at a pH of 7.5, with a decrease in retention time of almost 3 minutes. Different elution profiles had no effect on the retention of 5-HIAA, a different result compared with the previous experiments. The retention of 2-METHIP was also reduced at this pH, although not as greatly as the other compounds. The peak shape of 2-METHIP was improved when compared to previous experiments (Fig. 2.3C).

The use of MeOH as the organic modifier at a buffered pH of 2.5 had no significant effect on the elution of FIGLU and PYRGLU for all gradient elution profiles used. The elution profile greatly affected the retention of 5-HIAA and 2-METHIP, with each gradient providing nearly the same peak shape and intensity for both compounds at this pH (Fig. 2.4A). The use of MeOH as the organic modifier at a buffered pH of 5.0 again had little effect on the elution of FIGLU and PYRGLU for nearly all gradient elution profiles used. The retention of FIGLU increased at this pH and the peak shape was greatly improved while PYRGLU remained unchanged. The use of different gradient elution profiles again greatly influenced the elution of 5-HIAA and 2-METHIP with no significant observable change in peak shape or intensity (Fig. 2.4B). No positive effects on the elution of FIGLU and PYRGLU were observed at a buffered

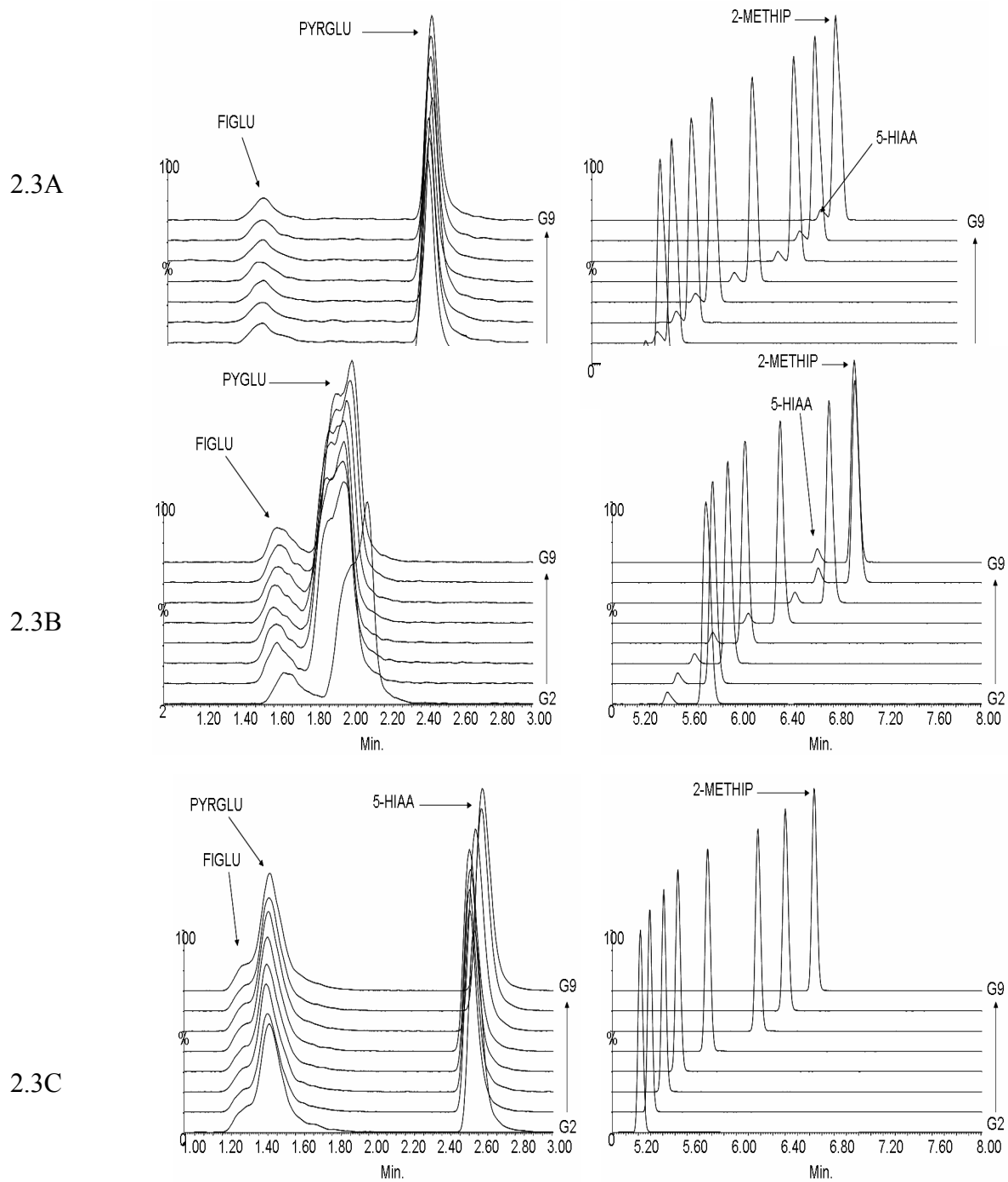


Figure 2.3: Chromatograms of all analytes eluted at mobile phase pH values of 2.5 (A), 5.0 (B), and 7.5 (C) using AcN as the organic modifier on a C-8 reversed phase column

pH of 7.5 for all elution profiles used. The retention of PYRGLU was slightly reduced while the overall peak shape of FIGLU diminished. Interestingly, increasing pH had little effect on the retention of both 5-HIAA and 2-METHIP compared with other pH experiments using MeOH as the organic modifier. In a similar observation, increasing pH had negligible effects on the overall peak shape of both compounds (Fig. 2.4C).

The experimental variation of both pH and organic modifier produced significant observable changes in the chromatographic profile for all four analytes. Specifically, the effects of pH were dependant upon the organic modifier used. Increasing pH greatly affected the retention properties of FIGLU, PYRGLU, and 5-HIAA when using AcN as the organic phase. The affects of increasing pH on the retention for all compounds using MeOH as the organic modifier was far less dramatic by comparison. Also, the use of MeOH for elution greatly increased both retention and peak shape for all compounds as apposed to the use of AcN. Therefore the optimized conditions taken from the above experiments clearly involve the use of MeOH for elution. Based upon the experimentally observed chromatographic properties of each elution profile at varying pH, the Waters gradient #2 at a buffered pH of 5.0 provided the best overall peak shape, retention, and intensity. Therefore these chromatographic conditions were considered to be optimum for the separation of all compounds when using a C-8 reversed phase column.

2.2.2 Buffered separations using a C-18 reversed phase column at a pH of 2.5, 5.0, and 7.5

The separation of all four analytes was observed using a C-18 column at a buffered pH of 2.5, 5.0, and 7.5. These conditions were tested by various gradient elution profiles with both AcN and MeOH individually evaluated as the organic phases. A shorter (50 mm in length)

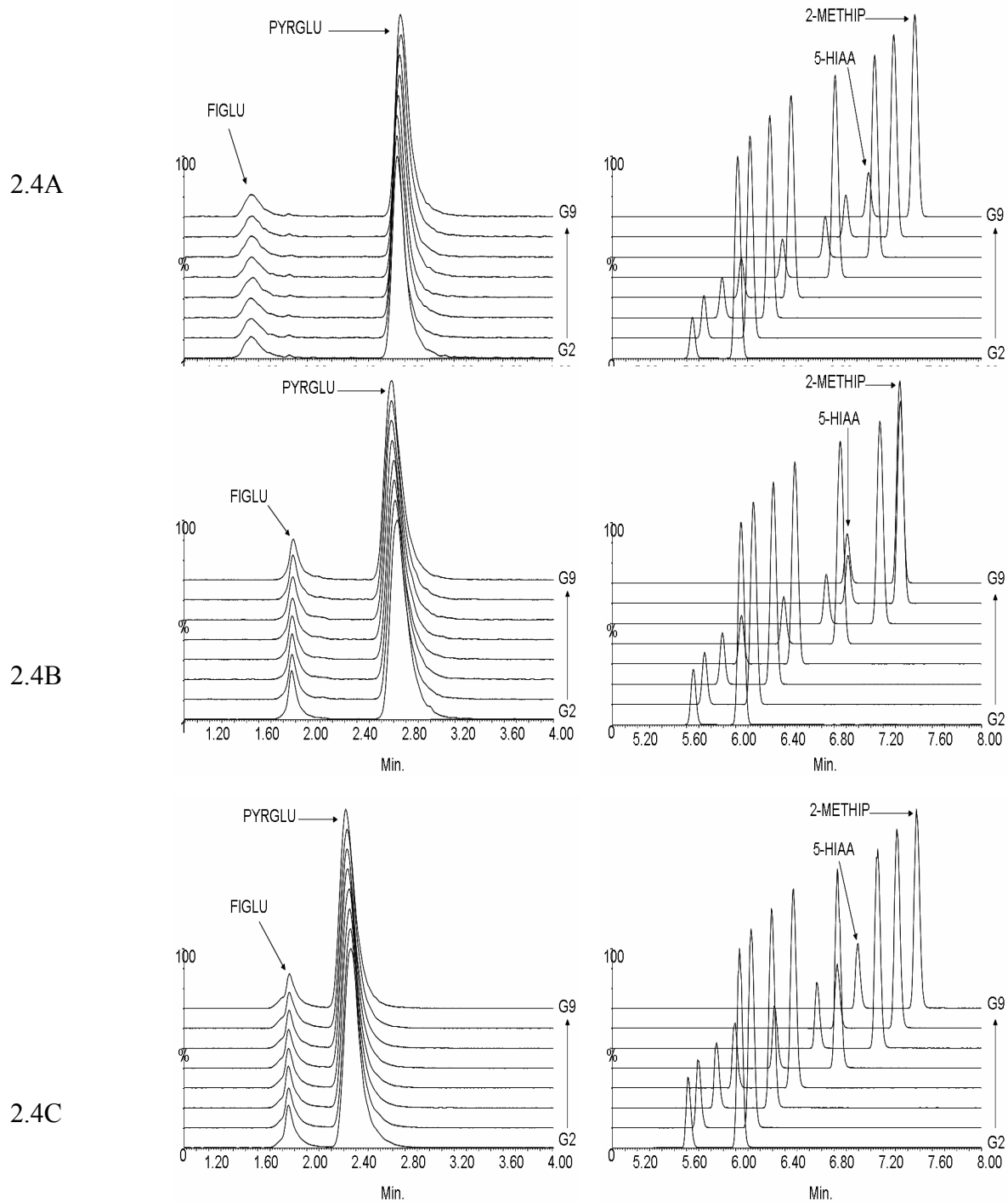


Figure 2.4: Chromatograms of all analytes eluted at mobile phase pH values of 2.5 (A), 5.0 (B), and 7.5 (C) using MeOH as the organic modifier on a C-8 reversed phase column

analytical C-18 column was used for these experiments to minimize the back pressure on instrument. The use of AcN as the organic modifier at a buffered pH of 2.5 had little effect on the elution of FIGLU and PYRGLU for all gradient elution profiles used. The elution profile greatly affected the retention of 5-HIAA and 2-METHIP, although similar peak shape and intensity was observed for both compounds at this pH (Fig. 2.5A). The use of AcN as the organic modifier at a buffered pH of 5.0 again had little effect on the elution of FIGLU and PYRGLU for nearly all gradient elution profiles used. PYRGLU was retained much less, eluting near the void, at this pH and the peak shape was negatively affected. 5-HIAA at a pH of 5.0 was greatly affected by the use of different gradients, resulting in a drastic decrease in peak shape and intensity, worsening from Waters Gradient #2 to #9. At this pH, the retention of 2-METHIP decreased slightly, and the peak shape also diminished from Waters Gradient #2 to #9 (Fig. 2.5B). Increasing the pH provided no positive effects on the elution of FIGLU and PYRGLU for all elution profiles used. The retention of both compounds was greatly diminished, both eluting near the void. 5-HIAA was dramatically less retained at a pH of 7.5, with a large decrease in retention time. The effect of different elution profiles on the retention and peak shape of 5-HIAA was not seen, a different result compared with the previous experiments. The retention of 2-METHIP was also reduced at this pH, although not as greatly as the other compounds. The peak shape of 2-METHIP was extremely diminished when compared to previous experiments, again worsening as the gradient profile differed from Waters Gradient #2 to #9 (Fig. 2.5C).

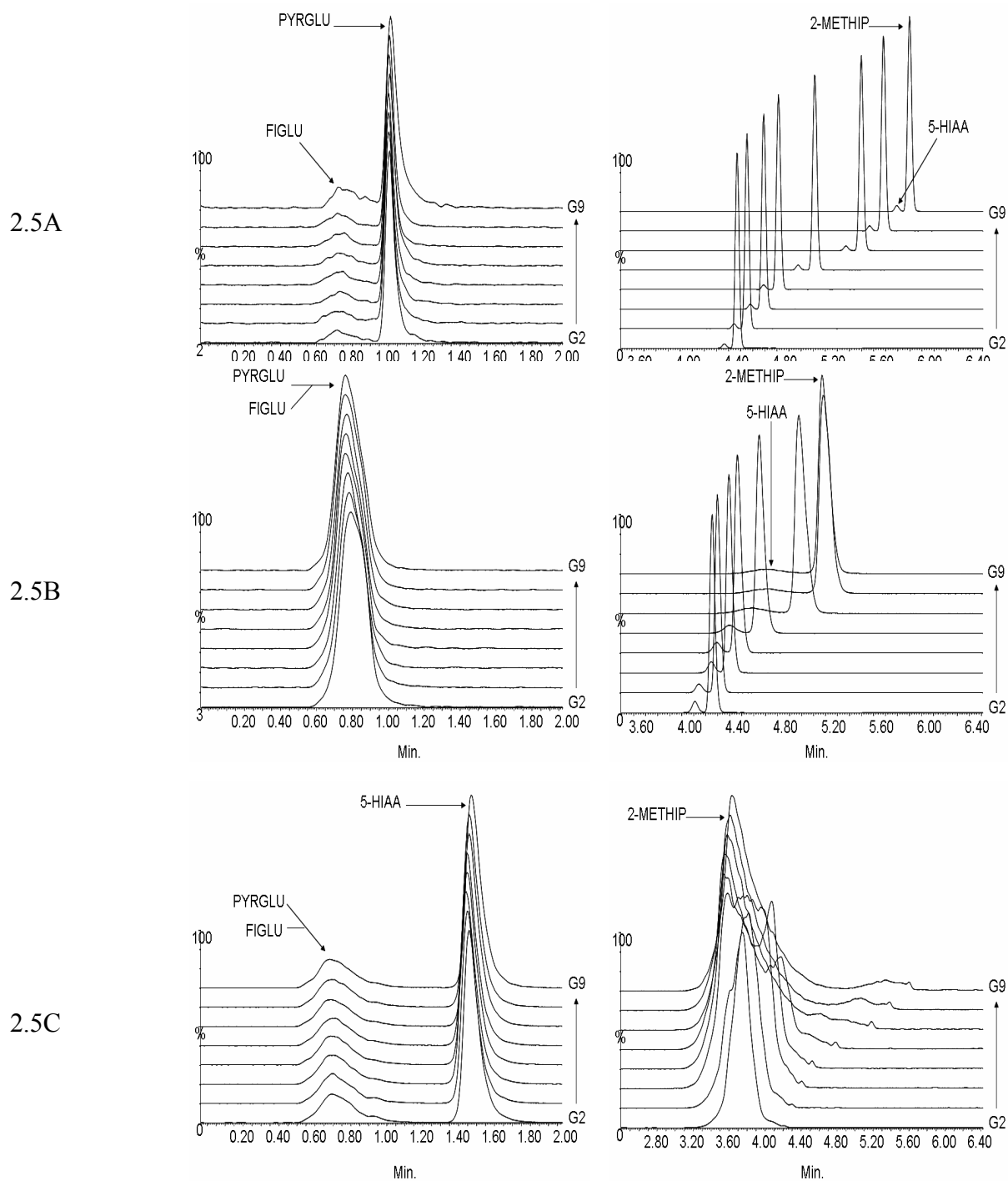


Figure 2.5: Chromatograms of all analytes eluted at mobile phase pH values of 2.5 (A), 5.0 (B), and 7.5 (C) using AcN as the organic modifier on a C-18 reversed phase column

The use of MeOH as the organic modifier at a buffered pH of 2.5 had no significant effect on the elution of FIGLU and PYRGLU for all gradient elution profiles used. The elution profile greatly affected the retention of 5-HIAA and 2-METHIP, with each gradient providing nearly the same peak shape and intensity for both compounds at this pH (Fig. 2.6A). The use of MeOH as the organic modifier at a buffered pH of 5.0 again had little effect on the elution of FIGLU and PYRGLU for nearly all gradient elution profiles used. The retention of PYGLU decreased at this pH and the peak shape was slightly improved while FIGLU remained unchanged. The use of different gradient elution profiles again greatly influenced the elution of 5-HIAA and 2-METHIP with no significant observable change in peak shape or intensity (Fig. 2.6B). No positive effects on the elution of FIGLU and PYRGLU were observed at a buffered pH of 7.5 for all elution profiles used. The retention of FIGLU and PYRGLU were slightly reduced and the overall peak shape diminished for both compounds. Interestingly, increasing pH had a largely negative effect on the retention and peak shape of 5-HIAA compared with other pH experiments using MeOH as the organic modifier. For both 5-HIAA and 2-METHIP the peak shape worsened as the elution profile transitioned from Waters Gradient #2 to #9 (Fig. 2.6C).

