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## STEROID ANALYSIS BY PH-MEDIATED STACKING MEKC

by

Liliya Bykova

Dissertation submitted to the Eberly College of Arts and Sciences at West Virginia University in partial fulfillment of the requirements for the degree of

> Doctor of Philosophy in Chemistry

> > Approved by

Lisa A. Holland, Ph.D., Committee Chairperson Christy M. Foran, Ph.D. Fred L. King, Ph.D. Ronald B. Smart, Ph.D. Björn C. Söderberg, Ph.D.

**Chemistry Department** 

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Keywords: steroids, MEKC, pH-mediated stacking, cyclodextrin, endocrine disruption, fish Copyright © 2009 Liliya Bykova

#### Abstract

#### STEROID ANALYSIS BY PH-MEDIATED STACKING MEKC

#### by Liliya Bykova

This dissertation is based on research that led to the development and application of capillary electrophoresis method for the analysis of sex steroid hormones in the blood or plasma of fish species. The analysis of steroid hormones is essential to reveal chemical compounds that are suspected in endocrine disruption, but it is challenging due to the similarity of the chemical structures of steroids, their low concentrations, and limited volumes of plasma or blood samples in fish available for analysis. There is therefore an acute need to develop reliable accurate and systematic analytical methods applicable for the analysis of structurally similar steroid hormones at very low concentrations. The method developed here is based on micellar electrokinetic chromatography, a type of capillary electrophoresis that incorporates secondary equilibria. The method utilizes pH-stacking for steroid preconcentration to improve the detection limits. This method of pH-stacking is accomplished by using charged derivatives of cyclodextrin which become neutral (protonated) or anionic (deprotonated) based on the pH of the sample buffer. Preconcentration by means of pH-stacking occurs upon introduction of the sample into the capillary at the pH junction, resulting in a fast and efficient separation analysis of steroids. Using the developed method the separation of eight targeted steroids, that include  $\alpha,\beta$ dihydroxyprogesterone, ethynylestradiol,  $17\beta$ -estradiol, estrone, hydroxyprogesterone, 11ketotestosterone, progesterone, and testosterone is achieved in less than 4 min which is substantially faster than steroid analysis by immunoassay, GC-MS or LC-MS. For all targeted steroids, the within-day and day-to-day reproducibility in migration time is <1 and <2 % relative standard deviation (RSD), respectively. The reproducibility in peak area obtained in aqueous samples is below 6 % and 22 % (RSD) within-day and day-to-day respectively. The limits of detection range from 2 to 14 nM using a 60 s electrokinetic injection. The method is validated by measuring the recovery of standard steroids added to the aqueous or fish plasma samples prior to sample preparation. The recovery of testosterone and  $17\beta$ -estradiol added to the fish plasma prior to sample preparation range from 74 % to 102 %. The method is successfully applied to the

determination of sex steroid levels in blood plasma of yellow perch captured from natural aqueous habitats. The results are compared to radioimmunoassay.

#### **DEDICATION**

#### To those who I love and those who love me.

To my dearly beloved husband Dr. Peter Maksymovych for his loving, caring and encouraging attitude, and more importantly for not letting me get away with loafing around while writing this dissertation. If it has not been for him being in my face now and then, reminding me that I needed to finish, I don't know if I would have ever done it at all.

I would like to express my sincere gratitude and love to my mom Alla Bykova for her long distance love and overwhelming support and patience during these past few years. She always believes that I can do anything even when I cannot fairly believe in myself. Thank you for being able to let me go from under your wing to the other side of the world and do what I do and be who I am although I know how hard that was for you to let me go. I miss you and I dedicate this dissertation to you.

In loving memory of my brother Viktor Bykov (1973-1995), an irresistible optimist and altruist, whose memory will never be erased as years may come and go.

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### LIST OF SYMBOLS / NOMENCLATURE

- 1. Å angstrom  $1 \times 10^{-10}$  meter;
- 2. A area;
- 3. AD androstenedione;
- 4. AVE average;
- 5. BGE background electrolyte;
- 6. °C degrees Celsius;
- 7. cm centimeter;
- 8. CAPS 3-[cyclohexylamino]-1-propanesulfonic acid;
- 9. CD Cyclodextrin;
- 10. CE capillary electrophoresis;
- 11. CHES 2-[ cyclo-hexylamino]-ethanesulfonic acid;
- 12. CMC critical micelle concentration;
- 13. CMCD carboxymethyl-β-cyclodextrin;
- 14. CPM counts per minute;
- 15. CZE capillary zone electrophoresis;
- 16. E applied electric field;
- 17. E1 estrone;
- 18. E2  $17\beta$ -estradiol;
- 19. E3 estriol;
- 20. EDC endocrine disrupting compound;
- 21. EE ethynyl estradiol;
- 22. EOF electroosmotic flow;
- 23. D diffusion coefficient;
- 24. 17,20 aDHP dihydroxyprogesterone;
- 25. 17,20 $\beta$ DHP dihydroxyprogesterone;
- 26. DNA deoxyribonucleic acid;
- 27. g gram;
- 28. GC-MS gas chromatography mass spectrometry;
- 29. GnRH gonadotropin releasing hormone;

- 30. GtH gonadotropin;
- 31. HCl –hydrochloric acid;
- 32. HP hydroxyprogesterone;
- 33. HPCD hydroxypropyl-β-cyclodextrin;
- 34. I current;
- 35. IA immunoassay;
- 36. id inner diameter;
- 37. kPa kilopascal;
- 38. 11KT 11-ketotesteosterone;
- 39. kV kilovolts;
- 40. L liters;
- 41. LC-MS liquid chromatography- mass spectrometry;
- 42. LOD limit of detection;
- 43. log P log partition coefficient;
- 44. LOQ limit of quantitation;
- 45.  $L_t$  total length of the capillary;
- 46.  $L_w$  length to the capillary window;
- 47.  $\Lambda$  conductivity;
- 48. M molar concentration, mol/L;
- 49. mAu milliabsorbance units;
- 50. MEKC micellar electrokinetic chromatography;
- 51. min minutes;
- 52.  $\mu_{eph}$  electrophoretic mobility;
- 53. µL microliter;
- 54. mL milliliter;
- 55. µm micrometer;
- 56. mm millimeter;
- 57. µM micromolar;
- 58. mM millimolar;
- 59. MOPS 3-N-morpholino-propanesulfonic acid;
- 60. n number of measurements;

- 61. NaOH sodium hydroxide;
- 62. ng nanograms;
- 63. nm nanometers;
- 64.  $\eta$  viscosity;
- 65. od outer diameter;
- 66. P partition coefficient;
- 67. P progesterone;
- 68. Pa pascal;
- 69. PDA photodiode array;
- 70. PMT photomultiplier tube;
- 71. psi pounds per square inch;
- 72.  $\rho$  resistivity;
- 73.  $\rho$  density of water;
- 74.  $R^2$  correlation coefficient;
- 75. R resistance;
- 76. q ion charge;
- 77. r ion radius;
- 78. R<sub>s</sub> resolution;
- 79. RIA radioimmunoassay;
- 80. mRNA messenger ribonucleic acid;
- 81. RP C18 reversed phase C18;
- 82. rpm revolutions per minute;
- 83. RSD relative standard deviation;
- 84. s second;
- 85. SAX strong anion exchange;
- 86. SD standard deviation;
- 87. SDS sodium dodecyl sulfate;
- 88. SEF stacking enhancement factor;
- 89. SHBG sex hormone-binding globulin;
- 90. SHR steroid hormone receptor;
- 91. S/N signal-to-noise ratio;

- 92. T testosterone;
- 93. t migration time;
- 94. V volts;
- 95. V voltage;
- 96.  $v_e$  electrophoretic velocity.

#### **INTRODUCTION**

Rapid separation methods, such as capillary electrophoresis, are effective tools for screening the chemical composition of a wide variety of samples constituents. The following chapters describe the development of an effective analytical method for steroid analysis using capillary electrophoresis. Chapter 1 provides the literature background of endocrine disruption and discusses several known analytical methods used for steroid analyses. Chapter 2 proceeds with a detailed account of capillary electrophoresis, and describes the method developed in this work which is based on the micellar electrokinetic capillary chromatography (MEKC). Chapter 3 is devoted to in-capillary preconcentration methods, and much of the data in Chapter 3 is published in reference [1]. Chapter 3 includes a comparison of chromatographic preconcentration and pHstacking preconcentration using charged cyclodextrin. The comparison indicates the latter technique is much more reproducible for the targeted analysis. The method is evaluated using six representative steroid hormones. The limits of detection were measured and stacking enhancement factor was calculated and compared to the steroid analysis at non-stacking conditions. Finally, Chapter 4 presents the application of the stacking-MEKC method to the analysis of steroids in fish blood or plasma samples. Data from this chapter comprises a manuscript in preparation. The flushing protocol used to regenerate the inner surface of the capillary in analysis of standard steroid samples needed to be modified in order to achieve reproducible results when used for plasma sample extracts. The analysis of the plasma samples required the development of an effective purification scheme to extract steroids while eliminating interfering compounds. The method was validated by recovering steroids spiked in the aqueous standard steroid solutions and in the fish plasma samples prior to extraction. To investigate the effect of the differences among the fish species on the stacking, four different fish species were used for analysis. The developed and optimized method was applied to the determination of steroids in plasma of yellow perch fish. The MEKC results were compared to the results independently measured by radioimmunoassay. The developed method allowed for determination of the steroids in plasma samples with the improved limits of detection obtained by the stacking preconcentration.

