

ESTROGEN ANALYSIS BY LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

by

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ABSTRACT

ESTROGEN ANALYSIS BY LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

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Estrogens are well known as female hormones associated with both health and disease. Although they have been reported as potential carcinogens, their use as therapy in the treatment of neurodegeneration has recently received a great deal of attention. The main goal of this work was to advance estrogen analysis using liquid chromatography – mass spectrometry (LC-MS) and associated techniques in order to support pre-clinical and clinical research on the neuroprotective effects of estrogens.

Native estrogen analyses were performed in different types of biological samples, including human cerebrospinal fluid (CSF), horse serum, phosphate buffered saline – bovine serum albumin (PBS-BSA), mouse serum, and mouse brain. A derivatization step with dansyl chloride was used in sample preparation prior to the introduction of LC-MS to enhance the electrospray ionization efficiency of native estrogens, since they are poorly ionizable in aqueous liquid phases. The four dansylated estrogens were successfully analyzed in cerebrospinal fluid (CSF) using a heart-cutting two dimensional reversed-phase (2D RP) LC-tandem MS system; detection limits in the part-per-trillion concentration range were achieved. Interestingly, later elution and 2D-separation were considered to be factors that reduced interferences in the analysis of 17 α - and 17 β -estradiol from CSF matrices. The matrix effects were further studied in other biological samples for these two estrogens, which are of most interest for their neuroprotective effects, using two sequentially connected C18 columns coupled with tandem MS.

The data revealed that matrix effects were observed in mouse brain and gelded horse serum but not in mouse serum and PBS-BSA.

In direct support of clinical trials, the stability of Premarin® Intravenous, a potential first response treatment for traumatic brain injury, was investigated under the conditions that mimic ambulance environment. The drug is a cocktail of estrogen metabolites mainly in the form of sulfates, generated from natural sources of pregnant mare's urine. The experiment was designed in a time frame of six months and Premarin® Intravenous was studied in the form of dry powder and reconstituted solutions. Although individual major components of the drug in reconstituted samples significantly varied at elevated temperatures of 100 and 120°F, they were considered to be fairly stable in the form of dry powder with the variation $\leq 10\%$. It was noted that the variation was larger for reconstituted samples stored at elevated versus room temperature.

Based on the diversity of estrogens and their metabolites, they were used as a model to study the mechanism of hydrophilic interaction chromatography (HILIC) in the conjunction with MS. The retention behavior of estrogens and their metabolites was investigated on five polar stationary phases. Two interaction models were applied, the partition and the surface adsorption model. Retention of the compounds, especially estrogen glucuronides, on the amide- and diol-bonded stationary phases could be best fit the surface adsorption model while mixed modes were observed on most stationary phases. The advantages of electrospray (ESI) – MS detection in conjunction with HILIC were also emphasized. The combination is considered to be ideal for providing an increased sensitivity over reversed phase separation approaches in many cases. A comprehensive literature review was carried out on this topic.

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CHAPTER 1

INTRODUCTION

Estrogens (Figure 1.1.) are best known for their role as sex hormones, responsible for the development and maintenance of secondary sexual characteristics, as well as some other systemic effects such as pregnancy and long-bone maturation.¹ They interact with estrogen receptors (ERs), members of nuclear hormone receptors, and exert diverse and important biological effects in animals and humans, such as mitogenic effects.^{2,3} Estrogen metabolism has long been recognized as the source for endogenous formation of estrogen conjugates. Both endogenous and synthetic exogenous estrogens are readily bio-converted into estrogen conjugates, mainly sulfates, followed by glucuronides.⁴ These conjugates are inactive for ERs and; therefore, do not promote ER-mediated activity.³ However, contrary to the initial intuition of more readily excreted estrogen conjugates, they are actually present for a much longer half-life than the native estrogens.⁴⁻⁶ These conjugates are hypothetically transported and taken into target cells; then, enzymatically hydrolyzed to the native estrogens, which can trigger the ERs via ligand binding.^{3,4,6} Although both estrogens and their metabolites are very important for regulation of physiological processes, a close relationship between exposure to estrogens and various types of cancer such as breast, ovarian, and prostate cancer, has been reported.^{7,8} Moreover, such compounds have been also listed as endocrine disrupting compounds (EDCs), contributing to the issue of environmental pollution. They can be released to the environment from natural sources such as phytoestrogens found in many plants including rice, carrots, potatoes, and etc., or from synthetic sources used in contraceptives and for therapeutic purposes.⁹ Regardless of having both negative and positive effects, estrogens have been widely used in pharmaceutical applications for the treatment of hormone imbalance, prevention of osteoporosis, and birth control. As an added benefit, estrogens can prevent and decrease the incidence of stroke-related

mortality in postmenopausal women.^{10,11} The administration of 17 β -E2 has also been reported to reduce mortality rates and increase survival from ischemic injury.^{12- 15}

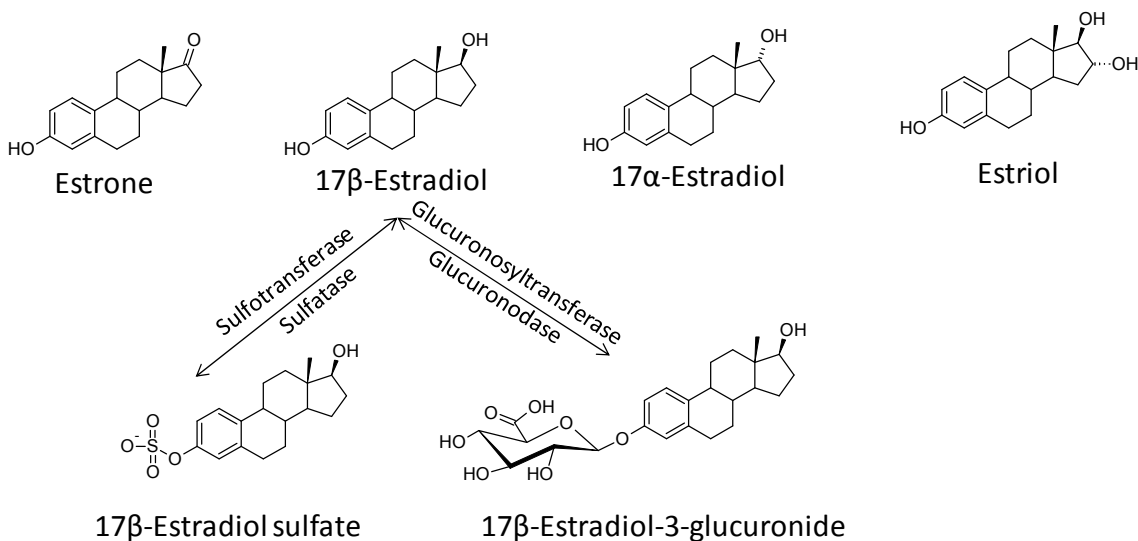


Figure1.1 Chemical structure of estrogens and their metabolites

Because of the complicated and important role of estrogens and their metabolites in health and environment, analytical research on these compounds has been recently emphasized, especially to support clinical and environmental studies. For many years, estrogens have been mainly determined by biological techniques known as enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays, which are highly sensitive methods (limits of detection in the pg/mL concentration range).^{1,16,17} However, these methods can require the use of radioactive materials, and artifacts have been reported to occur due to nonspecific binding of radioactive labels.^{15,18} Moreover, method reproducibility has been questionable in inter-laboratory assays.¹⁹ It is worthy to note that the College of American Pathologists has recommended that radioimmunoassays be improved with respect to analytical accuracy.¹⁵ Besides radiation detection, UV-Vis has been also used as a detection method for ELISA. Although this method is a cost effective technique and does not involve the use of radioactive material, overestimation of estrogen concentration is still an inherent issue.²⁰ As an alternative, mass spectrometry (MS) is a

good candidate for estrogen detection, especially when it is coupled with gas chromatography (GC) or high performance liquid chromatography (LC) separations.⁹ The substantial growth of LC-MS for the past decades has increased numbers of its applications in the field. The performance of this technique has largely improved by reducing the detection limits (pg-fg/mL) and enhancing analyte identification.^{9,21,22}

In this work, we present briefly the background of estrogen analysis by LC-MS (chapter 2) and the actual applications in our laboratory with various biological samples. The samples consisted of human cerebrospinal fluid (CSF), phosphorous buffer saline – bovine serum albumin (PBS-BSA), horse serum, mouse brain and serum, as well as Premarin®Intravenous (Pfizer/Wyeth Pharmaceuticals), an estrogen-containing drug from natural source of pregnant mare's urine. A method for trace level analysis of the four native estrogens by heart-cutting LC-tandem MS in CSF was fully validated and demonstrated (chapter 3).²³ This method will be used to support clinical research on neuroprotective effects of estrogenic therapy in humans. This clinical research is led and being carried out by Dr. Jane Wigginton' s team at The University of Texas – Southwestern Medical Center (UTSW; Dallas, USA), in collaboration with Dr. James W. Simpkins at the Univeristy of North Texas Health Science Center (UNTHSC; Ft. Worth, TX). A clinical trial was planned for one year with the participation of around 50 traumatic brain injury (TBI) patients; half of them being treated with a therapeutic estrogen-containing drug and the other half with placebo. The CSF samples from the patients are coded during the analysis and will be only decoded in the end of the project to compare the recovery of the two TBI patient groups (this process is ongoing at the time of preparation of this dissertation). Pramarin®Intravenous is used as estrogenic therapy for these patients; to be administered in a narrow therapeutic window (less than 3 hours) to be most effective.¹³ A six month stability study of the drug has been performed to support interest in the use of this estrogen-containing cocktail for intervention treatment in TBI cases through the Resuscitation Outcomes Consortium (ROC; <https://roc.uwctc.org/tiki/tiki-index.php>) (chapter 4).²⁴ Premarin®Intravenous is the only FDA-approved intravenous delivery of estrogens. To be effectively administered in the narrow time

window of effectiveness, Premarin®Intravenous should be stable stored in ambulances, which do not have refrigeration (in reconstituted form, if possible), over an extended time period. The stability study was performed on the dry powder and reconstituted solutions of the drug at different temperatures, which mimic the ambulance environment. In addition, the evaluation of matrix effects on estrogen analysis in other biological samples was also investigated to support pre-clinical studies and select appropriate surrogates for blank biological matrices in preparing standard curves when the availability of blank matrices is limited (chapter 5).²⁵ In related work, we focused some investigations on the fundamental area of chromatographic separations. With the emergence of hydrophilic interaction chromatography (HILIC) – MS in the field of metabolite profiling,²⁶ the advantages of this separation technique when coupled with MS were described in detail (chapter 6)²⁷ and estrogen conjugates were used as a model to study the mechanism of hydrophilic interaction chromatography (HILIC) (chapter 7).²⁸

CHAPTER 2

LITERATURE BACKGROUND

2.1 Structures and Metabolism of Estrogens

Native estrogens, consisting of estrone (E1), estriol (E3), 17 α - and 17 β -estradiol (17 α - and 17 β -E2), are steroid hormones with the general skeleton as shown in Figure 2.1, generated from cholesterol via testosterone and androstenedione in ovaries, brain, and body fat deposits.²⁹ They are synthesized in both males and females along with estrogen receptors (ERs), which act as ligand-activated transcription factors.²⁹ Estrogens are believed to interact with intracellular ERs and activate the expression of gene encoding proteins, which control important biological functions in animals and humans.^{4,30-33} Those receptors have higher affinity for 17 β -E2 than the other estrogens; thus, 17 β -E2 binds more vividly to the receptors and it is recognized as the predominant endogenous activator and initiator for a number of downstream ER-mediated cellular events.^{2,3} In contrast, 17 α -E2 is generally considered to be a less active and feminizing estrogen.³⁴

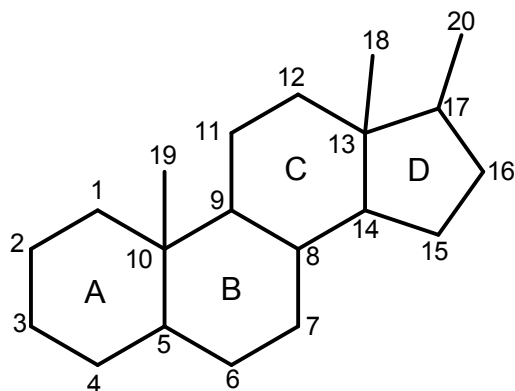


Figure 2.1 Steroid skeleton.

Estrogens are eliminated from the body by conjugation to hormonally inactive water-soluble metabolites, mainly the sulfates and glucuronides. Estrone sulfate is the most abundant

circulating estrogen at a concentration of 10 times higher than that of unconjugated estrone.^{3,35} This finding affirms the belief that sulfated steroid hormones serve an important role as steroid hormone precursors,³⁶ and sulfation/desulfation processes of estrogens may represent a vital endogenous system in regulation of biologically-active steroid hormones in target tissues.^{3,6,37} A current hypothesis is noted, that inactive estrone sulfate is transported to target tissues via the circulatory system and taken into target cells, most likely by anion transporters.³ After arrival in target cells, estrone sulfate is enzymatically hydrolyzed to E1 by intracellular membrane-bound sulfatases, and then, catalytically oxidized to active 17 β -E2 by 17 β -hydroxysteroid dehydrogenases.^{3,6,38} The transport of estrogen conjugates into target cells is not well understood, but it is believed that the process occurs by virtue of the high affinity of anion transporters for both estrogen sulfates and glucuronides.^{38,39} Estrogen glucuronides have received less attention as steroid hormone precursors, primarily because of their less abundance and more ready excretion from the body.⁶

Estrogen metabolism is presented in Figure 2.2. Estradiol and estrone are synthesized from the precursor of cholesterol through the formation of testosterone and androstenedione. Once formed, these three estrogens can be interconverted between each other or hydroxylated to estriol. All native estrogens can also be conjugated by sulfation or glucuronidation and vice versa.^{3,6,40}

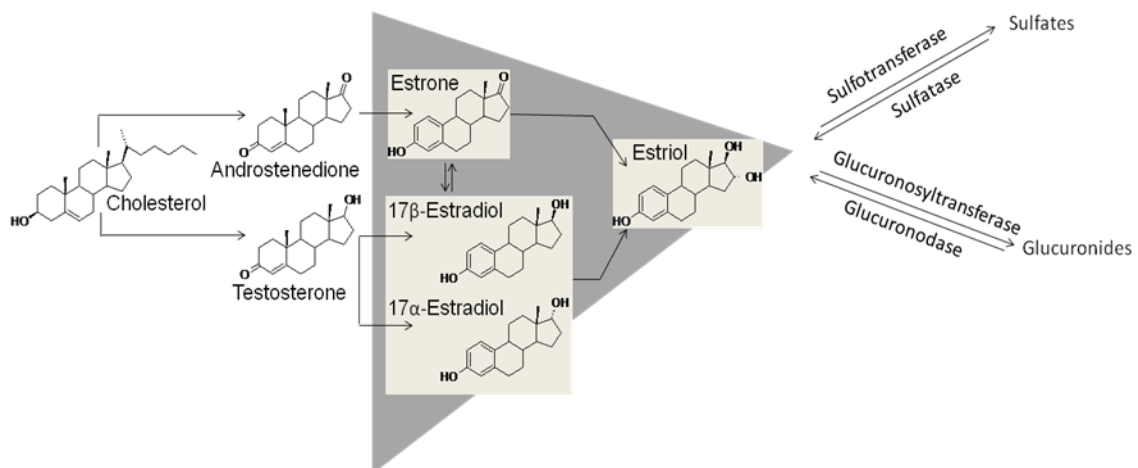


Figure 2.2 Estrogen metabolism (adapted from reference 39)

2.2 The Role of Estrogens in Health

Although estrogens have been long known as hormones that regulate reproductive functions in females, they also have protective effects on bone, the cardiovascular system, and the brain. They have been commonly used in the drug industry for contraception and hormone replacement therapy, where it has been recommended for menopause, menopausal symptoms, and in the prevention of osteoporosis and heart disease.⁴¹ Moreover, it has been demonstrated that estrogens, used in hormone therapy, can prevent and decrease the incidence of stroke-related mortality in postmenopausal women.^{10,11} The beneficial effects of estrogens in pre- and post-ischemic treatment have also been demonstrated in a number of laboratory studies;^{12,13,42,43} in these, 17β -estradiol (17β -E2) and its less bio-active metabolite, estrone (E1), have been the endogenous estrogens of greatest interest. The administration of 17β -E2 has been reported to reduce mortality rates and increase survival from ischemic injury,¹²⁻¹⁵ whereas 17α -estradiol (17α -E2) has been considered to be less active due to weaker binding affinity to estrogen receptors.⁴⁴ However, recent studies have indicated that 17α -E2 not only has strong neuroprotective activity, but also is locally synthesized in the brain.^{44,45} These findings support the notion that all native estrogen hormones may have beneficial effects in the brain and be a therapy for the treatment of stroke and traumatic brain injuries.

Unfortunately, the release of estrogens into the environment from either natural sources (phytoestrogens in plants) or synthetic sources (used in medicine) has negative impacts in humans and wildlife, even at very low concentrations from pg/L to ng/L levels.^{46,47} They are listed as endocrine disruptor compounds (EDCs),^{48,49} which are linked to reproductive abnormalities, such as decreased fertility and feminization,^{9,50} and related to various types of cancer, especially ovarian and breast cancer.^{7,8} Evidence for the estrogens associated with breast cancer has been reviewed in numerous studies including cell culture, animal, and human systems.¹ Their role in ovarian cancer has been also received more attention since its link to this disease was presented in epidemiology studies.¹ There are two mechanisms considered for how estrogens contribute to cancers. The first is prolonged exposure of target tissues or cells to excessive mitogenic

stimulation by estrogens, which increases cell proliferation.^{4,51} The second is the generation of genotoxic species such as free radicals from some estrogens.¹ For example, 4-hydroxyestradiol undergoes metabolic redox cycling, releasing superoxide and semiquinone/quinone intermediates. These metabolic intermediates may damage DNA, proteins, lipids, etc, induce cell transformation, and cause tumorigenesis (Figure 2.3).⁴

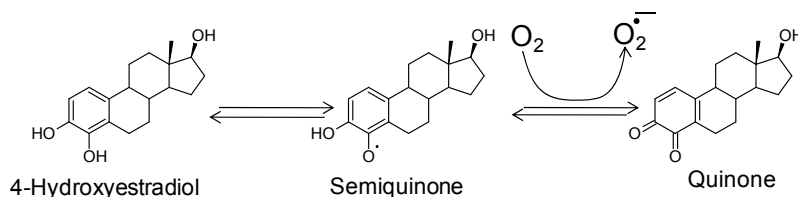


Figure 2.3 Metabolic redox cycling of 4-hydroxyestradiol

Although many articles have been published on the impact of estrogens in public health, effects of estrogens as neuroprotective therapy or carcinogens are still controversial. Much more effort and work is needed in the field, especially in the development of analytical methods for quantification and qualification of estrogens, for bio-monitoring in environmental and clinical research settings.

2.3 Methodology of Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

Many analytical methods have been developed and used to quantify and qualify estrogens, including immunoassays, as well as gas and liquid chromatography (GC or LC) coupled with electrochemical or mass spectrometry (MS) detection. Between these techniques, applications of LC-MS have grown rapidly in the past decade, owing mainly to significant instrumental developments in this field.^{9,48} Among LC-MS methodology, electrospray ionization (ESI) is extensively used as a primary ionization source interface to combine LC and MS, especially because of its simplicity, efficiency, and easy coupling with the flowing eluent stream generated by LC. However, there are still challenges in method development for estrogen analysis.

2.3.1 Analytical Challenges

The *first* challenge occurs when biological samples are analyzed. This is not an easy task because estrogens are usually present at extremely low concentrations (pg/mL to ng/mL) in complex biological matrices.¹ Matrix effects, ion enhancement and especially ion suppression, are commonly observed using ESI-MS. These effects are mainly caused by endogenous species in the background matrix. Acceptable degrees of matrix effects are debatable; in some laboratories, matrix effects are not acceptable but, in many circumstances, they may be tolerated depending on the validity and repeatability of the analytical method.⁵² As a consequence of ion suppression, sporadic reduction of signal intensity is possible and may lead to a decrease of method sensitivity.⁵² Ion suppression that varied by a factor of 8-10 between samples has been reported for steroid glucuronide analysis.⁵³ Not only that, the problem can be more complicated due to differential matrix effects caused by variability between individual subjects, the use of blank bio-matrix in varying lots (control samples obtained from different patients/subjects) to construct calibration curves, MS assays (cross-talk), sample preparation,⁵² and the ionization process.⁵⁴

The ionization efficiency of analytes during the ESI process is dependent on numerous factors, such as stability of mobile phases (pH, additives, solvent composition),²⁷ spray conditions (spray voltage, flow rate), and characteristics of the analytes.⁵³ Introduction of stable isotopically-labeled internal standards (SIL-IS)^{52,55,56} can mitigate substantial variation by partially correcting for signal irreproducibility⁵⁷ or recovery problems, but it does not completely eliminate these issues. Therefore, the analysis should be carefully performed in this type of samples with sufficient method validation⁵⁸ and matrix evaluation.⁵⁹⁻⁶¹

The *second* challenge is poor ionization efficiency of native estrogens in the liquid phase, which affects the response factor of the analytes and can result in low sensitivity of analytical methods, which feature ESI-MS detection. The *third* challenge is the structural diversity of estrogens; a wide variety of sometimes isomeric (and isobaric compounds) compounds exhibit a variety of hydroxyl, sulfate, and glucuronide functional groups. These estrogenic compounds have relatively different hydrophobicity or hydrophilicity as well as polarity, giving rise to

challenges in chromatographic method development. Measuring several of them at the same time is troublesome because analytical conditions are necessarily optimized for each type of functional groups to enable estrogen analysis at low levels of abundance in complex biological matrices.

2.3.2 Liquid Chromatography-Mass Spectrometry Method Development

Method development for estrogen analysis will be discussed in the context of partially resolving or overcoming analytical challenges mentioned in the previous section. Although hydrophilic interaction chromatography (HILIC)-MS has emerged as an important and complementary technique to reverse phase (RP) LC-MS, especially in the field of endogenous metabolite profiling,²⁶ RPLC-MS has been used as the most common separation method in estrogen analysis for many years.^{1,9,62,63} Developing a chromatographic methodology specific to certain estrogen analysis is the key to reducing interferences and matrix effects whereas sample preparation and tandem MS are also crucial factors.

2.3.2.1 Sample Preparation

Sample preparation for estrogen analysis in complex matrices, especially biological samples, requires sufficient selectivity for the analytes of interest in samples containing structurally similar compounds.⁶² These requirement can be achieved by using clean-up techniques to reduce interfering species and/or analyte derivatization strategies to enhance analyte signals.

Different extraction and clean-up techniques have been used such as solid phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PP). SPE is a favored approach in terms of specificity but it is time consuming and costly. Although the most popular SPE sorbent is C18,^{64,65} a major problem of this method is low organic washing volumes (20-30% methanol), which does not effectively remove hydrophobic interferences. Fortunately, companies such as Phenomenex, Inc. (Torrance, CA, USA) have recently commercialized special SPE phases dedicated for steroid analysis (e.g., Statra-X). This type of SPE provides

additional features, π - π and hydrogen bonding interaction, enhancing the retention of steroids with up to 70% methanol wash.⁶⁶ Another advantage of the SPE method is the ability to couple it online with LC-MS, offering increased speed, better analyte recovery, and higher sensitivity due to pre-concentration.^{63,67,68}

In contrast, LLE has low cost and does not require additional equipment; however, to set up an automated system is not likely. Extracts obtained from SPE and LLE are considered relatively clean while those from PP may contain more impurities and less analytes, leading to higher ion suppression and reduced recovery. Ion suppression is mostly due to co-eluted interfering compounds that affect ESI droplet desolvation processes.^{27,52} The effect can result in random variations in signals, slopes of standard curves, sensitivity, and reproducibility of the method.^{52,61} The low recovery is primarily attributed to analyte co-precipitation with proteins during PP.⁶⁹ Variation in PP recovery mainly depends on characteristic of precipitants and sample composition. Acetonitrile has been found to be one of the most effective precipitants, in comparison to others such as trichloroacetic acid, acetone, chloroform/methanol, and ammonium sulfate. PP is amenable to combination with other sample preparation strategies to further reduce interfering species. In these cases, PP is normally carried out first, but analysts should always take care to evaluate recoveries for each sample preparation step.⁶²

When poorly ionizable estrogens are analyzed by ESI-MS, chemical derivatization can be used to produce more ionizable or permanently-charged species, which enhances the sensitivity up to several orders of magnitude. Many derivatization reagents have been recently studied to aid in lowering the detection limit for estrogens.²¹ A selection of reported derivatization reagents are shown in Figure 2.4 and include dansyl chloride,^{14,70} 2-hydrazino-1-methylpyridine, 2-fluoro-1-methylpyridinium p-toluenesulfonate,⁵⁰ isonicotinoyl azide,⁷¹ and pyridine-3-sulfonyl chloride.⁷² For the major active estrogen E2, a limit of detection of 50 pg/mL has been achieved using dansyl chloride as derivatization reagent in combination with reverse phase LC- ion trap MS in the positive mode. The acetonitrile-water mobile phase was acidified to protonate the derivatized targeted molecules.¹⁴ The sensitivity can be improved to the fg/mL level when isonicotinoyl azide

is used, but this derivatization reagent is less selective than dansyl chloride in term of estrogenic derivatization. Isonicotinoyl azide reagent reacts with both phenolic and alcohol group while dasylation reaction only occurs at the phenolic group.

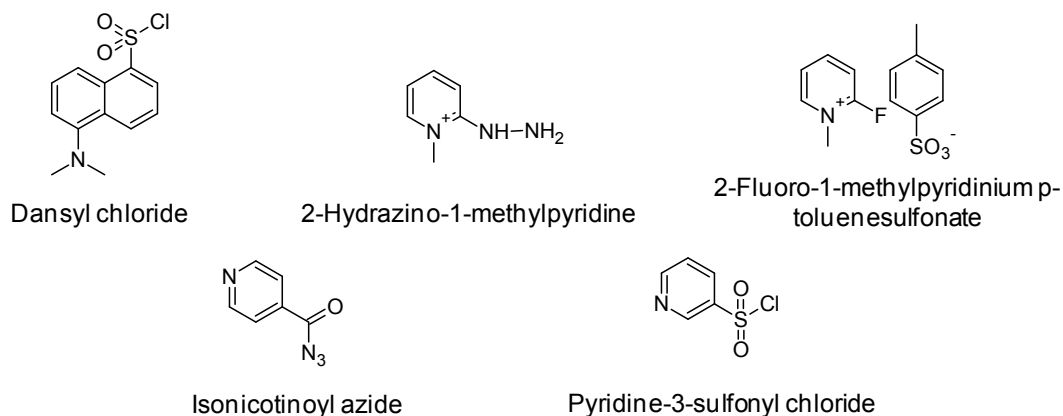


Figure 2.4 Chemical structures of derivatization reagents

2.3.2.2 Liquid Chromatography

Separation plays a vital role in method performance; since many ions observed in MS are common to different compounds, complete specificity without separation is not possible.^{63,73} Chromatographic stationary phases must offer sufficient retention and separation of the analytes and a good chromatographic method is mandatory to support MS detection. LC-MS in estrogen analysis is usually performed on reverse stationary phases (C18, C8, phenyl).⁶³ Monolithic columns have been used to improve the sample throughput with better separation efficiency at high flow rate compared to conventional LC columns. High sample throughput can be also achieved by using sub-2 μ m particle columns but these columns have high demands on LC system with operational pressure up to 1000 bar.⁶³

RPLC is very efficient in the analysis of native estrogens, which are less polar than estrogen conjugates. However, only a small amount of organic solvent in a predominantly aqueous mobile phase during RPLC separations can reduce ESI droplet desolvation, and

consequently, ionization efficiency.⁵⁵ In this scenario, HILIC is a complementary method to RPLC, using mobile phases containing high proportions of organic solvent. The mechanism of HILIC and the advantages of ESI-MS detection in conjunction with this chromatographic mode are discussed in detail in chapter 6.²⁷ Limit of detection of HILIC-MS was reported with one order of magnitude lower than those of RPLC-MS in the analysis of estrogen conjugates in human urine.⁷⁴

2.3.2.3 Tandem Mass Spectrometry

Tandem MS is becoming more indispensable to provide better selectivity and sensitivity in complex sample matrices. During the analysis, only selected product ions, from fragmentation of precursor ions, are transmitted and monitored in mass analyzers.⁷⁵ Most of other chemical noise is removed to help reduce false positive or false negative rates.⁶² The combination of LC with tandem MS is highly beneficial and widely applied for estrogen quantification in complex samples such as biological or environmental samples.^{8,62,63,75} The method has also been used to clarify unambiguous identification of unknown compounds or estrogens analogues that possess the same mass and similar retention time.^{76,77} For the analysis of estrogen conjugates, fragmentation of the analyte ions by loss conjugate groups (sulfate or glucuronide) has been observed at the orifice of MS.⁶² This leads to the decrease of sensitivity but the use of LC-tandem MS can overcome this by monitoring the total de-conjugated product ions produced from the fragmentation at both the orifice and mass analyzer. In spite of superior sensitivity and selectivity, the method is still limited due to economic constraints (tandem MS instruments are relatively expensive) and the persistent issue of matrix effects.

2.3.2.4 Two Dimensional Liquid Chromatography – Mass Spectrometry

Although applications of two dimensional (2D) LC-MS in estrogen analysis is extremely limited, the technique still provides a very promising method for analyses of complex samples in

terms of sensitivity and selectivity; more attention should be paid to this field. 2D-LC-MS can provide better separation and minimize matrix effects and interferences caused by co-eluting impurities, increasing a number of estrogens that can be resolved and identified.⁵⁷ This is a powerful technique but requires a sufficient orthogonality between the two separation dimensions. In other word, retention times of the analytes in the two dimensions must be non-correlated to some extent.^{78- 81} Two common 2D-LC modes have been described, including comprehensive and heart-cutting modes.

In comprehensive mode, the entire sample eluted from the first dimension is transferred and subjected to the second dimension. The transfers are performed in subsequent aliquot fractions collected in two alternating sampling loops or trapping columns in multiple repeated cycles.^{82- 85} A simpler form of this mode is the combination of restricted access material (RAM) and analytical column. It is based on column-switching technique, in which the analytes are retained on RAM as the first dimension while other impurities are washed off; then, the effluent from the first dimension is then switched and the retained analytes of interest are introduced to the analytical column as the second dimension. The method was applied previously to quantify native estrogens along with other EDCs in effluents and river water samples.⁵⁷

Even though comprehensive 2D-LC-MS has been widely applied in challenging analytical separations, such as those encountered in proteomics.^{86- 88} the most common technique is fraction heart-cutting mode, especially with applications in the field of small molecule analysis. In this mode, only selected fractions of the effluent from the first dimension are diverted and re-injected into the second dimension.^{82,89,90} Many combinations of separation methods have been reviewed with acceptable chromatographic orthogonality and compatible coupling with MS. RP is normally involved as one of the dimensions and the other dimension can be HILIC, size exclusion, normal phase, or even other RP.^{91- 94} The optimization of separation in this mode is less difficult than in the comprehensive mode, in which speed and efficiency allow real time analysis in the second dimension only during the period of time that the next fraction is collected from the first dimension.⁸² Since heart-cutting 2D-LC-MS can substantially improve the separation

and enhance sensitivity with fewer efforts in method development, it was used in one of our research projects to simultaneously quantify the four native estrogens in human cerebrospinal fluid (Chapter 3). In this work, two types of reverse stationary phases, C18 and amide-embedded C18, were used to separate four dansylated estrogens along with their deuterated internal standards in the conjunction with tandem MS.

CHAPTER 3
SIMULTANEOUS QUANTIFICATION OF FOUR NATIVE ESTROGEN HORMONES AT TRACE
LEVELS IN HUMAN CEREBROSPINAL FLUID USING LIQUID CHROMATOGRAPHY-TANDEM
MASS SPECTROMETRY

3.1 Introduction

Estrogens are best known for their role as sex hormones. They are not only important components of the reproductive system and bone health, but they also play a crucial role in neuroprotective processes.^{29,95} It has been demonstrated that estrogens, used in hormone therapy, can prevent and decrease the incidence of stroke-related mortality in postmenopausal women.^{10,11} The beneficial effects of estrogens in pre- and post-ischemic treatment have been demonstrated in a number of laboratory studies;^{12,13,42,43} 17 β -estradiol (17 β -E2) and its less bio-active metabolite, estrone (E1), have been the endogenous estrogens of greatest interest. The administration of 17 β -E2 has been reported to reduce mortality rates and increase survival from ischemic injury,¹²⁻¹⁵ whereas 17 α -estradiol (17 α -E2) has been considered to be less active due to weaker binding affinity to estrogen receptors.⁴⁴ However, recent studies have indicated that 17 α -estradiol not only has strong neuroprotective activity, but also is locally synthesized in the brain.^{42,45} These findings support the notion that all native estrogen hormones may have beneficial effects in the brain, particularly for the treatment of stroke and traumatic brain injuries. Thus, development of analytical methods that allow simultaneous measurement of all native estrogens (E1, 17 β -E2, 17 α -E2, and estriol (E3)) is important for supporting future research to investigate and further define these physiological effects.

Research on the neuroprotection effects of estrogen have been primarily performed on rodents^{13,42,96} due to limited availability of samples such as brain tissues, cerebral blood, or cerebrospinal fluid (CSF) from humans. Among these, CSF is the most accessible. CSF is mainly produced by the brain and contains components that reflect the physiological state of the central

nervous system.^{97,98} Understandably, obtaining significant quantities of CSF for method development and analysis can still be difficult.