As observed with the C-8 analytical column mentioned previously, both pH and organic modifier produced significant observable changes in the chromatographic profile for all four analytes. Again the effects of pH were completely dependant upon the organic modifier used. Increasing pH greatly affected the retention properties of all analytes when using both AcN and MeOH as the organic phase. The retention properties of both FIGLU and PYRGLU were negatively affected with increasing pH regardless of the organic phase. While similar effects

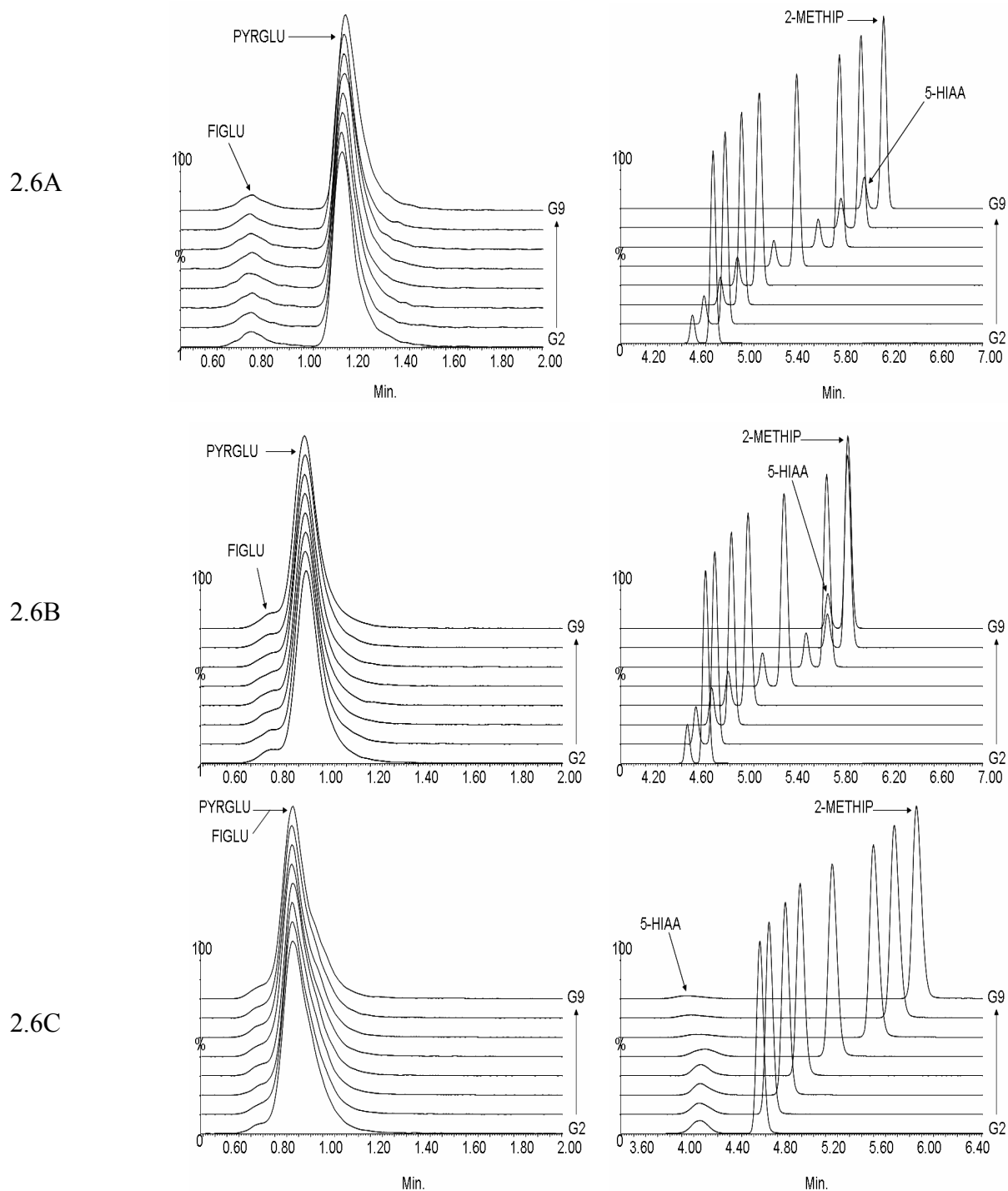


Figure 2.6: Chromatograms of all analytes eluted at mobile phase pH values of 2.5 (A), 5.0 (B), and 7.5 (C) using MeOH as the organic modifier on a C-18 reversed phase column

were observed for 5-HIAA and 2-METHIP, the use of MeOH seemed to delay the on-set of these effects as the pH increased for all experiments. Unlike the experiments performed using the C-8 stationary phase, experiments performed using the C-18 analytical column did not produce results that were optimum for all compounds. In general, the use of a C-18 stationary for the separation of all analytes proved to be unsuccessful for all test conditions.

2.2.3 Optimized Chromatographic Method for Reversed Phase Separations

The chromatographic method that provided the best overall separation was chosen based upon the experimental data collected from the variation of stationary phase, pH, organic modifier, and gradient profile. The C-8 reversed phase analytical column was found to possess the best overall chromatographic properties. While the use of an aqueous mobile phase buffered at a pH of 5.0 in concert with MeOH as an organic modifier provided the best retention of all analytes. The gradient elution profile that produced the best retention, peak shape, and intensity with the given chromatographic conditions utilized Waters gradient #2. The complete HPLC gradient timetable is listed in Table 2.2.

Table 2.2: Optimized instrument method

Time	A%	B%	Curve
0.00	100.0	0.0	1
1.00	100.0	0.0	1
3.00	20.0	80.0	2
6.00	20.0	80.0	2
6.01	100.0	0.0	2

Mobile phase A (A%) is 5mM ammonium acetate at a pH of 5.0

Mobile phase B (B%) is 0.2% acetic acid in acetonitrile

2.2.4 Method Validation

Linearity was evaluated based on the average of six calibrators (n=2) and a blank calculated from a standard curve. The curves were fit to a linear equation of slope and intercept ($y=mx+b$) weighted by $1/x$. Using statistical software, EP evaluator v.6, the validity of each calibration point was assessed for all compounds. The linearity experiment failed for FIGLU and PYRGLU. (Appendix A1, A2) In both cases the high calibration samples did not produce a linear response. The precision of these points was also unacceptable. The linearity experiment passed for 5-HIAA and 2-METHIP. (Appendix A3, A4) The calibration points were both linear and precise throughout the calibration range.

The accuracy of each analyte was evaluated based on the percent recovery for two levels of spiked samples compared with a baseline of pooled urine. All compounds evaluated failed the accuracy experiment. The measured recoveries were greater than the acceptable value of 15% for all analytes. The precision of the recovered values varied greatly for each compound; PYRGLU was the most imprecise while 2-METHIP had the greatest precision. The results of this experiment are presented in Table 2.3 and Fig. 2.7.

Table 2.3: % Recovery of FIGLU, PYRGLU, 2-METHIP, 5-HIAA from pooled urine using traditional buffered reversed phase chromatography.

Spike Solution	% Spike Recovery (mean +/- S.D., n = 3)			
	FIGLU	PYRGLU	2-METHIP	5-HIAA
Mid-Level	0.000 +/- 0.00	125.2 +/- 1261.9	45.61 +/- 0.55	43.33 +/- 22.1
High-Level	57.90 +/- 6.41	151.8 +/- 146.0	48.74 +/- 0.79	64.37 +/- 4.16

Given the poor results observed for the linearity and accuracy experiments the remaining validation procedures were not performed. Both linearity and accuracy represent a significant portion of method validation, and as such, poor results indicate the need for another analytical approach.

2.2.5 Discussion

Various chromatographic parameters were evaluated to determine the most effective analytical technique for the separation of FIGLU, 2-METHIP, PYRGLU, and 5-HIAA. The experiments performed utilized a traditional buffer system as the mobile phase (MPA) using both MeOH and AcN as the organic modifier (MPB). The mobile phase pH was buffer at 2.5, 5.0, and 7.5 to evaluate the chromatographic effects of pH. A number of gradient profiles ranging from concave, linear, and convex were observed for each mobile phase system. The optimum chromatographic conditions were observed at a mobile phase pH of 2.5 using a convex gradient profile (Waters gradient #2) with MeOH as the organic modifier.

The optimized analytical method was validated according to linearity and accuracy. Both experiments were unsuccessful, therefore no further validation experiments were performed. The linearity experiment for FIGLU and PYRGLU failed based upon the deviation of individual calibration standards from the expected value. An acceptable deviation is no greater than 15% for any calibration level. The observed deviation for both FIGLU and PYRGLU was greater than 20% for high level calibration standards. The linearity experiment was successful for 5-HIAA and 2-METHIP with observed deviations less than 15% for all calibration levels. The

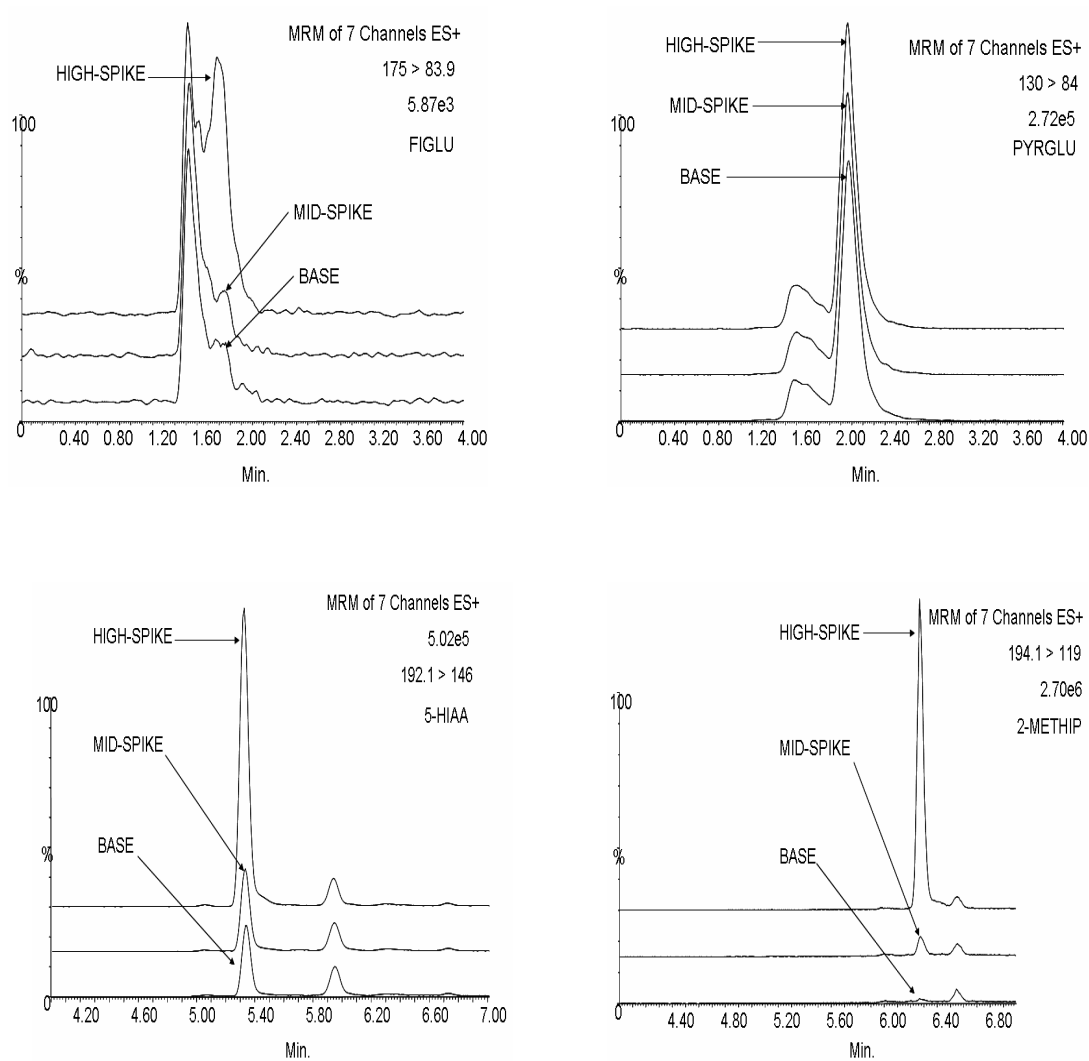


Figure 2.7: Chromatograms of accuracy experiment for FIGLU, PYRGLU, 5-HIAA, and 2-METHIP using traditional buffered reversed phase chromatography

accuracy experiments performed failed for all compounds. Acceptable results for accuracy are determined by the percentage of standard spike that is recovered based upon the experimental value observed. An accurate analytical method should recover within 20% of the theoretical (100%) recovery. For all compounds analyzed the observed spike deviated from the theoretical recovery by more than the allowable 20%. Therefore, based upon the results from both linearity and accuracy experiments the analytical method based upon a traditional buffered reversed phase separation is not valid.

This analytical method failed to validate based upon several reasons. First, the elution of FIGLU and PYRGLU is within or near the column void volume. The lack of retention can affect the reproducibility of the chromatographic peak. This in turn can decrease the level of precision for the analysis of these compounds. These inconsistencies are believed to be the cause of such poor experimental results observed in the linearity experiments. Secondly, matrix effects for the analysis of urinary metabolites by reversed phase chromatography are greatest near the void volume of the column. The mechanism of retention utilized by these separations is the result of hydrophobic interactions with the stationary phase. Highly polar hydrophilic compounds therefore elute in the column void along with other compounds of interest that maybe unretained. Signal suppression resulting from this elution pattern can cause severe degradation of analytical results. Given that FIGLU and PYRGLU elute in the column void, the presence of matrix effects most likely caused the failed recovery. The measured signal loss of standard in de-ionized water versus urine matrix is dependant upon the retention of the target analyte. As seen in Fig. 2.8, the signal loss observed for each compound due to matrix is greatest for FIGLU and least for 2-METHIP illustrating the effect of retention on signal suppression. Although, matrix effects are not localized to compounds eluting near the column void. The recovery of 5-HIAA and 2-

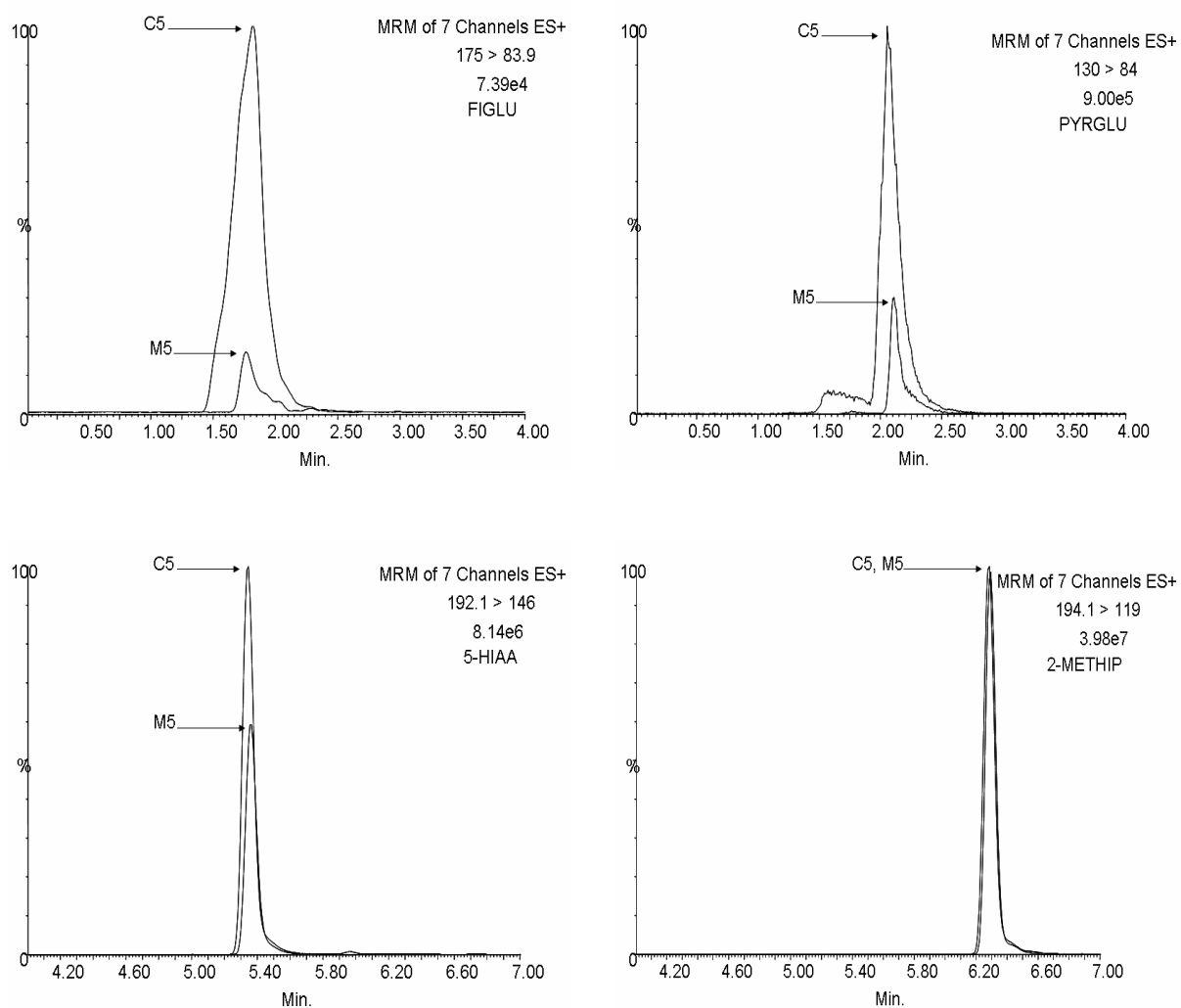


Figure 2.8: Chromatograms representing calibration solutions in de-ionized H₂O (C5) and urine matrix (M5) for all analytes using traditional buffered reversed phase chromatography.