# <u>CHAPTER 1</u>

# BACKGROUND AND SIGNIFICANCE OF ENDOCRINE DISRUPTION

#### **1.1. PHENOMENON OF ENDOCRINE DISRUPTION**

#### **1.1.1.** INTRODUCTION

The scientific interest in the occurrence of endocrine disruption dates back to the sixties and early seventies, when it was first reported that a synthetic estrogen, diethylstilbestrol, can have adverse effects on experimental animals and even humans (2). The pioneering work of John McLachlan, Howard Bern and other researchers has paved the way to the present field of endocrine disruption (*3-6*). However, the broad scientific and social recognition of the endocrine disruption problem was due to the work of Theo Colborn in the early nineties who found the evidence that certain non-hormone chemicals may also affect the sexual development of numerous wildlife species (*7*).

It is now known that a variety of environmental hormones and chemical pollutants in the environment disrupt human reproduction causing birth defects, sexual abnormalities, and reproductive failure. The threat of changes in the animal or human endocrine system by xenobiotic compounds (chemicals which are not normally produced or likely to be present in an organism but which are found in it) has become a major issue facing researchers today and continues to be a central topic at toxicological conferences, in the general press, as well as in the political arena (8). In particular, the problem of endocrine disruption has affected the state of West Virginia. In 2004, the researchers at the USGS (U.S. Geological Survey) reported 80 % of the male bass fish collected from the South Branch of the Potomac River (Hardy County, WV) had female tissue developed inside their gonodal tissue (9-12). Smallmouth bass are valued game fish in much of the United States and a high occurrence of testicular oocytes (intersex) has gained great public attention (13, 14). Endocrine disrupting chemicals have been identified in some areas of the Potomac Watershed, and it is suspected that they are contributing to these occurrences (15, 16).

#### **1.1.2.** ENDOCRINE DISRUPTION, SOURCES AND CONSEQUENCES

An endocrine disrupting compound (EDC) is a chemical compound that can potentially disturb the normal communication between the hormone and the cell receptor in the living organism, leading to the malfunctioning metabolism or catabolism of sex steroids, suppressed fertility and reproduction, and even complete sex-reversal in the organism itself or its next generation (*17*).

Among the significant sources of endocrine active chemicals affecting the environment are the agricultural waste products, atmospheric contamination, and sewage (*18-20*). The number of registered and potential endocrine disruptors is constantly growing. These include alkylphenols (*21*), heavy metals (*22*), pesticides (*23*), phthalates (*24, 25*), polycyclic aromatic hydrocarbons (PAHs) and dioxin (*26*), polychlorinated biphenyls (PCBs) (*27, 28*), natural and synthetic steroid estrogens (*29*), and pharmaceutical drugs (*30-32*).

All stages of the hormonal function of an organism can be affected including hormone synthesis, storage/release, transport/clearance, receptor recognition/binding, and post-receptor responses. The effects may be reversible or irreversible, immediate or latent. The intensity of the exposure depends on many factors including the dose of the pollutant, the interval of exposure, the body weight, stage of development etc. This complexity creates unique challenges in the detection and measurement of endocrine disruptors in the environment.

#### **1.1.3.** The Need for Biomonitors

The aquatic environment is exceptionally subject to xenobiotic damage. Water and sediment can act as a sink for many types of pollution from multiple sources. The pollutants can be retained in the sediments and subsequently absorbed into the aquatic organisms. To determine the safe maximum contaminant level in the environment one has to measure the rate of accumulation of pollutants by the aquatic organisms, explore the biochemical disruption mechanism and estimate the possibility of disruptions in the viability or fertility of the organisms and their offspring.

Biomonitoring is generally achieved by placing an animal into the test water containing a suspect chemical and observing its effects on the animal. A simple toxicity measure is the dose of a pollutant which kills 50 % of the test animals ( $LD_{50}$ ) (*33*). Laboratory toxicity tests commonly inspect the effects of a single pollutant for a relatively short period of time under controlled conditions. Exploring similar effects in the wildlife is equally important since the organisms are exposed to lower levels of complex mixtures, but over much longer time scales.

Fish accumulate pollutants in their fatty tissues such as liver and gonads, but the effects may become apparent only when concentrations in such tissues reach threshold level after several years. Thus the fish is a natural long-term biomonitor.

#### **1.1.4.** Endocrine System

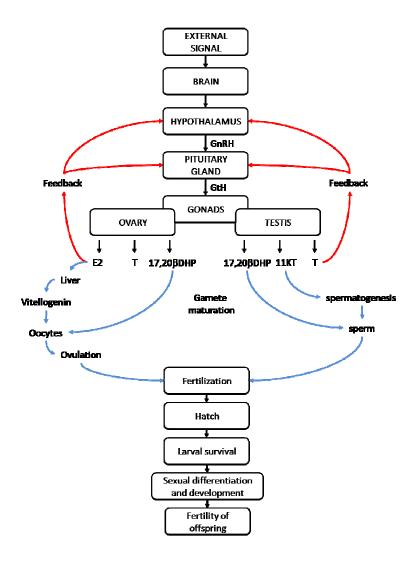
The endocrine system is the chemical communication system of the body comprising many glands such as ovaries, testes, pancreas, adrenal, thyroid, and parathyroid. The signals from the brain resulting from the external stimuli are modulated by the hypothalamic-pituitary system as changes in the hormone secretion. The hypothalamus produces gonadotropin releasing hormone which causes further release of gonadotropin from the pituitary gland. Gonadotropin is a peptide hormone that acts on the glands to stimulate secretion of sex steroid hormones and is the most concern in the context of reproduction. The secreted steroids in turn initiate changes in the secondary sexual characteristics, behavior, courtship, development and maturation of the gamets, and spawning (Fig. 1.1).

The endocrine signaling cascades similar to that in Figure 1.1 provide several sites where the endocrine signal can be regulated. For example, testosterone is secreted by the testis but regulates its own secretion by acting upstream at the pituitary gland and hypothalamic gland. Peptide hormones are commonly the intermediate messengers along a signaling cascade, while the terminal hormone is often of nonpeptide origin (i.e., steroids). Toxicologically the function of the terminal hormones appears to be most at risk of chemical disruption, where the foreign molecules bind to the nuclear receptors of these hormones in an agonistic or antagonistic manner (Section 1.1.6). The binding of a xenobiotic substance to the receptor results in abnormal receptor function with the associated toxicological outcome.

Steroid hormones are lipophilic in nature and can be transferred in the circulatory system from their point of origin to their target tissues only by specific carrier proteins such as sex hormone-binding globulin, corticosteroid-binding globulin, thyroxine-binding globulin (transthyretin), and albumin. Sex hormone-binding globulin (SHBG) binds testosterone, 17 $\beta$ -estradiol, and other sex steroids. Most steroid and thyroid hormones (> 95 %) are reversibly bound to proteins in the blood. Upon dissociation from the proteins, the steroid hormones can cross the cell membrane via diffusion due to their lipophilicity. Therefore, the vitally critical parameter is not the total hormone concentration in the plasma, but that of a free, unbound steroid.

The molecular mechanism of steroid receptor action described by Nelson and Cox (*34*) occurs as follows: after crossing the membrane of the target cell, the hormone binds to specific receptor proteins in the nucleus. The steroid hormone receptors (SHR) include the glucocorticoid, mineralcorticoid, progesterone, androgen, and estrogen receptors. The estrogen receptor is the major regulatory unit within the estrogen-signaling pathway. When functioning normally, the hormone binding changes the conformation of the receptor protein. It forms homo- or hetero-dimers with other hormone receptor complexes and binds to specific regulatory regions, called hormone response elements, in the DNA adjacent to specific genes. The binding regulates the transcription of the adjacent gene(s), increasing or decreasing the rate of the formation of mRNA. The hormone-regulated gene thus produces the specific cellular response to the hormone.

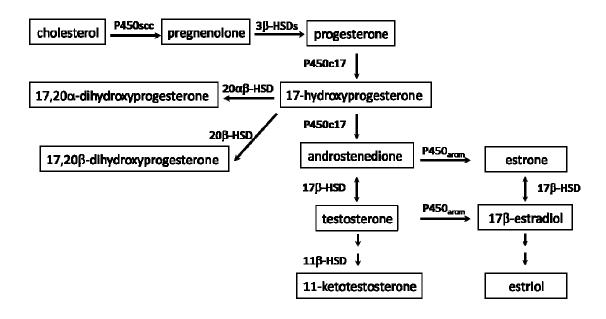
The bodily functions regulated by the endocrine system include reproduction, energy production, metabolism, fetal development, growth, and maturation. For example, ovaries release estrogen, which is necessary for growth, fetal development, and reproduction. The thyroid affects metabolism and brain development, and the pituitary controls other glands in the endocrine system. The endocrine system is very sensitive to very low levels of pollutants. Any anthropogenic substance, a substance caused by human activity, can potentially interfere with any of the steps described above to cause a disruption of the endocrine system.



**Figure 1.1.** Endocrine signaling cascade of the reproductive system in the fish. (GnRH – gonadotropin releasing hormone, GtH – gonadotropin, E2 –  $17\beta$ -estradiol, T – testosterone, 11KT – 11-ketotesteosterone,  $17,20\beta$ DHP – dihydroxyprogesterone). Reproduced with kind permission of Springer Sciences from (*35*).