Trace level quantification of small molecules in biological samples is challenging. Most biological matrices contain a wide variety of abundant species that can interfere with separation and detection of analytes of interest. Native, unmodified estrogen compounds represent an analyte class that can be present in low quantities in many biological matrices and do not exhibit appreciably-high absorption properties or mass spectrometric ionization efficiencies to allow for facile detection without some form of preconcentration.^{1,62} Radioimmunoassay is a highly sensitive method currently used for estrogen quantification in plasma (measurable in the pg/mL concentration range). However, this method requires use of radioactive materials, and artifacts have been reported to occur due to nonspecific binding of radioactive labels.^{15,18} Moreover, method reproducibility has been questionable in inter-laboratory assays.¹⁹ It is worthy to note that the College of American Pathologists has recommended that radioimmunoassays be improved with respect to analytical accuracy,¹⁵ and that no such assay has ever been reported validated for analysis of estrogens from CSF. As an alternative, mass spectrometry (MS) is a good candidate for estrogen detection, especially when it is coupled with gas chromatography (GC) or high performance liquid chromatography (LC) separations.⁹ The substantial growth of LC-MS for the past decades has increased numbers of its applications in the field. The sensitivity of LC-MS can be further enhanced to low pg/mL detection limits when chemical derivatization is employed in conjunction with the use of an electrospray ionization source,^{50,71,72,99} despite the additional sample preparation steps required. The introduction of protonated or charged moieties to poorly ionizable estrogens in liquid phase dramatically increases their detection sensitivity using ESI-MS. Among several derivatization reagents, dansyl chloride is the most popular reagent^{14,15,44,70,75} and has been originally used for the fluorometric detection of estrogens.¹⁰⁰ However, dansylated E1 and 17 α -E2 have not previously been chromatographically resolved on standard reversed phase columns (Fig.2 in ref.44) resulting in co-elution of the two compounds.

Additionally, their commercial deuterated standards are isobaric, making their analysis by stable isotope dilution and reversed phase LC-MS more difficult. Unless customized isotope standards are used, such as in the work reported by Toran-Allerand *et al.*,⁴⁴ complete separation of these compounds is totally necessary. As researchers work to achieve lower and lower detection limits, the use of derivatization reagents will maintain an attractive approach to do so.

In this work, we present the development and validation of a sensitive method, which involves liquid-liquid extraction, derivatization with dansyl chloride, and LC-MS/MS, to simultaneously quantify four native estrogens (E1, 17 α -E2, 17 β -E2, and E3) in human CSF. To address difficulties associated with chromatographic resolution of all four dansylated estrogens in reversed phase mode, a heart-cutting two-dimensional (2D) column set-up has been developed that is amenable for use with standard LC-MS instruments. Whereas comprehensive 2D LC instruments often require additional pumps, solvent reservoirs, switching valves, and degassing systems,^{101,102} this method can be used with only the addition of a single six-port valve to a standard binary gradient LC system. The heart-cutting approach requires initial separation of the sample on a commercial amide-C18 (a polar-embedded C18 phase), followed by separation of a critical pair on a commercial C18 phase. The method has been fully validated according to accepted bioanalytical method validation procedures.¹⁰³ From 1 mL of CSF, detection limits in the parts-per-trillion concentration range for each of the four estrogens are reported with satisfactory accuracy, precision, and recovery. To the best of our knowledge, this is the first reported method for separation of all four endogenous estrogens in their dansylated forms. The method was applied to the analysis of estrogens in CSF collected from patients admitted to the hospital following traumatic brain injury.

3.2 Experimental

3.2.1 Chemicals and Reagents

All estrogens and dansyl chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Internal standards consisting of estrone-2,4,16,16-d₄ (E1-d₄) and 17 β -estradiol-16,16,17-

d3 (17β -E2-d3) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Estriol-2,4-d2 (E3-d2) and 17α -estradiol-2,4-d2 (17α -E2-d2) were purchased from CDN isotopes (Quebec, Canada). Sodium hydrogen carbonate, sodium hydroxide, and dextran-coated charcoal were obtained from Sigma-Aldrich (St. Luis, MO, USA). Formic acid, acetone, and methanol, as well as HPLC-MS grade acetonitrile (ACN) and water, were purchased from J. T. Baker (Phillipsburg, NJ, USA).

3.2.2 Preparation of Charcoal-Stripped Cerebrospinal Fluid

Dextran-coated charcoal was used to strip free hormones from CSF for method validation and calibration. An amount of 2 g of dextran-coated charcoal was added to 100 mL of CSF. The mixture was shaken overnight at 4°C, followed by centrifugation at 2000 x g according to manufacturer guidelines. The CSF supernatant was filtered through a 13 mm syringe filter (0.2 μ m PTFE membrane).

3.2.3 Preparation of Stock Solutions, Calibration Standards, and Quality Control Samples

Stock solutions of estrogens and deuterated estrogens were prepared by accurately weighing and dissolving approximately 1.0 mg of each compound in 1.000 mL of methanol. Working standard solutions were prepared at 10 and 1 ng/mL by diluting the stock solutions with methanol. The calibration curve samples were prepared in duplicate by adding an appropriate amount of working standard solutions to 1 mL aliquots of charcoal-stripped CSF (CSCSF) to obtain standards of 20, 30, 50, 100, 150, 200, 250, and 300 pg/mL. Quality control samples were prepared in quintuplicate at three concentration levels (70, 110, and 220 pg/mL). Each of the four stable isotopically-labeled internal standards was spiked in all prepared and unknown samples at 125 pg/mL concentration, prior to processing.

3.2.4 Sample Preparation

A volume of 2.000 mL of ethyl acetate was added to 1.000 mL of each CSF sample. The mixture was vortexed for 30 seconds, shaken for 15 minutes, and then centrifuged for 20 minutes

at 2000 x g. Ninety percent (1.800 mL) of the organic supernatant was transferred into a 5-mL polypropylene centrifuge tube. The extraction was repeated a second time and the supernatants were combined. A total of 3.600 mL of supernatant was evaporated until dryness under a nitrogen stream at room temperature. The residue was reconstituted with 50.0 μ L of sodium bicarbonate buffer (100 mM, pH 10.14) and then dansylated by adding 50.0 μ L of dansyl chloride (1 mg/mL in acetone). The samples were incubated for 5 minutes at 60°C, loaded into autosampler vials with glass inserts, and then transferred to the autosampler for LC-MS/MS analysis.

3.2.5 LOD, Recovery, and Matrix Effect

Method validation, according to FDA guidelines,¹⁰³ was performed in charcoal-stripped CSF (CSCSF). Samples used for limit of detection were prepared in septuplicate at a concentration of 70 pg/mL. The accuracy and precision were determined by analyzing the quality control samples, in quintuplicate, at low, medium, and high concentrations. The recovery was evaluated by comparing the response of the analytes added to CSF before and after extraction. The relative slopes of calibration curves in CSCSF vs. no matrix were used to evaluate the presence of matrix effects in the experiment.

3.2.6 LC-MS/MS Conditions

The LC system (Thermo-Fisher Scientific, Inc., San Jose, CA, USA) was equipped with a Surveyor Autosampler and a Surveyor MS binary pump. The chromatographic system was arranged with the use of three analytical columns, including a Varian (Paolo Alto, CA, USA) Polaris Amide-C18 (2 x 100 mm, 3 μ m, 200 Å) and two Shimadzu (Tokyo, Japan) Shim-pack XR-ODS columns (2 x 100 mm and 2 x 75 mm, 2.2 μ m, 120 Å). This heart-cutting two-dimensional (2D) chromatographic configuration, shown schematically in Figure 3.1, was arranged to allow for heart-cutting to transfer sample components from the first to the second dimension using the built-in divert valve (2-position-6-port switching valve, VICI cheminert) on a LCQ Deca XP mass spectrometer (Thermo-Fisher Scientific, Inc., San Jose, CA, USA).

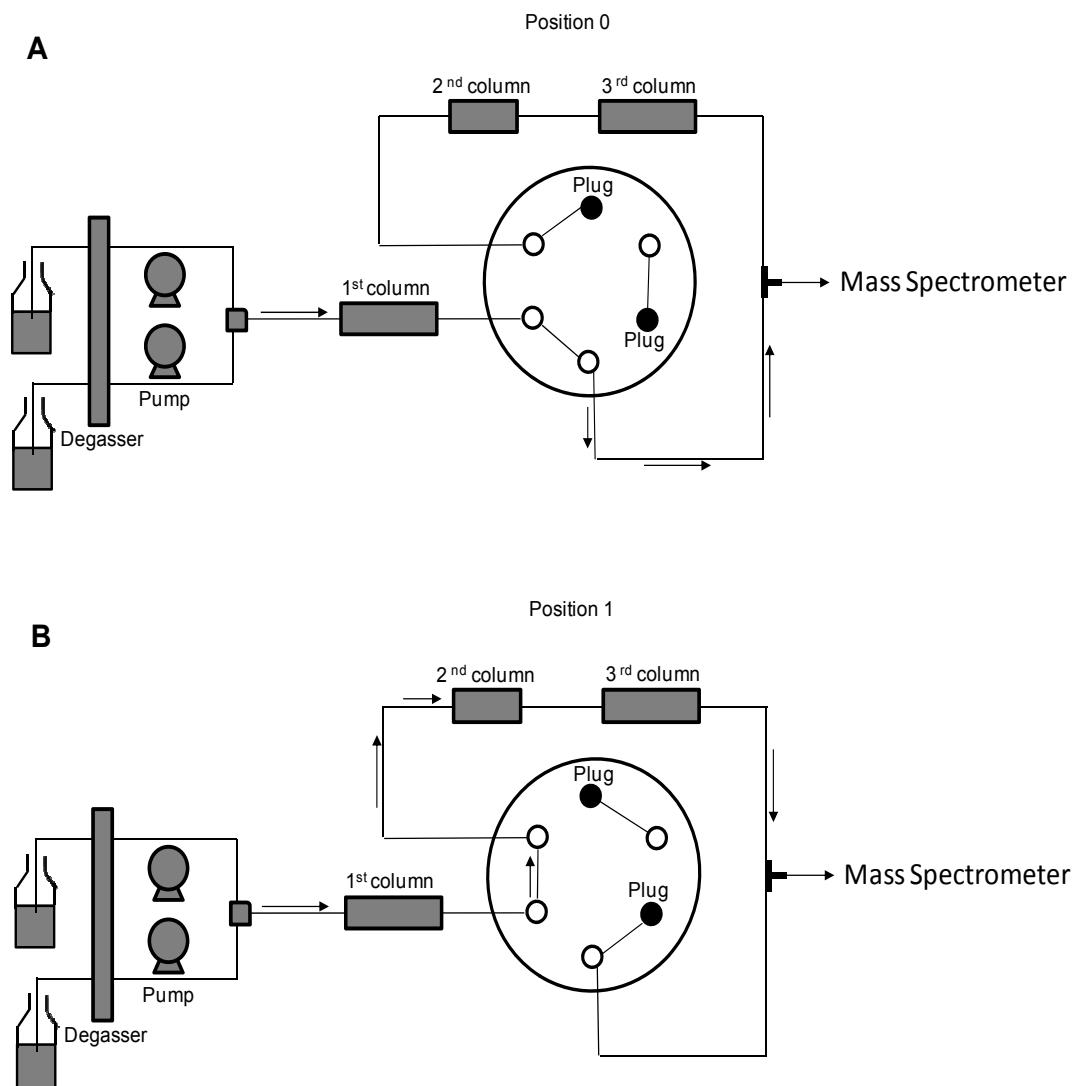


Figure 3.1 Schematic flow diagram of heart-cutting 2D-LC-MS system. (A) Position 0 and (B) Position 1 refer to divert valve settings.

The separation was performed with an elution gradient of mobile phase A ($\text{H}_2\text{O}/\text{ACN}/\text{formic acid}:95/5/0.1$) and mobile phase B ($\text{ACN}/\text{H}_2\text{O}/\text{formic acid}:95/5/0.1$). The LCQ was operated in the positive ionization mode with a conventional electrospray source. The separation period (45 minutes) was divided into 3 segments. Single reaction monitoring (SRM) MS/MS (parent $[\text{M}+\text{H}]^+ \rightarrow m/z$ 171 transition) was used in conjunction with two scan events for the analytes within each segment. The time course of the method, including mobile phase

compositions, flow rates, valve settings, and segmentation is presented in Figure 3.2. A detailed overview of instrument settings in each segment is given in Table 3.1. Data analysis was performed using Xcalibur (version 2.0) software.

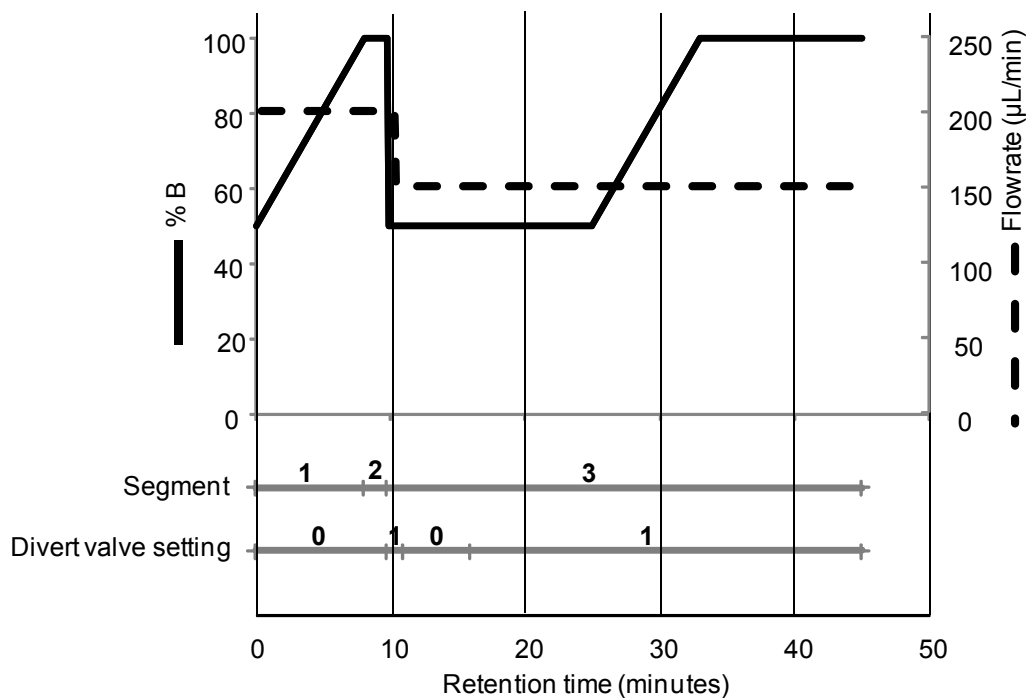


Figure 3.2 Flow rate, mobile phase, segment, and divert valve setting sequence event program for heart-cutting 2D-LC-MS system.

Table 3.1 Optimized instrumental settings for each segment of the heart-cutting 2D-LC-MS.

Analytical condition		Segment 1 (0-8 min)	Segment 2 (8-9.7 min)	Segment 3 (9.7-45 min)
Column		Polaris Amide C18	Polaris Amide C18	Shim-pack C18 XR-ODS
Mobile phase composition: (A) H ₂ O/ACN/Formic acid (95/5/0.1) (B) ACN/H ₂ O/Formic acid (95/5/0.1)		0-8 min: 50-100%B	8-9.7 min: 100%B	9.9-25: 50%B 25-33 min: 50-100%B 33-45 min: 100%B
Flowrate (200 μ L/min)		200	200	150
Valve position		0	0	9.7-11 min: 1 11-16 min: 0 16-45 min: 1
MS scan event		MS/MS	MS/MS	MS/MS
Event 1	Monitored compound	Dansylated E3	Dansylated E1	Dansylated 17 β - and 17 α -E2
	Parent mass (m/z)	522	504	506
	Isolation width (m/z)	1	1	1
	Collision energy (%)	42	42	42
	SRM range (m/z)	170.5-171.5	170.5-171.5	170.5-171.5
Event 2	Monitored compound	Dansylated E3-d2	Dansylated E1-d4	Dansylated 17 β -E2-d3 Dansylated 17 α -E2-d2
	Parent mass (m/z)	524	508	508.5
	Isolation width (m/z)	1	1	2
	Collision energy (%)	42	42	42
	SRM range (m/z)	170.5-171.5	170.5-171.5	170.5-171.5

3.3 Results and Discussions

3.3.1 Method Development

Although dansylation has been demonstrated to enhance the sensitivity during LC-MS in determination of estrogens, the inability to chromatographically resolve all four native estrogens in their dansylated form has been a significant limitation. This derivatization approach has been implemented in many recent publications to analyze estrogens in water⁵⁰ or different biological samples such as plasma, serum, or mouse brain;^{14,15,44,70,75} however, only two dansylated estrogens, E1 and 17 β -E2, were analyzed in most of the cases. These studies featured separations using reversed phase stationary phases including C12^{14,15} or phenyl⁷⁵ bonded phases. In our experience, dansylated E3 always elutes ahead of the other dansylated estrogens and can be easily separated. However, dansylated E1 and 17 α -E2 are co-eluted when standard C18 (Figure 3.3A) stationary phases are used (several different phases were tested; data not shown). In addition, their commercially-available deuterated forms, E1-d4 and 17 α -E2-d2, are isobaric, which makes quantitative analysis relative to isotopically-labeled internal standards difficult in this situation. Toran-Allerand *et al.*⁴⁴ have reported the measurement of dansylated E1, 17 α - and 17 β -E2 using customized isotopically-labeled internal standards, but the peaks of dansylated E1 and 17 α -E2 were still unresolved. Although the detection limits were in the low pg/mL range in their work, the description of specific values for detection limits for 17 α - and 17 β -E2 were a little ambiguous.

After screening several stationary phases consisting of C18, C6-phenyl, amide-C18, and modified cyclodextran phases, we determined that dansylated E1 and 17 α -E2 could be resolved on the Polaris Amide-C18 column. Unfortunately, dansylated 17 β - and 17 α -E2 co-eluted (Figure 3.3B) in this set-up, and since these compounds are also isobaric, the use of the Polaris Amide-C18 alone was not a viable option for simultaneous separation and quantification of all four estrogens. To address these problems, a heart-cutting 2D-LC arrangement was conceived, consisting of a binary pump, 3 columns, and a 2-position-6-port valve (Figure 3.1). The Polaris Amide-C18 column was used as the first dimension and two Shim-pack XR-ODS columns (175

mm total length) were used as the second dimension. To obtain baseline resolution of the critical pair, two C18 columns connected in series were required.

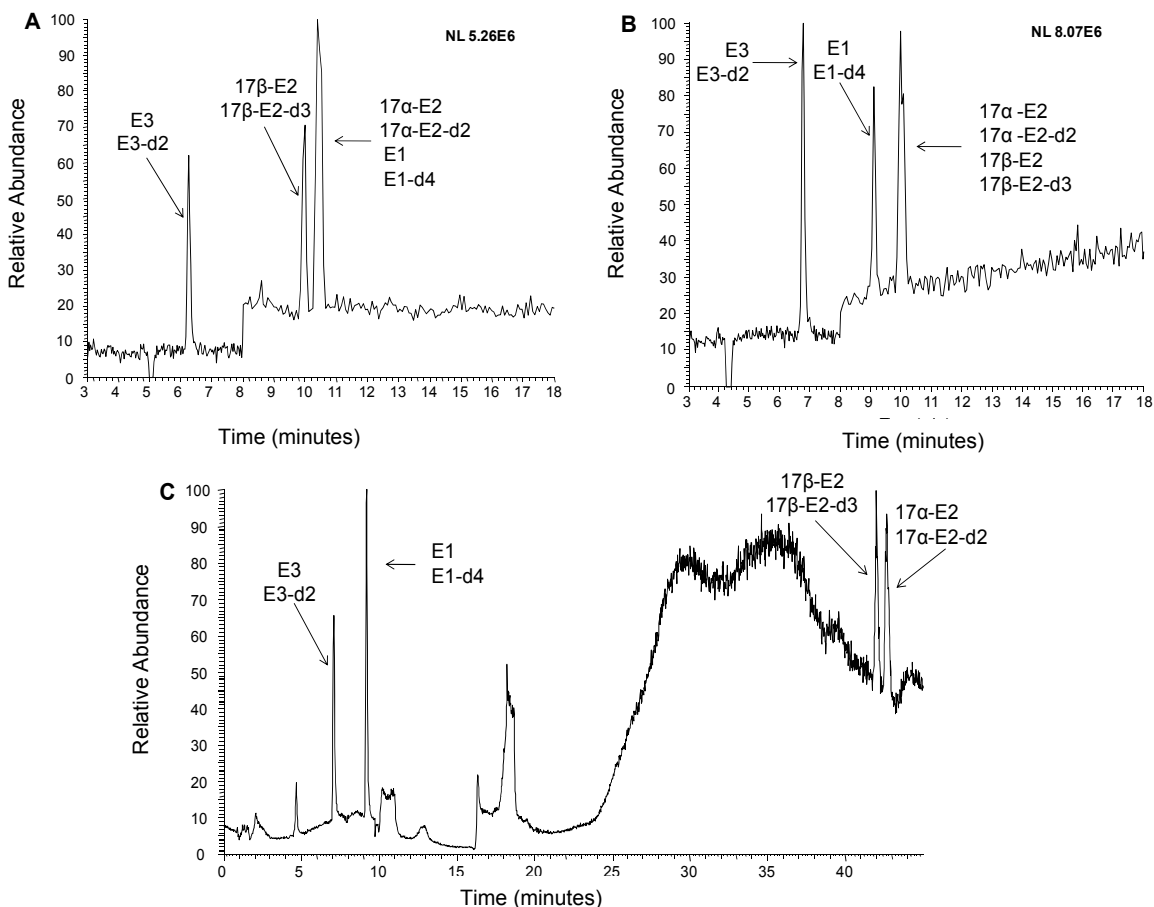


Figure 3.3 Ion chromatograms of the four estrogen analysis using (A) Shim-pack XR-ODS column (B) Polaris Amide-C18 column, and (C) heart-cutting 2D-LC-MS system (the base line variation from minute 16 to 40 was due to the valve switching and mobile phase gradient). The analyses were carried out in SIM mode according to the settings in different segments using 1 ng/mL of standard solution.

Samples were initially injected onto the amide-C18 column by the autosampler with the valve in position 0. All three columns were initially equilibrated with at least five column volumes of 50% B. In the first segment, dansylated E3 and E1 were separated on Polaris Amide-C18 column by a linear gradient elution from 50% to 100% B and detected by tandem MS. Dansylated E3 was eluted first at approximately 94% B and dansylated E1 was eluted later at 100% B. Dansylated 17β- and 17α-E2 coeluted last. The critical pair was diverted to the Shim-pack XR-

ODS columns by switching the valve to position 1 for a period of 1.7 minutes (segment 2). The diverted fraction was minimized to the smallest volume possible to limit the amount of high organic solvent mobile phase that was introduced into the second dimension, while simultaneously ensuring that any variation in retention for the critical pair could be accommodated. After the fraction was diverted, the valve was again switched to allow re-equilibration of the first dimension and eliminate the high organic content mobile phase (segment 3). After sufficient re-equilibration (5 min at 150 $\mu\text{L}/\text{min}$), all of the columns were again placed in line by switching the valve back to position 1. Dansylated 17 β - and 17 α -E2 were resolved with a linear gradient elution from 50% to 100% B (Figure 3.3C). The analysis was carried out in positive ionization SRM MS/MS mode. Different events were used to isolate the parent ion masses (for each estrogen and its related internal standard) (Table 3.1) prior to fragmentation. Each parent ion was monitored for its transition to m/z 171, signifying the common loss of the desulfonated dansyl moiety from each of the dansylated analytes. After optimization, the LC-MS/MS method, including baseline resolution of dansylated 17 β - and 17 α -E2, was able to be completed in 45 minutes.

3.3.2 Method Validation

The method was validated in CSCSF to determine the specificity, linearity, limit of detection (LOD), accuracy, precision, and extraction recovery. The specificity of the method was demonstrated by analyzing different pooled CSCSF samples. No interference was observed at the analyte retention and m/z . The cross-talk was examined by analyzing different samples with the addition of estrogen standards or deuterated internal standards. No cross-talk was detected between different MS events within a segment. The calibration range was established by analyzing standards ranging in concentration from 50 to 300 pg/mL for E3 and from 20 to 300 pg/mL for E1, 17 β - and 17 α -E2. The coefficient of correlation (R^2) in each case was determined to be greater than 0.996. The detection limits for E1, 17 α -E2, 17 β -E2, and E3 were 19, 35, 26, and 61 pg/mL , respectively. These detection limits refer to the use of maximum 1 mL of CSF per

sample for processing and analysis. The higher LOD of E3 may be the result of coeluting interferences from the matrix, which slightly suppress its ionization efficiency. The target values for mean accuracy and precision of quality control samples at each concentration level were less than 21% of the expected concentration for E3 and E1 and less than 14% for 17 α - and 17 β -E2. An extraction recovery of 91-104% was determined by comparing the response signal before and after the extraction at low, medium, and high concentration level quality control samples.

Although the detection limits were higher than the reported values from other methods,^{14,15,44,75} they can be improved if a higher sensitive mass spectrometry is used. As indicated in the study of Diaz-Cruz,⁹ a triple-quadrupole mass spectrometer can provide 5 times to one order of magnitude lower detection limits than a single-quadrupole. A newer version of triple-quadrupole can also give better detection limits than older units. Kushmir *et al.*⁷⁵ have reported the limits of quantification of E1 and 17 β -E2 in human serum as low as 1 pg/mL in positive ESI mode using dansyl chloride derivatization and 2D-LC-MS system equipped with an API 4000 triple quadrupole mass spectrometer (ABI-Sciex). However, similar limits of quantification were also achieved in negative ESI mode without derivatization using 1D-LC coupled to an API 5000 triple quadrupole mass spectrometer.¹⁰⁴ Our work has been performed on an ion trap instrument which is not ideal to obtain the most sensitive quantitative determinations. However, our configuration to reliably and reproducibly separate and quantify all four native estrogens in their derivatized form, and our focus on CSF as a new and important matrix for investigating neuroprotective effects of estrogens, are novel aspects. The method is also highly amenable to being improved in future work, with better-suited instrumentation, to achieve even lower detection limits.

3.3.3 Matrix Effects

Since dansyl chloride was used in excess for the derivatization reaction, interference from other side reaction products was judged to be relatively significant. The coefficient of variation for ten replicate injections of a 1 ng/mL estrogen standard solution was less than 14%

for the four estrogens without the biological matrix of CSCSF. Matrix effects were investigated by comparing the results of calibration performed in the absence of matrix with that from a CSCSF matrix, an accepted approach.^{60,105} The results obtained from calibration in CSCSF matrix were normalized to those in no matrix to establish a regression line (Figure 3.4). The experiment was performed three times on separate days. The slopes of the regression lines for the data were 0.71, 0.83, 0.95, and 1.04 for E3, E1, 17 β - and 17 α -E2, respectively, each with relative standard deviation \leq 7%. In the ideal case, a slope with a value of unity in such plots indicates the absence of matrix effects. The deviation of the slope to a value less than unity indicates ion suppression, whereas a slope greater than unity indicates ion enhancement in the presence of the matrix. A significant matrix effect was observed for E3 and E1 (29% and 17% reduction in signal, respectively, in the presence of CSCSF), but was negligible for 17 β - and 17 α -E2. The absence of a matrix effect for 17 β - and 17 α -E2 can be attributed to their transfer and separation in the second dimension of the heart-cutting 2D-LC method. Late elution and 2D-separation of these estrogens are speculated to reduce the interference from other matrix compounds.

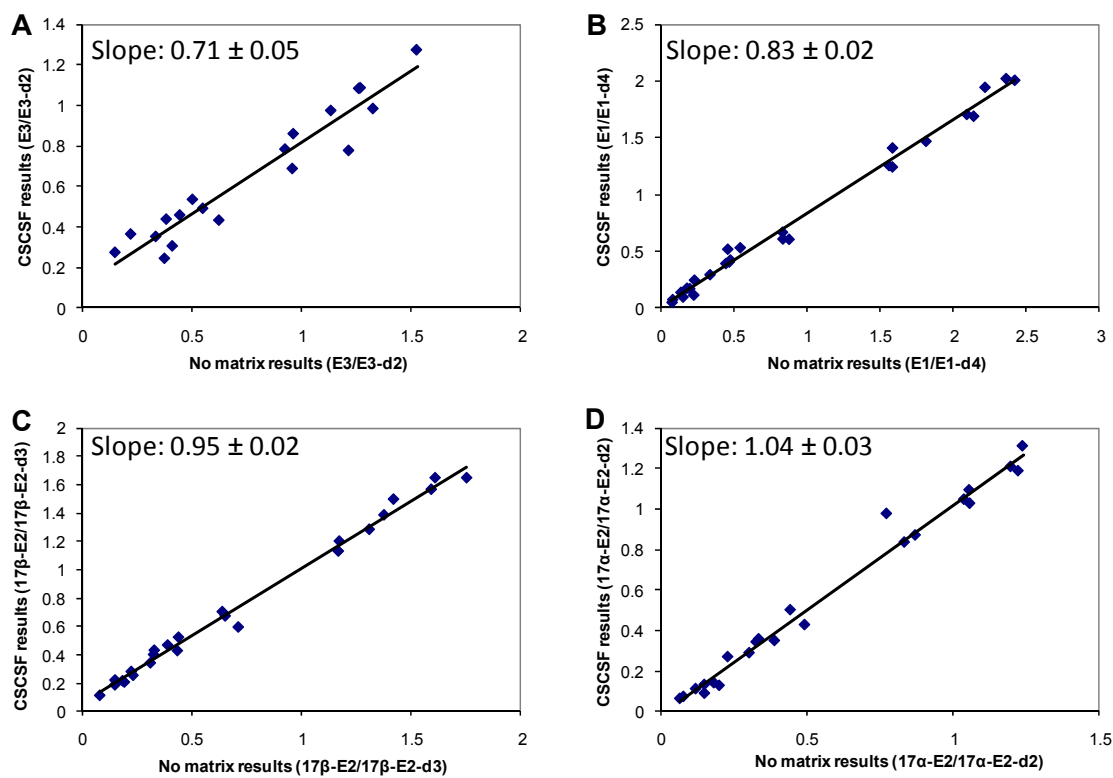


Figure 3.4 Evaluation of matrix effects using regression lines for (A) E3, (B) E1, (C) 17 β -E2, and (D) 17 α -E2.

3.3.4 Application

The method was applied to measure endogenous estrogens in the CSF of ischemic trauma patients. CSF was collected from brain-injured patients with a surgically placed ventriculostomy. From these traumatic brain injury patients, approximately 10 mL of CSF was collected from the buretrol under sterile conditions. After collection of the CSF, the samples were immediately placed on ice and centrifuged at 4°C for 10 minutes. The supernatant was snap frozen in the liquid nitrogen and the samples were stored at -80°C until analyzed. Three pooled samples from different patients were separately collected and analyzed. The results are given in Table 3.2 and LC-MS/MS data collected for sample 1 is shown in Figure 3.5. 17 β -E2 was detected in a significant amount in two of the samples. No 17 α -E2 was observed in any of the samples. E1 and E3 were present at low concentration or lower than LOD in one or more of the

three samples tested. Further research is underway to measure estrogens in CSF of ischemic trauma patients in connection with a phase two clinical trial. Samples of patients who have been administered estrogens (as Premarin®) or a placebo intravenously will be quantitatively evaluated by the LC-MS/MS method to support trials at Parkland hospital in Dallas, TX.

Table 3.2 Estrogen content in samples of CSF from human ischemic trauma patients.

Samples	E3 (pg/mL)	E1 (pg/mL)	17β-E2 (pg/mL)	17α-E2 (pg/mL)
1	< LOD	61 ± 2 *	184 ± 9	ND
2	ND	< LOD	ND	ND
3	ND	ND	208 ± 34	ND

*≤ LOQ

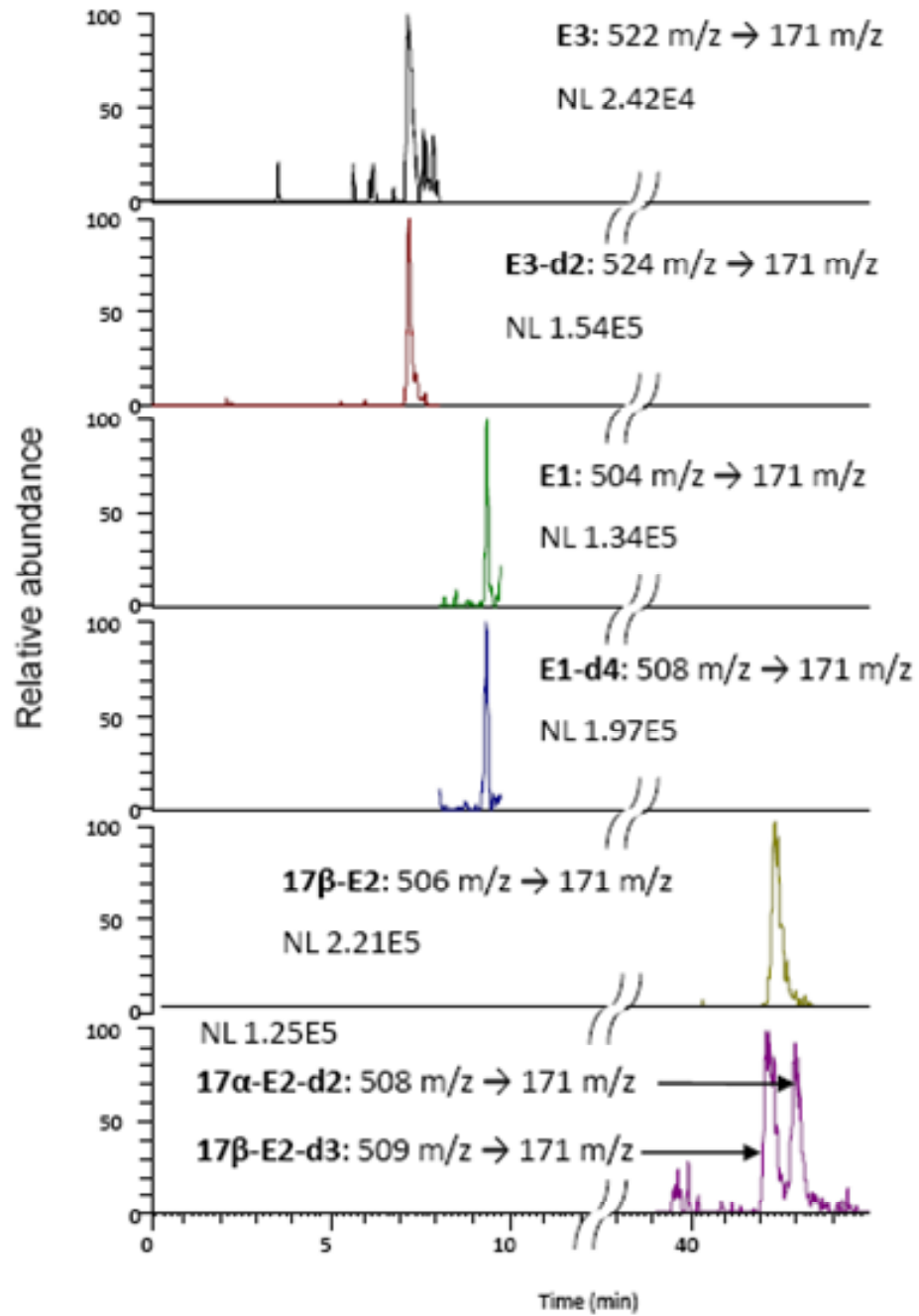


Figure 3.5 Ion chromatogram of the four dansylated estrogens and their internal standards in ischemic trauma patient CSF (Sample 1 from Table 3.2).