METHIP was also found to be unacceptable. The measured precision was greater for these compounds the accuracy, however, was not. This is also attributed to matrix effects that are present but not as severe as those observed for FIGLU and PYRGLU.

2.3 Reverse Phase Chromatography using an Ion Pairing reagent

Triethylamine (TEA) was evaluated as an ion pairing reagent for the reversed phase chromatographic separation of FIGLU, PYRGLU, 5-HIAA, and 2-METHIP. The chromatographic conditions used for these experiments were taken from the optimized method listed above. TEA was added to the mobile phases and to the internal standard solution. The amount of TEA added to mobile phases, expressed as a percentage (v/v), was evaluated to determine the best chromatographic conditions. Experimental samples used to develop working chromatographic conditions were prepared by the addition of 200 μ L of high calibrator (level 5) to a vial containing 600 μ L of the appropriate MPA. Experimental samples used for validation were prepared by the addition of 200 μ L of sample to a vial containing 600 μ L of internal standard solution. The internal standard solution was prepared using a solution containing mobile phase to match the initial on-column chromatographic conditions. Each chromatographic run was performed using a 25 μ L sample injection. The experimental results are discussed below.

2.3.1 Optimization of TEA Concentration in Mobile Phase

The addition of TEA as an ion pairing reagent to the mobile phases and sample vial can produce an increase in retention and acceptable peak shape when using a reversed phase chromatographic technique. The observed changes in the elution profile of target analytes maybe dependent upon the amount of ion pairing reagent used in the separation. Also the addition of TEA to mobile phases may have a negative affect on detector response depending upon the system used. LC/MS/MS instrumentation is sensitive to the addition of TEA, specifically to the amount of the ion pairing reagent present in the mobile phase. TEA can suppress ionization of compounds detected by ESI+ due to the positive charge and strong polar character of the molecule. If the concentration of TEA is too great, no detectable analyte signal will be observed. The concentration of mobile phase TEA was therefore optimized to provide the best chromatographic separation and signal intensity. Mobile phase concentrations of TEA were evaluated for 0.2, 0.05, 0.01, 0.0025, 0.001, and 0.0001% (v/v).

Loss of signal intensity for all analytes was clearly observed as the concentration of mobile phase TEA increased. Total loss of detectable analyte response occurred at 0.05% TEA for all analytes. FIGLU was the most sensitive to TEA with total loss of signal occurring at 0.01%. PYRGLU, 5-HIAA, and 2-METHIP were completely suppressed at a TEA concentration of 0.05%. The addition of TEA slightly increased the retention of 5-HIAA and 2-METHIP. The greatest affect on analyte retention was observed for FIGLU and PYRGLU, increasing retention on-column by over three minutes. From these results, TEA increases the interaction between polar compounds and the hydrophobic, non-polar stationary phase. Although retention is increased, observable interference from the matrix may still occur. Based upon the experimental results, the optimized concentration of mobile phase TEA was 0.0001% (Fig. 2.9). The

chromatographic conditions remained the same as those listed in section 2.2.3 with the addition of 0.0001% (v/v) TEA to both MPA and MPB. No other chromatographic parameters were altered.

2.3.2 Method Validation

Linearity was evaluated based on the average of six calibrators (n=2) and a blank calculated from a standard curve. The curves were fit to a linear equation of slope and intercept ($y=mx+b$) weighted by $1/x$. Using statistical software, EP evaluator v.6, the validity of each calibration point was assessed for all compounds. 5-HIAA and PYRGLU both failed the linearity experiment. (Appendix B2, B3) The high calibration samples did not produce a linear response for either compound. The precision of these points was also unacceptable. The linearity experiment was successful for FIGLU and 2-METHIP. (Appendix B1, B4) The calibration points were both linear and precise throughout the calibration range. The accuracy of each analyte was evaluated based on the percent recovery for two levels of spiked samples compared with a baseline of pooled urine. Acceptable spike recovery was observed for 5-HIAA. All other compounds evaluated failed the accuracy experiment. The measured recoveries were greater than the acceptable value of 15% for all analytes. The precision of the recovered values varied greatly for each compound; PYRGLU was the most imprecise while 2-METHIP had the greatest precision. The results of this experiment are presented in Table 2.4 and Fig. 2.10. The results obtained from the linearity and accuracy experiments were unacceptable therefore the remaining validation procedures were not performed. The poor validation results observed indicate the need for another analytical approach.

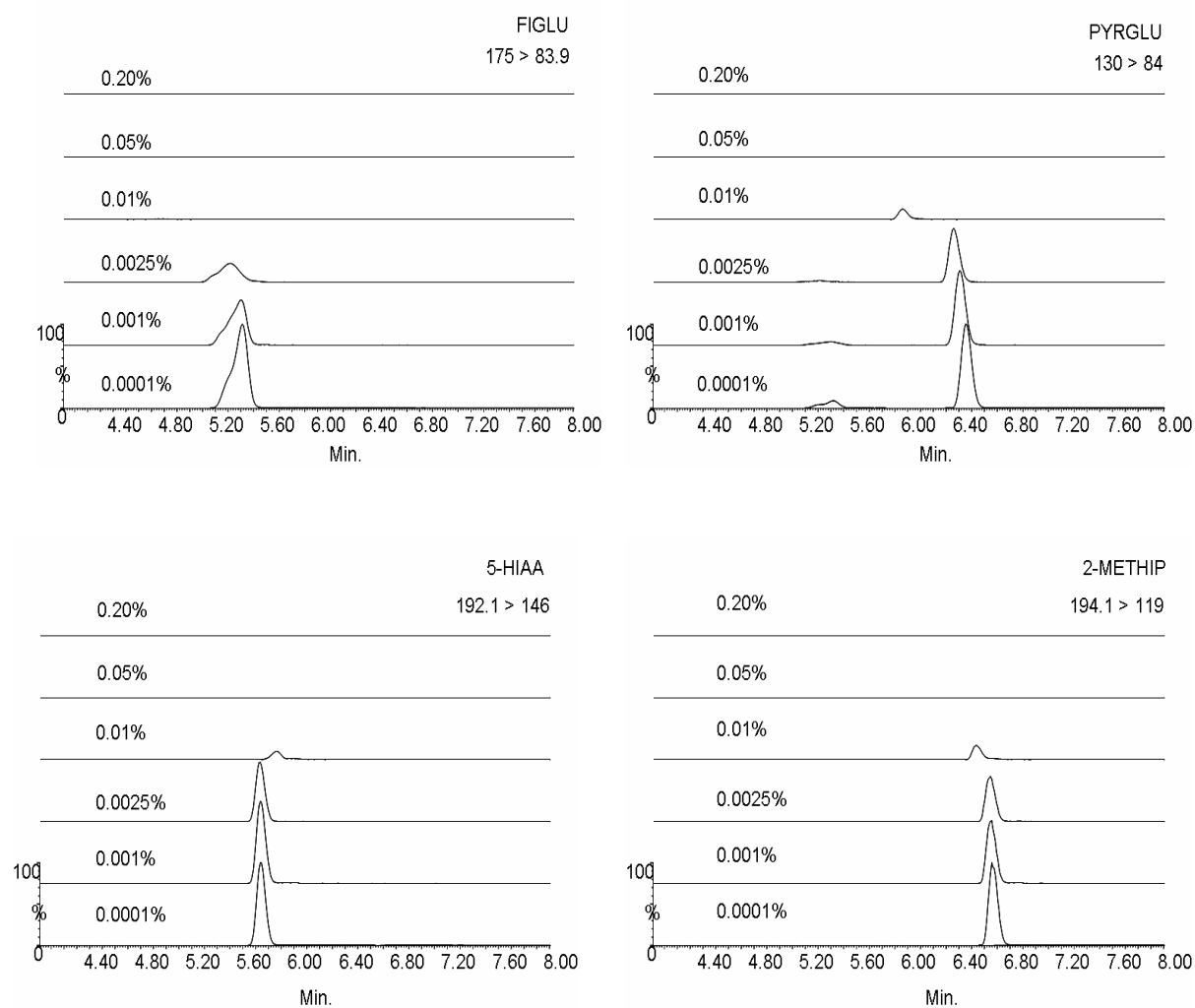


Figure 2.9: Chromatograms of each compound using increasing mobile phase TEA from 0.0001% to 0.20%

Table 2.4: % Recovery of FIGLU, PYRGLU, 2-METHIP, 5-HIAA from pooled urine using reversed phase chromatography with an ion pairing reagent

Spike Solution	% Spike Recovery (mean +/- S.D., n = 3)			
	FIGLU	PYRGLU	2-METHIP	5-HIAA
Mid-Level	25.88 +/- 1.62	30.83 +/- 32.72	45.49 +/- 0.63	105.8 +/- 25.3
High-Level	82.42 +/- 3.17	6.413 +/- 16.967	51.82 +/- 0.47	93.82 +/- 5.47

2.3.3 Discussion

The most suitable percentage of ion pairing reagent, TEA, was evaluated using increasing amounts in mobile phase and observing the change in chromatographic properties of retention and signal for each analyte. The optimized chromatographic method list in section 2.2.3 was used for the analytical evaluation of TEA as an ion pairing reagent. While all other chromatographic conditions remained the same, TEA was added to both MPA and MPB at the listed percentage (v/v). Complete loss of signal occurred for all analytes with TEA in mobile phase at 0.05%. The measured increase in retention was greatest for FIGLU and PYRGLU, while the retention of 5-HIAA and 2-METHIP only increased slightly.

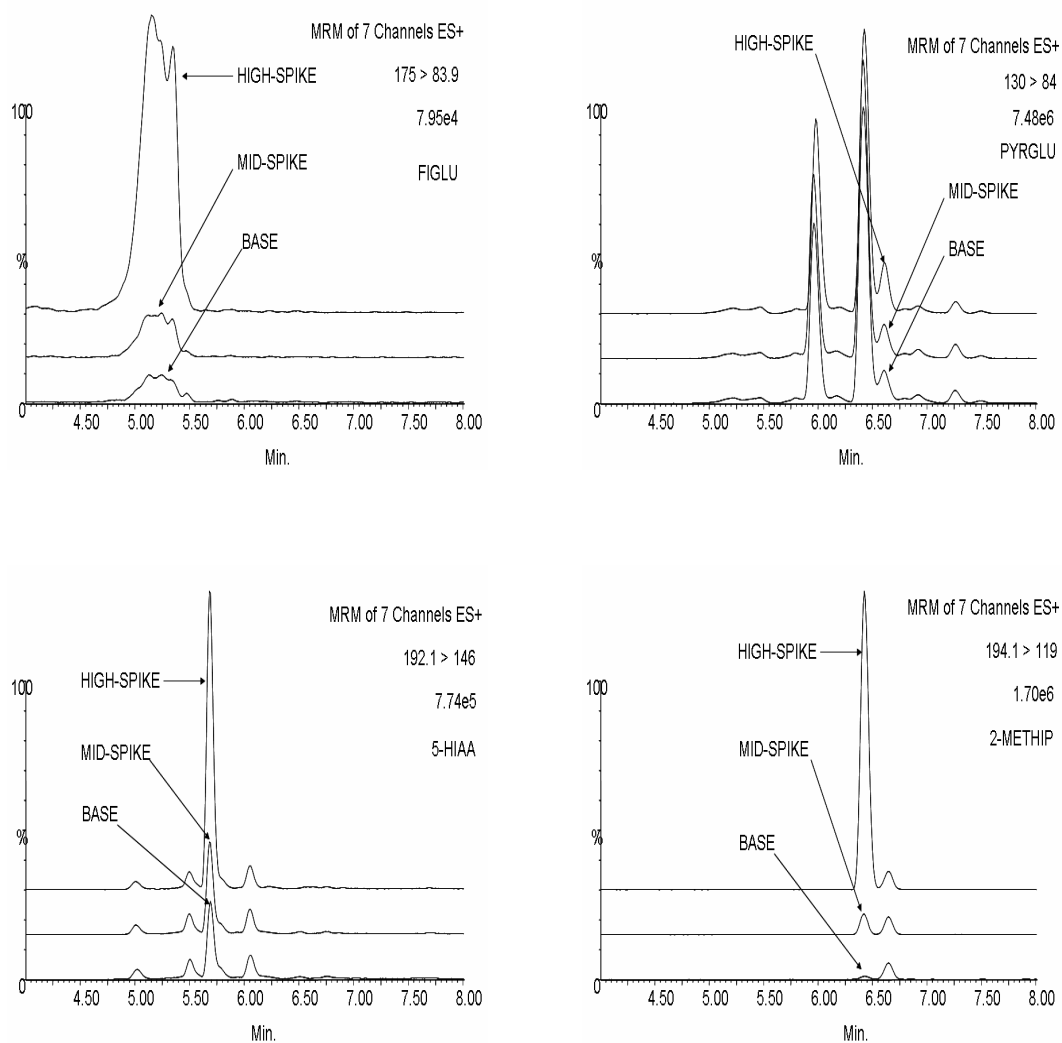


Figure 2.10: Chromatograms of accuracy experiment for FIGLU, PYRGLU, 5-HIAA, and 2-METHIP using reversed phase chromatography with an ion pairing reagent

Signal loss is observed using ESI when compounds compete for surface area on the Taylor cone that forms at the tip of ESI probe. The Taylor cone is formed as charged water molecules are dispersed outward by the electric potential created by the stainless steel capillary probe. The same electric potential causes ionized compounds in solution to form on the surface. The population of compounds occupying the surface of the Taylor cone is proportional to the amount of compound that will become ionized in the gas phase. In turn, the amount of compound ionized in the gas phase increases the detection of the analyte. Compounds in solution competing for the surface of the formed Taylor cone do so based upon individual charge and polarity. Any component of the mobile that is present in high concentration and is charged or polar, as in the case for TEA, will effectively compete with target analytes for the surface of the Taylor cone. This mechanism is responsible for the observed loss in signal as mobile phase concentration of TEA is increased.

The increased retention observed when using an ion pairing reagent for reversed phase chromatography relates to an increased interaction between the stationary phase and analyte. TEA was used as the ion pairing reagent in chromatographic study presented here. TEA is an organic molecule that contains a charged tertiary amine that responsible for the observed ion pairing interactions. The compound also displays non-polar and hydrophobic character by the presence of three ethyl groups bound to the amine. Both properties of TEA are useful for increasing the hydrophobic interactions between polar analytes and non-polar stationary phases. The general mechanism for this observed increased in chromatographic retention is thought to occur by the ionic interaction of TEA and the compound, which diminishes analyte polarity while increasing hydrophobicity. The formed complex between TEA and the analyte increases interaction with the non-polar stationary phase increasing chromatographic retention.

The chromatographic method developed using TEA as an ion pairing reagent was validated based upon linearity and accuracy. Neither experiment was successful for all analytes of interest; as a result no further validation experiments were performed. The linearity experiment for 5-HIAA and PYRGLU failed based upon the deviation of individual calibration standards from the expected value. An acceptable deviation is no greater than fifteen percent for any calibration level. The linear deviation for both 5-HIAA and PYRGLU was greater than 20% for nearly all calibration standards. The linearity experiment was successful for FIGLU and 2-METHIP with deviations less than 15% for all calibration levels observed. The accuracy experiments performed were only successful for 5-HIAA, all other compounds failed. Acceptable results for accuracy are determined by the percentage of standard spike that is recovered based upon the experimental value observed. An accurate analytical method should recover within 20% of the theoretical (100%) recovery. The calculated recovery of 5-HIAA was within 20% of the theoretical value indicating a successful recovery experiment. However, the observed recovery values for FIGLU, PYRGLU, and 2-METHIP deviated from the theoretical recovery by more than the allowable 20%. Therefore, the results from both linearity and accuracy experiments indicate that the analytical method based upon a reversed phase separation using TEA as an ion pairing reagent is not valid.

This analytical method failed to validate based upon several reasons. First, the presence of TEA in the mobile phase has a negative effect on analyte signal when using ESI. The result of these effects is believed to have caused the failed linearity experiments for 5-HIAA and PYRGLU. The relationship between analyte concentration and signal intensity was not linear as a result of the interference caused by TEA for these compounds. Secondly, matrix effects may not have been adequately removed even though analyte retention increased. The observed

matrix effects for FIGLU and PYRGLU using reversed phase chromatography have been attributed to the lack of retention and elution in the column void. This is believed to have a negative effect on compound response due to the large concentration of matrix interferences that are also un-retained, eluting in the column void. The lack of retention for both analytes and matrix compounds is due to their polarity and hydrophilicity. Sharing similar chemical properties it stands to reason that the effect of TEA would be comparable for both analyte and matrix components. Therefore, matrix interferences may also be retained longer as a result of ion pairing, causing interference throughout the chromatographic run. Finally, the action of TEA as an ion pairing reagent is dependent upon the concentration of all urine components which may vary from one individual to another. The ionic strength of the urine matrix may result in diminished interactions between TEA and target analytes. This effect would cause inconsistencies in analytical measurements for different samples and matrices. In order overcome these interactions an excess of TEA in the sample and mobile phase would be needed to help minimize the effect of matrix on ion pairing. However, as shown above, increasing the amount of mobile phase TEA has a drastic effect on analyte response and cannot be used in excess. For these reasons the use of TEA as an ion pairing reagent is not suitable for a valid analysis of FIGLU, PYRGLU, 5-HIAA, and 2-METHIP.