#### **1.1.5.** Steroid Hormones

The major difference between the endocrine system of the fish and the mammals are the steroid hormones. The main steroids in teleost fish, a large group of fishes with bony skeletons, their interrelationship via biosynthesis and the major enzymes are shown in Figure 1.2. The chemical structures of these steroids can be found in Table A1, Appendix 1.



**Figure 1.2.** Schematic demonstration of steroid biosynthesis in teleost fish (for simplicity only major pathways are shown). Reproduced with kind permission of Springer Sciences from (*35*).

Steroids are oxidized derivatives of sterols. They have the sterol nucleus but lack of alkyl chain attached to ring D of cholesterol, and they are more polar than cholesterol, for which the logarithm of the partition coefficient (log P) is 9.9. A steroid hormone molecule consists of a tetracyclic ring structure with substituents at specific sites within the molecule (Fig. 1.3.). The substituents are usually hydroxyl, carbonyl and alkyl groups. Very often steroids and their metabolites differ only by the presence or absence of one functional group or a variation in the steric conformation of the ring.

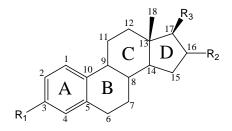


Figure 1.3. Basic structure of a steroid molecule.

The biosynthesis of steroid hormones starts from a common precursor, cholesterol, and proceeds via a complex sequence of biochemical reactions (Fig. 1.2). Kime (35) describes this process quite nicely, and briefly, cholesterol is converted to pregnenolone by the cholesterol side-chain cleavage enzyme (P450scc). Pregnenolone is then oxidized at position C-3 by 3 $\beta$ -hydroxysteroid dehydrogenases/ $\Delta^5$ - $\Delta^4$  isomerases (3 $\beta$ -HSDs) producing progesterone. The 17 $\alpha$ -hydroxylation of progesterone by 17 $\alpha$ -hydroxylase/C<sub>17-20</sub> lyase (P450c17) results in the formation of 17-hydroxyprogesterone. 17-hydroxyprogesterone is further converted to androstenedione by the lyase activity of P450c17. The final steps of estrogen production are controlled by P450arom and 17HSDs. Aromatase catalyzes the aromatization of the androgens to estrogens, such as androstenedione to estrone or testosterone to estradiol (36), whereas  $17\beta$ -HSDs are responsible for the interconversion between 17-ketosteroids, like and rostenedione and estrone, and  $17\beta$ -hydroxysteroids, e.g. testosterone and estradiol (37). Finally testosterone is converted to 11-ketotestosterone. In teleost fish, 11-ketotestosterone is important for sexual differentiation and male sexual development (38).

All steroid hormones act through nuclear receptors to change the level of expression of specific genes. Because hormones have very high affinity for their receptors, very low concentrations of hormones (nanomolar or less) are sufficient to produce responses in the target tissues of the fish. The exposure to hormonally active compounds does not necessarily lead to adverse effects. Sorting out adverse effects of endocrine disruptors is a scientific challenge but it is also practically important, since exposure to such compounds through food, air, water and many household products is everywhere and unavoidable. Therefore, in order to better identify the biological disruption, key steroids in the endocrine cycle must be profiled.

#### **1.1.6.** DISRUPTION OF HORMONAL REGULATION BY EXOGENOUS COMPOUNDS

The structural requirements and mechanisms of action of endocrine disruptors are complicated by the fact that there exist multiple ways to interfere with the endocrine system. The predominant mechanisms of disruption of hormone activity by xenobiotics involve: (1) mimicking steroids by binding to the receptor and inducing (agonizing) or inhibiting (antagonizing) the steroid response; (2) modulating endogenous steroid hormone levels.

To bind to the receptor, xenobiotics first compete with hormones for the binding to the carrier protein, which, as mentioned earlier, act as a vessel that distributes steroid hormones throughout the body. For example, xenobiotics estrogens can be both endogenous (from within an organism) which regulate the growth and development of their target tissues, e.g. estradiol, and exogenous (from outside of an organism) with estrogenic and/or anti-estrogenic activities that induce or inhibit estrogenic response and may disrupt the regulatory pathways, e.g. ethynyl estradiol.

#### 1.1.6.1. Hormone Receptor Agonists

A hormone receptor *agonist* is a compound that binds to and activates a hormone receptor. Endogenous hormones, for example  $17\beta$ -estradiol and  $5\alpha$ -androstan- $3\alpha$ ,  $17\beta$ -diol (commonly known as dihydrotestosterone), function as agonists to their respective receptors. Xenobiotics can act as receptor agonists and stimulate receptor-dependent physiological processes in the absence of the endogenous receptor ligand (hormone).

The estrogen receptor is most susceptible to the agonistic action of xenobiotics due to their diverse molecular structures. Compounds which bind to the estrogen receptor and induce receptor-mediated response, are termed estrogens or estrogen receptor agonists. Whether a xenobiotic can fit into the binding-pocket of the receptor and function as a receptor agonist is determined by the structure of the xenobiotic and its electrical charge. Some drugs are rather potent estrogens (i.e. diethylstilbestrol); however, chemicals with estrogenic activity are typically weak agonists with activity several orders of magnitude less than that of  $17\beta$ -estradiol. However, adult males, young individuals, and embryos all have been shown to exhibit endocrine toxicity resulting from xenoestrogen exposure due to the small amount of endogenous  $17\beta$ -estradiol in these individuals (*38*). The physiological consequence of the xenoestrogenic activity is typically feminization, for example, breast development in males (gynecomastia) (*39, 40*).

#### 1.1.6.2. Hormone Receptor Antagonists

Receptor *antagonists* are chemicals that bind to a hormone receptor but do not activate it. These chemicals inhibit receptor activity by preventing the endogenous hormone from activating the receptor. Estrogen receptor antagonists include certain phytochemicals (i.e., flavonoids) and PCBs. Estrogen receptor antagonism typically leads to de-feminization. In laboratory animal studies, estrogen receptor antagonists have been shown to disrupt estrous cycles and damage fertility in females (*41*).

Chemicals that known to bind to the androgen receptor in an antagonistic way include, for example, the heart failure drugs, spironolactone and cimetidine, and antiandrogen prostate cancer drugs, cyproterone acetate and hydroxyflutamide (42). Environmental chemicals that have been shown to act as androgen receptor antagonists include the metabolites of the agricultural fungicide vinclozolin, the DDT metabolite p, p'-DDE, some hydroxylated PCBs, and the organophosphate insecticide fenitrothion (43). Androgen receptor antagonism, unlike that of the estrogen receptor, may result in demasculinization.

#### 1.1.6.3. Mixed Agonists/Antagonists

Certain chemicals can function as either a receptor agonist or antagonist, and are referred to as *mixed agonists/antagonists*. The particular function depends on the level of endogenous hormone in the organism, the concentration of the xenoagonist, the binding affinity of the xenoagonist to the receptor, the concentration of the endogenous, and the binding affinity of the endogenous hormone to the receptor. For example, a weak agonist may bind to a receptor and stimulate some low-level receptor-mediated activity in the

absence of the endogenous hormone. However, in the presence of the hormone, binding of the xenobiotic to the receptor may prevent the binding of the endogenous hormone and suppress the hormonal activity if the xenobiotic is a weaker activator.

The drug tamoxifen functions as an estrogen receptor antagonist in reproductive tissue but functions as an agonist with respect to the preservation of bone mineral density and reducing serum cholesterol concentrations (44). Accordingly tamoxifen can function as a prophylactic against the growth of estrogen-responsive breast cancers and osteoporosis via two different mechanisms, estrogen receptor antagonism and agonism, respectively.

#### 1.1.6.4. Inhibitors of Hormone Synthesis

Endocrine toxicants can cause antihormone activity by lowering the levels of the endogenous hormones in the body. With steroid hormones, chemicals typically elicit this effect by inhibiting the enzymes necessary for the synthesis of the hormone. For example, cytochrome P450 enzyme (CYP19) is responsible for the aromatization of testosterone to form  $17\beta$ -estradiol. CYP19 inhibitors such as breast cancer treatment drugs, fadrozol, anastrozole, letrozole, can lower the endogenous  $17\beta$ -estradiol levels resulting in defeminization (45). Cytochrome P450s enzymes are also critical to various hydroxylation reactions that contribute to the synthesis of androgens and other steroid hormones. Inhibition of these enzymes can result in a variety of antisteroid hormone effects. For example, the agricultural and medicinal fungicides propiconazole, ketoconazole, and fenarimol are capable of inhibiting P450 enzymes and reducing synthesis and circulating levels of testosterone and other steroid hormones. Toxicological consequences of the

lowering of the endogenous steroid hormone levels are typically comparable to the effects elicited by antagonists of the hormone's receptor.

#### **1.2. LITERATURE METHODS FOR STEROID ANALYSIS**

#### **1.2.1.** INTRODUCTION

Although the concentration of specific pollutants may be analyzed at very low levels, no single method can predict or detect all EDCs present in an environmental sample. Ultimately, this prevents the analysis of complex biological mechanisms of endocrine disruption in one simple test. To address this environmental problem, reliable analytical methods must combine several types of analysis including in vivo and in vitro bioassays, and analytical chemistry for the determination of structurally similar steroid hormones at very low concentrations. Monitoring the key steroid hormones in the endocrine cycle of fish is essential to provide insight into the complex metabolism involving steroid hormones and identify a biological disruption. Hormones can cause endocrine disruption at very low concentrations. For example, steroid estrogens can be harmful to the fish at a concentration as low as 2.0 ng/L (46). Profiling circulating steroids in biologically relevant concentrations to determine how EDCs affect the endocrine system thus requires highly sensitive analytical methods. The separation of steroid hormones is further complicated by the high hydrophobicity of steroid molecules and their similar chemical structures. Finally, the analysis of steroids in fish plasma is difficult due to the presence of interfering compounds and small amount of the fish blood plasma available.