3.4 Conclusions

A heart-cutting 2D LC-MS/MS system for simultaneous measurement of the native estrogen hormones in human CSF has been developed and validated. The system provides a sufficient resolution for the four estrogens to allow for their simultaneous quantitative determination at ultra-trace level concentrations. Although the run time for each trial is fairly long (45 minutes), this method is amenable for use with a standard binary gradient LC system hyphenated to tandem MS. Analysis time can be reduced to less than 20 minutes with the addition of another binary pump to eliminate the mid-run re-equilibration in the third segment of the method. Furthermore, lower detection limits are likely to be achieved with the use of a triple quadrupole or other similar mass spectrometer systems.

CHAPTER 4
INVESTIGATION OF THE TEMPERATURE STABILITY OF PREMARIN USING LIQUID
CHROMATOGRAPHY-MASS SPECTROMETRY

4.1 Introduction

Neuroprotective effects of estrogens have been investigated in many studies.^{12,13,29,42,43} Pre-treatment with estrogens has been demonstrated to reduce ischemic damage in focal cerebral ischemia while post-treatment has also been shown to be beneficial for preserving brain tissues.^{12,13,42,43} Neuroprotective effects of estradiol were studied when administered after an ischemic insult on rats by Simpkins and coworkers.^{42,106} Beneficial effects were observed when the compound was administered within a therapeutic window of three hours after injury.⁴² Importantly, the study suggests that estrogens could be useful for treatment of traumatic brain injury (TBI) in humans. The promise of a clinical study to investigate the use of estrogens for treatment of TBI brings with it several points for consideration. Because patients are likely to be unconscious, the drug needs to be administered intravenously. Additionally, because a narrow therapeutic window exists (with diminishing returns even over the 0 – 3 hr time span), it is desirable to administer the treatment as early as possible.

Premarin® (Wyeth/Pfizer, PA, USA), in the form of Premarin®Intravenous, was considered for use as an estrogenic therapy for TBI in clinical trials, because it has been approved and widely used since 1942 (www.fda.gov/Drugs/DrugSafety). Premarin®Intravenous, for injection, contains a mixture of conjugated estrogens, present as sodium salts of water-soluble estrogen sulfates from natural sources and derived from pregnant mares' urine. However, approved protocol requires that the drug be stored in a sterile lyophilized form at 2 to 8°C, and that it be used immediately after reconstitution with sterile water (www.wyeth.com). Obviously, this requirement does not support the need for immediate treatment if a significant time delay exists between injury and arrival at the hospital. Instead, Premarin®Intravenous should ideally be

stored in the ambulance so that the treatment can be given as soon as possible. However, most modern ambulances do not have refrigerators and the stability of Premarin® Intravenous under ambulance temperature is unknown. Although synthetically prepared alkali metal estrogen sulfates were found to be unstable and hydrolyzed under prolonged exposure to the atmosphere,¹⁰⁷ the data for the stability of the drug are not publicly available while it is certain that many studies have been performed by the manufacturer over the years.

The analysis of Premarin® or estrogen conjugates is mainly carried out by reverse phase liquid chromatography – mass spectrometry (RPLC-MS),^{108,76,77} despite gas chromatography (GC) has been a predominant method in the past. Wyeth have reported 17 estrogens and United State Pharmacopeia (USP) have recognized 10 estrogenic components using GC.⁷⁶ However, an analysis, regarding the work of Reepmeyer *et al.*,¹⁰⁸ revealed the presence of more than 100 steroid conjugates in the drug. They claimed that the actual number of estrogenic component to be determined depends on the level of detection. Structure of estrogen conjugates in the drug is very similar; some of them are isobaric, differing only one double bond position. Thus, LC-MS alone do not provide enough evidence to identify these analytes. In this case, tandem MS has been used to clarify the structure of estrogen conjugates via fragmentation pathway. The technique has been applied in identification of several estrogen conjugates in the form of equilin, equilenin⁷⁷ and their derivatives.⁷²

In this work, the stability of solid and reconstituted forms of two batches of Premarin® Intravenous was investigated at different temperatures for 6 month periods using LC-tandem MS. Sampling was performed at several time points during the course of experiments. Eight of the highest abundance steroidal components of the drug were monitored. Variation of these components in the reconstituted solutions was observed, but was insignificant in the solid form. Interestingly, total steroidal amount was not highly changed in any of the samples at different temperatures, which suggests inter-conversion between components in the reconstituted ones. In order to more fully investigate the possible inter-conversion between steroid hormones, a series of storage studies were performed using pure standards. These studies indicated that

changes in degree of saturation in the aromatic rings are likely responsible for inter-conversion. Higher order tandem mass spectrometry experiments in an ion trap were used to further validate this finding and assist in the qualitative assignment of isobaric species.

4.2 Experimental

4.2.1 Chemicals and Reagents

All conjugated estrogen standards were purchased from Organics, L.L.C. (Northbrook, IL, USA), including sodium sulfate conjugates of equilenin (EN-S), 17 α - and β -dihydroequilenin (ADHEN-S and BDHEN-S), equilin (E-S), 17 α - and β -dihydroequilin (ADHE-S and BDHE-S), estrone (E1-S), 17 α - and β -estradiol (AE2-S and BE2-S). The structures of these compounds are shown in Figure 4.1. Premarin® Intravenous (conjugated estrogens, USP) was obtained from Wyeth/Pfizer (Philadelphia, PA, USA). Amonium acetate was from Sigma-Aldrich (St. Luis, MO, USA). Water and acetonitrile (ACN) were LC-MS grade and obtained from J. T. Baker (Phillipsburg, NJ, USA) and Burrdick and Jackson (Muskegon, MI, USA). Bacteriostatic and sterile water were purchased from Hospira, Inc. (Lake Forest, IL, USA).

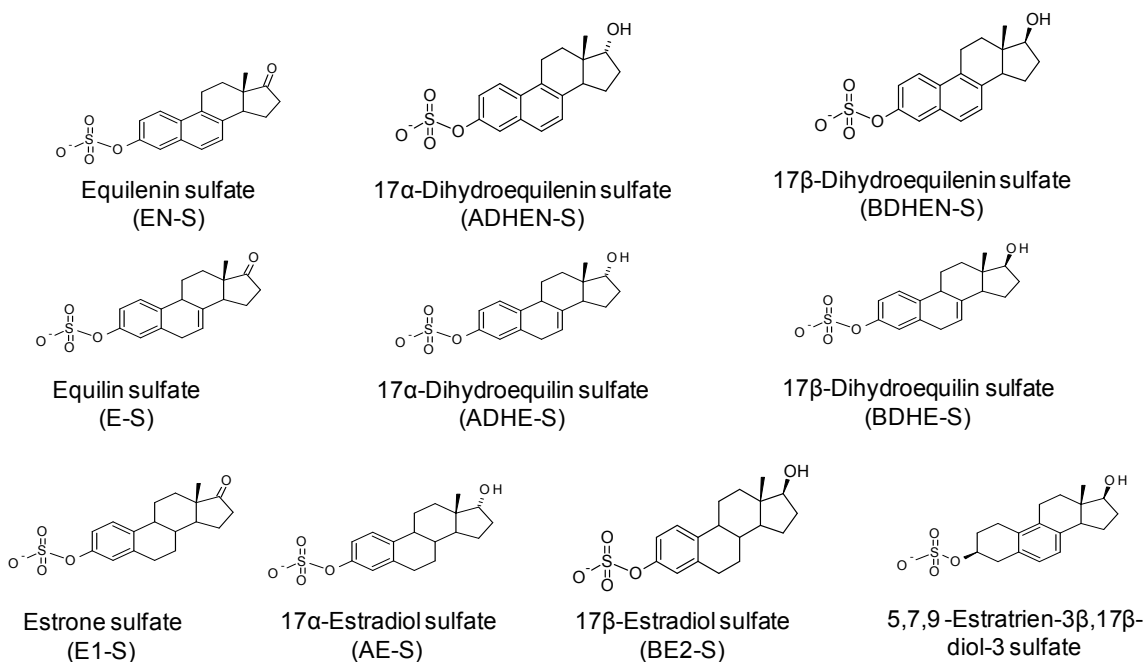


Figure 4.1 Chemical structures of studied estrogen conjugates.

4.2.2 Experimental Design

Temperature stability of Premarin® Intravenous was investigated in dry powder and reconstituted forms. Two batches of the drug were used for two consecutive experiments. Both dry and samples reconstituted with sterile water were studied at room temperature (RT) and at 100°F in batch 1, whereas only reconstituted solutions were tested at RT and at an elevated temperature of 120°F, each with and without benzyl alcohol as a preservative, in sterile water in batch 2.

4.2.2.1 Batch 1

Total nine Premarin® Intravenous vials from the same lot were removed from their vials, combined, divided, and accurately weighed into 8 vials. The samples in six vials were reconstituted in sterile water to the concentration of 47.5 mg/mL, which is the same concentration which would be present in a single vial when reconstituted according to manufacturer specifications. Three of these vials were labeled as R1, R2, and R3 and stored at room temperature while the other three vials were labeled as H1, H2, and H3 and stored at 100 °F. The samples in the remaining two vials were stored as dry powder. They were labeled as D1 and D2 and stored at room temperature (RT) and at 100°F, respectively. All samples were stored in the dark for the duration of the study, except during regular sampling for analysis.

4.2.2.2 Batch 2

Total eight Premarin® Intravenous vials from the same lot were selected and each of them was reconstituted with 5 mL of sterile water, which was injected through the rubber cap of the vials, according to manufacturer specifications. The samples in four vials were dissolved in sterile water containing 0.9% benzyl alcohol (BnOH), while those in the other four vials were mixed with sterile water without preservative. Two of each were stored at RT and labeled as R1 and R2 for non-preservative sterile water, and as R3 and R4 for the bacteristatic water. The other

four vials were stored at 120 °F and labeled similarly for non-preservative sterile (H1 and H2) and bacteristatic water (H3 and H4), respectively.

4.2.3 Sampling

Sampling was performed at monthly time points during a period of 6 or 7 months to determine the variation of sample components. A volume of 10.0 µL of each reconstituted solution was diluted to 10 mL with LC-MS water, followed by LC-MS analysis. For dry samples, they were first dissolved in sterile water at the concentration of 47.5 mg/mL and, then, subjected to the same dilution and analytical procedure for the reconstituted samples described above. Freshly-made solutions from dry Premarin® Intravenous, stored at 4°C, were used as control samples. The data were processed, based on the response for each monitored peak collected from the samples normalized to those recorded from the controls.

4.2.4 LC-MS Conditions

The experiments were performed on a Shimadzu LCMS-IT-TOF (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), equipped with two pumps (LC-20AD) in a high pressure mixing arrangement, an autosampler (SIL-20A HT), and a electrospray ionization (ESI) - ion trap - time of flight mass spectrometer (IT-TOF). The chromatographic analysis was carried out on a Shimadzu (Tokyo, Japan) Shim-pack XR-ODS column (2 x 100 mm, 2.2 µm, 120 Å) with an elution gradient of mobile phase A (20 mM ammonium acetate in H₂O) and mobile phase B (ACN) at the flowrate of 250 µL/minute. The run was started at 20%B (held for 5 minutes), followed by a linear gradient to 40% over 30 minutes and then to 98% over 20 minutes (and held for 10 minutes). The gradient was eventually returned to the initial condition for 5 minutes, for re-equilibration between runs. The IT-TOF was operated in the negative ionization mode with a spray voltage of -3.5 kV, and a scan range of 100-600 *m/z*. The nebulizing gas flow rate was set to 1.5 L/min and the drying gas was set to 75 kPa. The temperature of the curved desolvation line

and block heater was set to 250°C. LCMS Solutions software, version 3.4, was used for data collection and analysis.

4.2.5 Stability of Pure Standards in Solution

The stability of four pure standard solutions (E1-S, E-S, ADHE-S, and BDHE-S) was investigated. Solutions, at a concentration of 0.50 mg/mL, were prepared in 200 µL aliquots and stored in the oven at 120°F for three months. Sampling was performed at day 0, 14, 28, and 95. Each standard sample was diluted to 1.0 µg/mL and analyzed by LC-MS using the conditions described in section 2.4.

4.2.6 Tandem Mass Spectrometry Fragmentation

The fragmentation was performed in negative ion mode for ADHEN-S, BDHEN-S, and EN-S. Source conditions were consistent with those used for LC-MS analysis. Collision energy during collision-activated dissociation (CAD) was optimized for each compound at each tandem MS stage (MS^2 and MS^3) by infusing 1.0 µg/mL sample solutions at a flow rate of 10 µL/minute into the IT-TOF mass spectrometer with a syringe pump (NE-1010, New Era Pump System, Inc. NY, USA). Collision energies were optimized to maximize the generation of fragments for subsequent tandem MS steps. Once optimized, the standard solutions of ADHE-S, BDHE-S, and E-S, stored at 120 °F for 3 months were subjected to LC-MS³ under the optimum fragmentation conditions.

4.3 Results and Discussions

4.3.1 Peak Identification

A representative total ion chromatogram of Premarin® Intravenous samples is presented in Figure 4.2 with the eight most intense components selected for tracking through the storage experiment.

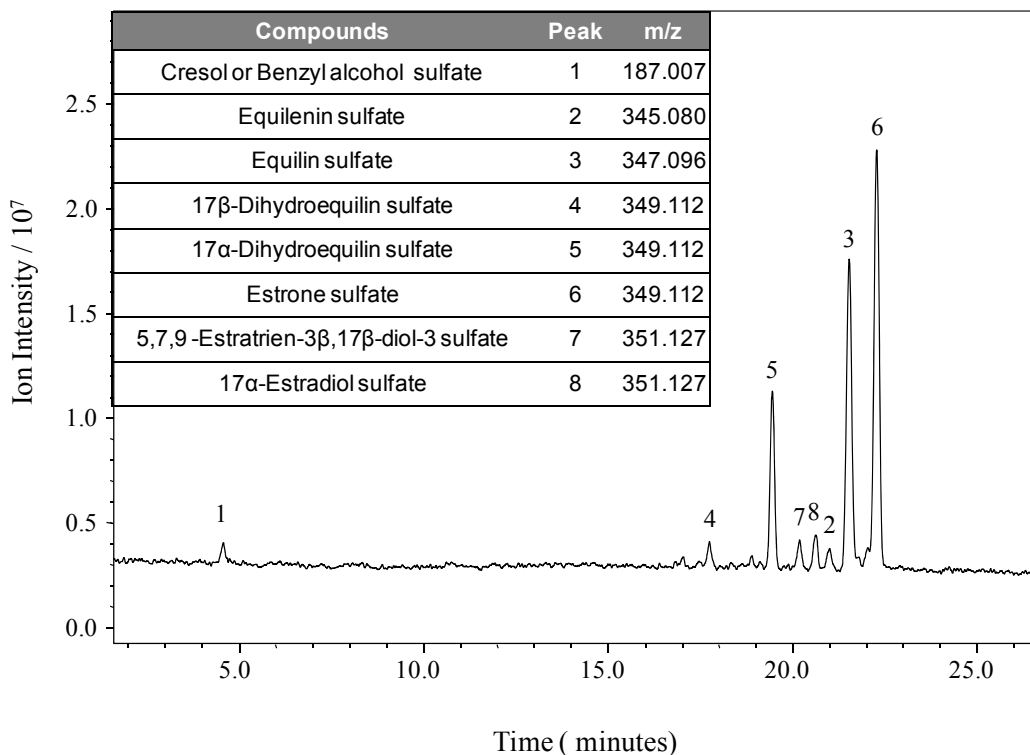


Figure 4.2 Total ion chromatogram of Premarin® Intravenous with the eight most intense components.

The peak investigation was based on matching m/z values and retention times between Premarin® Intravenous samples and pure standards. Six of the eight compounds (peaks 2-6, 8) were positively identified. Assignments for peaks 1 and 7 are tentative. According to Hill *et al.*,⁷⁶ peak 1 could be cresol or benzyl alcohol sulfate, but the latter is most likely. Peak 7 (m/z 351) can be 17 β -estradiol sulfate, 5,7,9-estratrien-3 β ,17 β -diol-3-sulfate, all of which are isobaric and have been reported to be present in Premarin by Wyeth-Ayerst. The retention time of peak 7 did not match that of 17 β -estradiol sulfate, indicating that peak 7 likely is 5, 7, 9-estratrien-3 β ,17 β -diol-3-sulfate. However, the standard of this compound was not commercially available to confirm its identification.

4.3.2 Premarin®Intravenous Stability

Stability of Premarin®Intravenous was studied in two consecutive batches. Batch 1 was carried out first. Batch 2 was performed to confirm and clarify results collected from batch 1.

4.3.2.1 Batch 1

Figure 4.3 displays the cumulative results for the dry samples. The data show that the main components in samples D1 (room T) and D2 (100°F) increase slightly for the first 93 days. This may be due simply to moisture desorption over time, or due to instrumental variation. A peak ratio of 1 indicates that the abundance of a component in the storage sample is equivalent to that determined for the freshly prepared control sample. These two dry samples were not significantly changed after day 93 over the remainder of the experiment. From day 93 – 175, the variations of each component were $\leq 10\%$ (most were between 2 – 5% variation), indicating that these non-reconstituted samples were fairly stable during six month time frame when stored at RT or at 100°F.

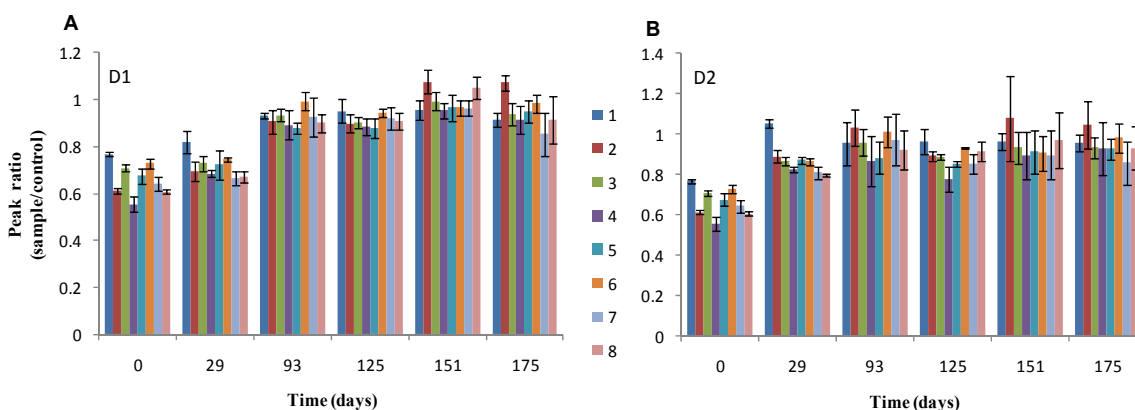


Figure 4.3 Variation of dry Premarin®Intravenous within 175 days for (A) D1 at RT and (B) D2 at 100°F.

The variation of reconstituted samples are shown in Figure 4.4. Samples R1-R3 (presented as an average in Figure 4.4A) show a slight increase for the first month, but more importantly, the relative abundance of the tracked components varied to a greater degree after day 151. Compared to samples R1-R3, samples H1-H3 (stored at 100°F; average peak ratios in

Figure 4.4B) showed an even more variable composition after 93 days. In both sample sets, compound 2 dramatically increased while some other compounds, such as compound 4 and 7, decreased. Interestingly, the variation of total signals for steroidal compounds (sum total of relative peak intensities from peak 2-8), at RT and 100°F, was not significant ($\leq 15\%$). This may be considered as being stable for total steroids, although individual components quantitatively varied. An unidentified microbial growth in most of the reconstituted samples (especially in R2), stored at RT, was noted during sampling on day 175.

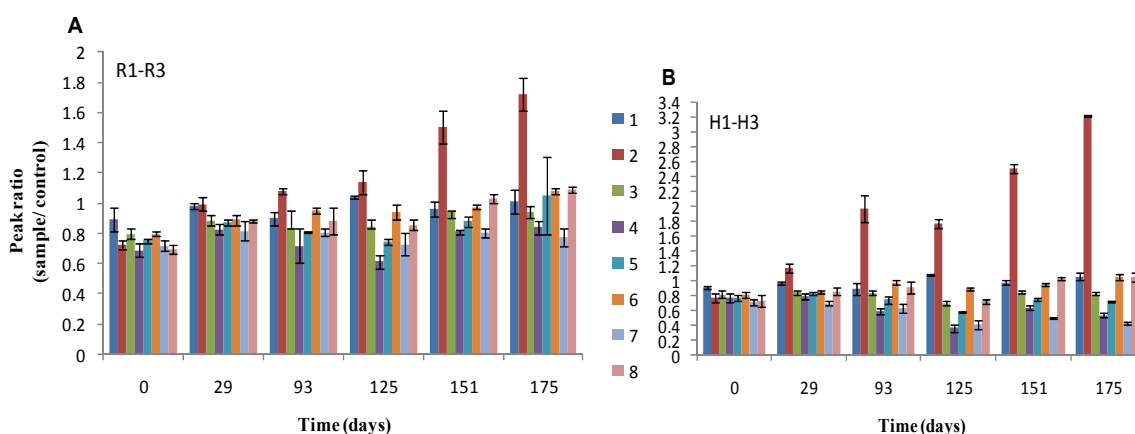


Figure 4.4 Variation of reconstituted Premarin® Intravenous solutions over 175 days for (A) R1-R3 at RT and (B) H1-H3 at 100°F. The results were averaged from triplicate of R1-R3 and H1-H3.

4.3.2.2 Batch 2

Since the microbial growth was observed in the reconstituted samples at RT in batch 1, the experiment with reconstituted solutions was repeated in batch 2 with a better control condition. The samples were dissolved with sterile water via injection through vial rubber caps to avoid the direct contact between Premarin® Intravenous samples and the ambient environment. No precipitation or bacterial growth was observed in reconstituted samples at RT but small rod-shaped particles were detected in those at 120°F after day 62. The shape of these particles was different from the shape of microbial growth observed in batch 1, which was a furry amorphous shape. The presence of the rod-shaped particles in batch 2 might be caused by precipitation under high temperature condition rather than bacterial contamination.

The variation of steroid hormone components in reconstituted batch 2 samples at RT, as shown in Figures 4.5A and 4.5B), was not significant for the first 148 days ($\leq 13\%$ for each tracked compound), however some marked variation of some components was observed after this timeframe. The composition of reconstituted solution stored at 120°F (Figures 4.5C and 4.5D) changed to a great degree over 215 days. Compound 2 continuously increased after day 40. Even so, the variation of total steroids was not substantial with the error percent of 12%, although it was slightly increased with time. At both temperatures, there was no different in composition variation between samples with and without the addition of BnOH.

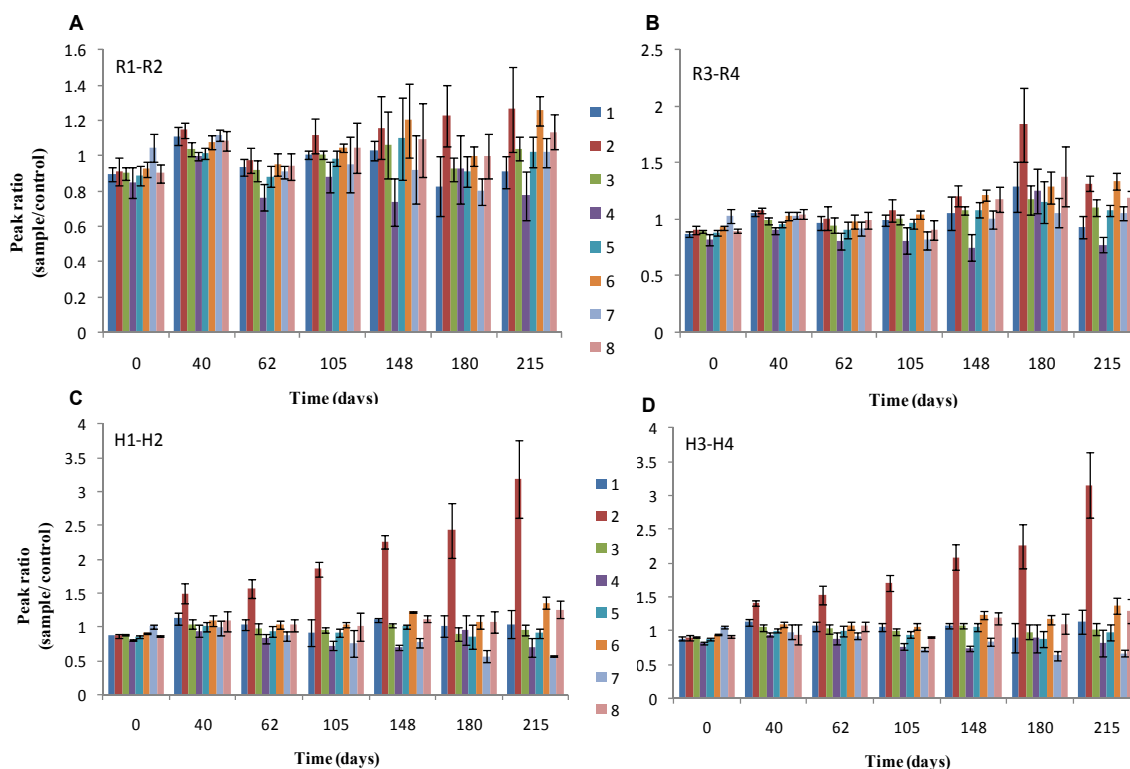


Figure 4.5 Variation of reconstituted Premarin® Intravenous samples during 215 days for (A) R1-R2 (no BnOH), (B) R3-R4 (with BnOH) at RT and for (C) H1-H2 (no BnOH), (D) H3-H4 (with BnOH) at 120°F.

In summary, Premarin® Intravenous was considered to be fairly stable in solid form at RT and even at an elevated temperature of 100°F. Variation of steroidal compositions in reconstituted samples at RT was not significant when the experimental conditions were well-

controlled and the sample was not exposed to ambient contamination. However, there was a marked change in the abundance of a number of the major components in the drug when high temperature was applied. The increase of compound 2, decrease of some other steroids, and low variation in the sum of total signals led to a hypothesis that inter-conversion among steroidal components, not degradation of sulfates as might be expected, was responsible for the observed variation in individual components. This hypothesis was investigated by studying the stability of different pure steroid hormone sulfate standards.

4.3.3 Steroid Hormone Sulfate Conjugate Stability in Solution

The increase of compound 2 levels and decrease of the other components in reconstituted samples could be caused by conversion between different components into more stable forms. Compound 2, EN-S, contains conjugated A-B aromatic rings; therefore, it is likely more stable than other compounds bearing unsaturated A-B rings such as compound 3, 4, 5, and 7. Possible conversion from the others into compound 2 could be the reason for its increasing levels over time under elevated temperature condition. This phenomenon was further studied using pure standard solutions. Total four standards, comprising of E1-S and three equilin sulfates (E-S, ADHE-S, and BDHE-S), were investigated at the concentration of 0.5 $\mu\text{g/mL}$ at 120°F. These compounds were selected because they contain unsaturated A-B rings, which were hypothesized to convert into saturated forms under high temperature over a period of time. After being stored at 120°F for 3 months, additional unknown peaks appeared in the LC-MS chromatograms of the pure standard solutions (Figure 4.6). The retention time and m/z values of these peaks matched those of corresponding equilenin sulfate compounds; thus, the conversion of equilin into equilenin was possible. No additional peak was detected with E1-S. Although these unknown peaks were not observed with freshly purchased and prepared standards, they were present at very low levels in freshly-made reconstituted samples after storage at -20°C for 1.5 years.

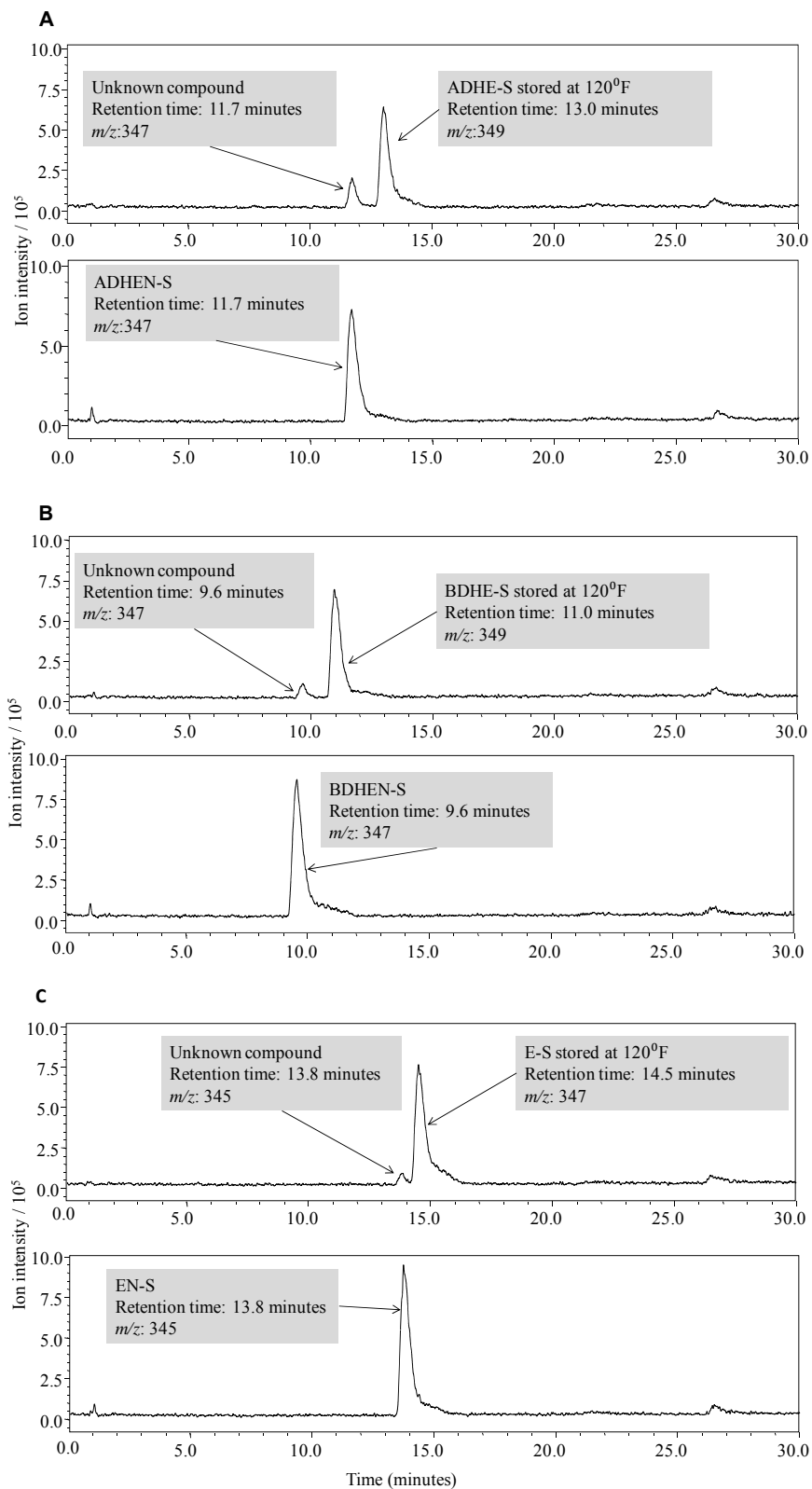


Figure 4.6 Chromatograms of (A) ADHE-S stored at 120°F and ADHEN-S, (B) BDHE-S stored at 120°F and BDHEN-S, and (C) E-S stored at 120°F and EN-S.

The unknown peaks were suspected to form from the conversion of equilin species into their corresponding equilenin forms. A supplementary study was implemented to clarify this using tandem mass spectrometry, including MS² and MS³. Total three equilenin compounds (ADHEN-S, BDHEN-S, and EN-S) were investigated and compared with the unknown peaks from their corresponding equilin. All compounds lost their sulfate group during MS² and the desulfated ions were further fragmented in MS³. CAD spectra of ADHEN-S, BDHEN-S, EN-S and the unknown peaks from their corresponding equilin storage study during MS³ are shown in Figure 4.7-4.9. Fragmentation patterns of these unknown peaks were consistent with that of the corresponding equilenin standards. The fragmentation during MS³ of ADHEN-S provided more product ions compared to ADHEN-S and EN-S. Peaks at m/z of 181 were dominant for ADHE-S, BDHE-S, and their corresponding unknown species while peaks at m/z of 193 were the most abundant for EN-S and its corresponding unknown. Although the same collision energy was applied for the two epimers of ADHEN-S and BDHEN-S (18 % collision energy in MS² and 16% in MS³), peaks at m/z of 169 and 237 were detected only in the mass spectrum of ADHEN-S but not in that of BDHEN-S. This phenomenon was also observed with their corresponding unknown species. Consequently, the conversion of equilin compounds into their corresponding equilenin was confirmed. It was noted that peaks at m/z of 196 (ADHEN-S and BDHEN-S), 210 (EN-S) and 222 (ADHEN-S and EN-S) were not isotopic species of those at m/z of 195, 209, and 221, respectively, since the ratios of ion intensity (196/195, 210/209, and 222/221) did not match the theoretical isotope distribution and was also observed to change with the variation in collision energy.