2.4 Reversed Phase Analysis of Butylated FIGLU, PYRGLU, 5-HIAA, and 2-METHIP

Other solutions designed to increase the retention of hydrophilic compounds on reverse phase columns require derivatization or chemical modification. These techniques require the use of common organic reactions to change the chemical structure and therefore chemical nature of target analytes. These procedures often involve extensive sample preparation and harsh chemical reagents. One of the more common methods of derivatization involves the butylation of carboxylic acids by Fischer esterification. The result of this procedure is a reduction in the hydrophilic nature of the compound making it more suited for hydrophobic retention. Fischer esterification is a relatively simple organic procedure only requiring a small amount of catalytic acid and heat. The derivatized sample can undergo further clean-up or be used directly for analysis.

2.4.1 Sample preparation

Butylated samples were prepared using a method adapted from [55]. Samples were prepared by the addition of 300 μ L of sample/calibrator, 200 μ L I.S., 100 μ L acetyl chloride, and 3 mL of 1-butanol to a 16 x 100 mm glass tube. The sample was vortex mixed for 30 sec. and blown to dryness under nitrogen at a temperature of 70°C for 30 min. The dried sample was reconstituted in 300 μ L of AcN and 300 μ L of de-ionized H₂O. Reconstituted samples were placed into sample vials using a transfer pipette and capped.

2.4.2 Chromatographic conditions

The separation was performed on a Waters (Milford, MA, USA) YMC-AQ C18 column, 150 x 2.0 mm, 5 μ m maintained at 30°C throughout the experiment. The mobile phases used consisted of 0.15% formic acid in de-ionized water (MPA) and acetonitrile with 0.15% formic acid (MPB). The sample injection for all chromatographic analyses was 25 μ L. The gradient program used for elution is presented in Table 2.5:

Table 2.5: Instrument method for the separation of butyl esters

Time	A%	B%	Curve
0.00	50.0	50.0	1
0.50	40.0	60.0	6
1.00	0.0	100.0	6
2.00	0.0	100.0	6
2.01	50.0	50.0	6

Mobile phase A (A%) is 0.15% formic acid in de-ionized H₂O

Mobile phase B (B%) is 0.15% formic acid in acetonitrile

2.4.3 Mass Spectrometer conditions

All compounds were detected in electrospray positive ionization mode, with the desolvation gas set to 800 L/hr. To increase sensitivity the nebulizer gas was not used. Capillary voltage was maintained at 3.5 kV, with source and desolvation temperatures at 150 °C and 350 °C, respectively. Each MRM was collected at unit mass resolution with a dwell time of 0.1 s.

The cone and collision settings were established individually for each compound for multiple reaction monitoring (MRM) detection. The conditions for detection of all analytes were obtained by direct infusion of a standard solution in line with the HPLC at initial mobile phase conditions. The MRM transitions and appropriate detection settings are presented in Table 2.6.

Table 2.6: MRM transitions and detection settings for all butylated analytes and internal standards

Analytes and I.S.	Parent ion [M+H]	Product ion	Cone potential (V)	Collision energy (eV)
FIGLU	287.2	83.9	30	35
PYRGLU	186.1	84.0	20	15
5-HIAA	248.1	146.1	20	15
2-METHIP	250.1	119.1	20	10
D3GLU	261.0	85.0	25	25
NBA	250.1	105.0	20	20
5-FIAA	250.1	148.1	20	15

2.4.4 Standard and working solutions

Standard stock solutions were prepared as follows: Working stock solution A (StkA) was made by the addition of 0.0028 g of FIGLU, 0.0020 g of 2-METHIP, 0.005 g of 5-HIAA, and 0.040 g of PYRGLU to a 100 mL volumetric flask and brought to volume with de-ionized water. The internal standard solution was prepared by the addition of 0.001g of NBA, 0.001 g of D3GLU, and 0.002 g of FIAA to a 500 mL volumetric flask and brought to volume with de-ionized water.

2.4.5 Calibration standards

Calibration standards were prepared by serial dilution of StkA. Five calibration standards, including a blank, were prepared for each analyte as follows: FIGLU, 0.0, 0.1, 1.0, 2.0, 3.0, 4.0 mg/L; PYRGLU, 0.0, 2.0, 20, 40, 60, 80 mg/L; 2-METHIP, 0.0, 0.1, 1.0, 2.0, 3.0, 4.0 mg/L; 5-HIAA, 0.00, 0.5, 5.0, 10, 15, 20 mg/L.

2.4.6 Chromatography

The chromatographic method was developed to provide the greatest efficiency for butylated analytes. The initial mobile phase conditions were composed of 50% organic to compensate for the added retention of butylated products, reducing the overall run time. The gradient profile used for this separation was a simple linear gradient with a one minute hold at 100% AcN. The chromatographic method provided good separation and overall peak shape for all analytes as seen in Fig. 2.11.

2.4.7 Method Validation

Linearity was evaluated based on the response of five calibrators and a blank calculated from a standard curve. The curves were fit to a linear equation of slope and intercept ($y=mx+b$) weighted by $1/x$. Using statistical software, EP evaluator v.6, the validity of each calibration point was assessed for all compounds. The linearity experiment was successful for FIGLU, PYRGLU, and 2-METHIP. (Appendix C1, C2, C4) However, the experimental results for

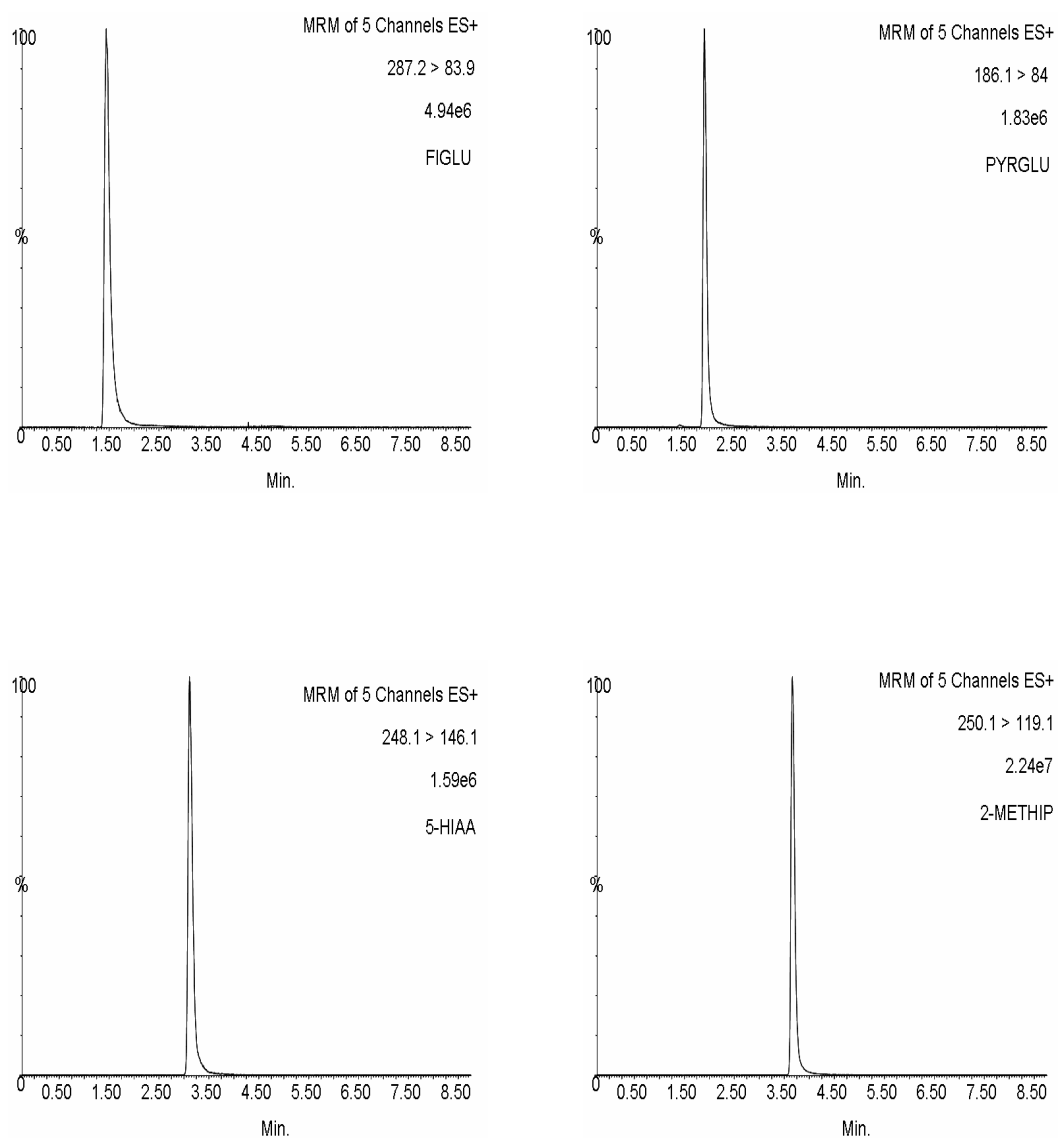


Figure 2.11: Chromatograms of StkA for butylated FIGLU, PYRGLU, 5-HIAA, and 2-METHIP

5-HIAA were unsatisfactory (Appendix C3). The magnitude with which the experimental results deviated from the expected linear plot was troubling. Given that all of the other analytes produced good results for this experiment, the data collected for 5-HIAA warranted further investigation. No further validation experiments were performed as a result of this inconsistency.

2.4.8 Discussion

In this analytical method a derivatization procedure involving the formation of butyl esters by Fischer esterification was evaluated for the separation and quantization of FIGLU, PYRGLU, 5-HIAA, and 2-METHIP. The sample preparation was adapted from previously published method and utilized a excess of 1-butanol catalyzed by the addition of acid and heat to produce butyl esters of compounds containing carboxylic acids. The butylation of target analytes was confirmed by mass spectral analysis. The parent ion of each compound increased in by 56 m/z for single butylation products and by 112 m/z for compounds containing two carboxylic acid function groups. The identification of butylated products was further confirmed by the observed daughter ions for each analyte, which corresponded well with the fragment ions of underivatized compounds. The samples were analyzed by reversed phase chromatography using a C18 stationary phase and mobile phase conditions that contained a high percentage of organic solvent to increase efficiency and signal intensity.

The validation of this analytical method did not proceed beyond the evaluation of linearity. The linearity results were acceptable for all compounds except for 5-HIAA which failed for all levels of calibration. Given the observed results for all other compounds, the lack of linearity for 5-HIAA was investigated further. The calibration solutions were remade and the

experiment was reevaluated for which the results were the same as those of previous experiments. The calibration solution was analyzed using the mass transition (MRM) for underivatized 5-HIAA with the same chromatographic method utilized for the linearity experiments. The results for the underivatized sample were linear, indicating that the calibration solutions were diluted correctly (Fig. 2.12). Therefore the most probable cause of the failed linearity experiment for butylated 5-HIAA was the sample preparation. The presence of strong acid and prolonged heating are believed to have caused the break down of 5-HIAA during sample preparation. Based all of the experiments performed, the analytical method of butylation was found to be unacceptable for the analysis of 5-HIAA and therefore no further experiments were evaluated.

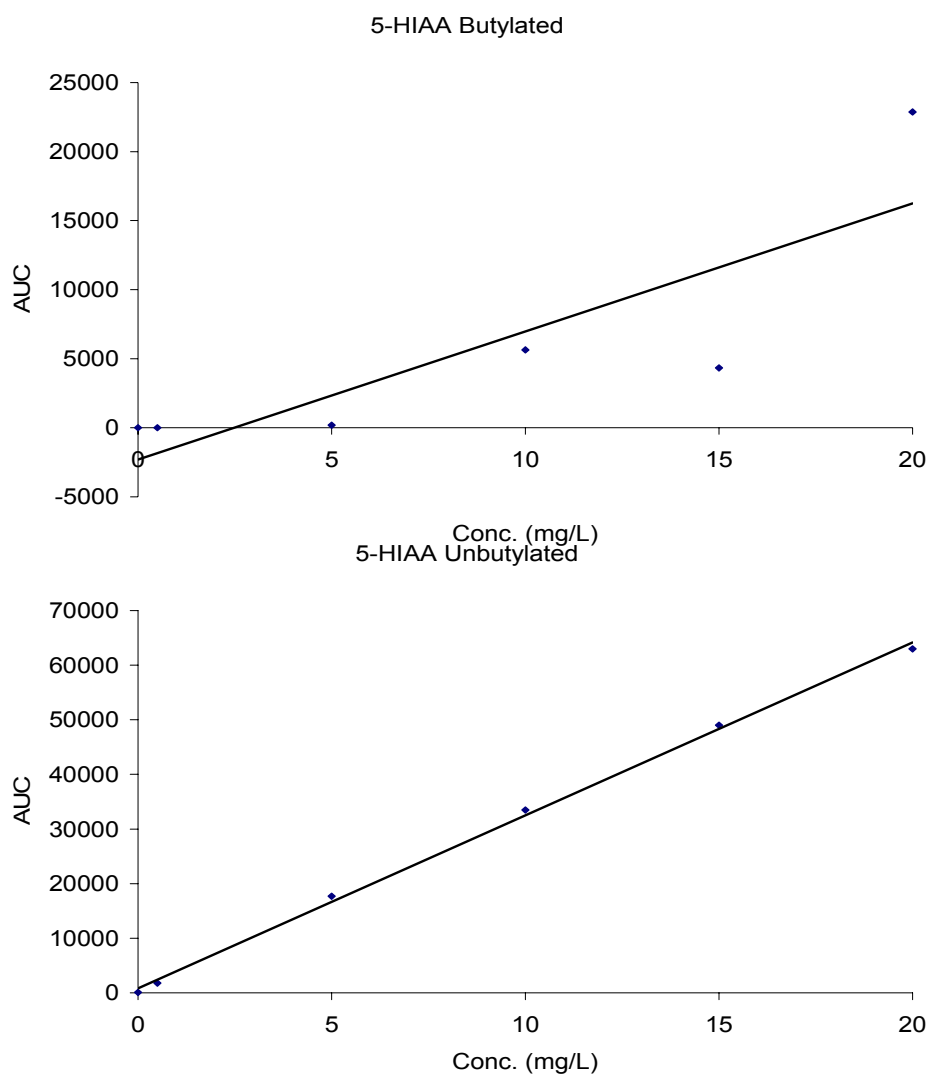


Figure 2.12: Linear plots of derivatized and underivatized 5-HIAA

Chapter 3: Weak-anion exchange chromatographic analysis of FIGLU, PYRGLU, 5-HIAA, and 2-METHIP

3.1 Introduction

LC separations are commonly performed using reversed phase chromatography, even though retention of small polar biological compounds is difficult without derivatization [56]. A major disadvantage of analyte derivatization is the increased sample preparation, which may be less cost effective for clinical settings. Many methods which do not require derivatization, rely on changes in mobile phase pH or ion pairing reagents to promote retention. In either case, the mobile phase additives have been shown to reduce sensitivity on LC/MS/MS systems [36]. Ion exchange chromatography offers an alternative approach, which can provide excellent retention of small polar biological compounds. However, classical ion-exchange methods require the use of mobile phases that contain high concentrations of non-volatile salts or other counter-ions, which can lead to ion suppression [57].

The method presented utilizes weak-anion exchange chromatography for compound separation. Four small biological compounds are separated on an amino stationary phase using a pH gradient with an organic modifier. The underivatized compounds require little sample preparation and are adequately retained on column to provide separation from interferences and to minimize loss of sensitivity due to matrix effects. This method was validated for clinical use and was found to be rapid, robust, and reproducible.

3.1.1 Chromatographic conditions

The separation was performed on a Phenomenex (Torrance, CA, USA) Luna Amino column, 50 x 2.0 mm, 5 μ m maintained at 40°C throughout the experiment. The three mobile phases consisted of 0.2% formic acid in de-ionized water (MPA), 20.0 mM ammonium formate in de-ionized water adjusted to pH 5.0 with formic acid (MPB), and acetonitrile with 0.15% formic acid (MPC). 25 μ L of the sample was injected under these initial mobile phase conditions: 40% MPA, 35% MPB, and 25% MPC at a flow rate of 0.3 mL/min. A concave gradient was employed over the first 2 minutes (0 – 2 min) to 25% MPA, MPB 0%, and 75% MPC. These conditions were held for one minute (2 – 3 min) following the gradient. Finally, the mobile phase composition was returned to the initial conditions after three minutes (3.01 – 8), and the flow rate increased to 0.5 mL/min to minimize total run time (0 - 8 min). The gradient employed in this method utilized a present instrument gradient, Waters #8, to perform a concave gradient from initial conditions to the final elution conditions (0 – 3 min). The gradient changes were concave up for MPA and MPC. However, the MPB concentration changes from 35% to 0% were concave down. The overall elution profile results in a change in mobile phase composition from a buffered environment to one that is un-buffered, acidic, and containing a large proportion of organic phase.

3.1.2 Mass Spectrometer conditions

All compounds were detected in electrospray positive ionization mode, with the desolvation gas set to 800 L/hr. To increase sensitivity the nebulizer gas was not used. Capillary voltage was maintained at 3.5 kV, with source and desolvation temperatures at 150 °C and 350 °C, respectively. Each MRM was collected at unit mass resolution with a dwell time of 0.1 s.