Natural and synthetic sex steroid hormones can be analyzed by liquid or gas chromatography coupled to mass spectrometry, immunoassay, or capillary electrophoresis. A number of papers (47-60) reported the analysis of one or several steroids simultaneously present in water, urine, or sediments. There are fewer reports dealing with the blood or plasma (61-88). A brief introduction to the benefits and limitations of the available methods for steroid analysis is presented in the following sections.

#### 1.2.2. LC-MS AND GC-MS OF STEROIDS

Natural and synthetic sex steroid hormones have been analyzed by liquid or gas chromatography coupled to mass spectrometry. Mass spectrometric detection allows for the structural elucidation of steroids and steroid metabolites, eliminating the need for predictive identification of steroids in the sample and enabling identification of steroid metabolites for which no immunoassay is available. Isotope-dilution GC-MS is a reference measurement procedure for plasma steroids listed in the database of the Joint Committee for Traceability in Laboratory Medicine (89). It has been widely used to validate immunoassays for steroid analysis as they require more thorough validation due to their cross reactivity (90). Despite isotope-dilution GC-MS being a de-facto gold reference method for steroid analysis, isotope-dilution LC-MS/MS has been proven to demonstrate the same accuracy for steroid analysis (91, 92) and is also used by clinical laboratories for the analysis of steroid hormones. Both LC-MS and GC-MS provide very low detection limits for LC-MS  $(2.8 - 7.1 \cdot 10^2 \text{ ng/L})$  (47-57, 61-71, 93) and GC-MS  $(1 - 10^2 \text{ ng/L})$ 1.4.10<sup>3</sup> ng/L) (58, 59, 72-79). These methods cannot, however, be used for fast steroid profiling. It takes 20-45 min by LC-MS (48, 50, 54, 56, 57, 61-65, 93), and 14.5-35 min by GC-MS (58, 72-74) to obtain a steroid profile, excluding sample preparation time. This is a considerably long time, especially when several hundred analyses or large-scale screening

have to be performed. In addition, isotope-dilution methods require isotope labeled steroid standards which are expensive and not readily available for all the steroids of interest. Another shortcoming is the large sample injection volumes required by chromatography. For example, a typical sample injection volume in LC-MS is  $2 - 100 \mu$ L, and  $1 - 2.5 \mu$ L in GC-MS. Additionally, liquid chromatography uses large volumes of flammable, expensive, and toxic mobile phases for analysis. While liquid chromatography does not require sample derivatization, gas chromatography method requires volatilization of the steroid analyte which can be obtained by derivatization of the analyte for  $40 - 130 \min (72, 73, 75, 76, 78)$ . Also, the use of gas chromatography for thermally degradable chemicals such as steroid hormones is questionable.

## **1.2.3.** Immunoassay

Immunoassays are widely used in clinical laboratories for steroid analysis in the serum or plasma. Immunoassay measures the concentration of the analyte using the unique molecular recognition properties of an antibody (94). Immunoassays are widely applicable, specific, and sensitive (LOD < 0.3 - 64 ng/L) (80-88, 96, 97). The instrumental equipment is relatively cheap and accessible. However, the analysis can be affected by the cross-reactivity of antibodies with steroid metabolites from the sample matrix as many of the immunoassays are preformed directly on a non-purified plasma or serum samples to decrease the analysis time or on a sample pretreated with the extraction or chromatographic purification prior to incubation with an antibody (95). Immunoassay cannot be used for steroid profiling, since the method is typically used for analysis of one steroid at a time. To analyze several steroids in a single sample one would need to partition the sample and

incubate each portion of the sample with a specific steroid antibody. Typically immunoassay requires 10-200  $\mu$ L of a reconstituted plasma sample to be introduced in each well of a 96-well mirotiter plate for incubation (*81, 85-87, 95, 97*). Since the volume of plasma sample available from a single fish is typically from 10  $\mu$ l to < 1000  $\mu$ l, the entire sample of fish plasma is often used to determine one steroid only. Given the likelihood of variations of steroid concentrations between fish plasma samples, profiling using multiple fish plasma samples is error prone, not to mention the increased analysis time since the incubation time in immunoassay ranges from 45 – 90 min up to several hours (*80-82, 84-88, 96*). Finally, the immunoassay analysis should be confirmed using GC-MS or LC-MS. For example, isotope dilution GC-MS does not suffer from cross-reactivity and does not depend on the presence of interferences in plasma samples as a result it has been used as a "gold" reference standard method for validating immunoassays (*89*). Figures of merit for steroid analysis reported in the literature are summarized in Table 1.1.

## **1.2.4.** CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) provides rapid and unsophisticated method development, versatility, high efficiency and resolution, various modes to vary selectivity, simple separation mechanism and extremely small sample volumes required (98-101). CE separations are faster comparing to other methods discussed above. For example, previous works reported steroid analysis separation times less than 14 min using conventional capillaries (102-112) and less than 7 min with microfluidic devices (102). In addition, in CE the same instrument can be used for the separation and analysis of a wide range of analytes such as large biomolecules (proteins and nucleic acids), metal and organic ions,

enantiomers and neutral molecules. These advantages of CE can help overcome the shortcomings of LC-MS, GC-MS, and immunoassay for steroid analysis discussed earlier.

## **CONCLUSIONS**

The goal of the research presented within the framework of this dissertation is the development of an analytical technique which would allow for the high-throughput determination of the low concentrations of endogenous steroids in fish blood or plasma in order to be able to control the cause and effects of the endocrine disruption in aquatic fauna. As discussed above, many analytical methods have been developed for the analysis of steroids in environmental and biological samples. Despite the advantages of the low detection limits these methods are time consuming and are difficult to apply to volume-limited plasma samples. These technical difficulties suggested a need for a new approach to hormone analysis compatible with stringent demands of high throughput screening in real life applications dealing with small sample volumes and complicated matrix composition.

Chapter 2 discusses the fundamentals of capillary electrophoresis, which was chosen as the baseline approach for analysis of steroid hormones. Development of the separation protocol using micellar electrokinetic chromatography under acidic conditions to rapidly separate steroids is outlined in this chapter as well. The developed method is evaluated with respect to the selectivity and migration times for analyzed steroids. **Table 1.1.** Figures of merit for steroid analysis by literature methods. Literature search was limited to the publications published by 2008 and reported analysis of at least two (or one for immunoassay) of the following steroids: progesterone (P), testosterone (T), estrone (E1), estradiol (E2), and estriol (E3). Sample was either blood serum or blood plasma.

#	Steroid	Method	Sample matrix	Sample prep.	Derivati zation	Sample volume (mL)	Sample injection volume, (µL)	Analysis time (min)	LOD (ng/L)	LOQ (ng/L)	RSD (%)	Linear range (ng/L)	Recovery (%)	Ref.
1	Various steroids including E3, E2, E1, P	HPLC- UV-VIS diode array	Human plasma	SPE	NO	2	20	10 (most steroids); 43 (P)	$4.0 \cdot 10^3 - 1.6 \cdot 10^5$	NR	NR	$ \begin{array}{c} 1.0 \cdot 10^{5} - \\ 3.0 \cdot 10^{6} \\ (R^{2} \geq \\ 0.995) \end{array} $	85.2 - 99.9	61
2	T, P, E1, E2, E3	LC-UV	Human serum	SPE	NO	0.08	NR	45	NR	NR	4	$10^4 - 10^5$	NR	62
3	Various steroids including E3, E2, E1, T, AD	HPLC - DAD	Serum	SPE	NO	1	20	30 (multistep gradient); 40 (linear gradient)	NR	NR	NR	$1.5 - 30, \\ 10^4$	NR	63
4	Various steroids including E2, E1, T, AD, P	HPLC - PDA	Serum; gonodal tissues	SPE	NO	10	25	30	NR	NR	NR	NR	NR	65
5	E2, E1	LC- (APCI)- MS/MS	Human plasma	Solvent extraction	YES	0.5	15	5	3.5 (E1); 2.8 (E2)	11.9 (E1); 6.3 (E2)	Inter- assay: 4-20 (n = 20)	$\begin{array}{c} 10-600 \\ (R^2 \ge \\ 0.995) \end{array}$	93-108 (E1); 100-110 (E2)	66
6	Deuterated E2 and E1	LC- MS/MS	Mouse plasma	Solvent extraction	YES	0.05	NR	NR	NR	50	Intra-, inter-day precision 12.9	$50-2.10^4$ (R <sup>2</sup> = 0.995)	83	69
7	E1, E2	LC- (APCI)- MS/MS	Serum; urine	Solvent extraction	YES	0.01	10	5	300 - 710	NR	NR	NR	93.8- 100.9 (serum); 93.5- 107.9 (urine) (n=2)	70