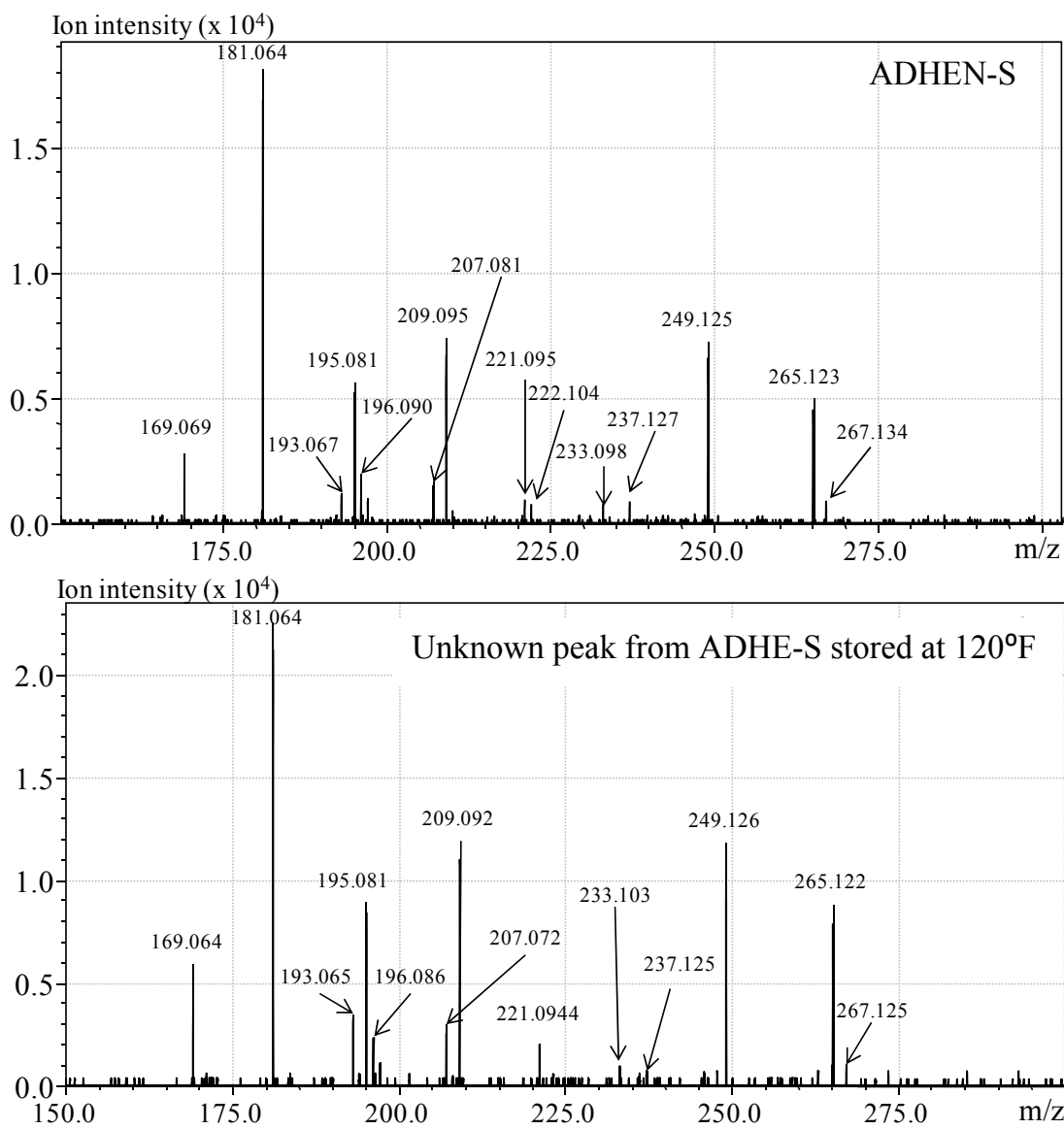


Figure 4.7 Negative electrospray with MS³ CID spectra of ADHEN-S and the unknown peak from ADHE-S stored at 120°F

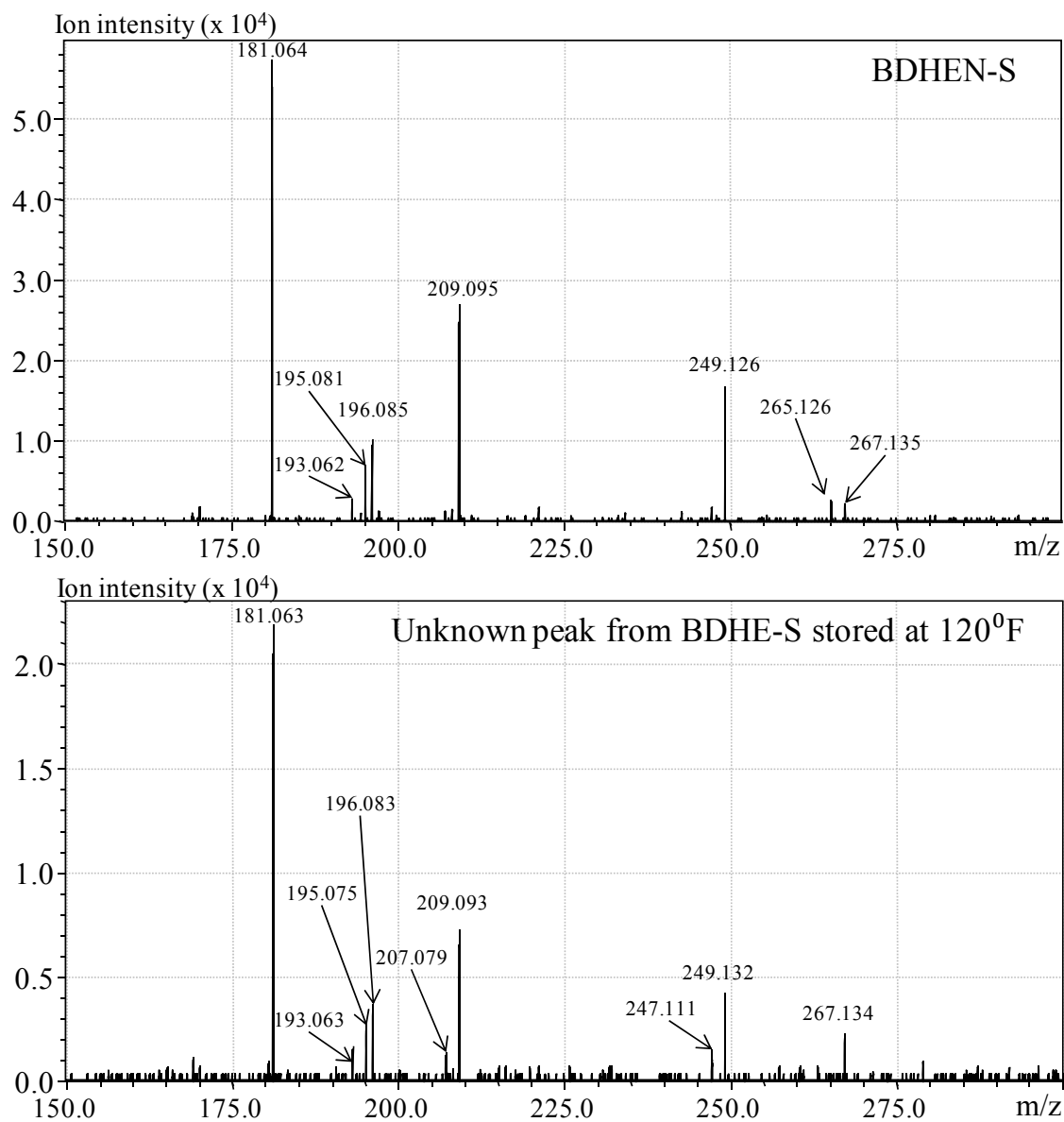


Figure 4.8 Negative electrospray with MS³ CID spectra of BDHEN-S and the unknown peak from BDHE-S stored at 120°F

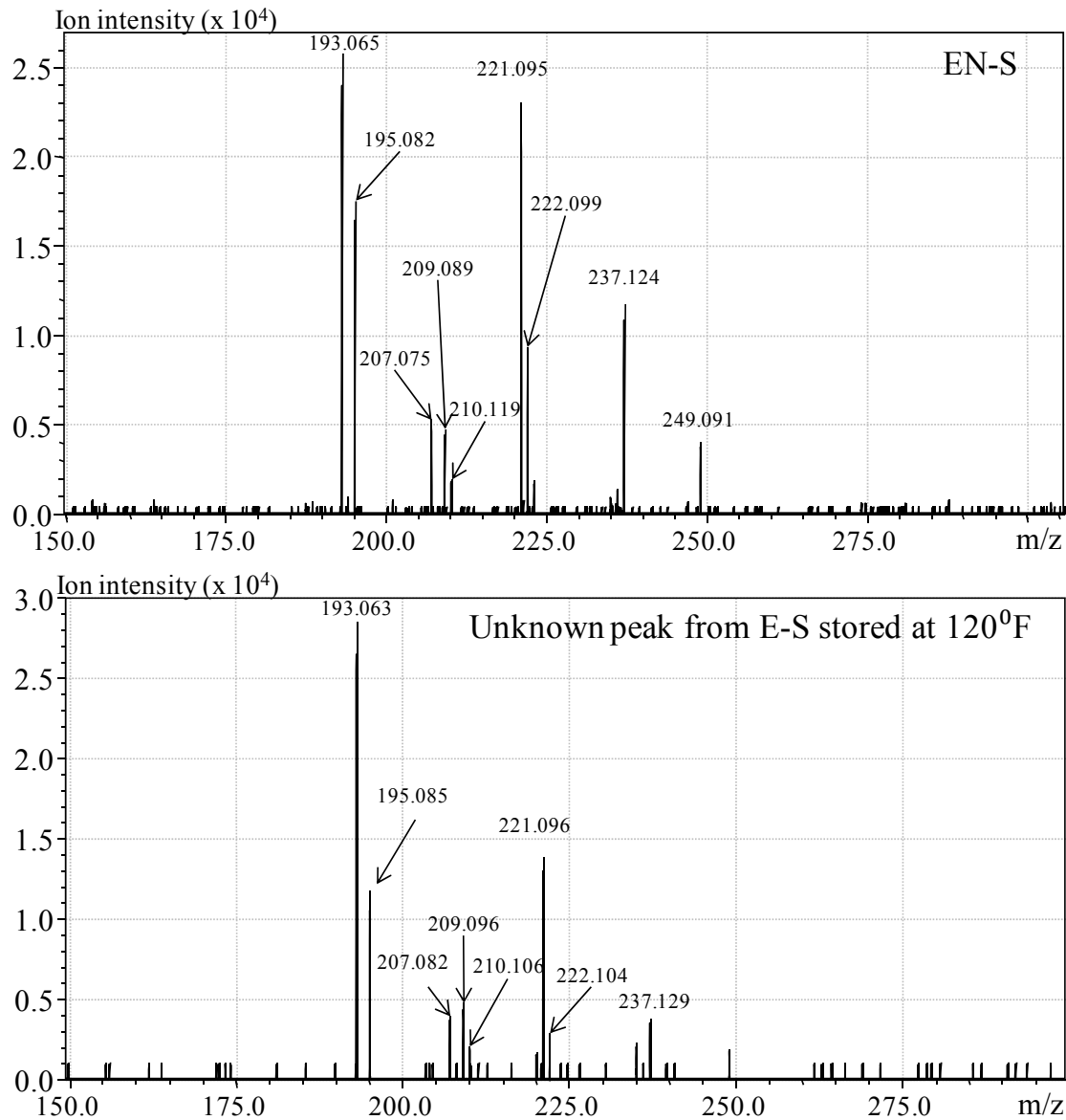


Figure 4.9 Negative electrospray with MS³ CID spectra of EN-S and the unknown peak from E-S stored at 120°F.

The fragmentation pathways for the three investigated compounds are suggested in Figure 4.10-4.12.^{47,77} Interestingly, the fragmented ions at m/z of 249 during MS³ of ADHEN-S and BDHEN-S could be distinguished from that during MS³ of EN-S with mass accuracy ≤ 16 ppm, using high mass accuracy IT-TOF instrument.

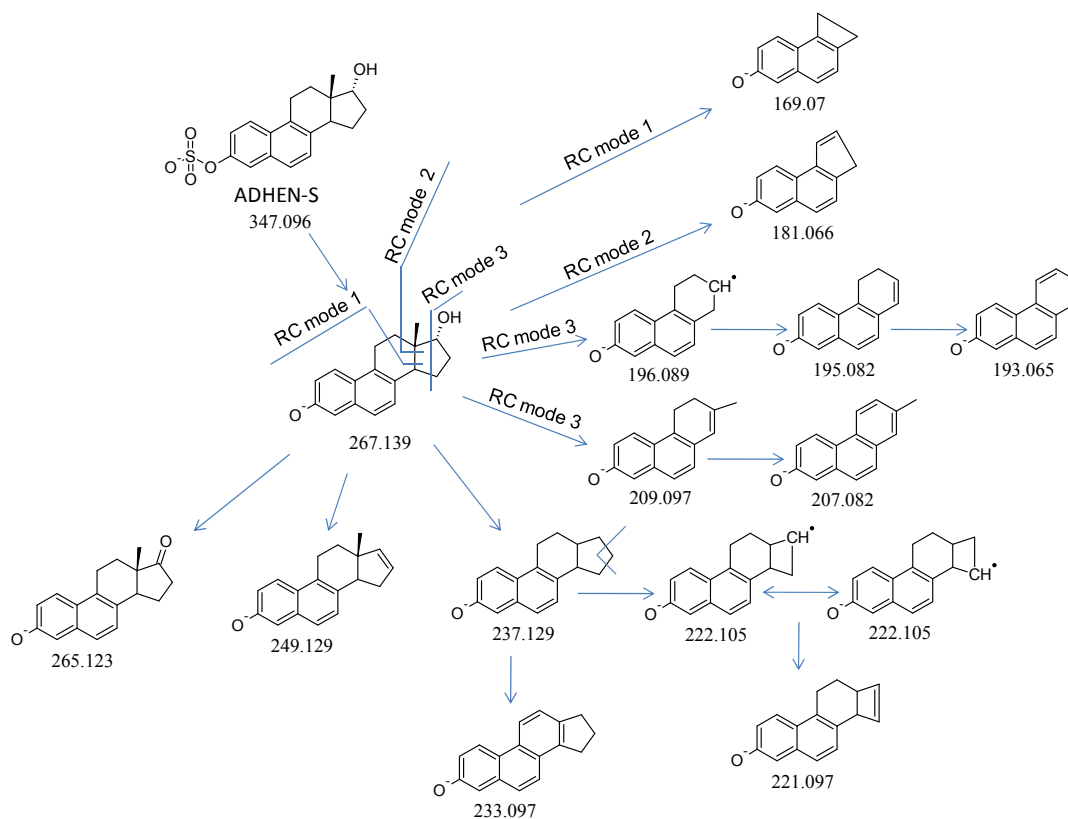


Figure 4.10 Fragmentation pathways of ADHEN-S with different retrocyclization (RC) modes.

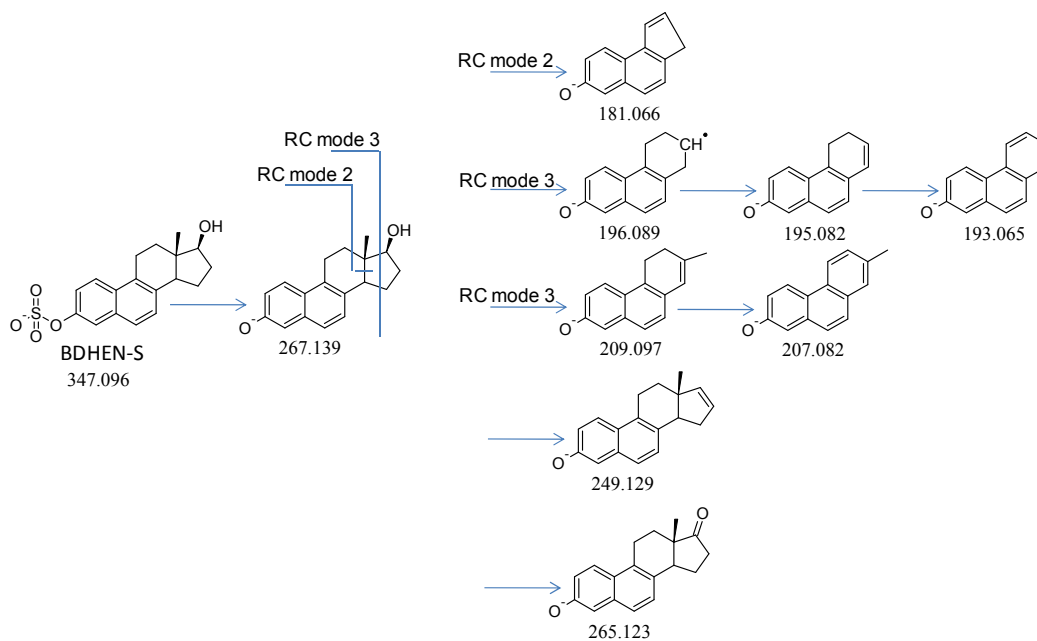


Figure 4.11 Fragmentation pathways of BDHEN-S with different retrocyclization (RC) modes.

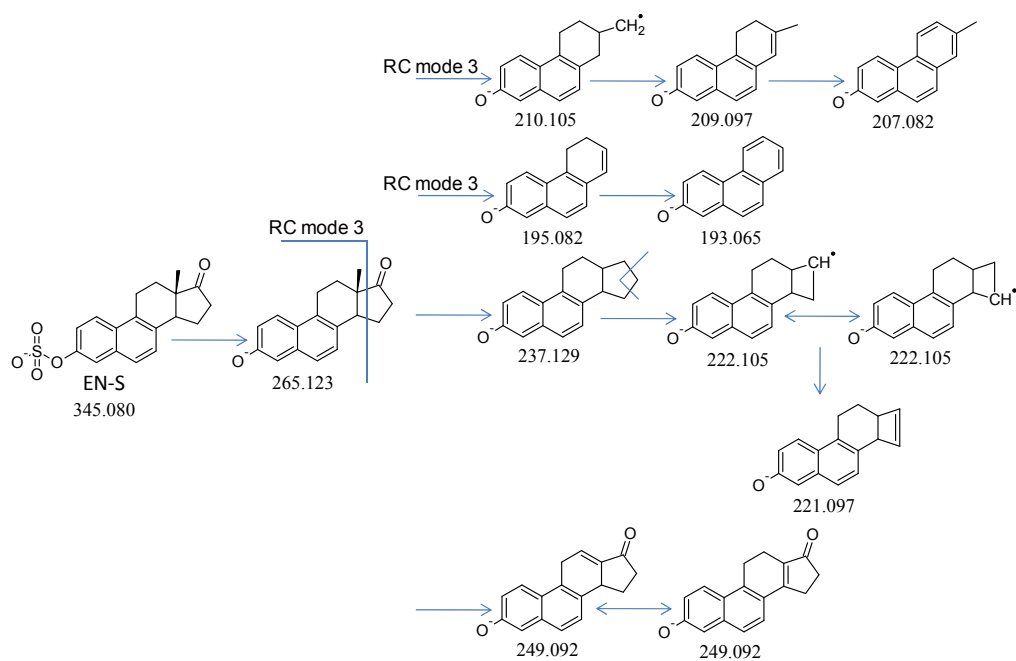


Figure 4.12 Fragmentation pathways of EN-S with different retrocyclization (RC) modes.

The variation of converted equilenin in equilin standard solution at 120°F was monitored for 95 days and presented in Figure 4.13. The peak area of the converted equilenin was normalized to total peak area of the converted equilenin and their corresponding equilin. The increase of ADHEN-S and BDHEN-S occurred for the first 14 days and leveled off after that while the amount of EN-S was constantly increasing during 95 days.

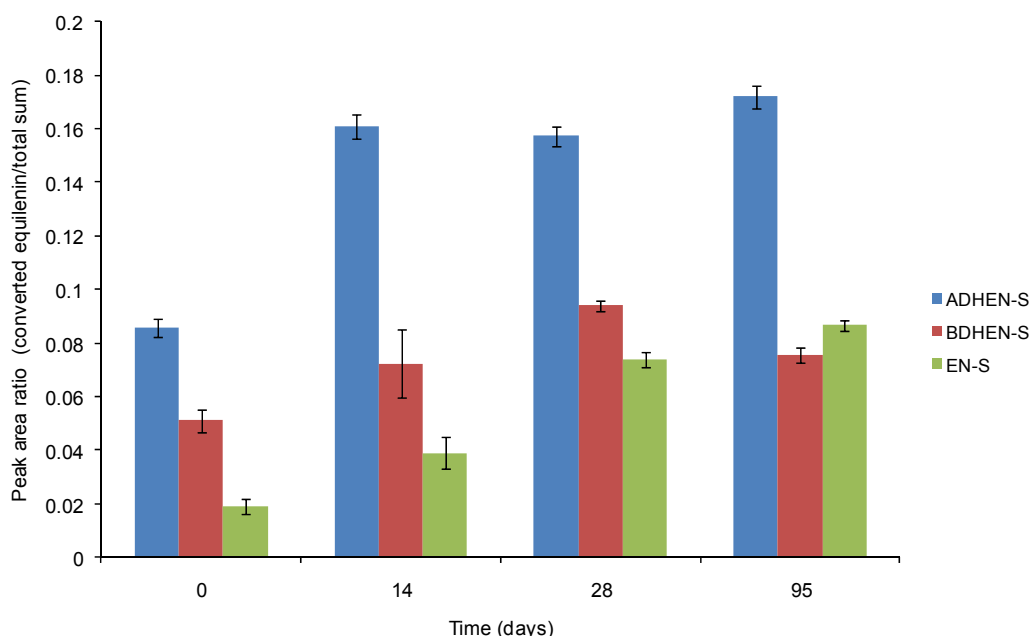


Figure 4.13 Variation of converted equilenin in pure equilin standard solutions at 120°F for 95 days.

The conversion from equilin into the corresponding equilenin occurred; thus, the transformation of E-S into EN-S partially explains the increase of EN-S in Premarin® Intravenous solutions. However, further oxidation of the hydroxyl group into ketone group at position C17 of ADHEN-S and BDHEN-S was not observed. In other words, these compounds were not completely oxidized to EN-S in pure standard solutions. The experiments with the pure standards did not fully explain the dramatic increase of EN-S and decrease of other components in Premarin® Intravenous solutions under high temperature over a period of time. The reason could be that, since this drug is originally from natural sources, there might be other unknown species in the drug possibly initiating or catalyzing the oxidation reaction at C17 of dihydroequilenin compounds and converting them into EN-S.

4.4 Conclusions

The major components in the dry samples seemed to be more stable than those in the reconstituted samples, especially at high temperature. The relative responses in the dry samples

D1 and D2) did not change to an appreciable degree after six months. The reconstituted samples, stored at RT, were considerably stable for the first three months and slightly varied after that for batch 2, which has better control against bacterial interferences. Although the reconstituted samples stored at high temperature varied significantly for each tracked compound, the variation of total steroids was $\leq 15\%$. This is not significantly higher than acceptable variation ranges of the two main components in Premarin®, estrone and equilin sulfate, recognized by USP.¹⁰⁹ The total of these two compounds should comprise 79.5 to 88.0% of the labeled content of conjugated estrogens,¹⁴ which is 10.2% variation. The conversion of equilin compounds into their corresponding equilenin forms occurred, partially explaining the increase of EN-S and decrease of other components in Premarin® Intravenous solution. If Premarin® Intravenous is to be stored in a reconstituted form in an ambulance for immediate response and use in TBI, the storage of such samples should ideally be performed at lower temperatures (e.g. in a cooler) and they should be replaced every month.

CHAPTER 5

EVALUATION OF MATRIX EFFECTS IN ESTROGEN BIOANALYSIS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

5.1 Introduction

Estrogens are believed to play an important role in regulating secondary sexual characteristics as well as productive functions in females.²⁹ They have been widely used as hormone therapy in the treatment of hormone balance¹¹⁰ and, recently, recognized by their neuroprotective effects.^{10,11} The beneficial effects of estrogens on the brain have been demonstrated in many studies.^{12,13,42,43} The most active estrogen, 17 β -estradiol (17 β -E2), has been reported to reduce ischemic damage in focal cerebral ischemia when administered before or even after the injury within a narrow therapeutic window of less than three hours.¹³ Although 17 α -estradiol (17 α -E2), an epimer of 17 β -E2, is considered less active since it binds less vigorously to estrogen receptors,⁴⁴ it has currently received more attention. 17 α -E2 is a less feminizing estrogen but still provides positive neuroprotection.^{44,45} The use of a less or non-feminizing estrogen as neuroprotective therapy can be advantageous in reducing the complications of feminizing side effects. For this reason, 17 α -E2 can be a good candidate in the treatment of neurodegenerative diseases along with 17 β -E2. The analysis of these two estradiols is, in turn, becoming the key in any pre-clinical and clinical research on neuroprotective activities of estrogens.

High performance liquid chromatography (LC) coupled to mass spectrometry (MS) has become a common chemical analysis technique for use in estrogen bioanalysis.^{1,14,15,21,22,44} However, trace-level analysis of physiological samples by ESI-MS is a challenge due to ion suppression or enhancement caused by the co-elution of interfering species from complex biological matrices.^{52,61} There are two issues attributed to the matrix effects; The first issue is the variation of matrix composition and the second one is the contrast in concentrations between

endogenous interferences and the estrogens. The compositional variation in biological matrices is related to the complexity of the samples, variability of sample matrices among individuals, diverse classes and concentrations of endogenous species in the samples.⁷⁵ Roberts *et al.*¹¹¹ have listed the distribution of over 70 endogenous compounds of small molecules in human adult serum with the span of concentrations over 10 orders of magnitude.⁷⁵ Biological samples generally contain substantial amount of proteins, carbohydrates, lipids, and salts, whose concentrations vary depending on the type of samples.¹¹²⁻¹¹⁶ For example, high concentrations of proteins are found in serum/plasma (60-90 mg/mL)^{112,113} whereas considerable amounts of lipids are present in tissues such as brain (0.13-0.16 mg/mL).¹¹⁴ These obstacles make the analysis become more challenging because estrogens are usually present at trace levels of pg/mL (commonly at 5 pg/mL) but concentrations of various interfering species in background matrices are 3-4 orders of magnitude higher.

Since characteristics of biological matrices highly affect the analysis, careful selection of blank biological matrices, in which standard curves are performed, is a must. Although using stable isotopically-labeled internal standards can reduce the degree of interference,^{55,56} the extent of matrix effects depends on the similarities in composition between real world samples (unknowns) and blank samples used to prepare standard curves. Ideally, these samples would have the same background matrix and the only difference is that the blank samples do not contain compounds of interest. However, the availability of appropriate blank biological matrices can be limited, which makes the search for a surrogate matrix necessary. In this scenario, questions about how to evaluate and select the appropriate matrix for a specific kind of sample are raised. The presence or absence of absolute matrix effects among different matrices needs to be demonstrated so that the matrices with similar effects can be used as surrogates to prepare calibration standards for quantitative analysis.

In this work, different matrices were evaluated and compared based on the slope and linearity of regression lines¹⁰⁵ in the analysis of 17 α - and 17 β -estradiol. The matrices studied included bovine serum albumin in phosphate-buffered saline (PBS-BSA), gelded horse serum,

mouse serum, and mouse brain. Various sample dilution and concentration schemes of serum samples, as well as solvent extraction types, were also taken into account. The data reveals that the matrix effects of PBS-BSA and mouse serum were not significant. Moreover, they presented similar effects, indicating that PBS-BSA may be an appropriate surrogate blank matrix for the estrogen analysis in mouse serum. In contrast, significant matrix effects were observed with gelded horse serum and mouse brain, especially in the analysis of 17β -E2.

5.2 Experimental

5.2.1 Materials

Estrogens, 17β - and 17α -estradiol (17β - and 17α -E2), and dansyl chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Internal standards consisting of 17β -estradiol- $16,16,17$ -d3 (17β -E2-d3) and 17α -estradiol- $2,4$ -d2 (17α -E2-d2) were from Sigma-Aldrich (Milwaukee, WI, USA) and CDN isotopes (Quebec, Canada), respectively. Sodium hydrogen carbonate, sodium hydroxide, and methyl tert-butyl ether (MTBE) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Formic acid, water and other solvents (acetone, methanol, acetonitrile (ACN), were purchased from J. T. Baker (Phillipsburg, NJ, USA) and Burdick and Jackson (Muskegon, MI, USA).

5.2.2 Preparation of Stock Solutions and Standard Curves

Stock solutions of estrogens and deuterated estrogens were prepared by accurately weighing and dissolving approximately 1.0 mg of each compound in 1.000 mL of methanol. Working standard solutions were prepared at 10.0 and 1.0 ng/mL by diluting the stock solutions with methanol. Samples were prepared by adding an appropriate amount of working standard solutions to a defined volume of samples to obtain standards with the spiked amount in the range of 0-350 pg. Isotopically-labeled internal standards were spiked in all samples at a concentration of 125 pg/mL. Solvent, in all samples, was evaporated under vacuum before further processes.

5.2.3 Sample Preparation

5.2.3.1 PBS-BSA and serum

Total three types of biological fluids were investigated, including PBS-BSA, mouse serum, and horse serum. The PBS-BSA was prepared by dissolving 40 mg BSA in 1.000 mL of 10 mM PBS. This yielded a PBS-BSA solution at pH 7.4 with the concentration of 40 mg BSA/mL, which is the normal value of BSA concentration in mouse serum. A volume of 1.000, 1.500, or 3.000 mL of solvent (ethyl acetate or MTBE) was added to a defined volume of 25.0, 250, or 500 μ L of biological samples, respectively. The mixture was vortexed for 30 seconds, shaken for 15 minutes, and then centrifuged for 20 minutes at 2000 x g. Ninety percent of the organic supernatant was transferred into a centrifuge tube and evaporated until dryness under a nitrogen stream at room temperature. The residue was reconstituted with 45.0 μ L of sodium bicarbonate buffer (100 mM, pH 10.14) and then dansylated by adding 45.0 μ L of dansyl chloride (1 mg/mL in acetone). Total sample volumes of 90.0 μ L were incubated for 5 minutes at 60 °C, loaded into autosampler vials with glass inserts, and then transferred to the autosampler for LC-MS/MS analysis. Four-time dilution was applied to the set of 25.0 μ L biological samples and 2.5-time and 5-time concentrated samples were respectively implemented for the sets of 250 and 500 μ L biological samples, according to the procedure mentioned above. These dilution and concentration ratios were selected based on common procedures reported in literature.^{14,15,70}

5.2.3.2 Brain tissue

The matrix of mouse brain was evaluated with a different sample preparation process since heavy precipitation occurred during derivatization after liquid-liquid extraction with ethyl acetate. Brains from 3-5 C57BL mice were pooled and homogenized in lysis buffer. The homogenates were centrifuged at 10^5 x g. The tissue lysate was collected and extracted with 6 mL of methylene chloride. The upper aqueous layer was discarded whereas the remaining solvent was evaporated under nitrogen stream. Since precipitation was observed during derivatization, an additional step of solid phase extraction (SPE) was used. The residue was

dissolved in 2 mL of MeOH/water (30/70), vortexed for 30 seconds, and passed through a Strata-X SPE cartridge (Phenomenex, Torrance, CA). After washing with 4 mL of water and 2 mL of MeOH/water (60/40), the analytes were eluted with 2 mL of ethyl acetate. The eluent was evaporated until dryness. The residue was then subjected to the derivatization reaction with dansyl chloride as described above.

5.2.4 Instrumental

The LC-MS system (Thermo-Fisher Scientific, Inc., San Jose, CA, USA) was equipped with a Surveyor Autosampler, a Surveyor MS binary pump, and a LCQ Deca XP ion trap mass spectrometer (Thermo-Fisher Scientific, Inc., San Jose, CA, USA). The chromatographic system was arranged with the use of two serially-connected Shimadzu (Tokyo, Japan) Shim-pack XR-ODS columns (2 x 100 mm and 2 x 75 mm, 2.2 μ m, 120 Å; 175 mm total length). The analysis was performed with mobile phase A (H₂O/ACN/formic acid:95/5/0.1) and mobile phase B (ACN/H₂O/formic acid:95/5/0.1). Chromatographic conditions are described in Table 5.1 with an analytical run followed by washing and equilibration steps.

Table 5.1 Liquid chromatography analytical conditions.

Step	Time (minutes)	Mobile phase composition	Flowrate (μ L/min)
Analytical trial	0-8	50-100% B	150
	8-20	100% B	150
Washing and equilibration	0-10	100% B	150
	10-22	IPA/water (95/5)	50
	22-35	100% B	50
	35-40	50% B	100

The analytes were separated and eluted in a period of 20 minutes. Single reaction monitoring (SRM) MS/MS (parent [M+H]⁺ \rightarrow *m/z* 171 transition) was used with three scan events monitoring *m/z* of 506 for the two estradiol compounds, 508 and 509 respectively for the

deuterated internal standards of 17α -E2-d2 and 17β -E2-d3. Data analysis was performed using Xcalibur (version 2.0) software.