The cone and collision settings were established individually for each compound for multiple reaction monitoring (MRM) detection. The conditions for detection of all analytes were obtained by direct infusion of a standard solution in line with the HPLC at initial mobile phase conditions. The MRM transitions and appropriate detection settings are presented in chapter 2, Table 2.1.

3.1.3 Standard and working solutions

Standard stock solutions were prepared as follows: Working stock solution A (StkA) was made by the addition of 0.032 g of FIGLU and 0.0015 g of 2-METHIP to a 500 mL volumetric flask and brought to volume with de-ionized water. Working stock solution B (StkB) was prepared by the addition of 0.08 g of PYRGLU and 0.005 g of 5-HIAA to a 100 mL volumetric flask containing 33 mL of StkA and brought to volume with de-ionized water. The internal standard solution was prepared by the addition of 0.001g of NBA, 0.001 g of D3GLU, and 0.002 g of FIAA to a 500 mL volumetric flask and brought to volume with de-ionized water.

3.1.4 Calibration standards

Calibration standards were prepared by serial dilution of StkB for the desired calibration range established from collected patient data. Six calibration standards, including a blank, were prepared for each analyte as follows: FIGLU, 0.00, 0.480, 0.960, 1.92, 2.88, 3.84, 15.4 mg/L; PYRGLU, 0.00, 25.0, 50.0, 100, 150, 200, 800 mg/L; 2-METHIP, 0.00, 0.0310, 0.0630, 0.125, 0.188, 0.250, 1.00 mg/L; 5-HIAA, 0.00, 1.56, 3.13, 6.25, 9.38, 12.5, 50.0 mg/L.

3.1.5 Urinary creatinine measurement

Urinary creatinine concentration was measured on a Cobas Mira Plus using a creatinine assay kit purchased from Roche (Quebec, Canada) following Jaffe's picric acid method [58].

3.1.6 Patient ranges

Adult patient ranges were established using intra-laboratory samples, following method validation. All concentration measurements were normalized to creatinine. Data was taken from approximately two hundred patient samples to calculate a working within laboratory range for both normal and elevated results. Normal patient ranges were established within the 95% confidence level. Any result outside the established normal range was considered to be elevated. The ranges established for each analyte are relevant to patients 13 years of age and older.

3.1.7 Preparation of quality control samples

Normal controls were prepared from pooled urine. The pooled sample was also used in the preparation of the elevated controls. Elevated controls were spiked with a known amount of standard salt which was dissolved in normal control urine. The amount of standard added elevated the normal value of each analyte to a level within the calibration range and above the observed normal patient range.

3.2 Method validation

The method was validated based upon linearity, accuracy, precision, and sample preparation stability. Linearity was evaluated using a six point calibration curve. Accuracy was established by measuring spike recoveries for all analytes in a pooled urine sample when spiked

with mid-level and high-level calibrators. The urine samples were spiked with no more than 10% of initial urine volume and calculated based on the average of three successive measurements for each level. Precision, within and between run, was calculated using normal and elevated (n=30) controls collected over a five day period. Sample preparation stability was evaluated from quantitative results of three samples taken over three days.

3.2.1 Linearity

The linearity of the calibration curve was evaluated by linear regression, including the intercept ($y=mx+b$), weighted by $1/x$. Linear curves were comprised of six calibration levels, run in duplicate and quantified from a standard curve to evaluate precision and accuracy. All calculations were performed using EP Evaluator 6 software, (RHOADS, Kennett Square, PA, USA).

3.2.2 Limits of detection (LOD) and quantification (LOQ)

The LOD ($S/N = 3$) and LOQ ($S/N = 10$) were determined using the regression approach based upon the linear regression of calibration from the established linear range [59]. The sensitivity of the present method was determined from these measurements.

3.2.3 Evaluation of matrix effects

The effects of sample matrix on calibration were measured using a simple matrix matching experiment. A pooled urine sample was prepared using intra-laboratory samples. To prepare calibration standards in matrix, a volume of pooled urine (500 μL) was added to 16 x 100 mm glass tubes and blown to dryness under a steady stream of nitrogen in a water bath

maintained at 50 °C. The dried urine was reconstituted in 500 µL of calibration solutions, absent of matrix, corresponding to the calibration range used to establish linearity [60]. The matrix matched calibration solutions were measured using the same method described in this report and compared with measured calibration solutions at the corresponding calibration levels [61]. The data from matrix matched and unmatched calibrators were taken to generate linear regression plots using Microsoft Excel (2003). Two plots were examined, the first comparing the area under the curve (AUC) vs. calibration concentration and the second comparing the response factor of analyte area corrected by internal standard area vs. calibration concentration. Within each plot, matrix effects were statistically evaluated by comparison of the slopes for each regression line using Student's t test.

3.2.4 Accuracy

Accuracy was evaluated by spike recovery from pooled urine samples. This baseline urine level was spiked with two levels of calibrators (mid-level and high-level). Both spiked samples were prepared by the addition of 10% v/v of the specific calibrate to the baseline urine. Mid-level solutions were spiked using calibrator level 5 (FIGLU 3.84 mg/L, PYRGLU 200 mg/L, 2-METHIP 0.25 mg/L, 5-HIAA 12.5 mg/L). High-level spikes were spiked using working stock solution B (FIGLU 15.36 mg/L, PYRGLU 800 mg/L, 2-METHIP 1.0 mg/L, 5-HIAA 50 mg/L). The baseline samples were prepared in a similar way i.e. made with 10% de-ionized water in the place of calibrate. The baseline, mid-level, and high-level samples were run in duplicate and quantified using a standard curve.

3.2.5 Precision

Precision was measured by the variation of normal and elevated control values for each analyte over a five day period. Within run data was evaluated statistically for each control group. Between run data was evaluated based upon values for all control group data.

3.2.6 Stability of sample preparation

Stability of the sample preparation was measured over a period of three days. A prepared sample of elevated control urine was measured once per day, starting with an initial measurement and evaluated at 24 h intervals for three consecutive days. During the course of the experiment, the sample was stored on instrument at 5°C. The preparation stability for each analyte was evaluated based upon the percent deviation of the analytes from the initial measurement.

3.2.7 Sample preparation

Urine samples were collected in tubes containing 20 μ L of thymol (0.05 mg/mL) as a preservative, and stored at -20°C. Samples were prepared by diluting 100 μ L of urine with 100 μ L of internal standard solution and 300 μ L of buffer solution matching the initial mobile phase conditions (40% MPA, 35% MPB, 25% MPC).

3.3 Results and discussion

3.3.1 Mass spectrometry

FIGLU, PYRGLU, 5-HIAA, and 2-METHIP are not conventional organic acids because each compound exhibits a zwitterion. This structural characteristic allows for both positive and negative electrospray ionization. The sensitivity for either mode of detection can be enhanced by mobile phase pH. Each analyte was evaluated in both modes at varying pH, from 2.0 to 11.0. The conditions that provided the greatest sensitivity for all analytes were electrospray positive at low pH (<4.0). Internal standards were chosen with similar chemical structures and ionization potential. The most dominant molecular ion formed at low pH, $[M+H]^+$, resulted from the ionization of the nitrogen containing functional group within the compounds. Solvent adducts were not observed. Stable product ions were formed for all analytes and internal standards. The proposed mechanism of fragmentation for each ion is displayed in Figure 3.1. The optimized mass spectrometer conditions provided good sensitivity for each analyte.

The selectivity of each analyte was evaluated by monitoring all MRM channels during individual injections of single analytes made from high concentration calibration solutions. No cross-channel interference was observed greater than 5% of the lower limit of detection. However, the selectivity of each analyte MRM in matrix was not adequate enough to negate the need for chromatography. Interferences were observed in the MRM's for PYRGLU, 5-HIAA, 2-METHIP, 5-FIAA, and NBA. Baseline separation of all analyte peaks from interferences was

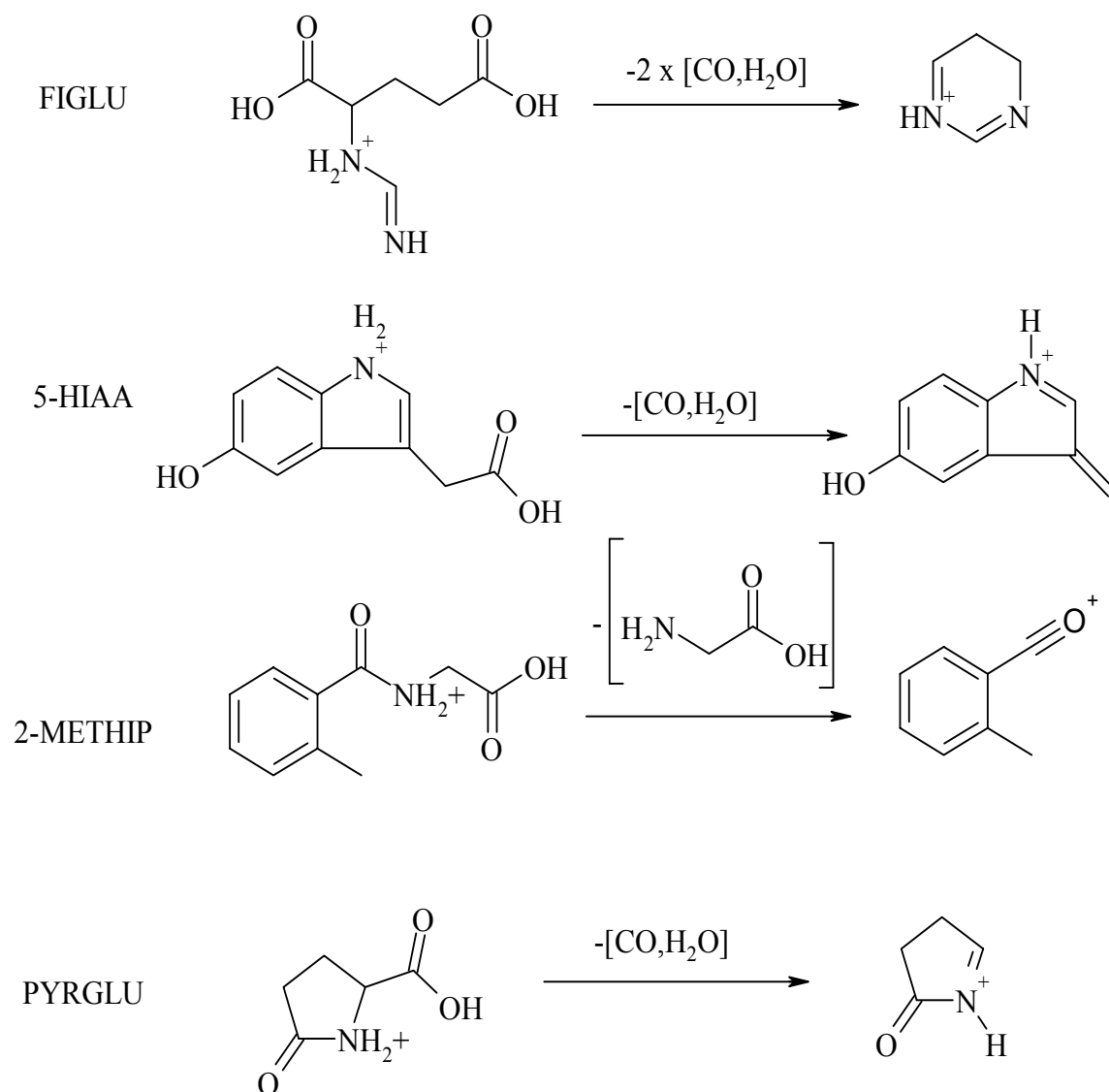


Figure 3.1: Prediction of fragmentation ions for FIGLU, 5-HIAA, 2-METHIP, and PYRGLU

achieved with the exception of PYRGLU and 2-METHIP. Resolution from the interferences of PYRGLU was achieved at 30% peak height and 2-METHIP at 10% peak height. Alternative MRM's were explored for all analytes that contained matrix interferences, however no MRM's were found to provide selectivity or sensitivity greater than those presented.

3.3.2 Chromatography

The separation of all target analytes and internal standards from interferences and the column void volume is important for the overall accuracy of the method. In developing this method, reversed phase chromatography was evaluated for retention, resolution, and efficiency. The reversed phase columns used varied in both hydrophobic character (C8, C18) and manufacturer. Experiments were performed using simple gradients with aqueous and organic mobile phases modified with formic acid to lower the pH (~2.5). The results of these experiments were similar for hydrophilic compounds, FIGLU and PYRGLU, yet varying slightly for compounds with more hydrophobic character, 5-HIAA and 2-METHIP. The retention of FIGLU and PYRGLU was not found to be adequate for any reversed phase column used. However, 5-HIAA and 2-METHIP were well retained and easily manipulated with gradients of organic mobile phase. In each case, the lack of retention for two of the compounds was unsuitable for accurate measurement, while the retention of the other analytes would require extended run times and column equilibration.

Ion exchange chromatography is well suited for the analysis of small biological compounds. However, the most common ion exchange methods require mobile phase additives that can drastically reduce sensitivity when using LC/MS/MS systems. A variation of traditional ion exchange relies on a change in pH and the addition of organic mobile phase to promote

elution. The retention of compounds, using weak anion exchange, is a result of electrostatic interactions between the carboxylic acid and the stationary phase. These interactions can be mediated by controlling the pH of the mobile phase. As the pH is lowered below the pKa of the carboxylic acid, the ionic character of the compound is decreased and retention shifts to a reversed phase mechanism. Compound retention is then mediated by hydrophobic interactions which can be manipulated by increasing the concentration of organic mobile phase. As a result, weak ion exchange chromatography can be accomplished without the use of salts or counter-ions. The resulting chromatography is illustrated in Figure 3.2.

3.3.3 Linearity

Linearity was evaluated based on the average of six calibrators (n=2) and a blank calculated from a standard curve. The curves were fit to a linear equation of slope and intercept ($y=mx+b$) weighted by $1/x$. All slopes had r^2 values greater than 0.995. Deviations from the standard values, based upon recovery, were less than 15% for all analytes. Residuals about the line of regression were less than 15% of the target value for all analytes.

3.3.4 Limits of detection (LOD) and quantification (LOQ)

The LOD ($S/N = 3$) and LOQ ($S/N = 10$) were measured for each analyte based upon the linear regression of calibration from the established linear range. The calculated values are shown in Table 3.1. Both LOD and LOQ indicate adequate sensitivity for this method given that clinically significant results are much higher than the LOQ.

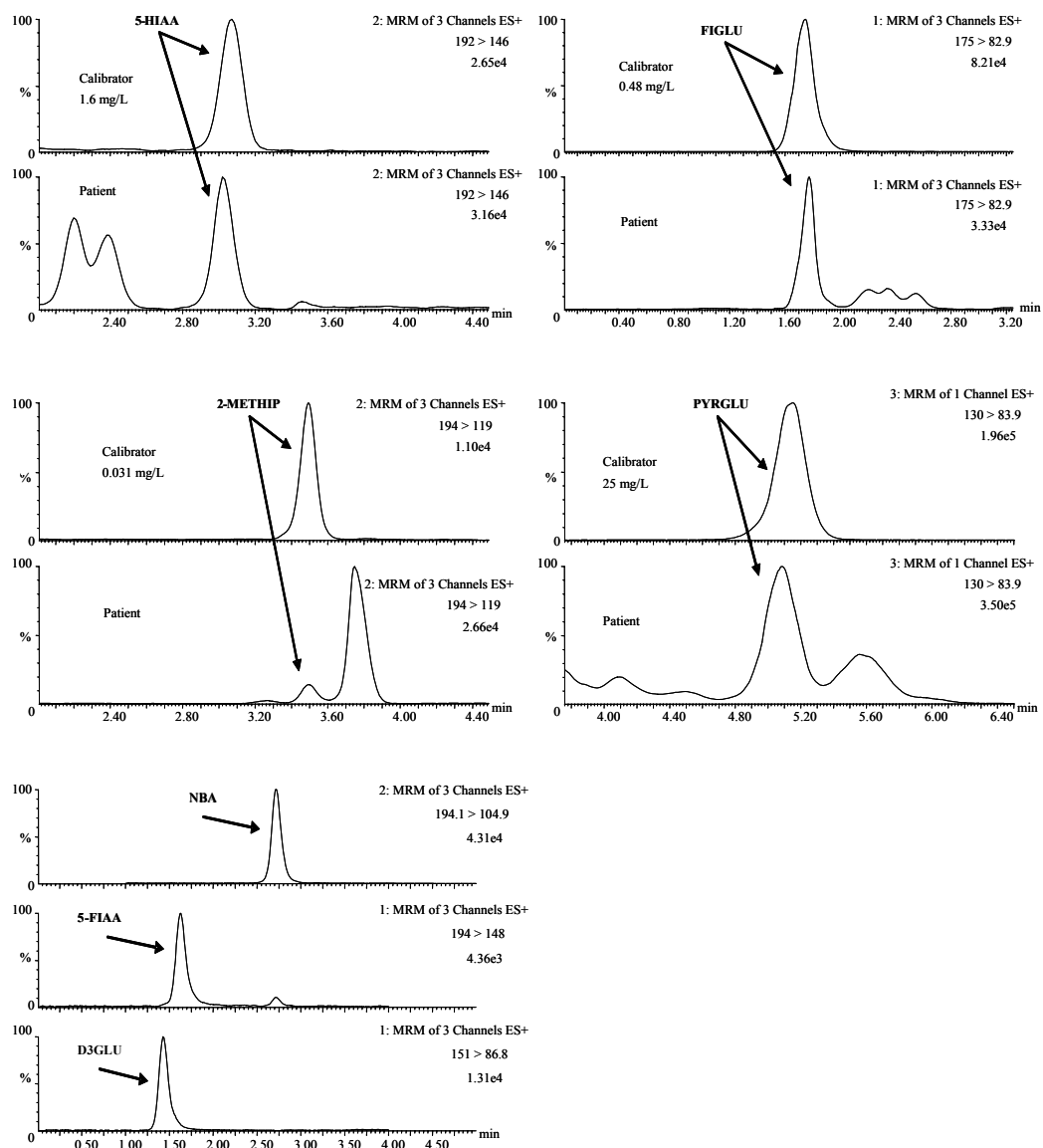


Figure 3.2: Representative chromatograms of 5-HIAA, FIGLU, 2-METHIP, PYRGLU, and internal standards. For the analyte chromatograms, the upper channels show a calibration standard and the lower channels show a normal patient. The internal standards shown are taken from the calibration blank.