#	Steroid	Method	Sample matrix	Sample prep.	Derivati zation	Sample volume (mL)	Sample injection volume, (µL)	Analysis time	LOD (ng/L)	LOQ (ng/L)	RSD (%)	Linear range (ng/L)	Recovery (%)	Ref.
8	E2, E3, E1, EE, diethylstilbest rol	LC- MS/MS, GC- MS/MS	Water, serum	SPE (C18 col.)	(GC-MS/MS): YES	1000 (water); 2 (serum)	NR (LC- MS/MS); 1 (GC- MS/MS)	20 (LC- MS/MS); 65 (GC- MS/MS)	NR	5·10 <sup>3</sup> (LC- MS/MS); (2 - 15) ·10 <sup>3</sup> (GC- MS/MS)	NR	NR	90	71
9	14 adrenal- cortical steroids incl. E1, AD, E2, T, P, E3	GC-MS	Blood serum	Solvent extraction	YES	1	2	14.5- separation (240 total time)	100-1000	NR	Intra- assay: 5.0-18.0; inter- assay: 6.0-19.0	NR	71.4- 100.0	72
10	Various steroids incl. E1, E2, T, AD, P	GC-MS	Fish plasma	SPE	YES	1 g	2.5	33	100 ng/kg	NR	NR	NR	85-118	73
11	EDCs, E1, E2, E3, EE, P, T	GC-MS	Serum	SPE- HPLC	NO	3.6	2	20	NR	NR	NR	NR	NR	74
12	E1, E2	GC- MS/MS	Serum	SPE; solvent extraction; semi-prep. HPLC;	YES	2	2	6	1	2	10	NR	24	75
13	Estramustine phosphate + metabolites: E1, E2	GC- (SIM)- MS	Human plasma	SPE + solvent extraction	YES	1	2	15	1.4·10 <sup>3</sup> (E1); 0.54·10 <sup>3</sup> (E2)	3.2·10 <sup>3</sup> (E1); 2.2·10 <sup>3</sup> (E2)	1.3 – 14 (E1); 2.1 – 11 (E2)	$\begin{array}{r} 1.4 \cdot 10^{3} - \\ 1.5 \cdot 10^{5} \\ (E1); \\ 0.54 \cdot 10^{3} \\ - 0.30 \cdot 10^{5} \\ (E2) \end{array}$	70 - 90	76
14	E1, E2, E3	GC-MS	Serum	Solvent extraction	YES	0.5	NR	4.5 (30 samples/ day)	NR	NR	Inter- assay: 3.42-3.73	100-500 pg	96.47 - 97.88	77

#	Steroid	Method	Sample matrix	Sample prep.	Derivati zation	Sample volume (mL)	Sample injection volume, (µL)	Analysis time	LOD (ng/L)	LOQ (ng/L)	RSD (%)	Linear range (ng/L)	Recovery (%)	Ref.
15	E1, E2, E3	GC- (EI)-MS	Serum	Solvent extraction	NO	0.2	NR	13	50 (E1, E2); 1000 (E3)	NR	NR	$50 - 5 \cdot 10^4$	NR	78
16	E2, P, T	RIA	Blood plasma	NR	NO	10 total	NR	3 – 4 hr at room T	8 (E2); 20 (P); 64 (T)	NR	NR	NR	NR	80
17	E1, E1- sulfate	RIA	Human serum	Extraction	NO	1	200	16 – 20 hr at 0°C	4.7 (E1)	NR	NR	NR	NR	81
18	E1, E2, E3, T, AD, 5α- dihydrotestost erone	RIA	Human plasma	Solvent extraction + column chromatog raphy + TLC	NO	1	NR	12 hr at ± 4°C	2.6 -15 pg/tube	NR	Intra- assay: 2.8-8.3; Inter- assay: 1.3-10.5	NR	61.2 -73.7 (n=90)	82
19	E1, E2 E1- sulfate	RIA	Blood plasma	SPE, HPLC	NO	2	200	NR	0.7 – 6	NR	Intra- assay: < 10	NR	55 - 70	83
20	E1, E2	EIA	Bovine plasma	Sephadex LH-20	NO	0.1	100	overnight at 4°C	< 0.3 (E1); < 1.2 (E2)	NR	Intra- assay: 4.8-5.6; Inter- assay: 1.9-8.8	0.25-1000 (E1); 1-5000 (E2)	94.5 (E1); 93.9 (E2)	85
21	E2	EIA	Human serum or plasma	NR	NO	NR	25	110 min	10	NR	Intra- assay: 4.1-24.1; Inter- assay: 6.4-26.7	0-1000; cross- reactivity: 2.10% (E1), 1.50% (E3)	89.2- 112.6	86
22	Т	EIA	Human serum	NR	NO	NR	10	110 min	50	NR	Intra- assay: 5.0-10.0; Inter- assay: 4.4-8.4	0-18000; cross- reactivity: 0.86% (DHT), 0.89% (AD)	70.4- 127.5	87

#	Steroid	Method	Sample matrix	Sample prep.	Derivati zation	Sample volume (mL)	Sample injection volume, (µL)	Analysis time	LOD (ng/L)	LOQ (ng/L)	RSD (%)	Linear range (ng/L)	Recovery (%)	Ref.
23	Р	EIA	Human serum or plasma	NR	NO	NR	25	110 min	300	NR	Intra- assay: 2.4-7.1; Inter- assay: 2.6-12.6	0-50000;	80.4- 118.6	88

NR-not reported

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# <u>CHAPTER 2</u>

## DEVELOPMENT OF STEROID SEPARATION

## **2.1. INTRODUCTION**

The long term goal of the research presented in this chapter was to develop a CE method for profiling steroids in blood plasma of fish which can assist in monitoring endocrine disruption. In order to fully understand the principle of the analytical approach used in this study a brief review of the capillary electrophoresis, micellar electrokinetic chromatography (MEKC) and cyclodextrin-modified MEKC based on introduction to the high performance capillary electrophoresis by Heiger (1) is presented below.

## 2.1.1. THEORY OF CAPILLARY ELECTROPHORESIS

*Capillary zone electrophoresis (CZE)* of ionic species using narrow inner diameter fused silica capillaries (< 100  $\mu$ m), high voltage up to 30 kV and on-line UV-visible absorbance detection was first demonstrated by Jorgenson and Lukacs in 1981 (2), who also investigated the relationships between the operational parameters and separation quality of CE (*3*, *4*). The separation of analytes in CE is affected by two transport processes: electrophoresis and electroosmosis. The net result of both is the separation of charged and neutral analytes. Separation of charged analytes is based on their respective charge-to-size ratios. Electrophoresis is the movement of a charged solute through a conductive solution toward or away from an electrode (anode or cathode) by the electric field. The movement of the solute depends on the mobility of the solute and the magnitude of the applied electric field and is called the electrophoretic velocity ( $v_e$ ):

$$v_e = \mu_e E \tag{2.1}$$

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where values  $v_e$ ,  $\mu_e$ , and *E* are ion velocity, electrophoretic mobility, and applied electric field, respectively. The electric field is a function of applied voltage and capillary length (in volts/cm). Electrophoretic mobility is dependent on the solute and the buffer properties:

$$\mu_e = \frac{q}{6\pi\eta r} \tag{2.2}$$

where q, r, and  $\eta$  are ion charge and radius, and viscosity of the solution, respectively. Cationic solutes with the largest charge-to-size ratio have therefore the highest net mobility and elute faster toward the cathode. For anions the trend is opposite while for neutral (uncharged) solutes electrophoretic mobility is zero. Figure 2.1 shows CE separation at normal polarity where the cathode and the anode are at the outlet and at the inlet of the capillary respectively. The opposite arrangement of the cathode and the anode will be referred to as reversed polarity in this dissertation.

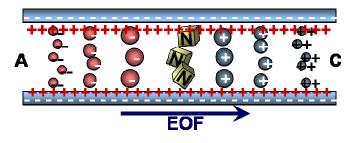


Figure 2.1. Schematic representation of separated analyte bands in CE and EOF.

*Electroosmotic flow (EOF)* is a distinctive element of CE. EOF is the bulk movement of the solvent being dragged by the positively-charged ions migrating toward the cathode. As mentioned above, CE is usually performed in capillaries made from fused silica. Surface charges on the interior of the capillary induce the formation of a double layer upon application of the electric field. In a fused silica capillary, the surface silanol (Si-OH) groups are ionized to negatively charged silanoate (Si-O<sup>-</sup>) groups at pH above three. These negatively charged silanoate groups attract positively charged cations from the buffer solution, which form an inner layer of cations (Stern layer) at the capillary wall. The density of these cations is not sufficient to neutralize all the negative charges, so a second, outer layer of cations (Gouy-Chapman layer) forms. The electric potential at the sheer plane of the double layer is called the *zeta* ( $\zeta$ ) *potential*. While the inner layer is tightly held by the Si-O<sup>-</sup> groups, the outer layer of cations is not tightly held because of its larger of cations is pulled toward the negatively charged cathode. Since these cations are solvated, they drag the bulk buffer solution with them, thus causing EOF, as represented in Figure 2.1. The existence of the EOF means that under normal polarity neutral and charged molecules, even negatively charged ones, will be swept along towards the cathode.

The magnitude of EOF is highly dependent on the pH of the running buffer because the  $\zeta$ -potential is largely controlled by the ionization of the acidic silanols on the capillary wall. At pH  $\leq$  3, the ionization is small and the EOF flow rate is therefore not significant. At pH  $\geq$  9 the silanols are fully ionized and EOF flow rate is high. For example, in 20 mM borate buffer at pH 9.0 in a 50 µm inner diameter capillary the velocity of EOF is approximately 4 nL/s. The magnitude of EOF also decreases with increased concentration of the running buffer. Increasing ionic strength causes double-layer compression, which results in reduction of the level of EOF. Additionally higher ionic strength will increase the current across the capillary and increase the likelihood of Joule heating and band broadening (5, 6). EOF can be suppressed by chemically coating the inner surface of capillaries (7-9) or by using acidic running buffer (10).