5.3 Results and Discussions

5.3.1 Absolute Matrix Effects

According to Matuszewski *et al.*,⁶⁰ the process efficiency (PE) is the product of the matrix effect (ME) and recovery (RE) of the extraction process ($PE = ME \times RE$). It requires that standards should be spiked *after* extraction to study the matrix effect and *before* extraction to study the process efficiency. The use of isotopically-labeled internal standards can compensate for the fluctuation of recovery during sample preparation processes; therefore, the relative contribution of RE to the PE is expected to be negligible in our study. Here, the results obtained from analysis of standards spiked *before* extraction was used to investigate matrix effects.

The matrix effects were evaluated by plotting the concentration-dependent responses recorded from analytes in biological matrices to those recorded from neat solution standards. The analyses were repeated two times in the range of spiked amount of 0-350 pg for each estradiol compound. The individual concentration levels from two sets of samples, those from neat solution standards and those from biological matrices, were alternately analyzed during a given sequence. This method was applied to limit the variation caused by instrumental error and carry-over at different time points. A regression line was constructed for each matrix. Peak area ratios (standard response normalized by internal standard response) were plotted; those obtained for the extraction from biological matrix on the y-axis, and those obtained for the analysis of neat solutions on the x-axis. The slopes of regression lines indicated the presence or absence of matrix effects. Deviation of the slope from unity is indicative of a matrix effect, which is exhibited as signal suppression (slope < 1) or signal enhancement (slope > 1) by the matrix.

The absolute matrix effects were investigated among different matrices of PBS-BSA, gelded horse serum, mouse serum, and mouse brain. Except for mouse brain samples, for which a different sample preparation scheme was used, all other biological fluid samples were

processed at an initial volume of 25 μ L and extracted using ethyl acetate. The regression lines obtained from the study are shown in Figure 5.1.

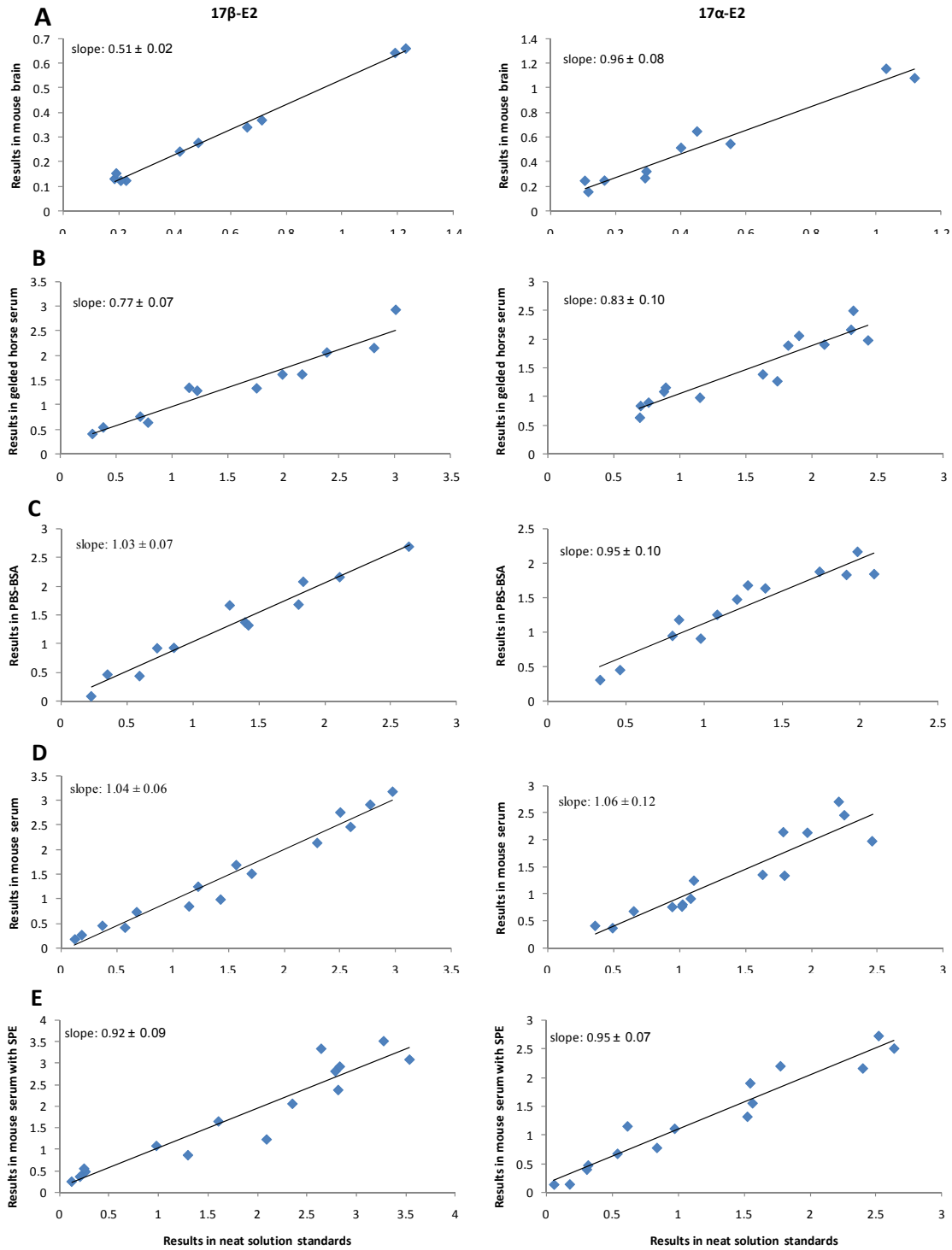


Figure 5.1 Matrix effects based on slopes of regression lines for 17 α - and 17 β -estradiol in (A) mouse brain, (B) gelded horse serum, (C) PBS-BSA, (D) mouse serum without SPE, and (E) mouse serum with SPE.

As presented in Figure 5.1A, the slope of regression lines in mouse brain, obtained for 17β -E2, was significantly deflected from the “ideal” value of 1 while that, obtained from 17α -E2, did not differ substantially from this value. This indicated the presence of matrix effects in mouse brain for the analysis of 17β -E2 but not 17α -E2. The reason could be attributed to the longer retention of 17α -E2 compared to 17β -E2 and the use of SPE in the sample preparation. Highly hydrophobic interferences might be removed by high organic content wash using Statra-X SPE. Consequently, matrix effects were possibly reduced for the longer retained analyte of 17α -E2.

Differently from the mouse brain matrix, the behavior of gelded horse serum was more complicated with the remarkable deviation of regression line slopes from the unity for both analytes (Figure 5.1B). In this case, LLE was used alone without the additional step of SPE as performed in mouse brain. For this reason, more endogenous compounds were possibly extracted and; thus, they could interfere the analysis in a stronger manner. These interfering compounds were not programmed to be detected by tandem MS but might be present at much higher levels, as several order of magnitude, than those of analytes of interest, potentially affecting ionization efficiency of the analytes.⁵⁹ Additionally, correlation coefficients of standard curves in horse serum were lower than 0.995, which did not meet the requirement for the analysis in biological samples.

In contrast, almost no matrix effects were observed in PBS-BSA (Figure 5.1C) and mouse serum (Figure 5.1D) since the slopes of regression lines in these two matrices were not significantly deviated from unity. The use of two serial connected columns for better chromatographic separation and isotopically labeled internal standards was able to reduce or eliminate the matrix effects. However, it was noticed that correlation coefficients of standard curves greatly varied in neat solution standards and most of them were lower than 0.995, when these standards were alternately analyzed with mouse serum samples. Furthermore, their linearity was even lower than that in mouse serum. This phenomenon was not encountered when the standard curves in neat solution standards were separately constructed after extensive column wash. It was suspected that interfering species were probably highly hydrophobic and

present at high concentrations. This might require an extensively long wash with different types of solvent to elute the entire interferences. However, this was not practical to have a more than 40 minute wash between analyses. As result of this, these species could continue to be eluted after the analysis in mouse serum and interfere the next analysis in neat solution standards. In other words, the background matrix might be similar for both analyses in mouse serum and neat solutions. This possibly resulted in small deviation from unity of the regression lines and caused false negative matrix effects. In this case, an alternate evaluation of the matrix effects was performed by including a SPE sample preparation step prior to LC-MS/MS to obtain cleaner samples and terminate the carry-over problem. However, the deviation from unity of the regression lines (Figure 5.1E) was not significant even with the additional SPE step. The low matrix effect in mouse serum was confirmed using the current sample preparation and analysis.

Although matrix effects were not expressed to a significant degree in mouse serum samples, likely due to the use of deuterated internal standards, signal suppression was observed during the analysis. Figure 5.2 shows full scan spectra (m/z of 100-2000) of the blank (Figure 5.2A) and mouse serum (Figure 5.2B) samples from 14.6 to 15.6 minutes, where the analytes were eluted. Both blank and mouse serum samples were subjected to dansylation reactions before the analysis. The background signals in the blank, the byproducts of the dansylation reaction, were highly responsive in the blank sample but appear to be significantly suppressed in the mouse serum sample. While the real analytical determination uses tandem MS in order to gain selectivity and reduce background noise, it cannot correct for interferences manifested during the electrospray process, where competitive ionization processes¹¹⁷ (between analytes, coanalytes, and interferences) modulate the overall ion abundance observed. For example, dansylated interferences, much like the dansylated estrogens, will still be quite responsive by virtue of the physicochemical nature of the dansyl group.

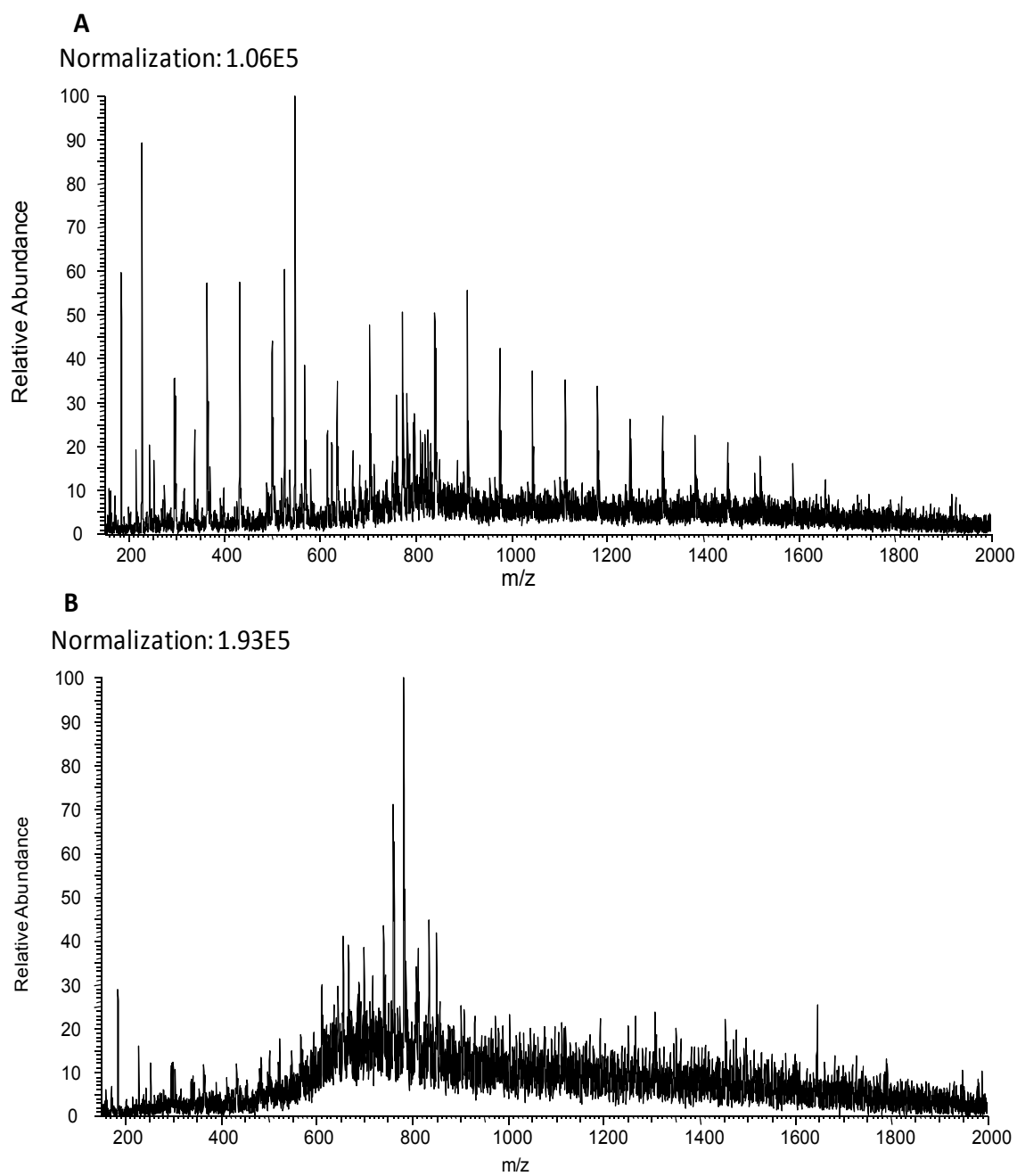


Figure 5.2 Full scan spectra of (A) blank sample and (B) mouse serum sample.

5.3.2 Serially-Concentrated Samples and Solution for Precipitation in Derivatization

The most common dilution ratio, used in serum or plasma sample preparation, is 4-time dilution^{14,70,104} (e.g., 25 μ L of initial serum to 100 μ L of final volume subjected to LC-MS/MS, as

shown above) while other serial concentrations of samples have also been reported, such as 2-time or 5-time concentrated samples.^{15,75} In some cases, with 4-time dilution of the samples, analyte concentrations in biological samples can be lower than the lower limit of quantification (LLOQ) of the method. Thus, larger volumes of samples and/or an appropriate sample concentration scheme would be required to bring the level of analytes into a quantifiable range. In this work, however, it quickly became apparent that precipitation during dansylation was observed using ethyl acetate as extraction solvent with concentrated samples. Precipitation, especially in the presence of hydrophobic analytes, such as estradiol, severely hampers analyte recovery and precision of the method (data not shown). As an alternative liquid-liquid extraction solvent, MTBE was investigated and, fortunately, no precipitation was observed.

The following experiments were carried out with horse serum. Sample volumes of 250 and 500 μL were used to create 2.5-time and 5-time concentrated samples in triplicate. Two sets of samples, those from neat solution standards and those from horse serum, were alternately analyzed by LC-MS at each concentration level, as before. Although an extensive washing and re-equilibration procedure was applied after each run, slopes of standard curves greatly varied among replicates for samples in horse serum and in neat solutions (Table 5.2). This was probably caused by the presence (and potentially, carry-over) of highly hydrophobic impurities (lipids, phospholipids, fatty acids, hydrophobic proteins, etc.), which co-eluted with the analytes and interfered with the ionization of the compounds. This same problem might have also occurred with the 4-times dilution samples run previously, but likely to a much lesser degree than for the concentrated samples. As a consequence, normalization of results in horse serum to those in neat solution could not be performed since the slopes of regression lines were not constant between replicates. All correlation coefficients of standard curves in neat solutions were higher than 0.995 for both sets. However, less satisfactory linearity ($R^2 < 0.995$) was observed for horse serum samples. Even in cases where samples are pooled and homogenized prior to sample preparation, the content of the co-eluting compounds might vary between different serum samples and cause poor linearity of standard curves. There will always be some heterogeneity to

the samples. If possible, the use of more concentrated samples to meet LLOQ should be avoided or extensive washing of stationary phases and clean-up processes prior to LC-MS/MS may be required. Another alternative would be to use a more time-consuming and expensive solid phase extraction step instead of or following liquid-liquid extraction.

Table 5.2 Slopes and correlation coefficients of standard curves for 2.5-time and 5-time concentrated horse serum samples and neat solutions.

Order of analysis	Initial horse serum volume	17 β -E2		17 α -E2	
		Slopes of standard curves	Correlation coefficients	Slopes of standard curves	Correlation coefficients
1	0	0.89	≥ 0.995	0.70	≥ 0.995
2	250	0.88	≥ 0.995	0.63	≥ 0.995
3	0	1.26	≥ 0.995	0.87	≥ 0.995
4	250	1.14	≥ 0.995	0.67	≤ 0.995
5	0	1.06	≥ 0.995	0.91	≥ 0.995
6	250	1.27	≥ 0.995	0.94	≥ 0.995
7	0	1.05	≥ 0.995	0.74	≥ 0.995
8	500	0.88	≥ 0.995	0.53	≤ 0.995
9	0	0.92	≥ 0.995	0.82	≥ 0.995
10	500	0.59	≤ 0.995	0.87	≥ 0.995
11	0	1.33	≥ 0.995	0.82	≥ 0.995
12	500	1.24	≤ 0.995	0.73	≥ 0.995

5.3.3 Choice of Extraction Solvents

Since the use of MTBE as an extraction solvent could mitigate and eliminate the precipitation during derivatization, recovery and matrix effects using this solvent were evaluated in comparison with those using ethyl acetate. The recovery using MTBE (85% for 17 β -E2 and 82% for 17 α -E2) was slightly higher than that using ethyl acetate (78% for 17 β -E2 and 75% for

17 α -E2). The matrix effects were still observed but less than those using ethyl acetate (Figure 5.3). Using MTBE was somewhat more beneficial in term of recovery and matrix effects and; moreover, avoiding the precipitation when samples were derivatized with dansyl chloride.

While the use of MTBE as a liquid-liquid extraction solvent was promising, it is not as commonly reported in the literature, relative to the use of ethyl acetate. Further studies should be performed for comprehensive method validation in this case, if MTBE is used to avoid precipitation in samples of serum. It was surprising to find very little mention of precipitation during liquid-liquid extraction procedures in the literature. It is possible that precipitation could have been overlooked, especially in samples which were subjected to minimal pre-concentration prior to analysis.

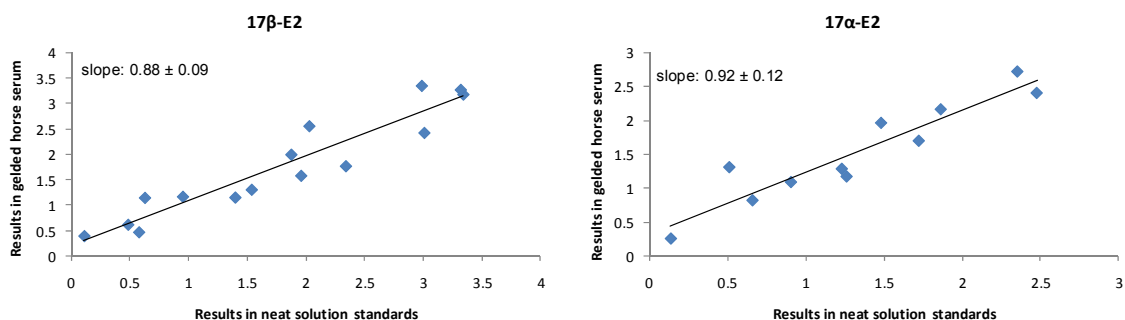


Figure 5.3 Matrix effects based on slopes of regression lines for 17 α - and 17 β -estradiol in gelded horse serum using MTBE as an extraction solvent.

5.4 Conclusions

Although different matrix effects and signal suppression were observed in biological samples, similar matrix effects between different biological samples were also revealed. The matrix effects were not significant in PBS-BSA and mouse serum (4-time dilution) when comparing the results from biological matrices and neat solution standards. PBS-BSA may be a good surrogate matrix for mouse serum with this dilution and preparation scheme. Further study of variations in concentration of PBS-BSA may be of interest since fine tuning of concentrations possibly provides similar matrix effects. Of course, matrix effects are dependent on the analyte and chromatographic method used; their occurrence should be independently evaluated for

different applications. Although precipitation was a problem during derivatization of samples concentrated prior to analysis, this was partially overcome through the alternate use of MTBE as an extraction solvent. Even so, contributions from carry-over during LLE were still a potential problem, regardless of an extensive column wash program (and the analysis of neat standard solutions) between analytical runs. In these cases, online/offline reverse phase or strong cation exchange SPE¹¹⁸ may also be a necessary alternative to LLE, despite higher cost and time consumption.

CHAPTER 6

THE ADVANTAGES OF ESI-MS DETECTION IN CONJUNCTION WITH HILIC MODE SEPARATIONS: FUNDAMENTALS AND APPLICATIONS

6.1 Introduction

Electrospray ionization (ESI) is a soft ionization technique used to transfer ions from solution into the gas phase at atmospheric pressure. Although the technique was first introduced in 1968 by Dole, it was not successfully applied for analysis of biological molecules until further development by the Fenn group in the 1980s.¹¹⁹ Conventional ESI is an extremely sensitive technique, and moreover, low flow-rate and reduced dimensionality variations such as microelectrospray and nanospray have been proven capable of excellent performance with remarkably high sensitivities (several orders of magnitude over conventional ESI).^{120,121,122} Since ESI is a flow device, it is ideal for interfacing high performance liquid chromatography (HPLC) with mass spectrometry (MS). The eluent from HPLC can be introduced into the ESI ion source to induce formation of gas phase ions, which are, in turn, led into the high vacuum of a mass spectrometer detector. The common mode of HPLC coupled with electrospray ionization-mass spectrometry (ESI-MS) is reversed phase HPLC (RPLC). This is a very powerful separation mode but the major limitation is the lack of adequate retention of highly hydrophilic, ionic, and polar molecules on a RP stationary phase. In this case, normal phase HPLC (NPLC) can be used to satisfy the separation condition. However, there are significant limitations to interfacing NP mode with ESI and hydrophilic analytes may not be soluble in NP solvent systems. Furthermore, ionization efficiency from totally organic and non-polar eluents, and consequently sensitivity, is diminished. This problem can be addressed by modifying the ionization conditions through direct¹²³ or post-column¹²⁴ addition of polar solvents and modifiers or using a make-up solution at the tip of the electrospray probe.¹²⁵ However, these modified techniques require additional set-up, such as pumps or mixing chambers and are inconvenient for routine analysis. Ionization

efficiency is still likely to be sub-optimal and the introduction of extra dead volume can limit separation efficiency. To address the separation of hydrophilic compounds under conditions suitable for interfacing with ESI, a “mixed mode” was introduced by Alpert in 1990,¹²⁶ reflecting the simultaneous presence of different interactions in which predominant hydrophilic interactions are accompanied with other interactions such as hydrophobic or electrostatic interactions. A new term, hydrophilic interaction chromatography (HILIC), was proposed to describe the combination of a polar stationary phase with an aqueous/polar organic solvent mobile phase, in which water is introduced to play the role of stronger eluting solvent. The assembly of HILIC and ESI-MS overcomes the mismatch between NPLC and ESI-MS. Advantages include increased sensitivity in ESI-MS, better on-column retention for highly hydrophilic, ionic and polar compounds and significant time savings due to feasibility of using higher flow rates, an advantage obtained from lower back pressures encountered when using low aqueous/high organic solvent mobile phases.¹²⁷

The growth of HILIC-ESI-MS has been steadily drawing attention for the last few years. A substantial amount of effort has been made to optimize HILIC mode separation for compatibility with ESI-MS. In this review, we present a brief description of both HILIC and ESI mechanisms, highlighting their mutual complementarities. Examples of applications from the literature are also presented to demonstrate the current state-of-art of HILIC-ESI-MS and its growing use.

6.2 Mechanism of HILIC

The mechanism of HILIC was first proposed by Alpert in 1990.¹²⁶ In this account, HILIC is introduced as a variant of normal phase chromatography but its mechanism is different from other variants, which include adsorption of analytes directly on the stationary phase. The mechanism of HILIC involves the partitioning of an analyte between a predominantly polar organic mobile phase and a water-enriched layer of the mobile phase that is partially immobilized on the stationary phase. However, Hemström and Irgum,¹²⁸ in their recent review, considered contributions from

both partition and adsorption processes when describing the mechanism of HILIC separations (Figure 6.1).

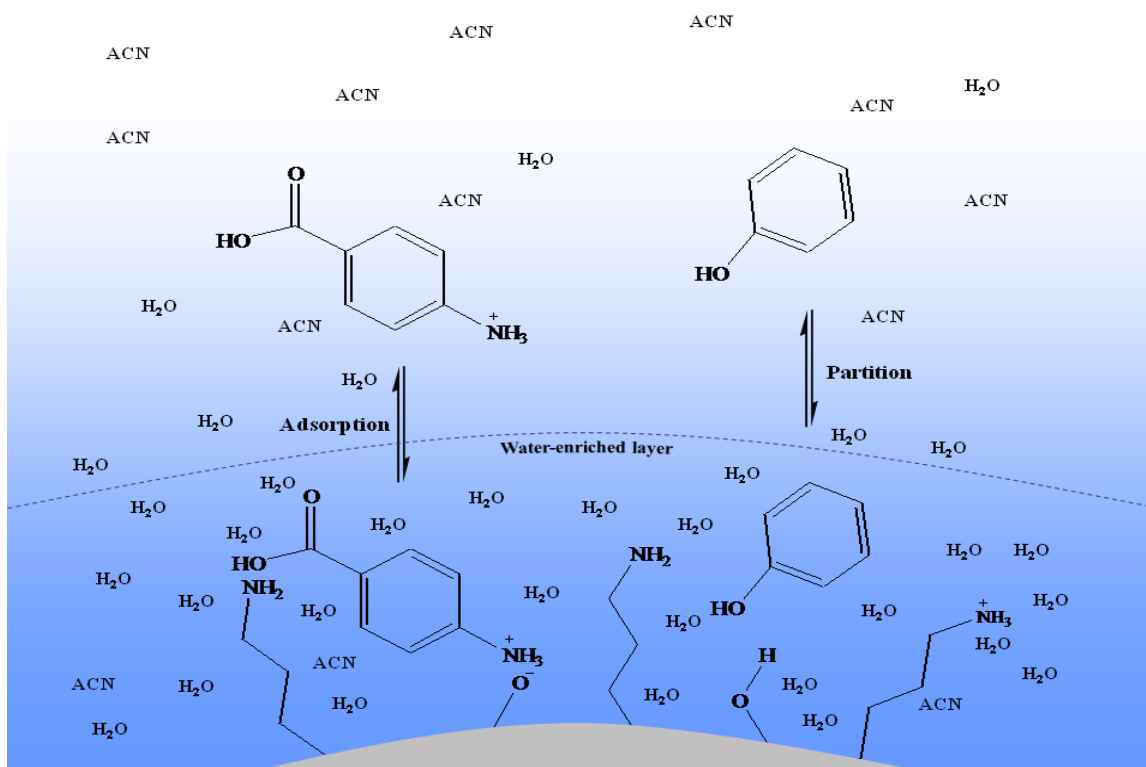


Figure 6.1 Mechanism of HILIC separation including partition and adsorption driven by hydrophilic and electrostatic interactions on an aminopropyl-bonded silica stationary phase with an aqueous-acetonitrile mobile phase.

The HILIC separation mechanism, as in any mode of chromatography, is due to the differential interaction of solutes between mobile phase and stationary phase. The interaction forces govern not only the retention but also the selectivity in HILIC separation. These include hydrogen bonding, which depends on the acidity or basicity of the solutes, electrostatic interactions, and dipole-dipole interactions, which rely on the dipole moments and polarizabilities of molecules.¹²⁹ To verify the HILIC mechanism, the effects of mobile phases and stationary phases have been examined.

6.2.1 The Effect of Mobile Phases

In HILIC mode, the mobile phase usually contains between 5 and 50% water for the elution of compounds from any of many different types of relevant stationary phases. The polar organic solvent is normally acetonitrile but other mixtures, such as acetone-ethylacetate¹³⁰ or dichloromethane-methanol,¹³¹ have also been used.

Methanol is considered as a relatively strong elution solvent; however, the use of alcohols as weakly eluting solvents may be useful when the analytes are not soluble in acetonitrile.¹²⁸ The chromatographic behavior of epirubicin **1** and its analogues was investigated by Li and Huang¹³² with the use of different organic solvents including methanol, isopropanol, tetrahydrofuran (THF), and acetonitrile as weakly eluting solvents in conjunction with a 10% aqueous buffer. Analytes chromatographed with methanol showed essentially no retention, owing to its strong hydrogen bonding character. Compared to methanol, isopropanol provides for longer retention due to its longer alkyl chain, which decreases its hydrophilic character. On the other hand, acetonitrile was shown to offer superior separation character, although based on the solvent strength (ϵ^0),¹³³ THF ($\epsilon^0 = 0.48$) should induce stronger retention than acetonitrile ($\epsilon^0 = 0.50$). This was not observed because THF behaves as a better hydrogen bond acceptor than acetonitrile. The analyses highlight the importance of hydrogen bonding, not only between the analyte and stationary phase, but also between the analyte and mobile phase components, during HILIC mode separations.

According to Neue *et al.*,¹³⁴ HILIC mode retention of ionizable compounds is a function of the pH and solvent composition of the mobile phase. Relying on this report, Ali *et al.*¹³⁵ found that ion exchange partially contributes to HILIC separation. A similar effect was also investigated by the group of Yoshida.¹²⁹

Besides the effect of pH, salt concentration also plays an important role in the HILIC retention mechanism, especially when a mixed mode of hydrophilic and ion exchange occurs using PolyHydroxyethyl A or Polysulfoethyl A stationary phases.¹²⁶ The PolyHydroxyethyl A column was reported to provide low capacity and limited cation exchange interaction for basic

compounds due to negatively charged functionalities as result of the manufacturing process or residual silanol interactions;^{126,136} therefore, a low concentration of salt is required for elution in HILIC mode. The study of Yoshida¹²⁹ indicates that broad peaks and variable peptide retention times may occur in the absence of salt. However, the solubility of salts and highly polar compounds in mobile phases containing a high percentage of organic solvent, as in the HILIC mode, should be taken into consideration as well.

Instead of increasing the salt concentration, the solvent composition can be varied by decreasing the organic solvent fraction. A comparison between HILIC and RPLC modes was carried out in peptide separation on an amide column using acetonitrile as organic solvent. The results showed that the peptides containing hydrophilic side chains eluted together in RPLC, while they were well retained and separated in HILIC. The peptide recovery and reproducibility of the HILIC separations were thus judged satisfactory.¹²⁹

6.2.2 *The Effect of Stationary Phases*

Various hydrophilic columns can be employed in HILIC mode, depending on specific applications. They are basically divided into two categories: silica-based columns and polymer-based columns.¹³⁷

Although a large amount of work has been published using non-bonded silica columns, irreversible adsorption of polar analytes on the column surface is a major problem. It is difficult to use these columns in bio-analytical separations and other applications, in which analytes of interest are present in complex matrices. In the HILIC mode, water shields the column surface but it cannot completely prevent the activation of silanol "hot spots" inducing a cation exchange mechanism for basic analytes. The addition of electrolytes is, thus, required to control the separation.¹²⁸ To overcome the drawbacks of non-bonded silica columns, polar-bonded silica phases have been widely employed. These include aminopropyl-, amide-, poly(succinimide)-, diol-, cyanopropyl-, and sulfoalkylbetaine-bonded silica phases (Figure 6.2). The properties of

these stationary phases and their application have been discussed in the recent HILIC review by Hemström and Irgum.¹²⁸

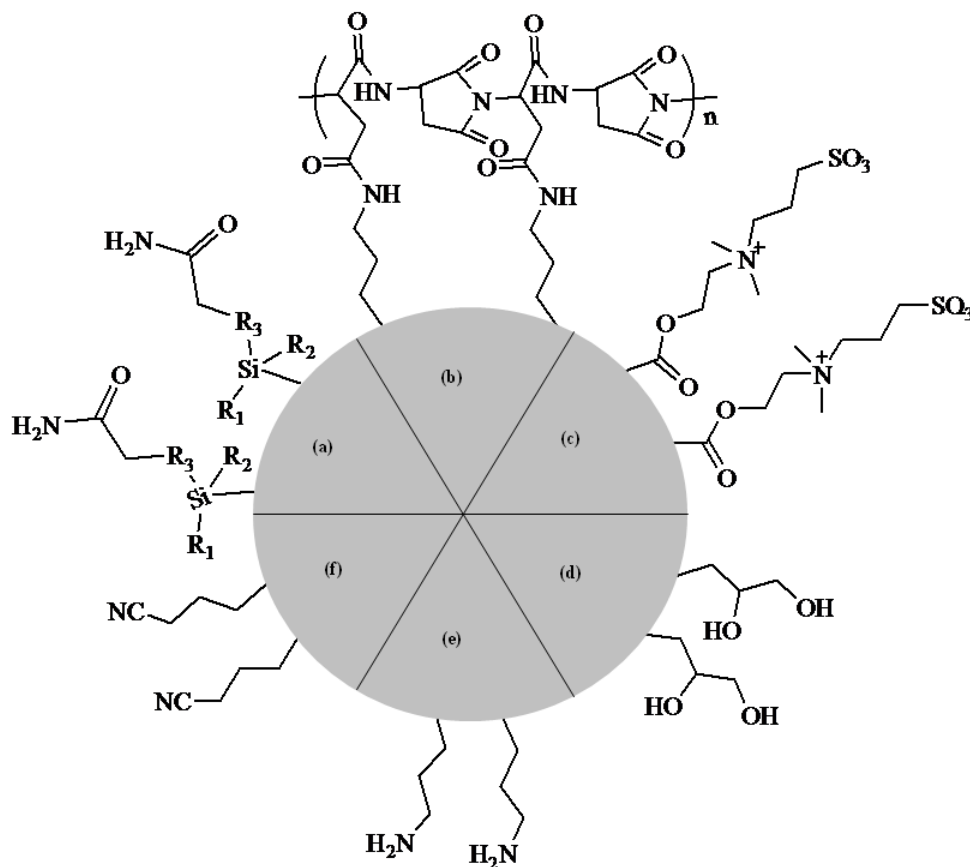


Figure 6.2 Common types of silica-based HILIC stationary phases: (a) amide-
 (www.separations.us.tosohbioscience.com/Products/HPLCColumns/ByMode/NormalHydrophilic/
 TSKgel+Amide-80.htm. Accessed 10/2007), (b) poly(succinimide)-,^{126,128} (c) sulfoalkylbetaine,¹³¹
 (d) diol-, (e) aminopropyl-, and (f) cyanopropyl-bonded silica columns.