Table 3.1: The calculated LOD and LOQ for all analytes

Analyte	LOD* (mg/L)	LOQ** (mg/L)
PYRGLU	0.437	1.46
2-METHIP	0.0172	0.0574
5-HIAA	0.280	0.933
FIGLU	0.286	0.953

* LOD was calculated at S/N = 3

** LOQ was calculated at S/N = 10

3.3.5 Evaluation of matrix effects

Two linear regression plots were generated for all analytes comparing AUC and the response factor of the analyte vs. calibration concentration. The variations observed in the slopes of matrix matched and unmatched calibrators within each plot were evaluated to determine the existence of any significant matrix effects. The assessment of plotted AUC data was to determine if any inherit matrix effects were present that would significantly alter analyte response. The data taken from graphs of response factor were used to determine if the internal standards appropriately correct for analyte specific matrix effects. The variations between the mean (n=2) slopes of matrix matched and unmatched calibration plots were evaluated using Student's t test at the 95% confidence level (Table 3.2). Figure 3.3 shows the average linear regression of both AUC and response plots for 2-METHIP to illustrate the effect of matrix and internal standard correction on the slope of each plot. FIGLU and 2-METHIP have significant statistical variations in slope ($t_{\text{calculated}} > t_{\text{table}}$) for AUC plots, indicating that matrix effects exist for these analytes. These variations were not observed for the AUC plots of PYRGLU and 5-HIAA, indicating that no significant matrix effects were present for these analytes. For all analytes, no significant statistical variations in the slopes for response plots were observed.

Table 3.2: Statistical comparison between the slopes of matched and unmatched calibrators

Analyte	Cal. Solution	Slope (AUC vs. Conc.)	t-value*	Slope (Resp. Factor vs. Conc.)	t-value*
FIGLU	H ₂ O Matrix	1076 ± 32 1256 ± 15	7.16	0.2457 ± 0.0023 0.2772 ± 0.0205	2.16
PYRGLU	H ₂ O Matrix	661 ± 10 537 ± 56	3.07	0.0219 ± 0.0006 0.0230 ± 0.0002	2.45
5-HIAA	H ₂ O Matrix	1891 ± 44 1530 ± 274	1.84	0.8918 ± 0.0051 0.9571 ± 0.0510	1.80
2-METHIP	H ₂ O Matrix	8570 ± 487 5840 ± 96	7.78	0.2831 ± 0.0032 0.2452 ± 0.0245	2.16

* Calculated t-value at 95% confidence level (n = 2); table t-value at 95% confidence level (n = 2) is 4.303. The difference is significant if $t_{\text{calculated}} > t_{\text{table}}$.

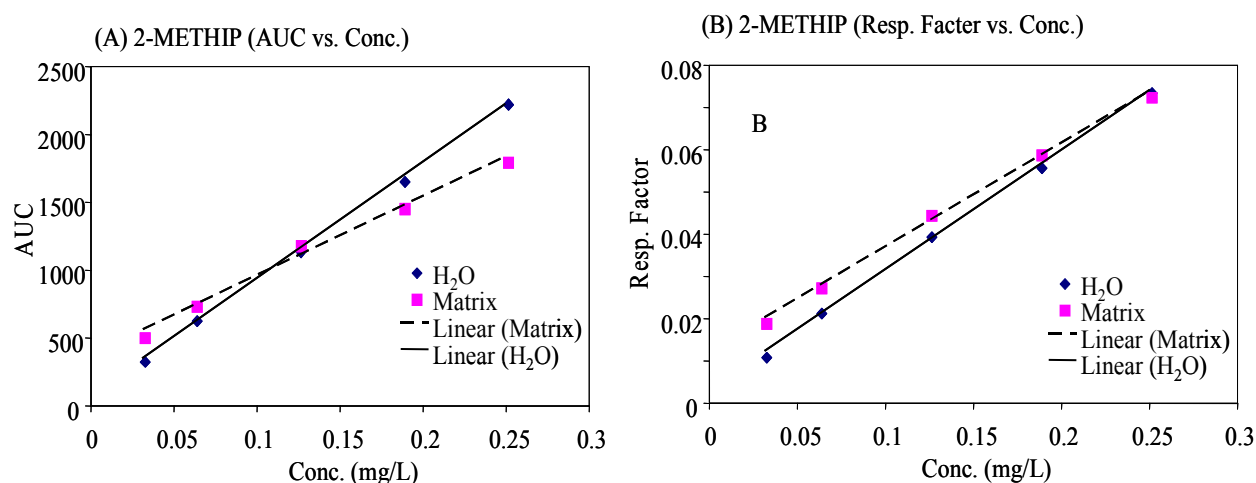


Figure 3.3: The plots of calibration solutions in H₂O and in urine matrix. Figure 3A is the linear regression plot of AUC vs. Conc. used to determine the effects of matrix. Figure 3B is the linear regression plot of Resp. Factor vs. Conc. used to determine the correction of internal standard on the slope. The variations in slope between each curve in 3A and 3B were determined using the Student's t test at the 95% confidence level.

These findings indicate the effectiveness of internal standards to correct for any matrix effects. Therefore, matrix effects were observed for some analytes, however, the use of response factors as a ratio of analyte area to internal standard area adequately compensated for any error that may have resulted from these effects.

3.3.6 Accuracy

The accuracy of each analyte was evaluated based on the percent recovery for two levels of spiked samples compared with a baseline of pooled urine. The percent deviation from the theoretical value for the recovered spike was less than 15%. The average recoveries for each analyte are found in Table 3.3.

Table 3.3: % Recovery of FIGLU, PYRGLU, 2-METHIP, 5-HIAA from pooled urine

Spike Solution	% Spike Recovery (mean +/- S.D., n = 3)			
	FIGLU	PYRGLU	2-METHIP	5-HIAA
Mid-Level	96.92 +/- 0.01	105.1 +/- 0.3	96.0 +/- 0.1	116.3 +/- 0.2
High-Level	101.3 +/- 0.1	107.9 +/- 4.0	97.7 +/- 0.1	112.0 +/- 0.3

3.3.7 Precision

Precision was calculated from two control values, normal and elevated, over five days. To establish precision, ten samples of each control were evaluated in one batch. Five samples of each control were run once per day for the remaining 4 days of the study. The with-in and between run precision for all days are displayed in Table 3.4. The within-run precision was less than 8% for all normal controls and no greater than 8.1% for elevated controls. The between-run

Table 3.4: Precision of normal and elevated controls in urine

Analyte	Control	Within-run*		Between-run**	
		Conc. (mg/L)	%CV	Conc. (mg/L)	%CV
FIGLU	NC	0.293 ± 0.022	7.57	0.291 ± 0.024	8.33
	EC	2.19 ± 0.17	7.96	2.22 ± 0.22	9.78
PYRGLU	NC	40.0 ± 1.9	4.75	39.6 ± 2.5	6.35
	EC	292 ± 14	4.86	295 ± 19	6.44
5-HIAA	NC	2.96 ± 0.13	4.26	2.91 ± 0.33	11.2
	EC	29.0 ± 1.6	5.66	28.7 ± 2.2	7.49
2-METHIP	NC	0.0329 ± 0.0025	7.66	0.0331 ± 0.0033	10
	EC	0.321 ± 0.012	3.86	0.320 ± 0.015	4.79

* Mean concentration (n = 5) of control values with S.D.

** Mean concentration (n = 30) of control values with S.D.

precision was less than 10% for FIGLU and PYRGLU. 5-HIAA and 2-METHIP had the greatest between run variation but were no more than 11.2%.

3.3.8 Sample stability

The data corresponding to sample preparation stability is presented in Table 3.5. All analytes deviated less than 14% from the initial values over a 72 h period. The deviation observed for all analytes was not great enough to affect the clinical relevance of the measurement. Given the precision of the collected data along with no negative observable trend in stability suggests that each analyte was stable on instrument for 72 h at 5°C.

Table 3.5: Sample preparation stability, % deviation over 3 days.

Analytes	24h	48h	72h
FIGLU	-10.2	-13.3	-11.7
PYRGLU	-8.62	1.03	-4.48
5-HIAA	-6.16	3.86	0.389
2-METHIP	-3.14	-0.314	-3.77

3.3.9 Patient ranges

Adult patient ranges were taken from approximately two hundred intra-laboratory samples. The values for normal and elevated results were taken from these ranges. The established ranges, corrected for creatinine, are presented in Table 3.6. Although no current patient ranges for FIGLU, PYRGLU, and 2-METHIP have been reported, urinary ranges for 5-HIAA are well established. The normal patient ranges determined by this method for 5-HIAA compare well with previously reported ranges [62,63].

Table 3.6: Normal and elevated ranges ($\mu\text{g}/\text{mg}$ Crea.), (n~200)

	Normal	Elevated
FIGLU	0.00 - 2.90	> 2.90
PYRGLU	0.00 - 96.0	> 96.0
5-HIAA	0.75 - 8.70	> 8.70
2-METHIP	0.00 - 0.10	> 0.10

3.4 Conclusions

An analytical method for the evaluation of four urinary metabolites using weak ion-exchange chromatography and tandem MS detection was developed and validated. The analytes were resolved using a pH gradient without high concentrations of counter-ion or buffer. Sample preparation was minimal, requiring only a simple dilution before analysis. Prepared samples

were found to be stable on instrument for up to 72 h, insuring that large patient batches can be assayed accurately. The method was found to be accurate and precise. The method is rapid allowing for high-through-put analysis and screening for metabolic disorders and toxicity.

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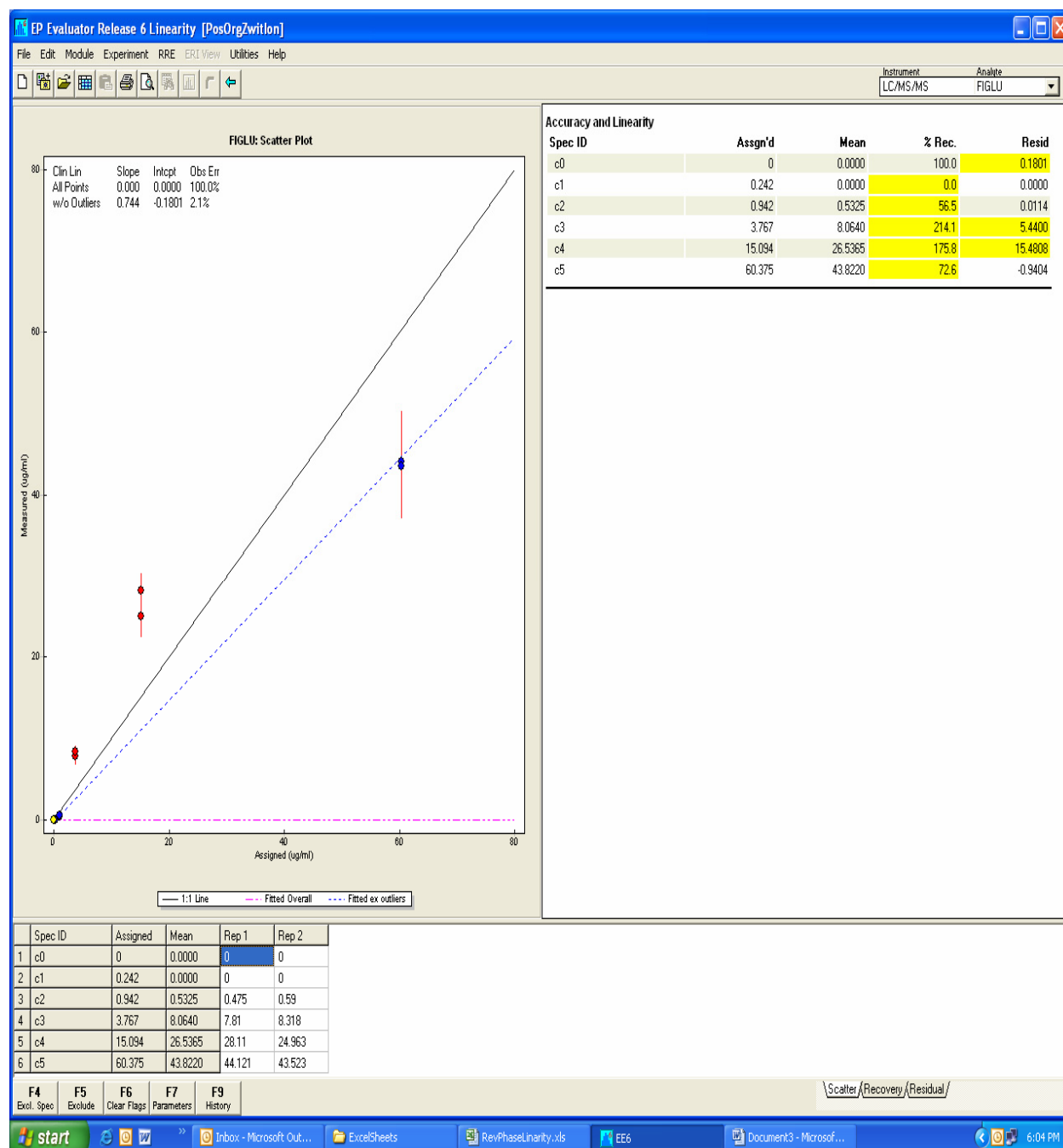
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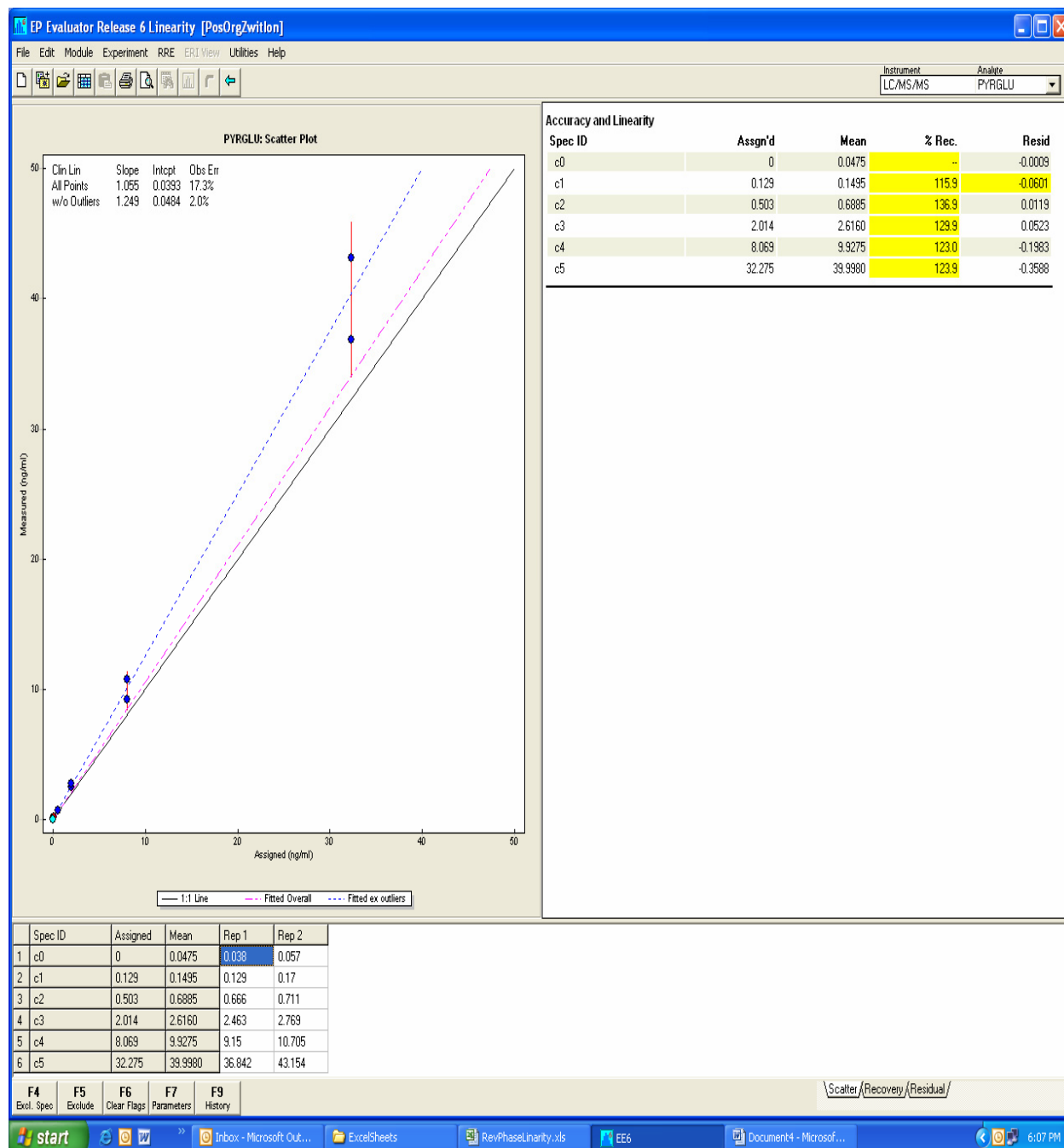
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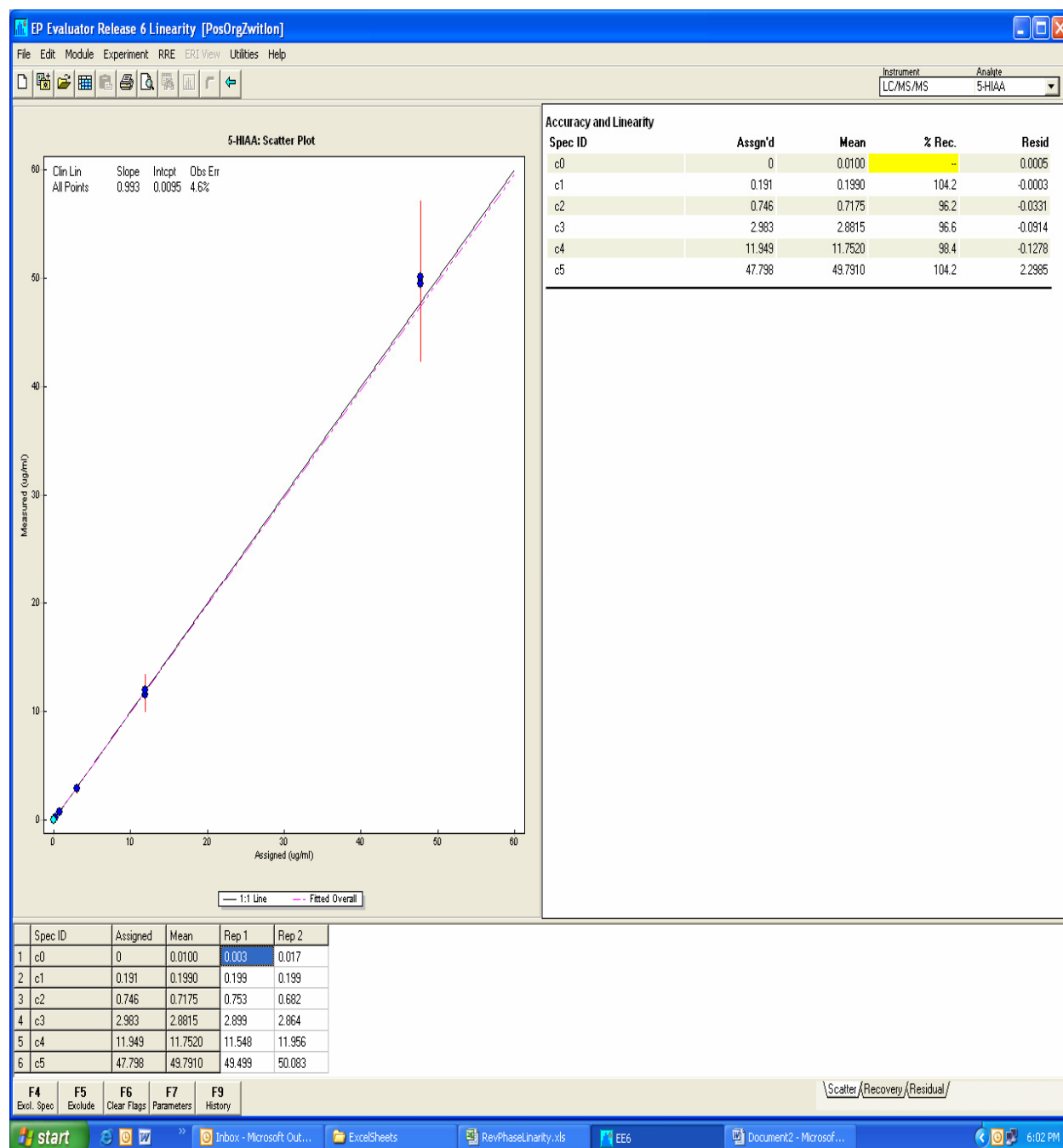
Appendix A1