EOF is generated throughout the entire length of the capillary and is characterized by a flow profile that is plug-like in nature. Consequently, solutes are swept along at the same rate regardless of their radial position in the capillary minimizing sample dispersion. This is an advantage compared to the laminar flow encountered in pumped systems such as in HPLC. In laminar flow, the solution is pushed from one end of the column. The solution at the column walls moves slower than the solution in the middle of the column due to the shear effect resulting in different solute speeds across the cross-section of the column. Therefore, the laminar flow tends to broaden the peaks as they travel along the column. The apparent mobility  $\mu_a$  of a solute is the sum of its electrophoretic mobility and the electroosmotic mobility:

$$\mu_a = \mu_e + \mu_{EOF} \tag{2.3}$$

Under normal polarity the apparent mobility of cationic species is therefore generally greater than EOF (Fig. 2.1). Neutral species do not have charges and therefore migrate as a single peak at the same velocity as EOF. Apparent mobility of anionic species is smaller than EOF since the anions have electrophoretic attraction toward the anode while EOF is driven by the cathode attraction. If EOF is strong enough, all species will migrate towards the cathode allowing single run detection of all analytes (Fig.2.1).

## 2.1.2. INSTRUMENTATION

CE offers high efficiency, selectivity, and low cost operation. CE instrumentation is schematically shown in Figure 2.2-A. The key component is the separation capillary filled with running buffer. The length and inner diameter of a separation capillary typically range from 30 to 100 cm and 10 to 100 µm respectively. Both ends of the capillary are submerged into buffer vials also containing platinum electrodes. The sample is introduced at the inlet site by applying a high voltage potential (electrokinetic injection) or by pressure (hydrodynamic injection). Migration through the capillary is driven by the applied field, and analytes are detected on-capillary as they pass a detection window at the capillary outlet. The high voltage power supply is capable of supplying up to 30 kV voltage across the capillary to assist sample injection and separation. Most commercial instruments use UV-visible absorbance detection. This is accomplished in-capillary by burning off a portion of the polyimide coating from the fused silica to form a detection window. The output of the detector is a plot of detector response versus time and is called an electropherogram (Fig. 2.2-B).

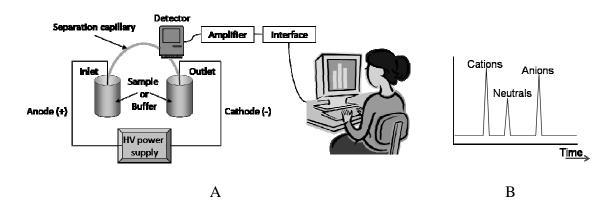


Figure 2.2. Schematic diagram of a CE instrument (A), and a representative CE electropherogram (B).

In CE the range of separated molecules is limited to those carrying a charge. The co-elution of neutral analytes in the CZE is one of the major limitations of electrophoresis. Steroid molecules are neutral and therefore require a pseudo-stationary phase to assist separation by means of secondary equilibria. Micellar electrokinetic chromatography is an extension of capillary electrophoresis which is used to separate neutral molecules (*11-17*).

#### 2.1.3. MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

*Micellar electrokinetic chromatography (MEKC)* for the separation of neutral compounds by adding surfactants such as *sodium dodecyl sulfate (SDS)* to the running buffer was first introduced in 1984 by Terabe and co-workers (*11*). Micellar electrokinetic chromatography combines the phenomenon of EOF with a form of partition chromatography to expand the range of separated molecules including neutral molecules. EOF drags all the neutral molecules through the capillary with essentially the same rate. Partition chromatography in its simplest form consists of a stationary phase and a mobile phase where the separation of neutral analytes can be achieved by introducing charged additives, such as surfactants, soluble polymers, crown ethers, cyclodextrins, antibodies, and proteins into the running buffer solution. The most popular additive is a surfactant.

The surfactant molecules are amphiphilic with both hydrophobic (e.g., alkyl moiety) and hydrophilic (polar or charged) groups, and they behave as solvated monomers at very low concentrations. When the concentration is increased beyond a minimum level, termed the *critical micelle concentration (CMC)*, these monomers spontaneously start to form micelles. A micelle is an ordered aggregate of surfactant monomers enclosing their

hydrophobic tails and exposing polar head groups toward the water or other polar solvents (Fig. 2.3) (18).

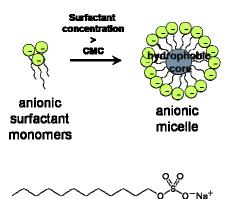


Figure 2.3. Micelle formation (top). Sodium dodecyl sulfate (SDS) monomer (bottom).

The micelles in MEKC are the equivalent of the stationary phase in HPLC and often called pseudostationary phase. Because micelles are not stationary, they are subject to both EOF and electrophoresis. Separation in MEKC is thus based on the different ability of the analyte molecules to partition between the solution and the inside of the micelles. In the presence of EOF, all the analyte molecules and the micelles will pass through the detector window because the negatively charged micelles are attracted to the anode, but the greater magnitude of the EOF sweeps them toward the detector at the cathode end of the capillary. In reality, solutes are distributed in MEKC between the pseudostationary and aqueous phase and a partition coefficient (P) is defined as:

$$P = \frac{C_{PS}}{C_s} \tag{2.4}$$

where,  $C_{PS}$  and  $C_s$  are the molar concentrations of a solute in the pseudostationary and aqueous phase respectively. Both, CMC and partition coefficient are temperature dependent. An increase in temperature causes an increase in CMC, hence a decrease in micellar concentration which consequently reduces the retention factor. On the other hand, higher temperatures lower the partition coefficient and also reduce the retention factor (19). It is well known that temperature increase reduces the viscosity of the running buffer which ultimately increases electrophoretic and EOF velocities and reduces the migration time. Therefore in order to maintain a reproducible MEKC separation temperature control is critical (20).

In all separation techniques, the primary goal is to achieve good resolution,  $R_s$ . In CE, the resolution can be calculated from an electropherogram using the following equation,

$$R_s = \frac{2\Delta t}{\omega_1 + \omega_2} \tag{2.5}$$

where  $\Delta t$  is the difference in migration time of two analytes, and  $\omega$  is the peak baseline width in time units. The resolution in MEKC can be improved by optimizing efficiency, selectivity, and retention factor. The latter is the easiest to control since in general, there is a linear correlation between an increasing concentration of the surfactant. However, a potential problem when using too concentrated surfactants is an increase of Joule heating due to an increase in the effective resistance, thus the dissipated power (V·I = I<sup>2</sup>·R) of the running buffer (21). Even with the narrowest capillaries, an efficient cooling is necessary to eliminate the excess heat generated by extremely high electric field. Another way of improving the resolution is to extend the elution window. In the separation of neutral analytes, all analytes elute between  $t_{EOF}$  and  $t_{micelle}$ . Hydrophobic analytes totally retained in micelles elute with them at  $t_{micelle}$ . On the other hand, very hydrophilic analytes do not interact at all with micelles and elute with EOF. Even though the elution window is often fairly small, the peak capacity can be very high because of the high efficiency. It is also easy to manipulate the selectivity by changing the physical nature of the micelle such as size, charge, and geometry and by using different surfactants. The use of surfactants may have dramatic affect on the EOF by interacting with the capillary wall. When EOF is suppressed only charged pseudostationary phase migrates through the aqueous phase.

Unfortunately, CE separations of native steroids cannot be accomplished with the use of only micellar hydrophobic pseudostationary phase because lipophilic steroidal compounds readily partition inside of SDS micelles in aqueous solutions and often comigrate. The separation of steroids by MEKC requires the secondary equilibrium by means of the introduction of another additive to the running buffer. One such additive is cyclodextrin which is used to control the high affinity of steroids to the micelles and thus the separation resolution and efficiency (22-24). The background on cyclodextrin chemistry is briefly discussed below.

#### 2.1.4. CHEMICAL AND PHYSICAL PROPERTIES OF CYCLODEXTRINS

Cyclodextrins are well-known in host-guest chemistry. Cyclodextrin (CD) was first mentioned in 1891 by Villiers; however, Schardinger gave the first detailed description of the preparation and isolation of this group of macrocyclic molecules and helped develop 45 the fundamentals of CD chemistry. Later in the 1930s, Pringsheim significantly advanced the progress of CD research when he discovered that organic compounds have a tendency to form complexes with crystalline CDs (25). The compounds are industrially synthesized from the degradation of starch mediated by the simple enzymatic conversion. CDs are nontoxic and can be widely used in food, medication, and cosmetics (26).

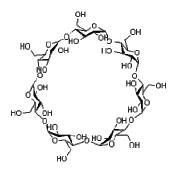


Figure 2.4. Chemical structure of a  $\beta$ -cyclodextrin molecule.

CD is a cyclic oligomer of  $\alpha$ -D-glucose. CD normally used in the analytical separations are  $\alpha$ -cyclodextrin (six glucose units);  $\beta$ -cyclodextrin (seven units), and  $\gamma$ -cyclodextrin (seven units). The CD molecule resembles a doughnut and is typically represented as a shallow truncated cone (Fig. 2.4). For a molecule with m glucose units, one cavity opening is contoured with m primary hydroxyl groups and has a reduced diameter compared to the other cavity opening contoured with 2m secondary hydroxyl groups. The interior of the cavity is decorated with a row of CH groups (C-3 carbons) then a row of glycosidic oxygens, and then a row of CH groups (C-5 carbons).