Although silica has limited solubility (~ 100 mg/L) in aqueous solvents between pH 2-7 at room temperature, it becomes more soluble at pH above 8 and below 2,¹³⁸ and the solubility proportionally increases with the increase of water fraction.¹³⁷ In HILIC mode, a significant fraction of water is commonly used to elute the analytes. This may cause the stationary phase to dissolve if extreme pH conditions are employed. Under these conditions, non-silica-based columns provide a viable alternative.

The supporting material of non-silica-based stationary phases is commonly made from organic polymers.^{139- 143} The amino packing based on organic polymers has been shown, as expected, to be more stable than aminopropyl-bonded silica under strongly acidic and basic conditions. Several applications were reported using these types of columns including HILIC separation of oligosaccharides, fluorouracil **2**, taurine **3**, and methionine **4**. The S-DVB resins, after being highly sulfonated, can also be applied for HILIC separation.¹²⁸

6.2.3 The Effect of Mixed Modes

The separation of analytes on polar stationary phases using low aqueous/high organic mobile phases involves not only hydrophilic interaction but also other interactions such as hydrophobic or electrostatic interactions. This phenomenon has been described as giving rise to a “mixed mode” mechanism in HILIC separation.

Alpert has mentioned the mixed mode effect reflecting the presence of both hydrophobic and hydrophilic interactions when analyte retention on a PolyHydroxyethyl A was studied.¹²⁶ This work indicates that hydrophobic interactions are negligible at a high level of organic solvent while hydrophilic interactions are negligible at a low level of organic solvent, except for strongly hydrophilic analytes such as organic bases. The phenomenon of the latter case was considered as a mixed mode, causing tailing of these compounds due to electrostatic interactions with residual silanols.

The mixed modes of ion exchange and HILIC were first recognized by Zhu *et al.*^{144,145} in the separation of peptides. They found that the technique was competitive or even better than RPLC in some cases. However, the addition of a non-volatile salt buffer prevented its combination with ESI-MS. To address this problem, Strega *et al.*¹⁴⁶ studied the mixed mode of anion-cation exchange and HILIC using ammonium acetate as additive. Another phenomenon was also observed by the group of McKeown.¹⁴⁷ They showed that columns prepared from ultra-high purity silica gave lower retention and less selectivity than the columns made from metal containing silica. This was caused by the higher acidity of the silanols activated by the metal

impurities in their vicinity, mainly aluminum and iron. The discussion focused mainly on combined RP and ion exchange interactions, but Hemström and Irgum argue that these findings should translate to a mixed mode mechanism of cation exchange and HILIC, as well.¹²⁸

6.3 Mechanism of ESI

Although the detailed picture may be very complex, the ESI process can be simplified to provide a pragmatic overview in light of addressing concerns with the use of solutions commonly employed in HILIC separations, including the potential for ionization from non-aqueous mixtures. In general, electrospray ion generation is achieved through the following steps: Production of charged droplets from electrolyte dissolved in a solvent by application of a high voltage to a spray capillary; shrinkage of droplets by solvent evaporation and droplet disintegration; emission or production of ions and ion clusters from the droplets; and extraction of the ions (solvated ion clusters) into the mass spectrometer.¹¹⁷ The analyte may be charged by one or a combination of four general processes: i) Charge separation in solution; ii) adduction (association) of a charged species; iii) gas phase (e.g. charge transfer) reactions; or iv) electrochemical oxidation/reduction.¹⁴⁸ The entire process can be envisioned as a special kind of electrolytic cell, where part of the circuit is completed by the transport of charged droplets and gas phase ions across the source chamber. Figure 6.3 provides a general schematic of the process.

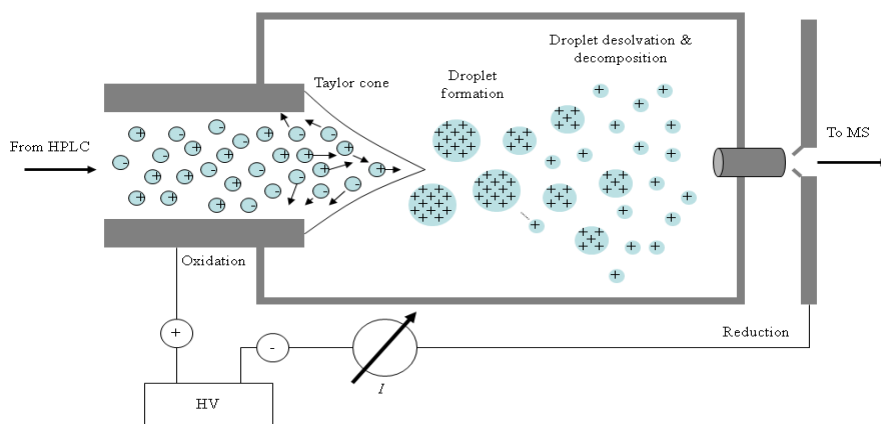


Figure 6.3 Schematic depiction of the electrospray process in positive ionization mode. Figure adapted from reference 117 and 149.

The process begins with the dissolution of analytes and electrolytes in solution. The solubility of the various species is controlled by their respective solvation energy in the given solvent or solvent mixture. Charge separation is incurred for ionic species as a function of the dielectric constant of the bulk solvent. A host of descriptors can be used to describe the solvent-solvent and solvent-solute interactions, which prevail to cause dissolution. In general, solubility is dictated by the ability of the solute to reorient solvent molecules (loss of entropy) and disrupt solvent-solvent interactions (loss of enthalpy). Though energetically unfavorable, this is offset (and made favorable for soluble solutes) by the corresponding solvent-solute interactions which are formed.¹⁵⁰ With regard to electrostatics, the dielectric constant of the medium will dictate the ability of charges (in the form of ionized solvent components) to separate in the solution. In HILIC mode separations, compounds are typically eluted and introduced into the ESI source in a lower dielectric medium than that commonly encountered in RPLC.

The solution is passed through the spray capillary which is held at high voltage ($\sim \pm 2 - 5$ kV). For the sake of discussion, we will address here the formation of positively charged ions through the application of a positive voltage to the spray capillary (as shown in Figure 3). Through charge separation, induced by the high potential on the capillary, positively charged ions in solution are repelled away from the capillary and form a Taylor cone.¹⁵¹ The cone is formed as the positive charges, which are trying to reach the counter electrode, are impeded by the presence of solvent. Droplets enriched in positive charge are formed following jet expansion of the charged liquid into a spray plume. To achieve this condition, a strong electric field is needed at the tip of the capillary. Equation 6.1 describes the magnitude of the electric field (E_c) with respect to the source configuration:¹¹⁷

$$E_c = 2 \frac{V_c}{r_c} \ln \left(\frac{4d}{r_c} \right); \quad (6.1)$$

where V_c is the potential applied to the capillary, r_c is the radius of the capillary, and d is the distance to the counter electrode. The magnitude of the electric field needed for charged droplet

formation is described by the onset voltage (V_{on}) and is a function of r_c , d , and the surface tension (γ) of the solution, as shown in Equation 6.2:

$$V_{on} \approx 2 \times 10^5 (\gamma r_c)^{1/2} \ln \left(\frac{4d}{r_c} \right). \quad (6.2)$$

Table 6.1 shows the onset voltage and surface tension for some common solvents (when $d = 4$ cm and $r_c = 0.1$ mm). Interesting to note is that the non-aqueous solvents listed in Table 6.1 require a significantly lower potential for the onset of droplet formation. This is due to their lower surface tension compared to water and one of the major reasons for employing hydro-organic solvent mixtures to achieve efficient ionization.

Table 6.1 Onset voltages and surface tension for common solvents.³¹

	CH₃OH	CH₃CN	(CH₃)₂SO	H₂O
V_{on} (kV)*	2.2	2.5	3.0	4.0
γ (N/m²)	0.0226	0.030	0.043	0.073

*Assuming $d = 4$ cm and $r_c = 0.1$ mm

Once charged droplets have formed, they begin to traverse the source chamber on their way to the counter electrode. In this period, the droplets shrink in size through the combination of solvent evaporation, induced by the convective forces of air molecules in the atmospheric pressure chamber, and droplet disintegration, induced by the columbic repulsion of charges at the droplet surface overcoming the force of surface tension trying to hold the droplet together. The efficient conversion of droplet charge to gas phase ions is aided by the production of very small and highly charged droplets. Droplet radius, which is a function of flow rate (V_f), surface tension (γ), and the density of the solution (ρ), can be described by Equation 6.35:

$$R \propto (\rho V_f^2 \gamma)^{1/3}. \quad (6.3)$$

Although current (I) is also proportional to flow rate, Equation 6.3 shows that trying to increase ion current by increasing flow rate is counterproductive. The current will increase, but ion intensity decreases as larger droplets are formed which are less efficient for forming gas phase ions. The role of density and surface tension in the sprayed solution relative to droplet size means that smaller droplets may be formed if solvent systems are employed where these characteristics are minimized. Many common organic solvents have lower density and give rise to decreased surface tension compared to water, further supporting the advantages of HILIC elution solvents in the context of increased ionization efficiency. Additionally, the rate at which solvent evaporates from the droplet surface is a function of the vapor pressure of the solvent; again favoring organic solvents with higher volatility. Early studies by Cole and coworkers investigating the effect of source temperature on ionization from droplets containing chlorinated solvents in the negative ionization mode have corroborated this effect.¹⁵²

Droplet disintegration is another important part of the electrospray process. The condition where coulombic repulsion on the charged droplet surface overcomes the force of surface tension holding the droplet together is called the Rayleigh stability limit.¹⁵³ This is described mathematically by the Rayleigh equation:

$$Q_R^2 = 64\pi^2\epsilon_0\gamma R_R^3. \quad (6.4)$$

Q_R is defined as the charge (Coulombs) on the droplet surface which is just sufficient to overcome surface tension (ϵ_0 is the permittivity of vacuum and R_R (meters) represents the size of the droplet carrying sufficient charge to satisfy Rayleigh instability). For pure methanol, $\gamma = 0.0226 \text{ N/m}^2$, therefore:

$$Q_R^2 = 1.25 \times 10^{-10} R_R^3. \quad (6.5)$$

The coefficient in this expression for acetonitrile ($\gamma = 0.030 \text{ N/m}^2$) is only slightly larger. For water ($\gamma = 0.073 \text{ N/m}^2$), it is three times larger, meaning (for a given droplet volume)

significantly more charge can be carried on a charged water droplet compared to a charged methanol or acetonitrile droplet before decomposition takes place. In other words, for droplets with equal charge and volume, one based from polar organic solvents will decompose earlier; whereas one based from water must still shed volume through evaporation before reaching Rayleigh instability. This is in a sense an oversimplification, but it does describe a significant difference in droplet properties with respect to the droplet's composition. In practice, charged droplets are formed from solution containing a significant amount of electrolyte ions for conductance. This increase in ionic strength lowers the surface tension of the liquid in which it is present. Overall, during the disintegration process a number of droplet subdivisions take place which ultimately lead to the release of gas phase ions.

lavarone and Williams studied the effect of surface tension on multiple charging and "supercharging" of various moderate molecular weight molecules, including dendrimers and cytochrome *c*.¹⁵⁴ That found that as the surface tension of the liquid was increased, higher charge states could be achieved, because of the ability to hold more charges at the droplet surface prior to decomposition. They showed that adding *m*-nitrobenzyl alcohol (*m*-NBA) into solution increases charging when present in a solution which previously displayed lower surface tension. Solutions studied included various combinations of water, methanol, acetic acid, 2-propanol, glycerol, dimethyl sulfoxide (DMSO), *m*-chlorophenol, formamide, and 2-methoxyethanol.

The debate over the detailed mechanism for formation of gas phase ions from small charged droplets is beyond the scope of this review article. Many past and recent articles have specifically addressed this debate.^{117,148,154-157} To be brief, small ions are believed to be released through the process of ion evaporation (Ion Evaporation Model; IEM). Based from transition state theory, this model assumes that at some point, in a small charged droplet, the solvation energy of an ion in solution becomes sufficiently unfavorable so that the ion is released from the droplet into the gas phase. Large ions, such as proteins and high molecular weight polymers, are believed to

form gas ions through the Charge Residue Model (CRM).^{154,158} In this model, droplets desolvate and decompose until only a charged residue, the ion of interest, remains.

Focusing on the ionization of small and medium molecules, and assuming a mechanism based from the IEM, solvation energy of the analyte/ion of interest in the droplet is an important parameter to consider. Ionization of hydrophilic molecules could be expected to be more favorable in the presence of a non-aqueous or low-aqueous solvent environment, assuming they can be dissolved in the medium in the first place. This is particularly relevant for consideration in light of HILIC applications. Conversely, guidelines pertaining to the increased ionization of hydrophobic molecules from aqueous solutions would be less valid when considering a non-aqueous solvent system.¹⁵⁹ In such cases, a more accurate descriptor of ionization efficiency to consider would be solvophobicity, with respect to given solvent systems, for a given analyte. Even so, many of the molecules investigated by ESI-MS are ionic in solution, making solubility parameters based on non-ionic analytes in low polarity solvents less valid for assessing ionization.¹⁵²

The role of solvation energy also comes into play when considering the conductance of the sprayed solution. Suitable electrolytes must be incorporated into a non-aqueous system to provide conductance. In e.g., 100% MeOH, approximately 1 μ M electrolyte (a minimal amount) must be present to achieve “threshold” conductivity and a stable spray current according to Equation 6.6:

$$I \approx H\sigma^n; \quad (6.6)$$

where the current I is a weak function of conductivity σ ($n \approx 0.2 - 0.3$) and H is a constant determined by the experimental set-up.¹⁶⁰ The common thought is that the electrosprayed solution must contain a certain amount of water to provide adequate conductance for generation of ion current. Although conductance is increased in an aqueous environment, an aqueous environment is clearly not a necessity.

To further add to the picture, if we consider the use of organic solvents which have a lower dielectric constant than water, the association of charges leading to multiply charged ions would change. Since electrostatic forces are intimately linked to the dielectric constant in solution (shown by Equation 6.7), a lower dielectric medium might be expected to decrease available charge states due to decreased charge separation.

$$P = \frac{Q_1 Q_2}{4\pi\epsilon\epsilon_0 r}, \quad (6.7)$$

where P is the potential based on Coulombic interactions between two charges (Q_1 , Q_2), ϵ is the dielectric constant, and r is the distance between the charges. A protein ionized from a lower dielectric organic medium would likely have a narrower distribution of charge states compared to the same analysis performed in water. In general, it has been shown that solvent systems with lower dielectric constants and higher viscosities, favor a decrease in the number of charge states observed.^{152,161} Such an effect may be considered a drawback with respect to mass range limitations of some mass analyzers, but it may also be an advantage; increasing the summed signal to noise ratio for a given molecule by narrowing the distribution of charge states which it may access.

In line with a short discussion of ionization mechanisms by electrospray, another model has been proposed which focuses not on the way ions are formed, but on the relative ionization efficiency of analytes of interest. The Equilibrium Partitioning Model (EPM), developed by Enke and coworkers, addresses the way ions in a desolvating droplet compete for a limited number of charged sites on a droplet surface.¹⁶² The model predicts that analyte response will be proportional to concentration, but that the response factor will decrease as the amount of electrolyte in the solution increases. This is the basis for “saturation” or “ionization suppression” which is so commonly cited for loss of linearity in ESI-MS applications. Without going into too much detail, the charged droplet can basically be thought of as a two phase system. The charged surface of the droplet is distinctly different than the droplet interior. Inside the droplet,

analytes and electrolytes can migrate (through diffusion and electrophoresis) or partition from the bulk solvent interior to the droplet surface. It is from the droplet surface, which is characterized by an excess surface charge, where ions are formed. The excess charge controls the maximum rate of gas phase ion production and is related to the rate of solution phase charge separation. Therefore, the amount of gas phase ions produced cannot exceed the amount of excess charge on the droplet surface.¹⁴⁸ An intricate and covariant interplay of solvation energies, surface activities, and relative partitioning factors control the access by analytes in the droplet to the limited surface charge. Thus, the EPM can be used to predict the effect of ionic partition constants, excess charge, and concentration of analytes and electrolytes (all of which can be determined experimentally) on analyte response. All of the aforementioned variables (surface activity, relative partition constants, and solvation energy) are dependent on the solvent from which the charged droplet is composed. In general, several excellent publications have addressed systems of varying degrees of complexity with respect to the EPM.^{159,162-164}

Regardless of the mode of release from the charged droplet, the desired end product of the ionization process is a gas phase ion. This ion can be sampled into the mass spectrometer and is subject to processes governed therein. A comprehensive discussion of gas phase thermodynamics is beyond the scope of this review.¹⁶⁵ Of note with respect to ion formation, however: Regardless of the solvent medium from which the ion is formed, the nature of the gas phase into which the ion is transferred is essentially non-polar. In this respect, the droplet surface forms a phase boundary between the solvated environment with a given dielectric constant and the gas phase, which essentially has a dielectric constant equal to unity. Thus, those electrostatic interactions which are formed in solution, be they charge associations or formed non-covalent complexes, will be strengthened vis-à-vis Equation 9 when they enter into the gas phase. This certainty has given rise to a number of discussions relating to the solution versus gas phase correlation of equilibria measured by means of ESI-MS.^{160,166-171} The bottom line is that such correlation is usually system dependent, and care should be taken when trying to quantitatively correlate observed gas phase ion abundances with solution phase concentrations.

Also, the role of proton affinity (gas phase basicity) once ions have reached the gas phase should be considered.¹⁷² It is important to choose a solvent with a proton affinity lower than that of the analyte to avoid gas phase ion suppression when operating in the positive ionization mode.

In summary, the role of the solvent in the electrospray ion generation process is undoubtedly very important. The solvent provides a medium for dissolution of the analytes and electrolytes in the system (related to the solvent's dielectric constant). It provides conductance, with the aid of added electrolytes, for the flow of charge, both in the formation of charged droplets, but also related to the formation of charged ions and electrostatically-bound non-covalent complex or adduct ions. The physicochemical characteristics of the solvent (specifically solvent density, viscosity, surface tension, and vapor pressure) dictate droplet size, droplet desolvation, and droplet disintegration. Included with this character are effects on analyte solvation energy (solubility), surface activity, and relative partitioning within the droplet. Finally, the interplay of all of these processes and parameters lead to the emission of a gas phase ion. Thus, when considering ionization from a low aqueous or non-aqueous medium, it is the entire process from sample introduction to ion formation which must be considered. Specific effects based on instrumental parameters, such as applied voltage, nebulizer gas flow rate, solution flow rate, and the distance between the spray capillary and counter-electrode are also important.

6.4 Method Development of HILIC-ESI-MS

Although the extraordinary potential of ESI as an interface between HPLC and MS has been demonstrated since the early 1980s,¹²⁰ it was not employed to combine HILIC mode separations with MS detection until the 1990s. For the last several years, the interest in and application of this combination has shown a dramatic increase. Using the HILIC mode, highly hydrophilic, ionic, and polar compounds can be retained and separated in the presence of polar, NP-like, or ion exchange-type stationary phases.

The presence of buffer salts and acids in mobile phases used for HILIC mode separation has a strong impact on the behavior of the analytes. Electrostatic interactions induced between

the analytes and polar/ionic stationary phase (and residual silanols) are tempered by the presence of a buffer.¹⁷³ However, a high concentration of buffer can suppress the ESI signal¹⁷³ and cause problems when the solubility of many buffer salts decreases in HILIC mobile phases containing a large proportion of organic solvents.¹²⁹ Therefore, the buffer concentration should be optimized so that peak shape, efficiency, and retention can be improved without harming the ESI sensitivity. To obtain a good separation, in some cases, it is necessary to add certain acids such as TFA, acetic, or formic acid. These acids not only control the pH of the mobile phase but also provide ion pairs for basic analytes to improve their peak shape and separation efficiency.¹⁷⁴ Even so, significant signal suppression is still observed in ESI-MS with the addition of TFA to the mobile phase.¹⁷⁵ Recently, Naidong has indicated that the gain in sensitivity from using higher organic content in HILIC mode is large enough to compensate the loss of signal caused by adding a small amount of TFA (usually below 0.05%).¹²⁷ Later, this group also reported the negative effect of TFA and minimized it by adding 0.5% acetic acid or 1% propionic acid to mobile phases containing either 0.025% or 0.05% TFA.¹⁷⁴ A factor of two- to five-fold signal enhancement was achieved for eight basic compounds of interest. The method was successfully applied to the high throughput analysis of extracted biological samples to support pre-clinical and clinical studies. The signal enhancement can also be achieved by post-column addition of aprotic solvents or long chain alcohols, following HILIC separation.¹⁷⁶ The explanation was based on the principle that, since analyte ions must form through the transfer of a proton to another compound present in the droplet to become part of the excess charge, solvents with increased gas phase proton affinities may lead to enhanced response. It happens to be that the aprotic solvents and long chain alcohols exhibit slightly stronger gas phase proton affinities than water and short chain alcohols, like methanol or ethanol.^{177,178} Analytical conditions employed for HILIC-ESI-MS vary depending on analyte characteristics; however, in general, an amide-bonded column is often used in combination with ammonium formate or acetate buffers. It is noted that the buffer concentration should not exceed 0.05% to achieve reasonable efficiency without suppressing ESI

signals. Typical gradient conditions are employed starting at 5% aqueous and changing to 50% aqueous component over time, in acetonitrile.^{129,137,146,173}

6.5 Applications of HILIC-ESI-MS

6.5.1 Pharmacokinetics

Drug analysis in pharmacokinetics has been a challenge because the drug compounds are present in low abundance in complex biological matrices. HILIC-ESI-MS have been shown to be quite suitable for use in this situation. Since the mobile phase contains an aqueous component, the interference from the aqueous nature of the biological matrices is no longer a concern compared to NPLC.¹⁷⁹ Additionally, HILIC coupled with ESI-MS potentially allows a higher sensitivity because the higher percentage of organic solvent in HILIC mode contributes to more efficient desolvation during ESI.¹⁸⁰ Due to these advantages, a relatively large amount of work has been performed on this topic.

Since biological samples are normally in complicated matrices, pre-treatment of samples, in some cases, is necessary. Solid phase extraction (SPE) and liquid-liquid extraction (LLE) are usually used for this step. The widely used trapping mechanism for SPE is hydrophobic interaction and the most common SPE sorbent is alkyl-bonded silica (C2, C8, and C18 silica). This, of course, may result in poor retention of highly hydrophilic analytes,¹⁸¹ but still provides a mechanism by which complex matrices can be fractionated. Both online^{182,183} and offline¹⁸⁴⁻¹⁹⁶ sample preparation with HILIC-ESI-MS, primarily for the analysis of biological fluids, but also for tissues has been reported. For example, a small gel-filtration column has been employed for pre-purification of samples.^{197,198} This application was demonstrated for isolation, separation, and detection of N-glycans using ZIC HILIC-ESI-IT-MS in combination with fluorescence and ultraviolet detectors. However, this step can be skipped by simply employing a one-step protein precipitation procedure,¹⁹⁹⁻²⁰⁶ because HILIC separation can provide online sample clean-up, where most of the unwanted hydrophobic compounds are eluted with the solvent front. By

simplifying and minimizing sample preparation, degradation of analytes was reduced and percentage of recovery increased.

Naidong published a review on quantitative analysis of polar ionic compounds in biological fluids on non-bonded silica columns in HILIC mode coupled with ESI-MS/MS.¹²⁷ Contrary to common perception, the silica columns showed superior column stability in the HILIC mode and the analysis could be carried out at high flow rate because of the low backpressure generated from the silica column. Later, a variety of studies were performed, analyzing human and animal plasma using silica columns. A new method was developed using direct injection of SPE extracts for the analysis of isoniazid **5** and cetirizine **6** by HILIC-ESI-MS/MS.²⁰⁷ This method was then improved into a 96-well SPE format, which was also described by Kall *et al.*,²⁰⁸ and finally, the whole system was upgraded to an automated 96-well SPE format²⁰⁹ coupled with HILIC-ESI-MS/MS. The method eliminates the evaporation and reconstitution steps between SPE and HILIC separation. Good sensitivity was achieved when the aqueous/acetonitrile mobile phase was used with the addition of TFA (0.025%, v/v) and acetic acid (1%, v/v). For cetirizine analysis, the limit of quantitation was in the ppb range using only 100 µl of plasma and the run time was only 2 minutes. This method was successfully employed to measure cetirizine in human plasma for drug-drug interaction studies. In parallel, the addition of automated 96-well LLE to the HILIC-ESI-MS system was also developed.²¹⁰⁻²¹² Using either the combined LLE- or SPE-HILIC-ESI-MS analytical platform, the Naidong group successively measured many other compounds, including fluconazole **7**,²¹¹ omeprazole **8**,²¹² tobacco-specific nitrosamine metabolite (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol) **9**,²¹³ paroxetine **10**,²¹⁴ and atenolol **11**,²¹⁵ from human plasma.

Lee *et al.* developed a method for the determination of tiapride **12**,²¹⁶ levosulpiride **13**,²¹⁷ levofloxacin **14**,²¹⁸ gabapentin **15**,²¹⁹ and carvedilol **16**,²²⁰ in human plasma. The analysis utilized a non-bonded silica column and an aqueous/acetonitrile mobile phase with addition of ammonium formate, combined with ESI-MS/MS in the multiple reaction monitoring mode. The method was

also successfully applied to the analysis of drugs from human plasma for pharmacokinetic studies.

6.5.2 Drug Discovery

In the pharmaceutical industry, drug discovery is the main focus. Bioactive compounds extracted from natural products have been found to be vital and complementary to synthetic organic compounds as lead drug candidates. Strege has reported the analysis of polar compounds for natural product drug discovery by HILIC-ESI-MS.¹⁷³ In this study, amide-, polyhydroxyethyl aspartamide-, and cyclodextrin-based columns were shown to provide superior retention, while the use of cyano-based columns resulted in much less retention because of their relatively weaker polar character. The effect of mobile phases, including the effect of buffer salt concentration, buffer character and pH were also studied. Ammonium acetate at a concentration of 6.5 mM, and adjusted to pH 5.5, was determined to be the best buffer for HILIC separation in conjunction with ESI-MS. Two sets of standard mixtures were evaluated by both positive and negative ionization mode HILIC-ESI-MS. To demonstrate the feasibility of the method, a complex mixture of fermentation broth was analyzed after pretreatment with SPE. Seventy components appeared to be chromatographically resolved and, for further analysis, additional deconvolution provided by the generation of extracted ion chromatograms was necessary. Later, the Strege group also developed a mixed-mode anion-cation exchange/HILIC method to provide direct compatibility with ESI-MS for small molecule drug discovery.¹⁴⁶ The technique involved the use of silica-based small pore weak ion exchange resins eluted with a combined aqueous and pH gradient. The authors recommended that these methods were useful for the analysis of polar compounds in biological and synthetic complex mixtures.

Recently, HILIC-ESI-MS was successfully applied to the analysis of several drug related compounds from other natural sources such as snake venom, *Panax* plant, apple juice, and mulberry.^{221- 224} Qualitative identification and quantitation of spermine **17**, a linear polyamine, in the viper venom showed that this compound constituted approximately 1% of the dry venom

mass.²²¹ The analysis was carried out on a polymer cross-linked amino column (Grom-SIL 120 Amino-2 PA) with ammonium formate buffer adjusted to pH 3.5 in the aqueous phase. For quantitative analysis, HILIC-ESI-MS/MS was employed following fractionation by preparative size exclusion chromatography. Compared to spermine, underivatized dencichine **18** extracted from *Panax* medicinal plant species is more hydrophilic.²²² Optimum separation and retention of dencichine was achieved in the presence of 80 mM ammonium formate, which still permitted sufficient ESI-MS sensitivity. HILIC-ESI-MS was also used to investigate the retention behavior of oligomeric proanthocyanidins.²²³ The analysis was successfully performed on an amide column without the addition of buffer. In this condition, a mixture of natural oligomeric proanthocyanidins extracted from apple was separated based on the degree of polymerization up to decamers. The degree of polymerization was then confirmed by negative ionization mode ESI-MS. As a result, the investigators found that two main factors were contributing to the separation, specifically hydrogen bonding and solute hydrophilicity. Hydrogen bonding occurs between the amide groups on the stationary phase and the hydroxyl groups of the solute oligomer, while hydrophilic interaction is due to the higher order structure of oligomeric proanthocyanidins. Among these two, hydrogen bonding was concluded to be the most important interaction mode for HILIC separation of the polyphenolic compounds. A similar application was carried out by Neungchamnonng *et al.* for separation and quantitative determination of 1-deoxynojirimycin **19** in mulberry leaves on an amide column coupled with tandem MS.²²⁴

Besides the therapeutic compounds isolated from natural products, synthetically derived compounds are also of paramount importance in the pharmaceutical industry. Small quaternary amine compounds such as choline **20**, chlormequat **21**, acetylcholine **22**, betaine **23**, and mepiquat **24** are fairly significant in terms of biomedical and environmental applications. The feasibility of analyzing these compounds by HILIC-MS and their retention behavior were investigated separately by Guo and the Grumbach group. It was found that the sensitivity of the HILIC-MS method was 75 times higher than that of the RPLC-MS method. Even though the impact of biological matrices on the analytical performance has not yet been tested, the method

still holds a great potential for low-level analysis of choline, and other quaternary amine compounds.²²⁵⁻²²⁷

An advanced procedure for separation and analysis of a low molecular weight cardiac sodium/calcium exchanger inhibitor (NCX_{IF}) was reported by Khananshvili and coworkers.²²⁸ Three chromatographic modes were studied including RPLC, NPLC, and HILIC in combination with ESI-MS. For RPLC, the retention time of NCX_{IF} on the C18 column did not increase when ion-pairing reagents like TFA or heptafluorobutyric acid (HFBA) were added to the mobile phase. However, NCX_{IF} was successfully separated from other small hydrophilic molecules on the two HILIC columns, PolyGlycoplex and PolyHydroxy A.

6.5.3 Biomolecular Analysis

Limited work has been found on protein analysis by HILIC-ESI-MS.²²⁹⁻²³¹ The reasons are due to the condition of the HILIC separation more than the detection by ESI-MS. First at all, most integral proteins are well separated by common protein separation methods; therefore, there is less of an impetus to develop a new method. Secondly, there is the inherent issue of protein solubility in predominantly polar organic solvent mobile phase.¹²⁸

Although the analysis of amino acids and peptides by HILIC mode HPLC has been reported, HILIC separation has been mainly used as a purification process, without direct interface with ESI-MS. Very few publications can be found for HILIC separation of amino acids and peptides coupled online to ESI-MS. The determination of glutathione in plant somatic embryos was successfully achieved with low detection limit (0.5 nM), high selectivity and reproducibility, promising its application in microscopic cell structures and in single cell analyses.²³² Langrock *et al.*²³³ showed that a method could separate and detect three isobaric amino acids, Hyp-isomers, ILe, and Leu, allowing an unambiguous identification of amino acids. Interestingly, HILIC was combined with RPLC as an orthogonal separation prior to ESI-MS in this work. In fact, the orthogonal separation was first investigated for the analysis of peptides by Gilar and coworkers.²³⁴ Later, a similar approach was employed by Min *et al.*²³⁵ when they carried out

an experiment to determine oligosaccharides in glycopeptides using a fully automated HILIC-RP 2D HPLC setup combined with ESI-TOF-MS. Although the run time was long, the system appears to be useful for online oligosaccharide analysis. A simple separation of isomeric sialylated N-glycopeptides was demonstrated by Takegawa *et al.* using a ZIC-HILIC-ESI-IT-MS method.^{197,198} The retention of N-glycans was primarily attributed to hydrophilic interaction; however, the retention of sialylated N-glycans could be effectively tuned by electrolyte concentration, specifically ammonium acetate in this case, without losing the structure recognition capability of other isomers.

6.5.4 Food Analysis

Compounds extracted from mushrooms have been reported to be analyzed by HILIC-ESI-MS. Rotzoll *et al.*²³⁶ studied the attractive taste of morel mushrooms. They successfully determined (S)-malic acid 1-O-D-glucopyranoside (Morelid) **25** and γ -aminobutyric acid **26** as contributors to umami taste and mouth-drying oral sensation. It was noted that (S)-Morelid was isolated and detected in food products for the first time. The separation was carried out on an amide-bonded column with the mobile phase of acetonitrile and aqueous ammonium formate buffer. An ESI-quadrupole MS was employed and operated for detection in both positive and negative ionization modes. Using a similar approach, Chung *et al.*²³⁷ developed a method for determination of polar toxins including amanitins, phallotoxins, and muscarine in mushrooms. A mixed-mode hydrophilic and ionic interaction HPLC set-up on an amide-based stationary phase was used with the addition of methanol to the mobile phase as a modifier for fine tuning the magnitude of the system in terms of proton-donor and acceptor strength. For these compounds detected by ESI-MS, it was found that the negative mode showed a lower sensitivity than the positive mode. Tandem MS was used to diagnose the analyte structures. This combination enhances the specificity of the method over other methods which generate non-confirmative signals, such as UV-VIS spectroscopic detection or single quadrupole MS detection.

HILIC-ESI-MS was also employed to separate and characterize low molecular weight compounds in foodstuffs. An investigation by Schichtherle-Cerny *et al.*²³⁸ showed that free amino acids and small polar peptides can be successfully analyzed in complex food matrices such as deamidated wheat gluten hydrolysate and parmesan cheese. They also proved the possible identification of isobaric peptides due to the different fragmentation patterns obtained by collision-induced dissociation. Similar to that, Fenaille *et al.*²³⁹ accurately quantified another small peptide, dityrosine, in milk powders, using d₄-dityrosine as internal standard. The use of this stable isotope helped to determine if artifactual formation or breakdown of dityrosine occurred in the proteolysis process during sample preparation.