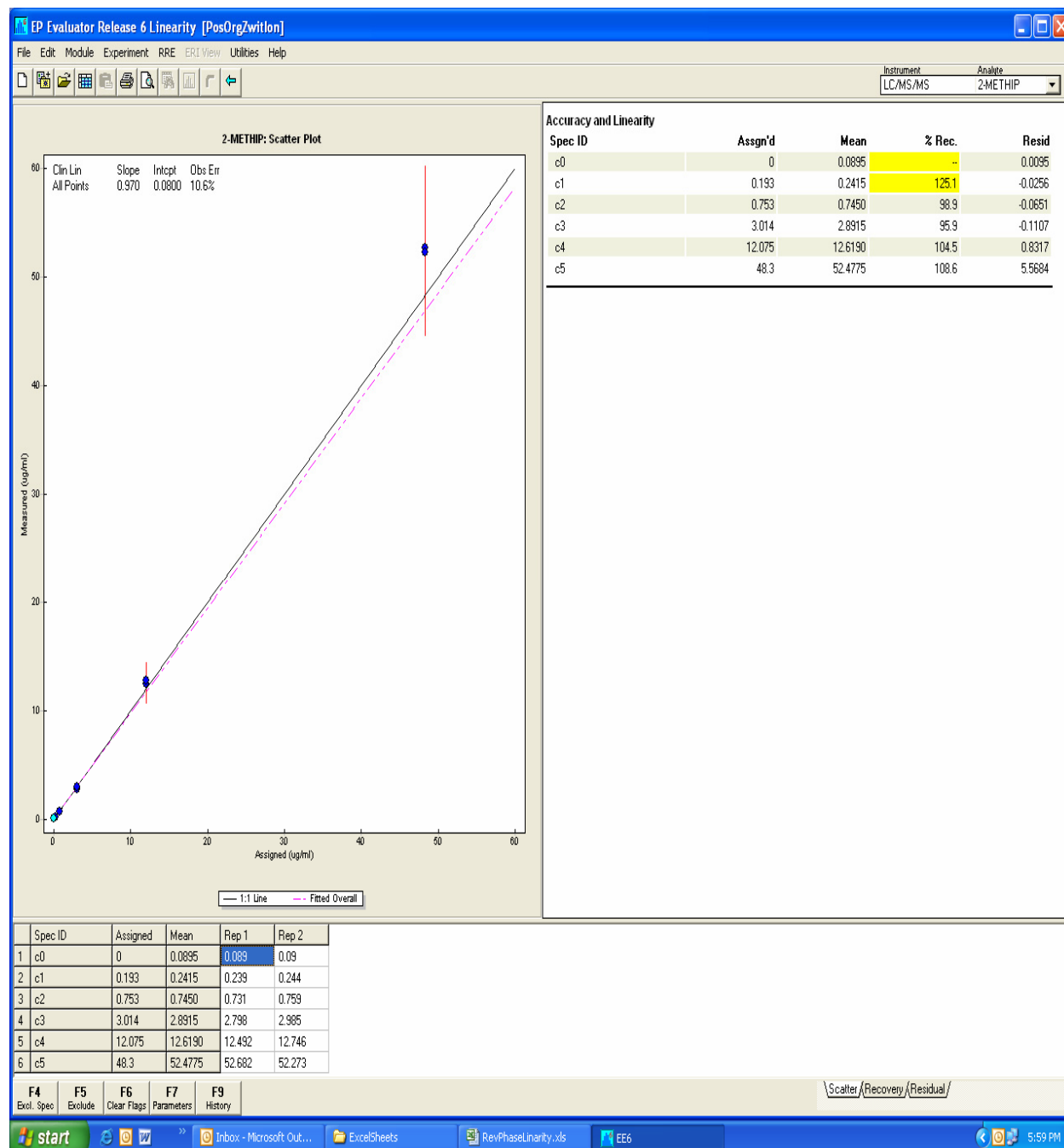
Appendix A2



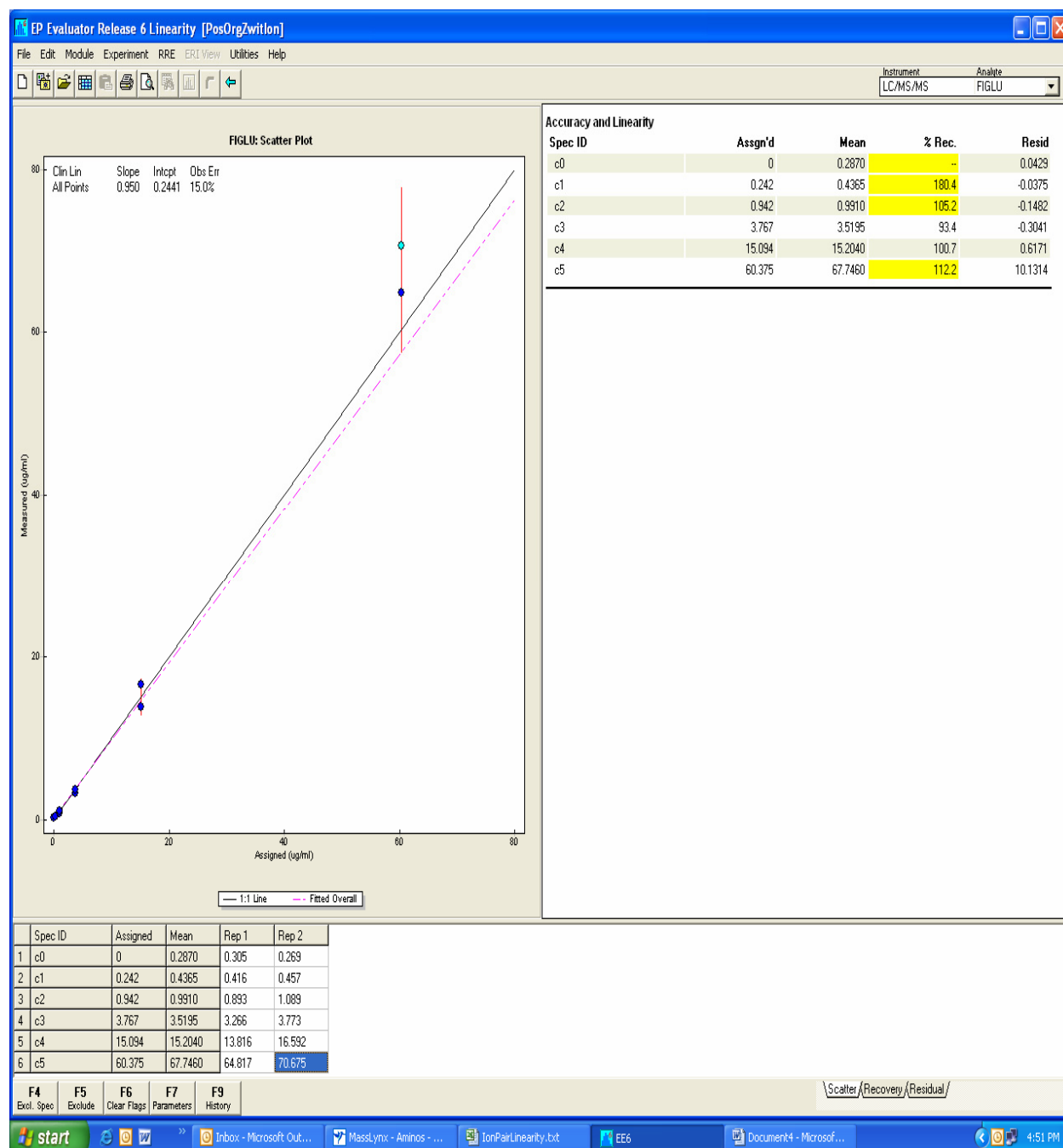
Appendix A3



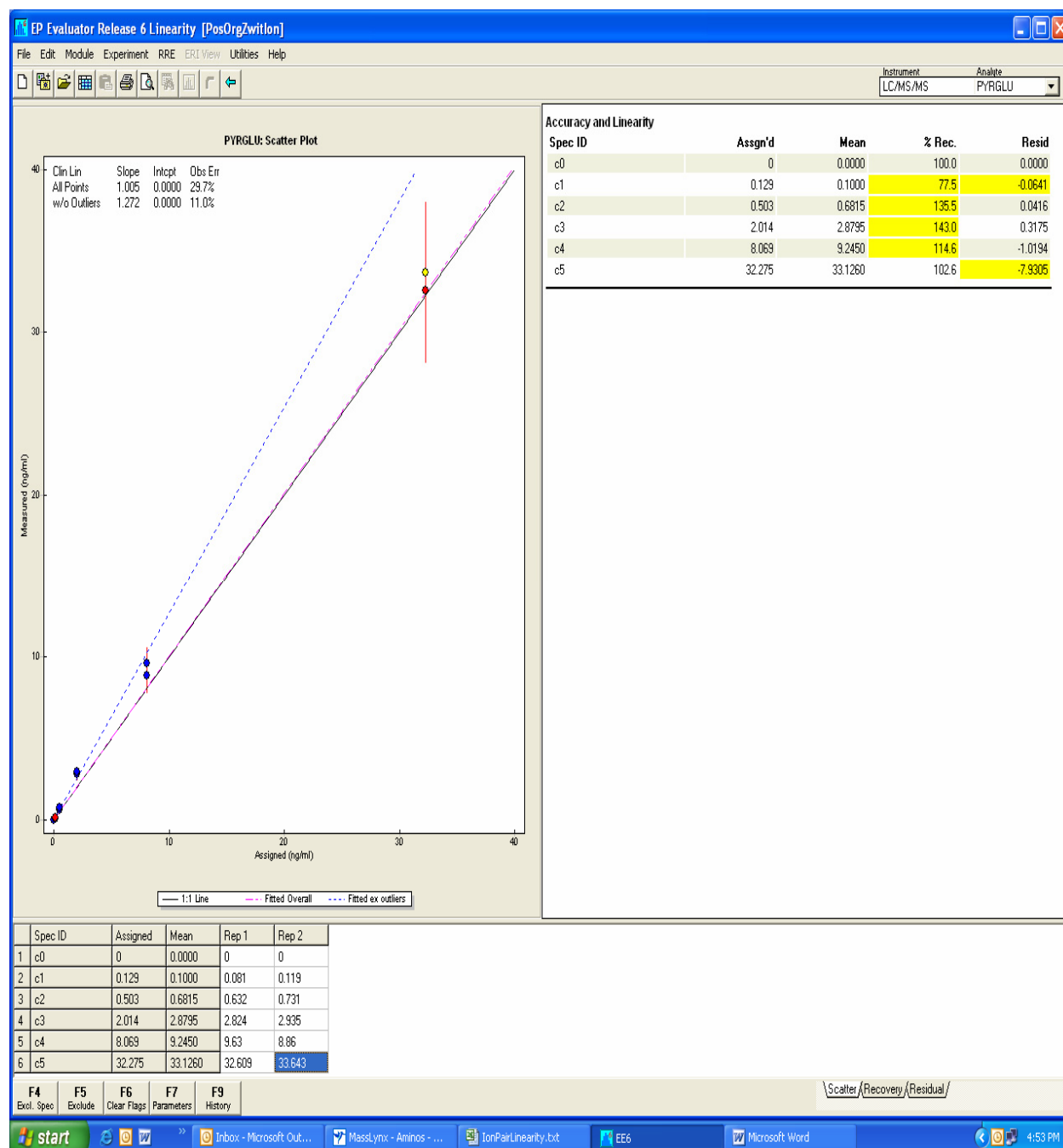
Appendix A4



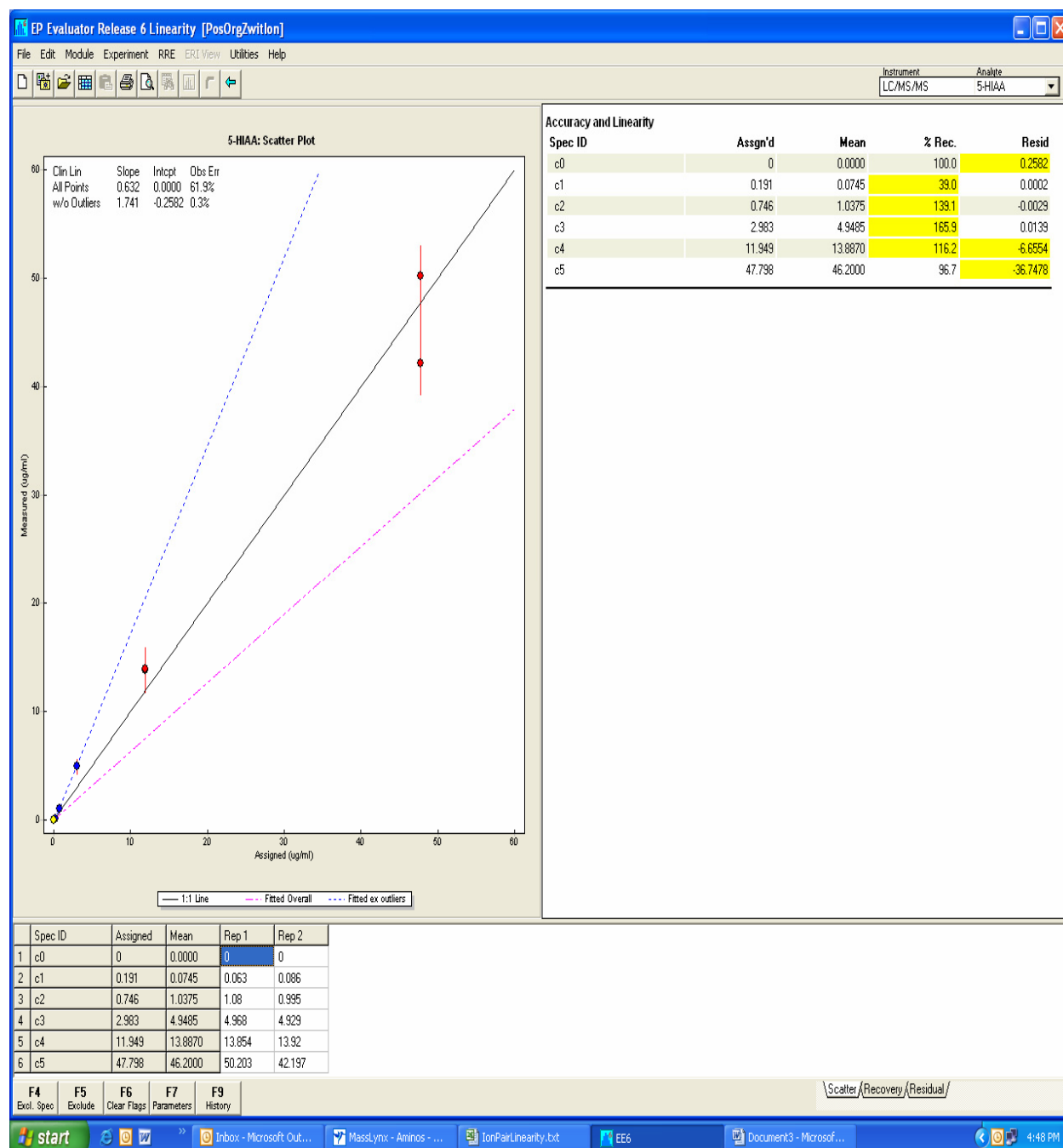
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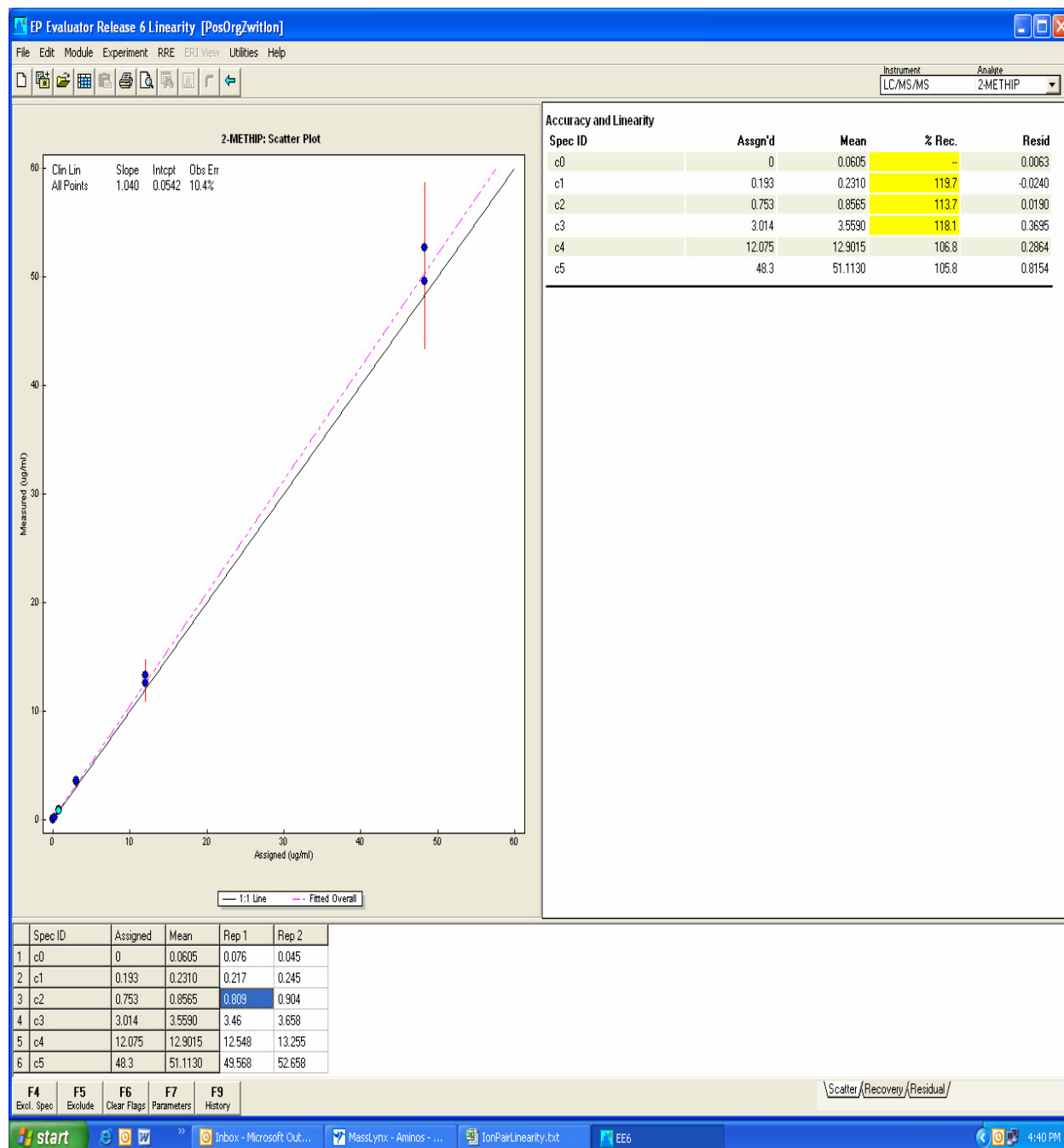