The hollow structure makes the cyclodextrin attractive subjects for chemical studies and applications. Whereas the interior of the cavity is non-polar, the exterior of the cyclodextrin is covered with hydroxyl groups making it soluble in water (27). At 25 °C, the least soluble is  $\beta$ -CD (1.85 g/100 mL), and most soluble is  $\gamma$ -CD (23.2 g/100 mL). These compounds are therefore studied as "hosts" for "guest" molecules capable of entering the cavity partially or completely and forming non-covalent host-guest inclusion complexes. Since cyclodextrins are neutral molecules, they move along with the EOF. In addition, they are inert and stable at a wide range of pH values, and have low UV absorptivity at short wavelengths. These properties make them very useful additives in chromatographic applications. Table 2.1 summarizes dimensions and physical properties of cyclodextrin molecules (28).

**Table 2.1.** Physical and chemical properties of CDs.

CD	# glucose units, m	MW, g/mol	Solubility in water, g/100 mL	A, Å	B, Å	C, Å
α	6	972	14.5	14.6	4.7 – 5.3	7.9
β	7	1135	1.85	15.4	6.0 - 6.5	7.9
γ	8	1297	23.2	17.5	7.5 – 8.3	7.9

To overcome the limited selectivity of MEKC the addition of cyclodextrins into the micellar phase is used to prevent the elution of highly hydrophobic analytes with micelles. This technique is referred to as CD modified MEKC.

## 2.1.5. Cyclodextrin (CD) Modified-MEKC

Due to the presence of a relatively hydrophobic cavity in CDs, they act as another pseudostationary phase and significantly contribute to the selectivity of the separation system. When a CD is added into the micellar system, the CDs and micelles compete to form inclusion complexes with the analyte. Because native CDs are neutral, the analytes having greater affinity to neutral CD migrate at the velocity of the EOF. The success of desired selectivity depends on the binding constant of the analyte with CD and the concentration of the CD, as the change in the concentration will shift the equilibrium toward to or away from the formation of the analyte-CD inclusion complex. Addition of a very high concentration of CD might, however, disturb the micelle formation (*29*).

The literature publications reported the MEKC analysis of sex steroid hormones are summarized in Chapter 1. Most works so far have, however, focused mainly on the optimization of the separation parameters and do not report required figures of merit, such as LODs, the reproducibility of the method nor its validation using biological samples. The analysis of steroids by cyclodextrin modified MEKC is the focus of this dissertation and will be discussed in details below in this chapter. The method is optimized to enable selective and rapid analysis of steroids. Two particular aspects are addressed: 1) how the affinity of targeted steroids either to the micelles or to CDs affects the migration order of the steroids in basic and acidic separation media, and 2) possible ways of manipulation and optimization of the steroid separation speed and resolution.

#### 2.2. EXPERIMENTAL

#### **2.2.1.** Chemicals and Materials

Sodium dodecyl sulfate (SDS), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), 2-[cyclo-hexylamino]-ethanesulfonic acid (CHES), n-decanophenone, 11ketotestosterone, and testosterone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxypropyl- $\beta$ -cyclodextrin (HPCD) was purchased from VWR (West Chester, PA, USA). Steroids, androstenedione, 17 $\beta$ -estradiol, estrone, 17-hydroxyprogesterone, and progesterone were purchased from Steraloids, Inc (Newport, RI, USA). 17 $\alpha$ -ethynyl estradiol was purchased from Cayman Chemical (Ann Arbor, MI, USA). Sodium phosphate monobasic salt was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methanol was purchased from EMD Chemicals (San Diego, CA, USA). Deionized water was purified using ELGA Purelab Ultra (Lowell, MA, USA).

Buffered solutions were prepared by weighing solid reagents, dissolving them in deionized water, and adjusting pH of the solution with aqueous solutions of 1N hydrochloric acid or 1N sodium hydroxide, as appropriate. Standard stock solutions of each sex steroid hormone were prepared by weighing out the solid steroid reagent and dissolving it in methanol. Before use, all the prepared steroid stock solutions were stored in a freezer at -20 °C and in the dark to avoid possible degradation. Stock samples were diluted to a desired concentration each day prior to use with the proper buffer. The CE background electrolyte and the samples to be analyzed were prepared fresh every day prior to use.

The presence of air bubbles should be eliminated as they can be injected into a capillary where they can cause large resistance during the electrophoresis leading to disruption or large fluctuations of the separation current during electrophoresis (from Ohm's law:  $V = I \cdot R$ , where V is the potential applied across the separation capillary in volts, R is the resistance in ohms, and I is the current in amperes). Micro-sized air bubbles can also be generated by Joule heating during electrophoresis, blocking the flow pathway and causing the current fail. In order to remove the dissolved air bubbles trapped in the solution the vials containing freshly made running buffers and samples were degassed using vacuum for 1 min for 2 mL vial or 15 sec for 250  $\mu$ L vial. Ultrasonication was also used for this purpose when necessary. All buffer solutions were filtered using 0.45  $\mu$ m porous filters before use to remove foreign particles which can clog the capillary or be injected and interfere with the separation analysis producing extra peaks in the electropherogram.

## 2.2.2. INSTRUMENTATION

CE measurements were carried out using a Beckman Coulter P/ACE MDQ capillary electrophoresis instrument (Beckman coulter, Fullerton, CA, USA) (Fig. 2.5). The instrument was equipped with a photodiode array UV-visible absorbance detection system (PDA) which provided simultaneous determination of steroids at multiple wavelengths in a single analysis or with photomultiplier tube (PMT) which allowed the detection of steroids at a specific wavelength.



Instrument

Inside

Capillary cartridge

**Figure 2.5.** Photographs of the Beckman® automated capillary electrophoresis machine used in the work. The light source is a UV-visible absorbance, with a PMT or PDA detector.

The electropherograms were collected at the wavelengths of UV-light where the analytes showed strong light absorption (200 nm for estrogens, 254 nm for androgens and progesterone, or 225 nm for all targeted steroids). All separations were performed using 26  $\mu$ m inner diameter (id), 360  $\mu$ m outer diameter (od), 30 cm long fused silica separation capillary (Polymicro Technologies, LLC, Phoenix, AZ, USA) with the detection window positioned 10.2 (short end separation) or 19.8 cm (long end separation) from the cathodic or anodic reservoir located at the sample inlet. The temperature of the capillary cartridge and sample storage trays inside the instrument was maintained at 25 ± 1 °C because the distribution coefficients are temperature sensitive.

## 2.2.3. MEKC AT PH 10 PROCEDURE

For this experiment, the running buffer containing 30 mM HPCD and 50 mM SDS dissolved in 50 mM CHES buffered at pH 10 was used. The steroid sample was prepared by adding aliquots of standard methanol stock solutions of six sex steroid hormones, estrone,  $17\beta$ -estradiol, estriol, testosterone,  $\alpha$ , $\beta$ -dihydroxyprogesterone and progesterone,

to the running buffer. The concentration of each steroid in the final dilution was 200  $\mu$ M. In order to avoid steroid precipitation the organic content was maintained at 20 % of methanol in the sample. However, the addition of small amounts of methanol increases the CMC of SDS. In fact, when the methanol content of the solution is 40% v/v or more the inverted micelles begin to form. In an inverted micelle, the polar head-groups of the surfactant monomers are centered in the interior while the lipophilic tails stand out into the nonpolar solvent (*30*). n-Decanophenone was used as the hydrophobic micelle marker. The sample was hydrodynamically injected into a silica capillary (effective length to the detection window  $L_w = 19.8$  cm) at 3.4 kPa (0.5 psi) for 4.0 sec. Separation was performed with a 16 kV potential drop across the capillary and normal polarity (cathode at the outlet), while being monitored at a detection wavelength of 225 nm where all steroids absorb light equally well and can be detected in a single run.

## 2.2.4. MEKC AT PH 2 PROCEDURE

The running buffer is comprised of 18 mM HPCD and 30 mM SDS dissolved in 10 mM phosphate buffered at pH 2.0. Standard solutions of steroids progesterone,  $17\alpha$ ,20β-dihydroxyprogesterone, testosterone, androstenedione, estrone,  $17\beta$ -estradiol, and estriol were prepared in the running buffer at the concentrations of 100 µM progesterone and  $17\alpha$ ,20β-dihydroxyprogesterone, 150 µM testosterone and  $17\beta$ -estradiol, 200 µM androstenedione, and 250 µM estrone with the addition of 20 % methanol. Steroid sample was injected into a bare fused silica capillary (effective L<sub>w</sub> = 10.2 cm) at 3.4 kPa (0.5 psi)

for 3.0 sec. CE was performed at 16 kV and reversed polarity (anode at the outlet), and the detection wavelength of 225 nm.

#### 2.3. RESULTS AND DISCUSSION

## **2.3.1.** Steroids Targeted for Analysis

The goal of the research presented in this dissertation chapter was to develop a rapid method to profile relative change in the steroid composition to better define physiological dysfunction following exposure to endocrine-disrupting chemicals. A simplified steroid metabolism pathway was used as a basis for the steroids targeted for analysis. The initial separation targets were progesterone, hydroxyprogesterone, 11-ketotestosterone, estrone, estradiol, and ethynyl estradiol. These analytes were selected because of their role in enabling or disrupting the chemical messaging in blood plasma samples. The presence of these steroidal compounds would therefore offer insight into the pathways involved in environmentally triggered endocrine disruption in fish. The molecular properties of the targeted steroids are summarized in Table 2.2.