Another application was reported by Lojkova and coworkers.²⁴⁰ Two imidazole compounds, 4(5)-methylimidazole **27** and 2-acetyl-4(5)-(1,2,3,4)-tetrahydroxybutyl-imidazole **28**, from ground coffee were separated with the isocratic mobile phase of 80% methanol and 20% 10 mM ammonium formate buffer. The analyte stream was then introduced to the ESI-MS operated under selected ion monitoring mode. The results showed a good reproducibility of retention times, peak areas, and analyte concentrations.

HILIC-ESI-MS was also extended to the field of drinking water and beverage analysis. Dichloroacetic acid was detected by Dixon *et al.*²⁴¹ for the first time in drinking water by using mixed-mode ion exchange and HILIC on an amino column in conjunction with ESI-MS/MS. Similarly, Person *et al.*¹⁴³ developed and validated a new procedure for the simultaneous determination of underivatized taurine **3** and methionine **4** in carbohydrate-rich-beverages. The advantage of this method was that no initial clean-up and no sample derivatization were needed. Although HILIC separation mode may be amenable to highly hydrophilic, ionic and polar compounds compared to RPLC, it is not always the best choice. Zywicki *et al.*²⁴² made a comparison between HILIC and RPLC coupled with ESI-MS in glycoalkaloid analysis from potatoes. RPLC was revealed to be more precise, accurate and rugged than HILIC in term of peak shapes for this particular application.

6.5.5 Metabolic Studies

Since the main purpose of metabolic analysis is to isolate and measure all metabolites in a biological system, the separation of metabolite mixtures is extremely important.^{128,243} HILIC-ESI-MS method has been recently recognized as a powerful tool for use in metabolomics.

Oligosaccharides have been known as important metabolites in biological systems. However, their metabolic studies are somewhat troublesome because they are usually available in a small quantity and difficult to separate by chromatographic techniques. Therefore, Darvill and coworkers attempted to employ the derivatization of oligosaccharides by reductive amination with *p*-nitrobenzylhydroxylamine (PNB) to form *N*-(*p*-nitrobenzyloxy)aminoalditol derivatives, which could be successfully separated by HILIC and detected by both UV and ESI-MS detectors.²⁴⁴ The use of combined detectors allowed higher confidence in discerning the derivatized forms of oligosaccharides.

The analysis of not only oligosaccharides but also several other metabolites from plants has been reported. Tolstikov and Fiehn used HILIC-ESI-MS to detect highly polar metabolites such as oligosaccharides, glycosides, amino sugars, amino acids, and sugar nucleotides in phloem exudates from petioles of fully expanded *Cucurbita maxima* leaves.²⁴⁵ HILIC separation was performed using a mobile phase consisting of aqueous ammonium acetate (6.5 mM, adjusted to pH 5.5) and acetonitrile. The best separation was achieved using an amide column compared to two other columns, non-bonded silica and PolyHydroxyethyl A. ESI was operated to detect both negatively and positively charged metabolites, when coupled to a quadrupole ion trap MS using continuous polarity switching. Unambiguous identification was possible by stepwise mass spectrometric fragmentation of the most intense ions. Due to the successful results, the research group concludes that HILIC-ESI-MS can be used for the qualitative and quantitative analysis to determine changes in different *Cucurbitaceae* species during their development and due to environmental alterations. This leads to the possibility in studying the function of such metabolites. Additionally, several other analyses of plant extracted metabolites have been performed, including phytosiderophores and their metal complexes on a ZIC column,²⁴⁶ and

glutathione on a silica column.²³² However, in some cases as indicated by Su *et al.*²⁴⁷ successful determination of metabolites is not enough to completely elaborate a complex metabolism mechanism.

Various approaches have been employed to efficiently quantify multiple known metabolites in biological systems. Bajad *et al.*²⁴⁸ and Lu *et al.*²⁴⁹ reported the determination of a large number of cellular metabolites using an amino column coupled with ESI-tandem MS. With a more complicated path, Kind *et al.*²⁵⁰ developed a comprehensive urinary metabolic method for identifying kidney cancer. They combined HILIC-ESI-MS and RPLC-ESI-MS to cover a very large portion of the urine metabolome, comprising both lipophilic and hydrophilic metabolites. From more than 2000 mass spectral features detected from the urine, several significant components were identified to discriminate between renal cell carcinoma patients and controls despite the relatively small sample size. Similarly, the metabolic fingerprinting of rat urine has been performed to diagnose particular diseases.^{251,252}

Of interest, HILIC-ESI-MS was also used as an alcohol testing method via detection of minor metabolites of ethanol such as ethyl glucuronide in hair,²⁵³ ethyl phosphate and ethyl sulfate in human urine.²⁵⁴

6.5.6 Environmental Analysis

HILIC-ESI-MS has been applied to investigate several reaction mechanisms including the photo-Fenton reaction and the aggregation of dissolved organic compounds into particulate organic matter.^{255,256} In the photo-Fenton reaction, HILIC-ESI-MS has been proposed to evaluate intermediates generated during the degradation of Diuron and Linuron herbicides. These compounds are not only toxic but also non-biodegradable, and consequently cause the pollution of freshwater when they are disposed into the environment. The study helped to optimize the amount of chemical reactants in the photo-Fenton reaction, which may affect the biodegradability during the treatment of herbicide-contaminated waste water.²⁵⁵ Another innovative technique was suggested by Cappiello *et al.* for investigation of underivatized oligosaccharides in mucilage

samples. The separation was performed on an amino-bonded column with unambiguous detection by ESI-tandem MS. The authors concluded that the application might open new perspectives to better understand the aggregation mechanism of dissolved organic compounds into particulate organic matter.²⁵⁶

As a concern of public health and safety, freshwater and brackish water bodies are potentially polluted by the production of toxins from living cells or organisms, such as cyanobacteria and dinoflagellates.^{257,258} Paralytic shellfish poisoning (PSP) toxins have been analyzed on amide- and ZIC-bonded HILIC columns. Using tandem MS detection, the amide stationary phase allowed separation and determination of all major PSP toxins within a 30-minute run with a high degree of sensitivity and selectivity. For these toxins, acetonitrile provided sharper peaks than methanol and, as pH increased, retention times and selectivity increased. On the ZIC stationary phase, the retention times were highly reproducible for each PSP toxin from different sample matrices. The method was concluded to be applicable for the routine analysis of seafood. Further, toxin profiles from different species were studied by using HILIC-ESI-MS alone or in combination with other methods. The Yotsu-Yamashita group was able to discover not only the distribution of tetrodotoxin, saxitoxin and their analogs,^{259,260} but also the presence of a new dideoxy analog of tetrodotoxin²⁶¹ from puffer fish using only HILIC-ESI-MS. Similarly, Dell'Aversano *et al.*²⁶² analyzed the assorted toxins produced by cyanobacteria. They found that the technique was suitable for a number of cyanotoxins, including saxitoxin, anatoxin, cylindrospermopsin, deoxycylindrospermopsin, and microcystins-LR and -RR. For saxitoxin and its analogs, selected reaction monitoring (SRM) was the preferred method due to its high sensitivity. For anatoxin and cylindrospermopsin, single ion monitoring (SIM) was adequate, but SRM provided additional sensitivity and specificity for confirmatory analysis. To obtain the more complete profiles of toxins from a specific species, several research groups assimilated data from HILIC-ESI-MS and from RPLC on a C₈ column coupled with ESI-MS^{263,264} or on a C₁₈ column with post column derivatization-fluorescence detection.²⁶⁵ Multi-toxin determination was possible even at trace levels,^{263,266} allowing a rapid, simultaneous screening of a wide range of toxins.²⁶²

A specific compound related to toxins, domoic acid, was also determined at low detection levels in Adriatic shellfish samples.²⁶⁶ Within these applications, amide columns with an ammonium salt additive in the mobile phase seemed to be commonly exerted in HILIC separation with ESI-MS/MS detection.

6.6 Conclusions and Perspectives

HILIC-ESI-MS has emerged to be a very promising technique for the analysis of highly hydrophilic, ionic and polar compounds. In the case of the separation of a complex mixture, it appears to be a complementary method of RPLC-ESI-MS to obtain a more informative data set for further studies in biological, natural product, or metabolic systems. However, the mechanism of HILIC separation is somehow complicated since it consists of not only hydrophilic interaction but also electrostatic and hydrophobic interactions. The mixed-mode, in some cases, gives better efficiency while, on the other hand, peak tailing and an inability to predict elution order are common side effects. These interactions can vary substantially with the variation of additives such as acids or salts, which may also be problematic in conjunction with combining HILIC separations to ESI-MS detection. Thus, the analytical system, especially the mobile phase composition, needs to be fine tuned to simultaneously achieve good separation efficiency without affecting ESI-MS signal intensity in a deleterious manner. Oftentimes, however, a significant increase in ESI-MS sensitivity may be observed by virtue of using a mobile phase with higher organic content. Overall, HILIC-ESI-MS will continue to garner attention as a viable choice for combining highly reproducible chromatographic separations with the capability for unambiguous sensitive detection in qualitative and quantitative analyses.

CHAPTER 7
RETENTION BEHAVIOR OF ESTROGEN METABOLITES ON HYDROPHILIC INTERACTION
CHROMATOGRAPHY STATIONARY PHASES

7.1 Introduction

Hydrophilic-interaction chromatography (HILIC) was first coined and described by Alpert in 1990,¹²⁶ although it has been widely used for the separation of carbohydrates since 1975.¹²⁹ The use of polar stationary phases in combination with aqueous-polar organic solvent mobile phases is favorable for the separation of hydrophilic compounds, which are poorly retained in reversed phase liquid chromatography (RPLC). Additionally, the advantages of HILIC mode separations coupled with electrospray ionization - mass spectrometry (ESI-MS) have recently been emphasized; HILIC mobile phases have high organic content that provide increased sensitivity when coupled to ESI-MS. This is due to the increased efficiency of droplet desolvation and subdivision processes at high organic solvent content, which is in contrast to the higher aqueous mobile phase content often encountered in RPLC separations.²⁷

Despite a growing literature base on the subject, the exact mechanism responsible for HILIC mode separations is still controversial. Alpert proposed the partition model, in which analytes partition between the bulk aqueous/organic mobile phase and a water-enriched layer, adsorbed on the stationary phase surface. Mixed-mode interactions, such as ion-exchange, electrostatic, or hydrophobic interactions, which can contribute to a lesser or greater degree depending on the properties of the analytes and the stationary phase used in the analysis, are also commonly present.^{27,126,129} More recent work by Hemstrom and Irgum examined the fundamentals underlying the separation mechanism in more detail.¹²⁸ Specifically, they discussed the contributions of adsorption and partitioning, and used mathematical equations that describe each of the models to study analyte retention. A wide range of the analytes and stationary phases which differ in nature were investigated and delineated based on their fit to either model. Most of

the work in their review indicated that the surface adsorption model was the most reasonable descriptor of retention in HILIC mode;^{140,267} however some applications did fit the partition model better,^{268,269} and some others remained questionable.^{270,271}

The retention behavior for different compounds in high performance liquid chromatography (HPLC) depends not only on the nature of the analytes and the stationary phase, but also on the composition of the mobile phases. It is generally understood that analytes characterized by higher hydrophilicity are more strongly retained in the HILIC mode. In addition to hydrophilic partitioning, prevalent adsorptive interactions can be characterized by hydrogen bonding, dipole-dipole, and/or Coulombic/ion-exchange interactions between the analytes and different stationary phases. These forces are also highly influenced by the organic solvent content and salt concentration in the mobile phases. Perhaps more than in RPLC, mobile phase compositions are extremely important for optimization of chromatographic retention, selectivity, efficiency, and reproducibility in HILIC.

In this work, the retention characteristics of estrogen conjugate compounds on different stationary phases were investigated. Estrogens are well known as stimulants of secondary sexual characteristics, pregnancy, and long bone maturation.¹ Recently, clinical studies involving estrogens have been amplified for two reasons. On the one hand, they have been found to be associated with carcinogenesis; their activity has been linked to ovarian, prostate, and breast cancer.^{7,8} On the other hand, they have been reported to exert neuroprotective effects in instances of ischemic trauma.^{12,13} These beneficial and deleterious effects have emphasized the importance of developing analytical methods for separation and speciation of estrogen compounds. This includes analyses for estrogens and their conjugates, including sulfated and glucuronidated forms, which are common metabolic products in the body.

A series of experiments was carried out with a focus on evaluating HILIC retention mechanisms for estrogen sulfates and estrogen glucuronides on five different commercial stationary phases (amide, amino, diol, acetylated β -cyclodextrin (CD), and native β -CD bonded phases). Partition and surface adsorption models were investigated with the variation of water

content in the mobile phase. Since native estrogens are poorly retained by HILIC stationary phase, the discussion centers primarily on the retention characteristics of the sulfated and glucuronidated forms. Between the two models, analyte retention could be more generally attributed to surface adsorption, and the analyte – stationary phase interaction was mainly considered to be due to hydrogen bonding. The effect of salt concentrations was also examined. The result revealed that the solvated salt ions, adsorbed on the stationary phases and associated with the analyte, significantly affected analyte retention and peak shape.

7.2 Experimental

7.2.1 Chemicals

LC-MS grade water and acetonitrile (ACN) were obtained from Burdick and Jackson (Muskegon, MI, USA). Five types of salts were used, including ammonium acetate (Acros Organics, Morris Plains, NJ, USA), tetraethylammonium acetate (Sigma-Aldrich, Steinheim, Germany), sodium chloride (Wal-Mart Stores, Inc., Bentonville, AR, USA), ammonium bicarbonate and sodium acetate (EMD Chemical Inc., Darmstadt, Germany). Eight estrogen and estrogen conjugate compounds were obtained from different suppliers. The estrogens (estrone (E1), 17 β -estradiol (BE2), and estriol (E3)) and estrogen glucuronides (estrone-3-glucuronide (E1-3G), 17 β -estradiol-3-glucuronide (BE2-3G), and estriol-3-glucuronide (E3-3G)) were from Sigma-Aldrich (St. Luis, MO, USA). Estrogen sulfates (estrone sulfate (E1-S) and 17 β -estradiol sulfate (BE2-S)) were from Organics L.L.C. (Northbrook, IL, USA). The structures of these compounds are shown in Figure 7.1.

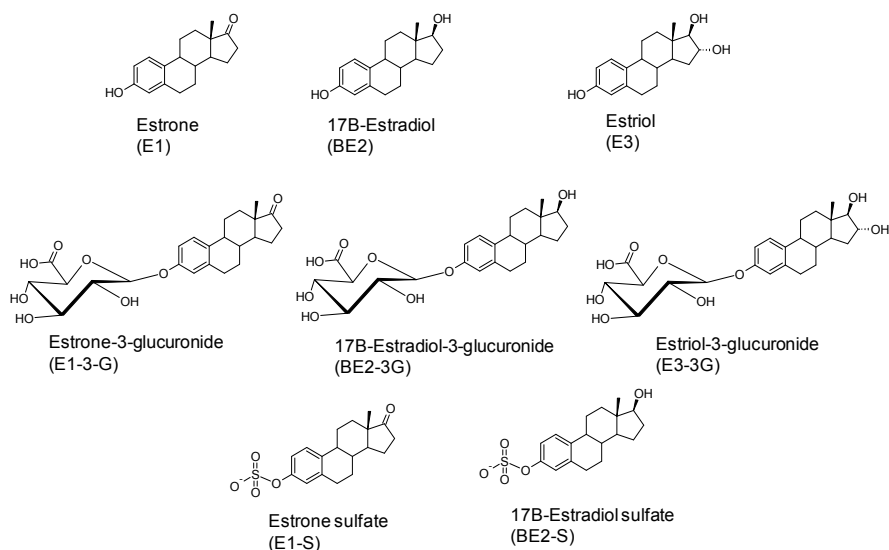


Figure 7.1 Structures of estrogen and estrogen conjugate compounds investigated in this work.

7.2.2 Apparatus

The experiments were performed on a Shimadzu LCMS 2010 (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), equipped with two pumps (LC-20AD) in a high pressure mixing arrangement, an autosampler (SIL-20A HT), and a ESI-single quadrupole mass spectrometer. Five columns with different stationary phases were tested, including Tosoh Bioscience (Kyoto, Japan) TSKgel Amide-80 (10 cm x 2.0 mm, 3 μ m d_p, 80 Å pore size), Varian (Palo Alto, CA, USA) Polaris Amino (10 cm x 2.0 mm, 3 μ m d_p, 200 Å pore size), Phenomenex (Torrance, CA, USA) Luna HILIC (10 cm x 2.0 mm, 3 μ m d_p, 180 Å pore size), Supelco (Bellefonte, PA, USA) Cyclobond I2000 (15 cm x 2.1 mm, 5 μ m d_p, 100 Å pore size), and Cyclobond I2000AC (15 cm x 2.1 mm, 5 μ m d_p, 100 Å pore size).

7.2.3 Experimental Conditions

All samples were dissolved in methanol at the concentration of 1.0 mg/mL and further diluted with ACN to the concentration of 50 μ g/mL for estrogens, 10 μ g/mL for estrogen glucuronides, and 1.0 μ g/mL for estrogen sulfates. All columns were washed with water/ACN (50/50) for one hour before equilibrating with initial method mobile phase conditions for 30

minutes. Variations in water content, from 5% to 40% and in salt concentration, from 0 to 20 mM ammonium acetate, in the mobile phase were investigated. Different types of salt were also studied using a UV-Vis photodiode array (PDA) detector. All analyses were performed in isocratic mode. The mobile phase flow rate was 200 $\mu\text{L}/\text{min}$. The ESI-MS was operated in the negative ionization mode with a spray voltage of -4.5 kV, and a scan range of 100-600 m/z . The nebulizing gas flow rate was set to 1.2 L/min and the drying gas was set to 6000 kPa. The temperature of the curved desolvation line and block heater was set to 250°C. LCMS Solutions software, version 3.4, was used for data collection and analysis. Least squares analysis of retention models was performed with Microsoft Excel. Retention factors (k') were calculated from the adjusted retention time of each analyte, divided by the dead time (t_0). The dead times for the columns used in this study, under the stated operating conditions, were: TSKgel-Amide, 0.8 min; Luna HILIC and Polaris Amino, 1.2 min; and Cyclobond I2000 and I2000AC, 1.3 min.

7.3 Results and Discussions

7.3.1 The Effect of Polar Stationary Phases

The effect of different stationary phases was explored by studying the retention behavior of a representative estrogen conjugate, E3-3G. Five polar stationary phases were involved in this study with different types of functional groups, including amide- (TSKgel Amide-80), amino- (Polaris Amino), diol- (Luna HILIC), and CD-bonded (Cyclobond I2000AC (acetylated CD) and I2000 (native CD)) phases. The water fraction was varied from 5 to 40 % with the addition of 5 mM ammonium acetate. Ammonium acetate was chosen because it is considered a standard ESI-MS friendly solvent modifier.

The capacity factor of E3-3G was plotted against the water fraction (up to 40%) in the mobile phase for each of the five columns (Figure 7.2A). Among the three non-CD columns, TSKgel Amide-80 provided the longest retention, while Luna HILIC provided the shortest. According to Liu *et al.*,²⁷² the analyte retention increases proportionally with the polarity of stationary phase, to some extent.

Luna HILIC is a cross-linked diol stationary phase, which has high polarity and can exhibit hydrogen-bonding properties.¹²⁶ The absence of dissociable moieties (under the pH conditions investigated), other than residual silanol groups (which are still important to consider), reduces mixed mode interactions with the stationary phase, which can affect the separation efficiency and, in some cases, cause irreversible retention. Phenomenex claims the use of Luna silica, which has high surface smoothness, improves bonding coverage and limits the interaction of analytes with the underlying solid support.

Compared to Luna HILIC, TSKgel Amide-80 retained the analyte better due presumably to the nature of the amide functional unit. The amide group possesses both hydrogen acceptor and donor sites, which facilitates increased retention of the acidic analytes. Technically, the diol phase also has hydrogen donor and acceptor properties, although the donor strength is significantly different than that for the amide, and is not directed. Additional differences in retention between the amide and diol phase may also be ascribed to differences in pore size and surface area. The TSKgel Amide-80 silica has a surface area of 450 m²/g (100 Å nominal pore size; 80 Å after bonding of the phase) and the Luna HILIC silica has a surface area of 200 m²/g (200 Å pores). Therefore, the increased retention of the analytes on the amide column can be ascribed to both the functional attributes of the phase and the increased surface area for analyte binding.

E3-3G was not retained as strongly on the Polaris Amino column, compared to the TSKgel Amide-80, in the range of 20-40% water in the mobile phase, however, it was not eluted at 5% water. This is believed to be due to the strong adsorption of the acidic analyte on the basic amino groups present on the Polaris column. The Polaris Amino phase is an aminopropylsilane-covered silica with 180 Å nominal pore size. Varian Polaris phases are marketed as polar embedded bonded phases which are designed to provide additional charge density in close proximity to residual silanols, and thus, reduce interactions between analytes and silanol groups. Similar retention effects, which mention contributions from ion-exchange and electrostatic interactions, have also been previously reported by other groups.^{272,273,274}

CD-bonded stationary phases have been recently investigated for their potential to be used in HILIC separations.²⁷⁵⁻²⁷⁷ In this work, at 15% water, E3-3G was retained on the Cyclobond I2000AC (acetylated β -CD) ($k' = 8.2$) slightly less than on the amide-bonded stationary phase ($k' = 14$), while the Cyclobond I2000 (native β -CD) provided significantly longer retention ($k' = 37$). The CD stationary phases provide sites for both hydrophilic (surface hydroxyl groups) and hydrophobic (internal cavity) interactions. CDs are well-known for forming inclusion complexes with hydrophobic analytes under (highly) aqueous conditions.^{277- 282} They have also been developed extensively for use in chiral separations.^{283- 287} The fact that the native CD column provided longer retention of E3-3G under HILIC conditions supports the importance of interactions between the analyte and the hydroxyl groups on the rim of the CD. When these groups are acetylated, the relative polarity of the phase is decreased, which resulted in a decrease in analyte retention. The Cyclobond I2000 column was the only phase in this study for which significant retention of estrogen sulfates was observed. Again, this increase in retention could also be partially attributed to the higher surface area present on the packing (100 Å pores).

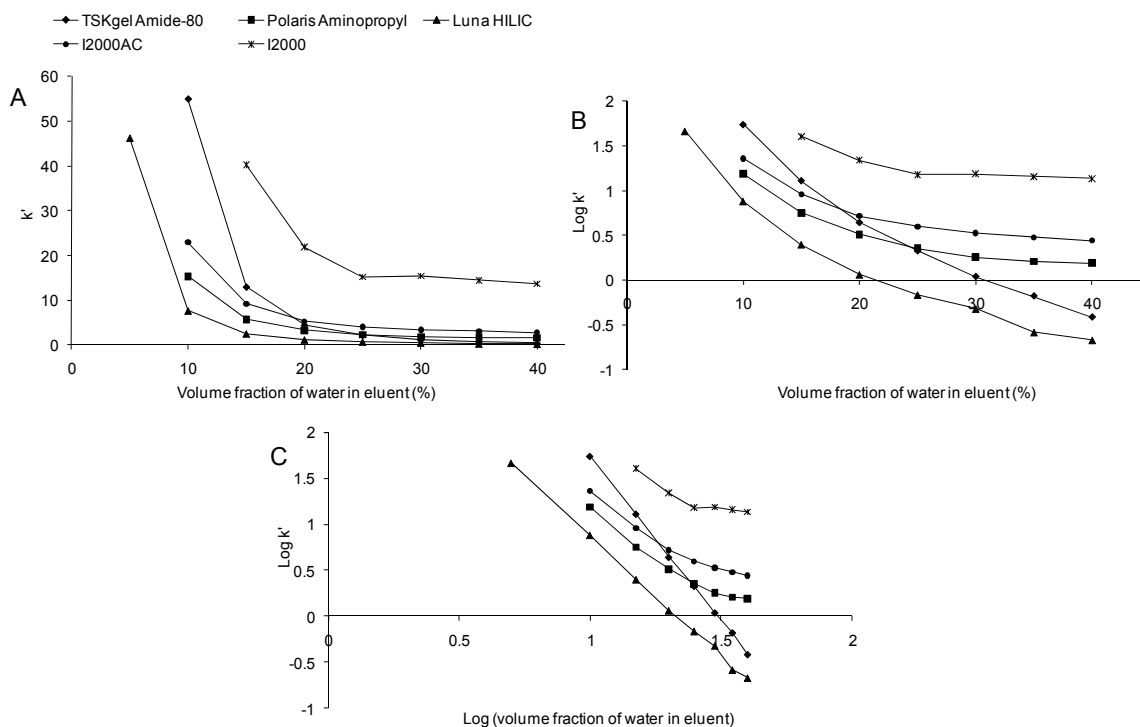


Figure 7.2 Retention behavior of E3-3G on different polar stationary phases in HILIC mode. (A) Plot of retention factor (k') vs. the volume fraction of water in eluent. (B) Plot of $\log k'$ vs. the volume fraction of water in eluent. (C) Plot of $\log k'$ vs. \log (volume fraction of water in eluent).

Many publications have recently explored the retention mechanism of HILIC.^{126,272,273,288,289} Two models, partition and surface adsorption, have been described, compared, and contrasted in these studies. In the partition model, analyte retention is achieved by partitioning between the aqueous/organic mobile phase and a water-enriched layer, adsorbed on the surface of stationary phase.^{126,290} In the surface adsorption model, the analytes directly interact with the surface of the stationary phase through noncovalent interactions, which can include hydrogen bonding, dipole-dipole, or Coulombic/ion-exchange forces.^{126,291} The mathematical functions which are used to describe these processes are well established and have been described elsewhere.^{128,290,291}

The HILIC mechanism was investigated, using the prescribed models, to determine whether the analyte, E3-3G, was retained by partition or surface adsorption processes. A plot of the untreated data (capacity factor (k') vs. water fraction) is shown in Figure 7.2A. Plots of $\log k'$

versus water fraction (log-lin) and log (water fraction) (log-log) were constructed (Figure 7.2B and 7.2C, respectively). Nonlinear plots were observed for all stationary phases in the log-lin plots, indicating the absence of a well-defined partition mechanism. A linear relationship was found in log-log plot for TSKgel Amide-80 and Luna HILIC with $R^2 > 0.9995$ in the range of 10-40% water and 5-25% water, respectively. The high correlation coefficient for these two columns in the log-log plot implies that surface adsorption, is most responsible for retention. The slight deviation observed for Luna HILIC in the lower k' range (Figure 7.2C) is likely due to the fact that the analyte is only minimally retained under these conditions. On the contrary, nonlinearity was observed for the other three columns in both log-lin and log-log plots. Mixed interaction modes, possibly including both adsorption and partitioning processes can be ascribed to this observation, so that neither of the models could be solely delineated.

7.3.2 Retention Behavior in HILIC

A mixture of estrogens and estrogen conjugates including estrone, 17 β -estradiol, estriol, and their derivatives, in sulfated and glucuronidated form, was investigated. In most cases, the estrogens and estrogen sulfates co-eluted near the dead volume; thus, their retention characteristics could not be included for the evaluation of most of the stationary phases. A focus was thus placed on the data collected from the three estrogen glucuronides, with the variation of water fraction in the water/ACN mobile phase containing 5 mM ammonium acetate. The elution order of these glucuronides did not vary from column to column (E3-3G > BE2-3G > E1-3G) but their retention behavior was significantly different.

7.3.2.1 TSKgel Amide-80

TSKgel Amide-80 is a silica-based phase, containing an amide group appended to a polyethylene-polysiloxane block copolymer. The glucuronides were well separated on this stationary phase with water content less than 20%. E3-3G had the strongest retention (Figure 7.3A). A similar study was recently performed by Qin and coworkers.²⁹² They successfully

determined seven estrogen metabolites in human urine with limits of quantification ranging from 2-1000 pg/mL using the TSKgel Amide-80 column. The order of retention reported in that work was consistent with our findings. Retention times between the glucuronidated analytes increased with the number of hydroxyl groups present in the parent structure (see Figure 7.1). A similar behavior was reported in the work of Yanagida *et al.*,²⁸⁸ where they found a high correlation between the logarithm of retention factors and the number of hydroxy groups in oligomeric proanthocyanin structures.

The data were visualized in plots of $\log k'$ against the linear and logarithmic function of water fraction (Figure 7.3B and 7.3C). Nonlinear plots were obtained with the log-lin treatment, while a straight line was observed in the log-log plot for E3-3G ($R^2 = 0.997$). It seems that different interactions occurred for E1-3G and BE2-3G at different ranges of the water fraction (10 - 20% and 20 - 40% water). The curves were disconnected at 20 - 30% water because of the presence of two peaks for each compound. In this region, it is possible that more than one set of interaction forces, perhaps due to more than one ionization state for the analyte, causes the observed peak splitting, as can be seen in Figure 7.3D. We do not believe that this is an overloading effect. This phenomenon has been previously reported by Guo and Gaiki²⁷³ and was reproduced extensively in our study using analyte concentrations that produced acceptable peaks under different conditions. A possible explanation is that a sample-matrix mismatch is present at this condition. If the analytes can access multiple protonation states, then different portions of the analyte populations may be prone to interact to different degrees with various adsorption sites (amide C=O, amide N-H, and residual silanols) on the stationary phase surface. In our opinion, this kind of retention behavior is not uncommon in HILIC separations, and can be observed for a wide range of analytes. Such effects increase the burden of method development needed to fully optimize conditions so that a single dominant interaction mode controls analyte retention.

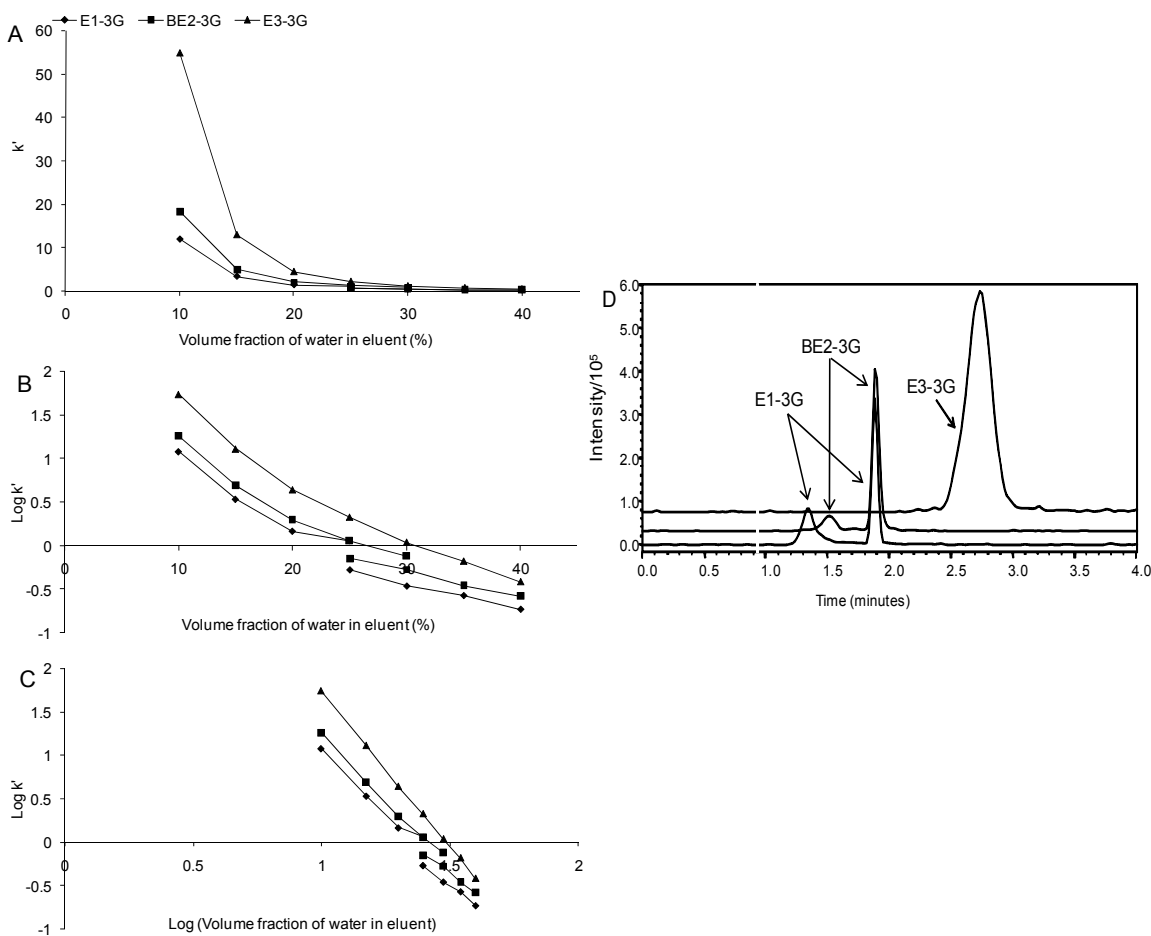


Figure 7.3 Retention behavior of estrogen glucuronides on the TSKgel Amide-80. (A) Plot of retention factor (k') vs. the volume fraction of water in eluent. (B) Plot of $\log k'$ vs. the volume fraction of water in eluent. (C) Plot of $\log k'$ vs. \log (volume fraction of water in eluent). (D) Overlaid extracted ion chromatgrams obtained for estrogen glucuronides in water/ACN (25/75) with 5 mM ammonium acetate.

7.3.2.2 Polaris Amino

Compared to the TSKgel Amide-80, the Polaris Amino is more basic, reactive, and can be prone to providing irreversible chemisorption of acidic analytes.¹²⁸ E3-3G was still retained the most and was well-separated from the other two estrogen glucuronides at a water content less than 25%. E1-3G and BE2-3G could only be retained and separated at water content less than 15%. Overall, the resolution and peak shapes were not as good as those observed with the TSKgel Amide-80 and slight peak tailing was observed. E3-3G, especially, was observed as a broad and asymmetrical peak. Both log-lin and log-log plots (Figure 7.4B and 7.4C) were

nonlinear. In other words, the data cannot be unequivocally ascribed to either model and it is difficult to draw a general conclusion about the retention mechanism for estrogen glucuronides on this phase. However, given the natures of the basic stationary phase and the acidic analytes, the simultaneous presence of both electrostatic interactions^{272,273} and hydrogen bonding, as well as partitioning processes, is possible.