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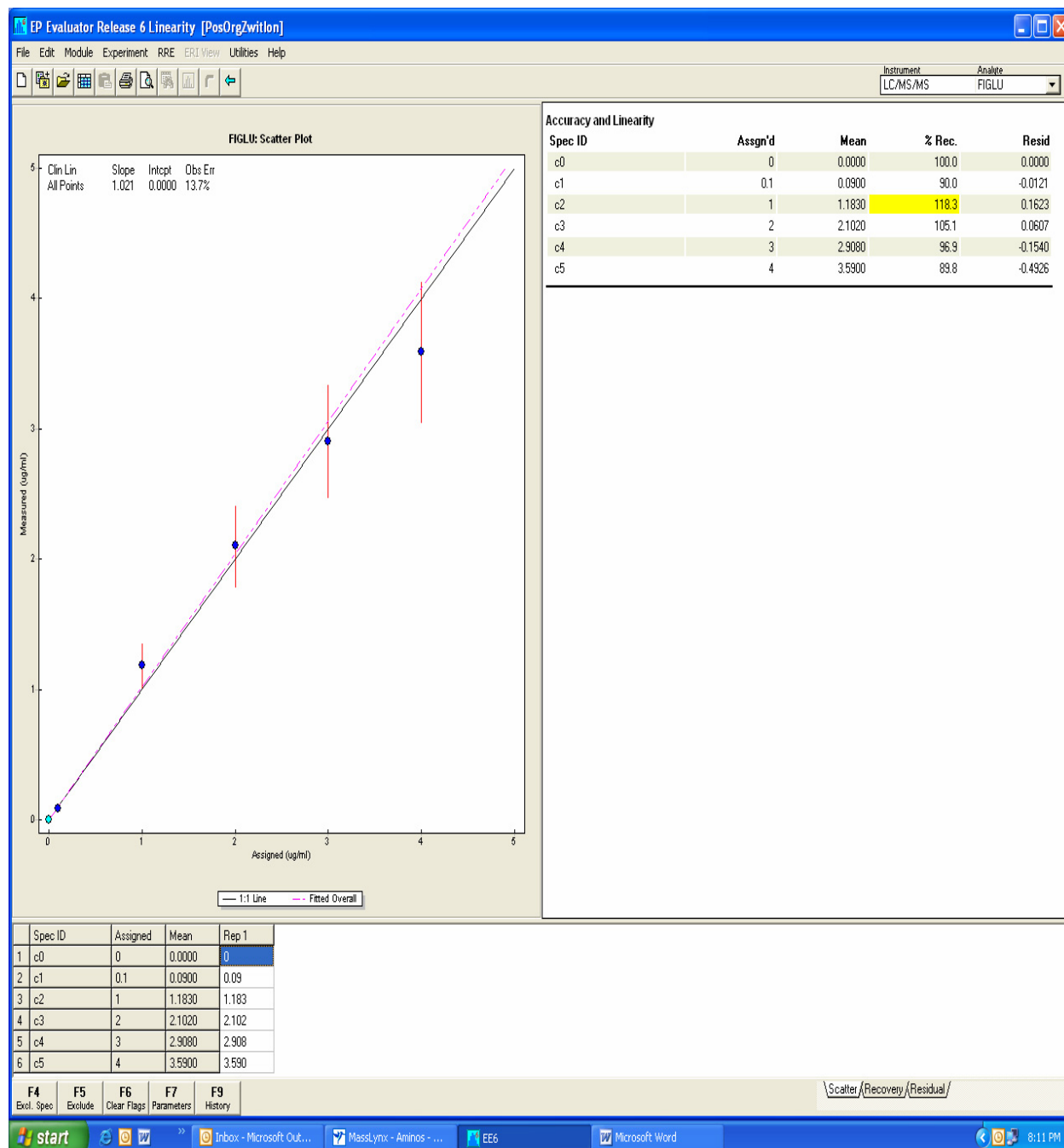
Appendix B3



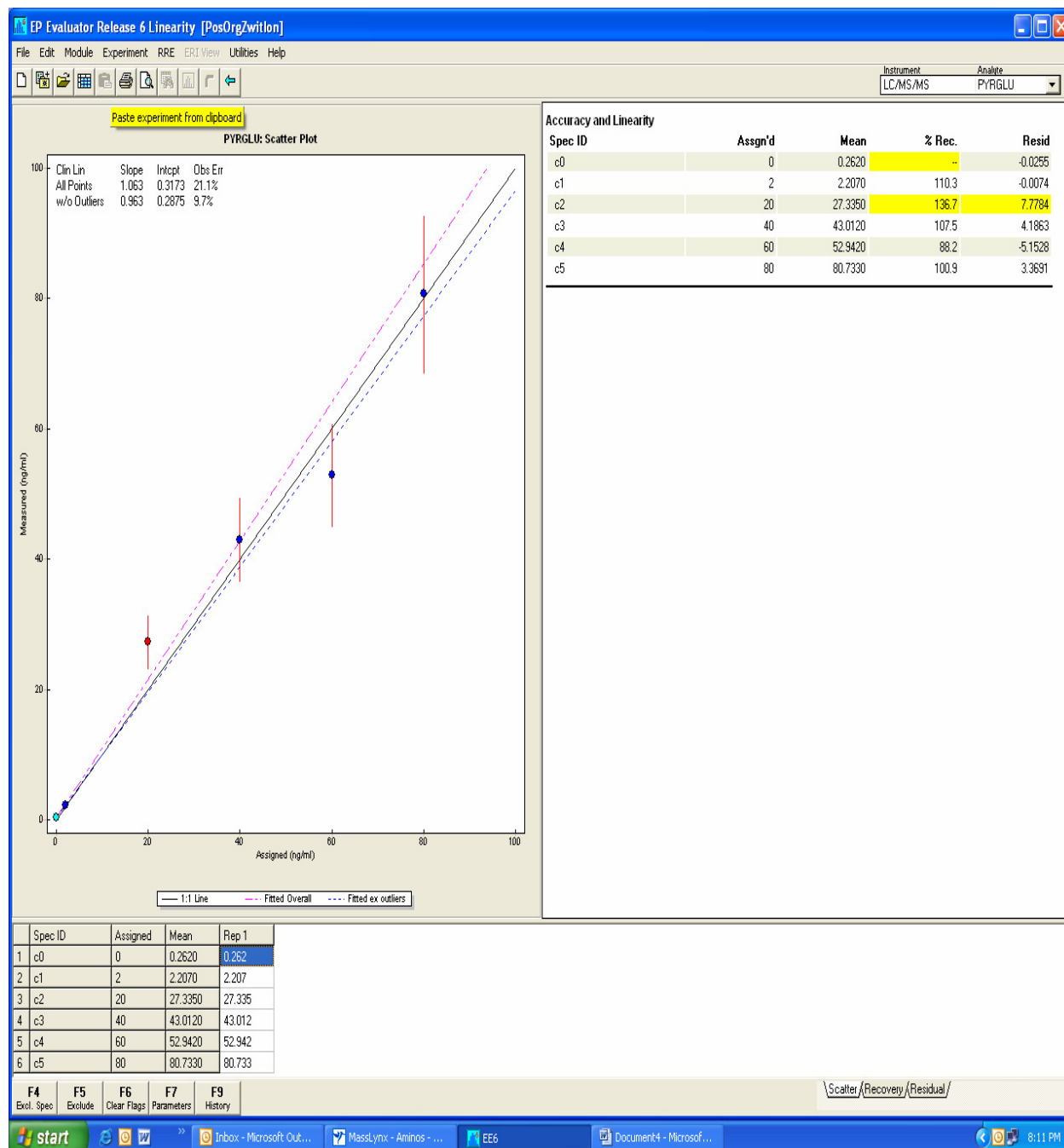
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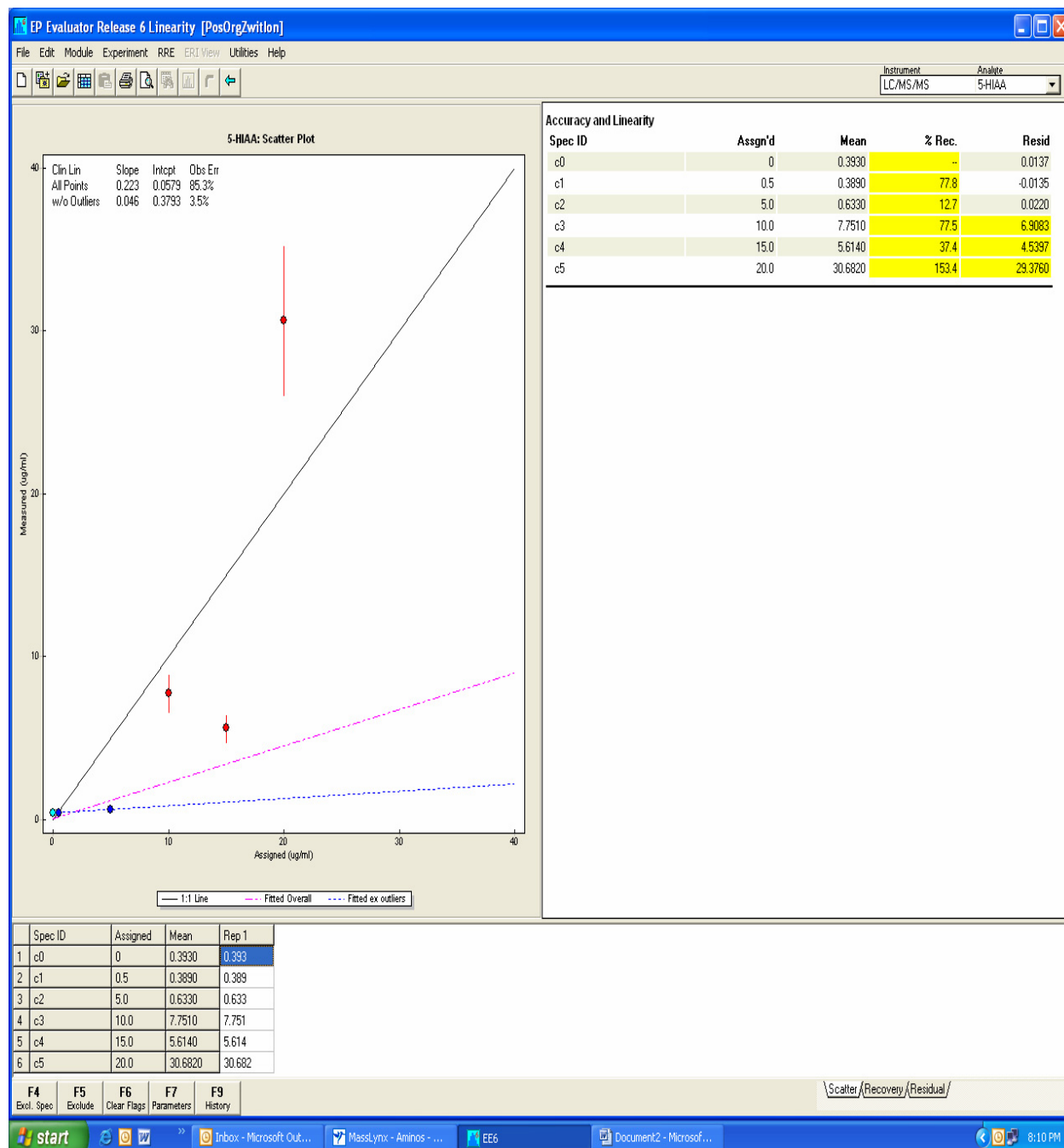
Appendix C1



Appendix C2



Appendix C3



Appendix C4