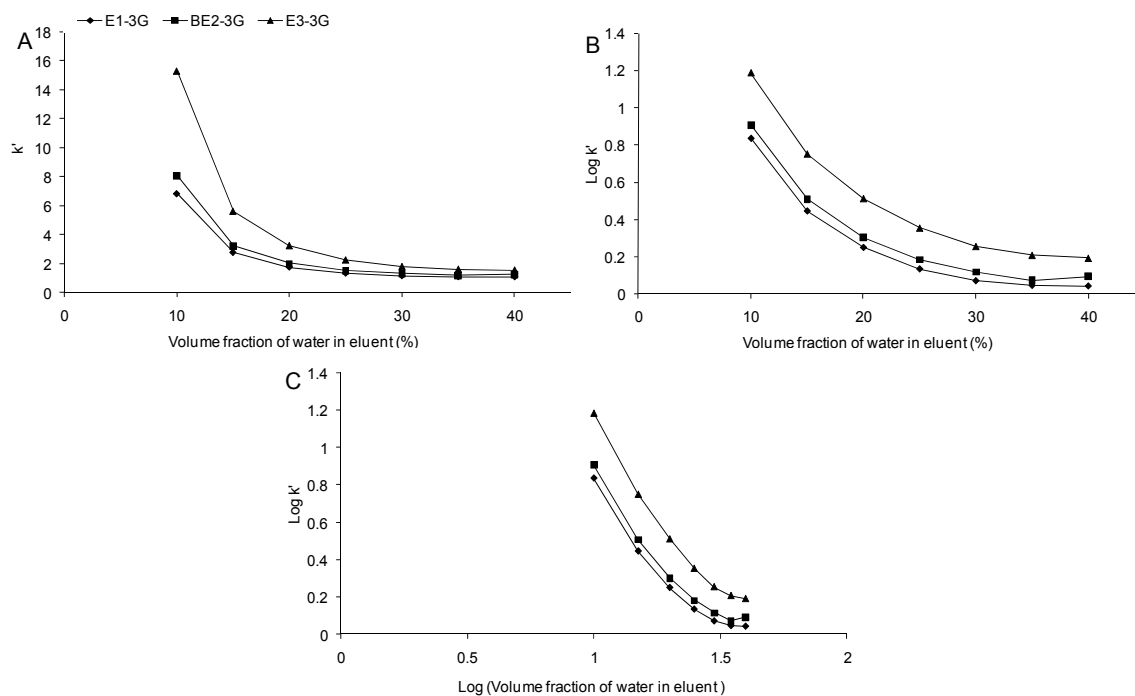


Figure 7.4 Retention behavior of estrogen glucuronides on Polaris Amino. (A) Plot of retention factor (k') vs. the volume fraction of water in eluent. (B) Plot of $\log k'$ vs. the volume fraction of water in eluent. (C) Plot of $\log k'$ vs. $\log(\text{volume fraction of water in eluent})$.

7.3.2.3 Luna HILIC

The Luna HILIC phase is a cross-linked diol phase with ethoxy bridges. The glucuronidated compounds were retained and separated on this phase with water content less than 15%. The data predominantly followed the adsorption model, as shown in (Figures 7.5A-7.5C). A straight line was obtained for a log-log plot returning $R^2 = 0.9997$ (water fraction $\leq 25\%$) for E3-3G, and $R^2 \geq 0.999$ (water fraction $\leq 20\%$) for E1-3G and BE2-3G. A notable phenomenon occurred when the mixture (all estrogens and estrogen conjugates) was analyzed at the water content of 25%. A broad and jagged peak was reproducibly observed for E1-3G (data not shown).

However, this was not observed when the analysis was performed with individual compound solutions. This might be explained by interactions among the early co-eluted analytes in the mixture at high water content. A combination of hydrophilic and hydrophobic interactions among the analytes and between the analytes and the stationary phase are believed to be responsible for this phenomenon; however, further experimentation is needed to draw firm conclusions about the nature of these observations.

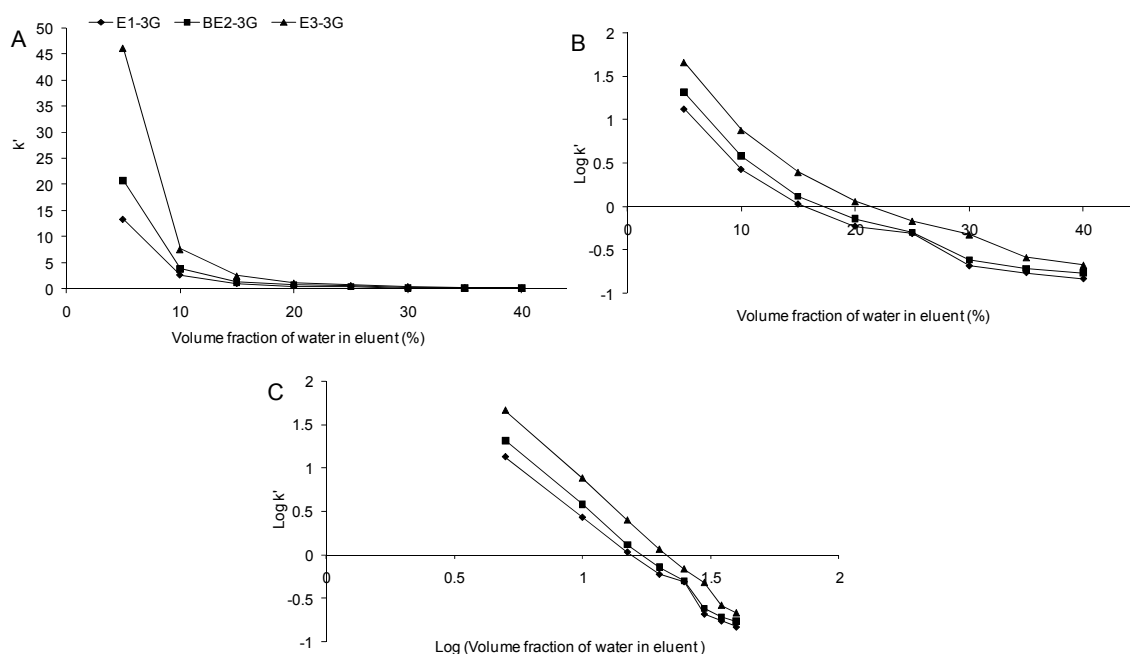


Figure 7.5 Retention behavior of estrogen glucuronides on Luna HILIC. (A) Plot of retention factor (k') vs. the volume fraction of water in eluent. (B) Plot of $\log(k')$ vs. the volume fraction of water in eluent. (C) Plot of $\log(k')$ vs. $\log(\text{volume fraction of water in eluent})$.

7.3.2.4 Cyclodextrin phases

Cyclobond I2000 and I2000AC columns have bonded native β -CD and acetylated β -CD forms on their stationary phases, respectively. β -CD contains seven glucose units resembling a toroidal structure with a hydrophilic rim and a hydrophobic interior cavity.²⁸⁰ The hydrophilic rim of the native β -CD phase is decorated with hydroxyl groups at the 2-, 3-, and 6-positions, while the hydrophilicity of the acetylated β -CD phase is due to the presence of acetyl groups at the 6-

position and hydroxyl groups at the 2- and 3- positions. The hydroxyl and acetyl groups exposed on the rim of the structure makes the CD phase attractive for use in HILIC mode.^{276,277}

In general, all three estrogen glucuronides were well-separated on the CD phases, and retention was maintained at a higher water content than the other stationary phases studied (Figure 7.6). The compounds were well-resolved, even at 30% water for Cyclobond I2000 and 20% water for Cyclobond I2000AC. However, all lines obtained in log-lin and log-log plots were nonlinear for both CD phase, and thus, retention could not be ascribed solely either a partition or an adsorption model.

The retention behavior of the analytes on Cyclobond I2000 was especially notable. At 30% water, all estrogen glucuronides and sulfates were retained and resolved. This stationary phase yielded an extremely long retention for most of the analytes in contrast with the other stationary phases. The retention of E1-3G and BE2-3G slightly decreased with increased water content in the mobile phase. Estrogen sulfates were well-retained in the entire range of water content studied. When k' was plotted against water fraction for E1-S and BE2-S, the curves trended upwards at both the lower and higher end of the water content ranges (Figure 7.7A). The steep deviation at the higher water content likely resulted from an increase in hydrophobic interactions between the analytes and the β -CD cavity. The data seemed to adhere to both models in the water content ranges of 25-40% but showed a better fit to the surface adsorption model ($R^2 \geq 0.9850$) (Figure 7.7B and 7.7C). Interestingly, the retention of BE2-3G behaved differently on the CD phases compared to the other phases. Normally, this analyte was immediately eluted after E1-3G and was well-separated from E3-3G on the other three columns. However, on the Cyclobond columns, the peak was shifted to longer retention, well resolved from E1-3G, and eluted just before E3-3G. The CD phases showed the most promise for achieving appreciable retention and separation of a broader range of estrogen metabolite analytes.

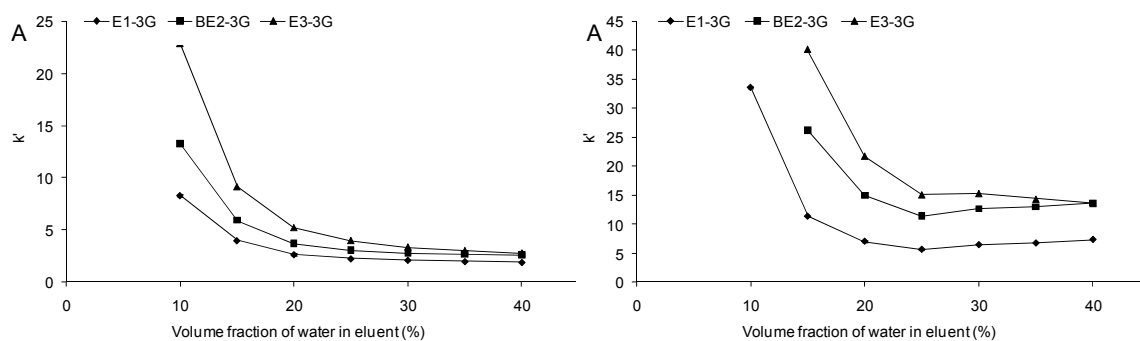


Figure 7.6 Retention behavior of estrogen glucuronides on (A) Cyclobond I2000AC and (B) Cyclobond I2000.

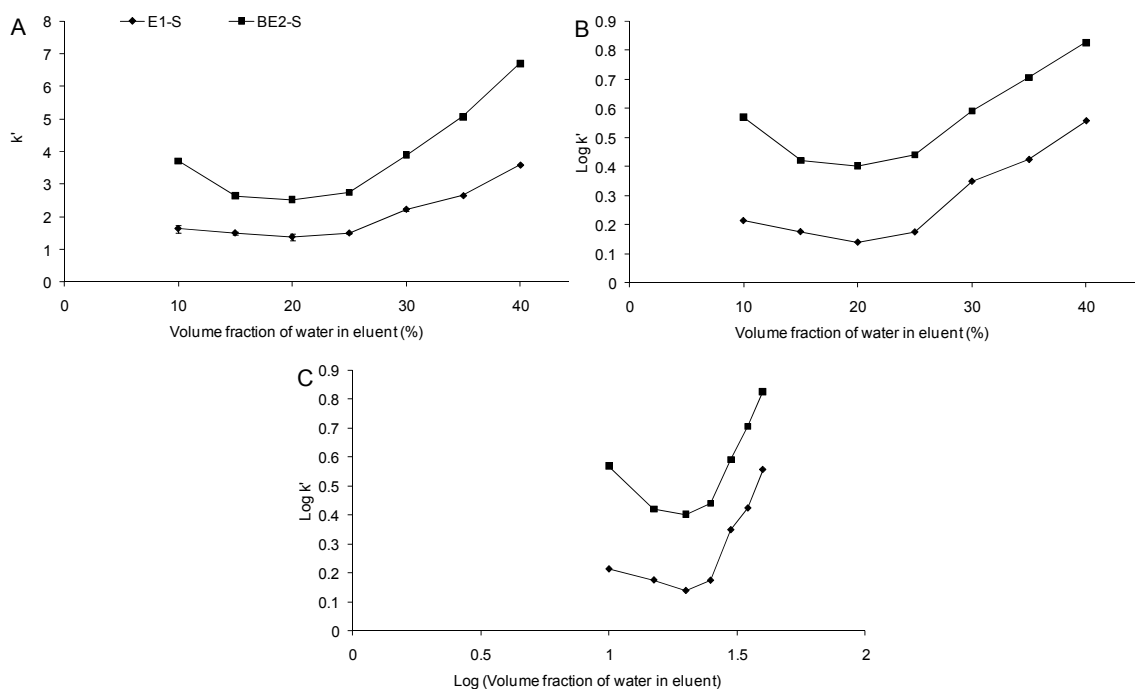


Figure 7.7 Retention behavior of estrogen sulfates on Cyclobond I2000. (A) Plot of retention factor (k') vs. the volume fraction of water in eluent. (B) Plot of $\log k'$ vs. the volume fraction of water in eluent. (C) Plot of $\log k'$ vs. $\log(\text{volume fraction of water in eluent})$.

7.3.2.5 Summary

Complementarity in the nature of the analytes and the stationary phases are crucial for achieving HILIC mode separations. Their mutual effects were investigated with the three estrogen glucuronides on different stationary phases in water/ ACN (15/85) mobile phase containing 5 mM ammonium acetate (Figure 7.8). Good selectivity was achieved with TSKgel Amide-80 and the

two cyclodextrin columns. E3-3G was well resolved from the other two glucuronides, but E1-3G and BE2-3G were not separated on Polaris Amino and Luna HILIC. Fronting peaks were observed for E3-3G on TSKgel Amide-80 and Cyclobond I2000AC while most peaks exhibited minor tailing on Polaris Amino, Luna HILIC, and Cyclobond I2000 phases. The tailing degree increased with the number of hydroxyl groups of the analytes (e.g. E3-3G > E1-3G). Cyclobond I2000 provided the longest retention, and consequently more band-broadening, while Luna HILIC provided the shortest retention, among the five columns. In general, TSKgel Amide-80 provided a better separation for estrogen glucuronides, in term of selectivity, efficiency, and run time, compared to the other columns under the conditions tested. The Cyclobond I2000 was shown to provide resolution of all estrogen metabolites studied, but strong retention resulted in substantial band broadening, which would require additional method development to optimize.

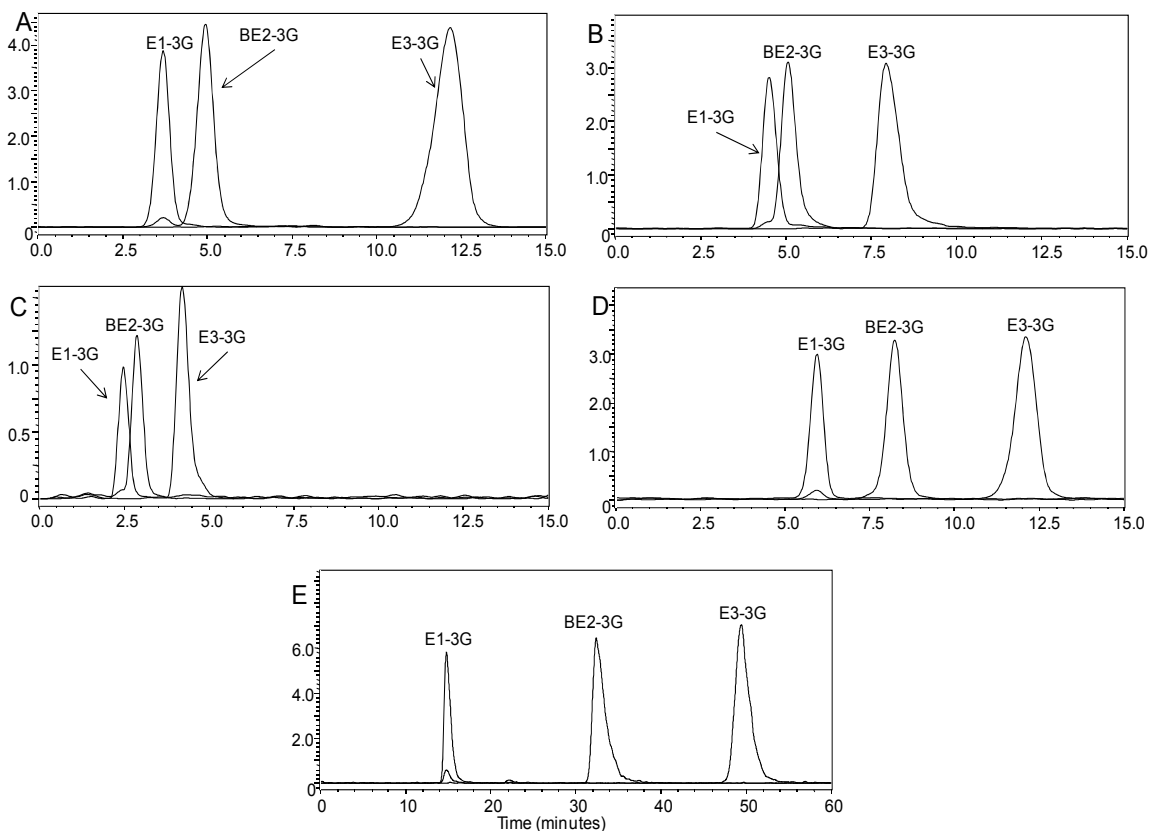


Figure 7.8 Chromatograms of estrogen glucuronides with different stationary phases: (A) TSKgel Amide-80, (B) Polaris Amino, (C) Luna HILIC, (D) Cyclobond I2000AC, (E) Cyclobond I2000, using 5 mM ammonium acetate in water/ACN (15/85).

7.3.3 The Effect of Salt Concentrations and Salt Types

Buffers and salts are commonly used in RPLC to improve analyte separation and peak shape. However, the use of salts in HILIC requires more attention since the solubility of salts in high organic mobile phases is poor and the effect of salt type and concentration on analyte retention is substantial. The most commonly-used salt in HILIC is ammonium acetate.^{27,128,129,273}

A series of experiments was performed to investigate the effect of salt concentration (0 – 20 mM ammonium acetate) on the retention behavior of estrogen conjugates. Retention was generally observed to increase slightly with increasing salt concentrations, except on the Polaris Amino and CD columns (Figure 7.9). The slight decrease in retention time of E3-3G on Polaris Amino is evidence for contributions to retention from electrostatic interactions. This influence of salt on electrostatic interactions was described in detail by Alpert.²⁹³ At higher salt concentration, electrostatic interactions are shielded, which allows other intermolecular forces, including hydrophilic partitioning and possibly, hydrogen-bonding, to be preferentially exerted. For the CD phases, the addition of salt was critical; with no salt present, the analytes were not eluted from the column.

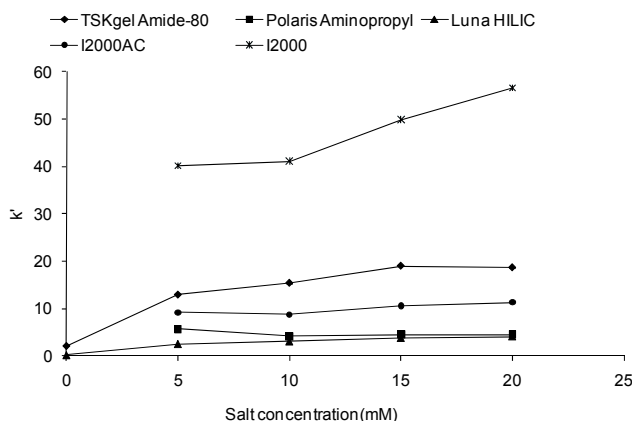


Figure 7.9 Retention factor vs. ammonium acetate concentration for E3-3G on five stationary phases. Mobile phase contains water/acetonitrile (15/85) with 0-20 mM ammonium acetate.

An increase in salt concentration is believed to facilitate and enhance salt bridge interactions between the analyte and the stationary phase, due to an enrichment of solvated salt ions around the stationary phase. Thus, hydrophilic partitioning and hydrogen bonding can be

supported, while less specific Coulombic interactions are shielded. This description is consistent with explanations provided previously in the literature. Guo and Gaiki argued that an increase in salt concentration “would result in an increase in volume or hydrophilicity of the liquid layer, leading to stronger retention of the solutes”.²⁷³ To study the salt bridge interaction between the analytes and stationary phases, different types of salt, including ammonium acetate, ammonium bicarbonate, tetraethylammonium acetate, sodium acetate, and sodium chloride, were investigated on TSKgel Amide-80 with the three estrogen glucuronides (Figure 7.10). A photodiode array (PDA) was employed as a detector instead of ESI-MS since some of these salts are not ESI-MS friendly. Among the acetate salts, the capacity factor of ammonium and sodium salts was significantly higher than that of the tetraethylammonium salt. Sodium ions have larger solvated radii, while ammonium ions can be hydrogen donors. These characteristics can support the formation of salt bridge between the analytes and the stationary phase, as well as the partitioning process. Although positively-charged tetraethylammonium ions can shield electrostatic interactions, they are neither hydrogen donors nor acceptors, and they are too bulky to be highly solvated. Analyte retention was observed to be drastically reduced in the presence of tetraethylammonium acetate. Furthermore, sodium chloride provided the longest retention and a good linearity was obtained in the log-log plot for E3-3G ($R^2 = 0.997$), indicating the adsorption model to be the best descriptor of retention. Yet, a mixed mode of partitioning and hydrogen bonding possibly occurred for E1-3G and BE2-3G since all curves observed in the log-lin and log-log plots were nonlinear. This further emphasizes the subtle, but important, role of analyte functionality on HILIC retention; the glucuronides differ only slightly in the number of hydroxyl groups present in their structures.

There was also a critical difference in the analyte retention when ammonium bicarbonate and acetate were tested as salt additives. The three estrogen glucuronides were co-eluted early at almost the same time using ammonium bicarbonate, while a good separation was observed with ammonium acetate as buffer. Guo and Gaiki²⁷³ also reported a similar effect. The bicarbonate salt detracts from the separation of acidic analytes when compared to the use of

different counter anions of ammonium salt, such as acetate and formate. We perceive this as the effect of the acidic hydrogen atom from the bicarbonate anion, making this anion a hydrogen donor which can be preferentially retained on the stationary phase and repels the acidic analytes. Through this systematic variation of salt types, we hypothesize that hydrogen bonding and salt bridge interactions, combined in some, but not all, cases with contributions from hydrophilic partitioning, is the dominant mechanism for HILIC retention of estrogen glucuronides on the amide-bonded stationary phase.

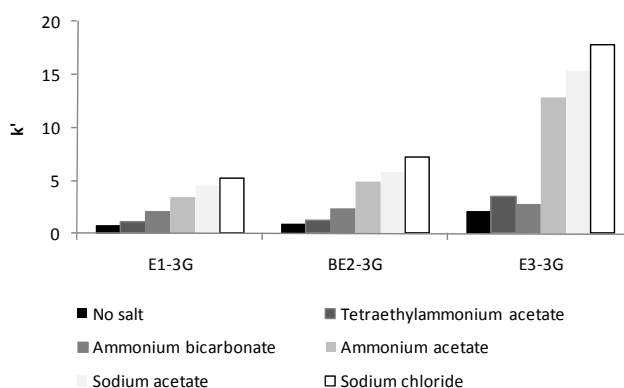


Figure 7.10 The effect of salt types on glucuronide retention on TSKgel amide-80 column.

7.4 Conclusions

The retention behavior of estrogen conjugates on different HILIC stationary phases is the result of a complex series of interactions. Hydrophobic unconjugated estrogen compounds were not retained in HILIC mode, however for estrogen metabolites, a number of interaction forces can be delineated, leading to retention and separation, depending on the complementarity between the analyte and the stationary phase. In several cases, the retention mechanism more-closely followed the surface adsorption model, while in others it was difficult to delineate between the importance of adsorptive and partitioning processes. Three dominant interaction forces were emphasized in this study, consisting of hydrogen bonding (and salt bridging), electrostatic, and partitioning, out of which hydrogen bonding was judged to be the most important. This agrees well with the intermediate theory by Berthod *et al.*,²⁷⁶ in which the HILIC mechanism involves both

partitioning and hydrogen bonding processes. It is reasonable to assume that hydrogen bonding, necessarily enhanced by the addition of salts, plays a vital role at the low water content in HILIC mode.

CHAPTER 8

CONCLUSIONS

Analysis of estrogens and their metabolites was performed using two different liquid chromatographic separation modes, reversed phase liquid chromatography (RPLC) and hydrophilic interaction chromatography (HILIC), coupled with mass spectrometry (MS). A primary goal of the work was the development of sensitive methods for ultra-trace quantitative analysis of estrogen compounds from complex biological fluids.

For native estrogens, derivatization with dansyl chloride was used in sample preparation prior to RPLC-MS to improve the sensitivity of the method since these compounds are poorly ionized in aqueous liquid phases. The method was successfully applied in two sub-projects. The first project involved the upgrade of the method into a heart-cutting two-dimensional RPLC-MS technique to analyze the four dansylated estrogens from cerebrospinal fluid (CSF); detection limits in the part-per-trillion concentration range were achieved. The second project was attributed to a matrix effect study on the analysis of 17α - and 17β -estradiol (17α - and 17β -E2) in various biological samples, including mouse brain, phosphate-buffered saline – bovine serum albumin (PBS-BSA), gelded horse serum, and mouse serum. Matrix effects encountered in the presence of an artificial PBS-BSA matrix were not significant and similar to those of mouse serum, making it a promising surrogate matrix to prepare standard curves for quantitative analysis in mouse serum. In contrast, horse serum and mouse brain exhibited considerable matrix effects, especially for 17β -estradiol, where significant signal suppression was observed.

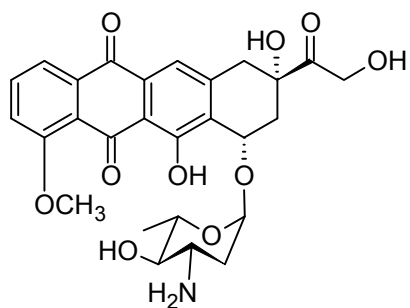
For analysis of sulfated and glucuronidated estrogen metabolites, LC-MS was applied without any additional derivatization step, because these compounds are present in anionic forms in slightly basic mobile phases. RPLC-MS was used in the stability study of Premarin® Intravenous, a mixture of estrogen sulfates, in the form of dry powder and reconstituted solutions under elevated and room temperatures. The variation of major

components, mainly equilin and equilenin sulfates, in dry powder was not significant at both elevated and room temperatures, while reconstituted solutions were also stable, but only at room temperature. The conversion of equilin compounds into their corresponding equilenin forms was observed in pure standard solution, partially explaining the increase of equilenin sulfate and decrease of other components in Premarin. The identification of these converted equilenin sulfates was pursued and verified based on fragmentation patterns generated by tandem mass spectrometry. This led to on-going project, in which different isobaric estrogens and their metabolites are being differentiated by higher order (MS^n) tandem MS fragmentation patterns, in combination with high mass accuracy, in an ion trap – time-of-flight mass spectrometer.

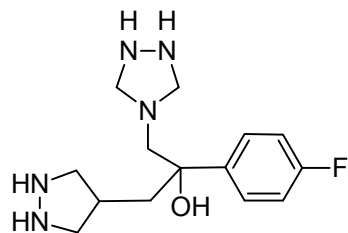
Besides fundamental MS studies, we also performed fundamental separation studies. Since estrogen glucuronides are the most hydrophilic estrogens, they were used as a model to investigate HILIC retention mechanisms. Although many interactions between the analytes and HILIC stationary phases might be involved (dipole-dipole, electrostatic, partition, etc.), hydrogen bonding was found to be the most important. The study has been extended to another on-going project, in which sensitivity between RPLC-MS and HILIC-MS is compared under the matrix effects of different solid phase extraction (SPE) fractions of human urine. Estrogen glucuronides were still a model of choice because they can be retained on both RPLC and HILIC mode. The preliminary data, obtained from the analysis of pure solution standards, agreed well with those published in literature with the observation of 10-times higher sensitivity in HILIC-MS versus RPLC-MS. We expect that the sensitivity would vary in HILIC-MS among different fractions because of the salt retention (from a urine matrix) on HILIC stationary phases, but it may not be highly affected in RPLC-MS because of the early elution of salts. Results from these fundamental studies will serve to guide the development of highly sensitive and efficient qualitative and quantitative methods for the determination of steroid hormones, particularly estrogens, and their metabolites, in a diverse range of biological fluid and tissue matrices.

APPENDIX A
STRUCTURES

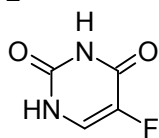
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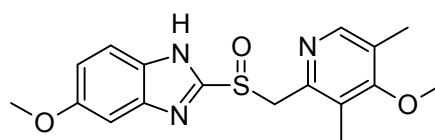
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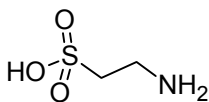
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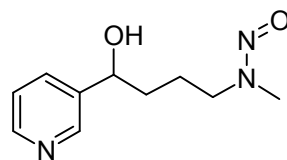
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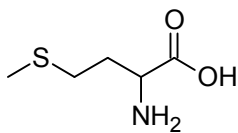
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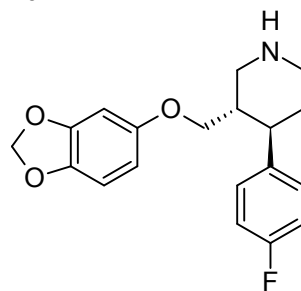
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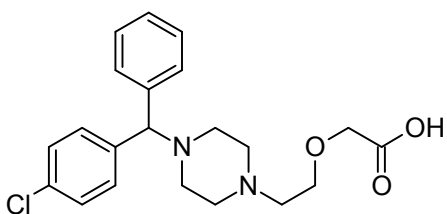
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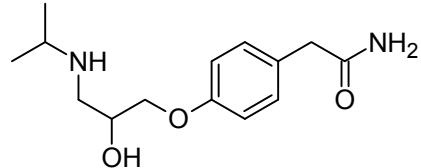
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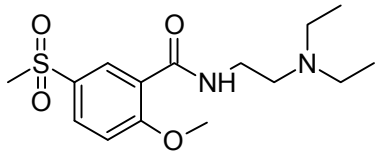
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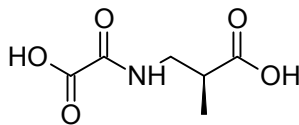
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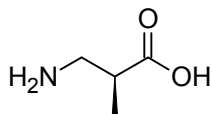
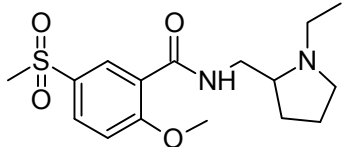


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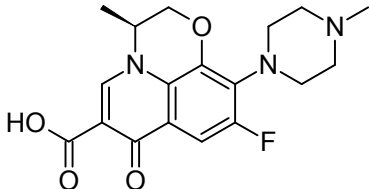
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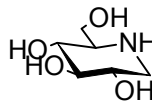


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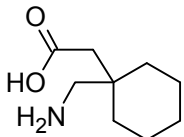
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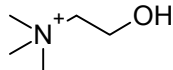
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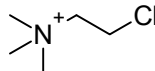
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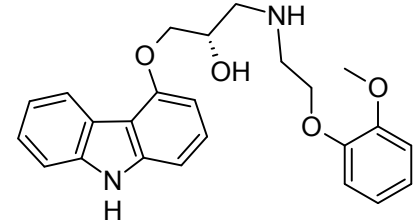
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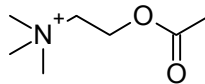
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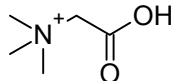
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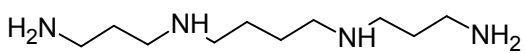
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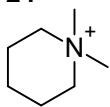
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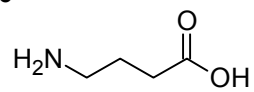
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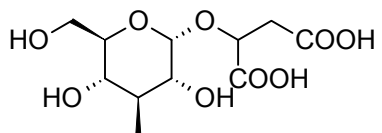
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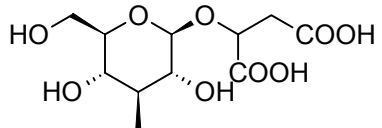
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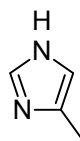


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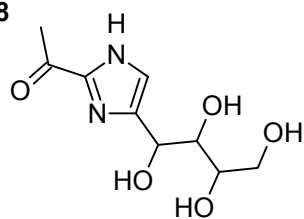


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BIOGRAPHICAL INFORMATION

Hien P. Nguyen (Nguyen Thi Phuoc Hien) was born in Hanoi, Vietnam, on February 24, 1977, but she spent most of her childhood in Ho Chi Minh City, also known as Saigon. She obtained her Bachelor's Degree in Chemical Engineering from Ho Chi Minh City University of Technology in 2001. She then attended Saxion Hogeschool Ijselland in The Netherlands and received her first Master of Science in Environmental Technology awarded by The University of Greenwich (UK) in January 2003. After that, she returned to her home country and worked at Nike for several months before pursuing other academic degrees. She came to the United States of America in May 2004 and started working on a graduate program in Analytical Chemistry at Texas Tech University. She graduated from Texas Tech with her second Master of Science in Chemistry in 2006. She then moved to Arlington, TX and started working on her PhD in chemistry at The University of Texas at Arlington under the supervision of Dr. Kevin A. Schug.