Steroid	CAS #	CAS name	Log P	HA	HD	MW
17-α-ethynyl estradiol	57-63-6	(17α)-19-norpregna-1,3,5(10)-trien-20-yne- 3,17-diol	4.5	2	2	296.40
17β-estradiol	50-28-2	(17β)-estra-1,3,5(10)-triene-3,17-diol	4.1	2	2	272.38
progesterone	57-83-0	pregn-4-ene-3,20-dione	4.0	2	0	314.46
Estrone	53-16-7	3-hydroxy-estra-1,3,5(10)-trien-17-one	3.7	2	1	270.37
Testosterone	58-22-0	$(17\beta)$ -17-hydroxy-androst-4-en-3-one	3.5	2	1	288.42
Estriol	50-27-1	(16α,17β)- estra-1,3,5(10)-triene-3,16,17-triol	2.9	3	3	288.38
αα-dihydroxy Progesterone	652-69-7	(20S)-17,20-dihydroxy-pregn-4-en-3-one	2.9	3	2	332.48
αβ-dihydroxy Progesterone	1662-06-2	(20R)-17,20-dihydroxy-pregn-4-en-3-one	2.9	3	2	332.48
17-hydroxy- progesterone	68-96-2	17-hydroxy-pregn-4-ene-3,20-dione	2.9	3	1	330.46
Androstenedione	63-05-8	androst-4-ene-3,17-dione	2.9	2	0	286.41
11-keto Testosterone	564-35-2	(17β)-17-hydroxy-androst-4-ene-3,11-dione	1.7	3	1	302.41

Table 2.2. Molecular properties of targeted steroids <sup>a</sup>

Log P – logarithm of partition coefficient; HA – number of proton acceptors; HD – number of proton donors. <sup>a</sup>Values reported by *Scifinder*, version 2006; Chemical Abstract Services: Columbus, OH, 2006; CAS #, MW, log D, HA and HD calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (© 1994-2008 ACD/Labs) (accessed on March 18, 2009).

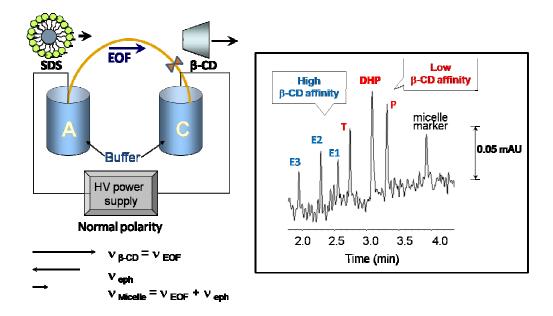
#### 2.3.2. MEKC SEPARATION OF STEROIDS IN AN ALKALINE MEDIUM

In the early phase of the method development, the separation of sex steroid hormones was performed in the basic medium by MEKC. The alkaline buffer consisted of CHES buffered at pH 10. SDS was used as the micelle forming agent due to SDS possesses high aqueous solubility, low critical micelle concentration (8 mM), low UV molar absorptivity, availability and small cost. HPCD was used as a cyclodextrin additive. Theoretically these separations are possible using any neutral  $\beta$ –CD; however, HPCD has higher aqueous solubility in contrast to an unsubstituted CD (*31*), and it was therefore used in this work. The molecular substitution of the HPCD was not controlled.

# 2.3.2.1. Separation Mechanism

EOF has a high velocity at pH 10. Neutral cyclodextrins migrate along with the EOF while SDS micelles migrate slower due to their anodic electrophoretic mobility. Neutral steroids are separated based on the difference in the distribution coefficients between a polar aqueous buffer and the non-polar micelle or unoccupied cyclodextrin cavities (*32*). Figure 2.6 illustrates a schematic diagram of the separation process as well as the acquired electropherogram for steroids separated via pH 10 method. All targeted steroids were separated in less than 3.5 minutes, substantially faster than standard immunoassay analysis. The elution order of the steroids in the basic medium corresponds to increasing affinity of the analytes to SDS (Fig. 2.6, right). Estriol,  $17\beta$ -estradiol, and estrone migrate first due to their preferential interaction with HPCDs while testosterone,

 $\alpha$ , $\beta$ -dihydroxyprogesterone, and progesterone migrate last due to their stronger partitioning into SDS micelles.



**Figure 2.6.** MEKC of steroids in alkaline medium. Schematic diagram of the separation process is on the right and the resulted electropherogram is on the left. E3 – estriol, E2 – 17 $\beta$ -estradiol, E1 – estrone, T – testosterone, DHP – 17 $\alpha$ ,20 $\beta$ -dihydroxyprogesterone, and P – progesterone, at 200  $\mu$ M each. Injection conditions: 0.5 psi, 4.0 sec, L<sub>w</sub> = 19.8 cm. Separation at 16 kV, normal polarity. Detection at  $\lambda$ =225 nm.

Separation of estriol,  $17\beta$ -estradiol, and estrone is due to the difference in their chemical structures, namely the presence of the hydrophilic hydroxyl and ketone groups attached to the ring D. According to the literature, hydrophilic hydroxyl groups remain exposed to the solvent even after forming a complex with cyclodextrin (28, 33). The aromatic hydroxyl group is an exception to the rule because it can penetrate deeply into the cyclodextrin cavity where it forms hydrogen-bonds to one of hydroxyl groups in cyclodextrin molecule (34). The host-guest stoichiometry is reported to be 1:1 (35, 36). The pH 10 separation data shows that estriol has the highest association with cyclodextrin

followed by  $17\beta$ -estraiol and estrone at the experimental conditions.

# 2.3.2.2. Figures of Merit

Calibration curves for the separation of sex steroid hormones using MEKC were determined from nine calibration curves measured 3 per day for 3 days. The linear range and correlation coefficients obtained for the pH 10 separation system are summarized in Table 2.3 below. The curves were measured separately for each analyte. Detection was performed by UV-visible absorbance at detection wavelength of 200 nm for estrone,  $17\beta$ -esradiol, and estriol, or 254 nm for androstenedione, dihydroxyprogesterone, and progesterone.

 E1
 E2
 E3
 AD
  $\alpha,\beta$ -DHP

 Linear range, μM
 (×10<sup>2</sup>)
 0.50-4.0
 0.50-16
 0.50-16
 0.50-16
 0.30-1.0

Table 2.3. Linear range of steroid detection via pH 10 MEKC separation.

0.993

 $\mathbf{R}^2$ 

# 2.3.3. MEKC Separation of Steroids in an Acidic Medium

0.999

If the pH of the running buffer is less than three, the velocity of the EOF approaches zero and thus does not assist the separation of the analytes. In this experiment, the separation of steroids under acidic conditions was performed at reversed polarity (anode at the outlet) because under suppressed EOF neither neutral cyclodextrins, which have the velocity of EOF, nor SDS micelles attracted to the anode would reach the detection

0.999

0.991

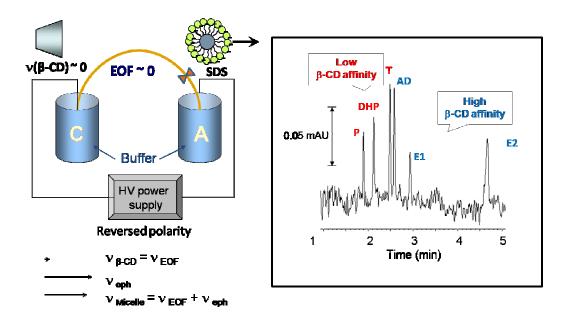
Р

0.50-16

1.00

1.00

window if the normal polarity (cathode is at the outlet) was used. Phosphate buffered at pH 2.0 containing SDS and CD was used as a background electrolyte. At the reversed polarity experimental conditions the SDS micelles carrying steroid molecules migrate faster than the EOF (Fig. 2.7, left). As a result, the steroids with higher affinity to the micelles will migrate earlier followed by the steroids with higher affinity to the cyclodextrins.



**Figure 2.7.** Electropherograms measured for steroids under acidic separation conditions. P – progesterone (100  $\mu$ M), DHP – 17 $\alpha$ , 20 $\beta$ -dihydroxyprogesterone (100  $\mu$ M), T – testosterone (150  $\mu$ M), AD – androstenedione (200  $\mu$ M), E1 – estrone (250  $\mu$ M), E2 – 17 $\beta$ -estradiol (150  $\mu$ M). Injection conditions: 0.5 psi, 3.0 sec. L<sub>w</sub> = 10.2 cm. Separation at 16 kV, reversed polarity. Detection at:  $\lambda$ =225 nm.

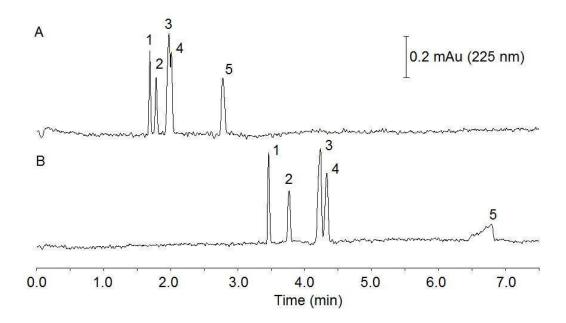
The resulting electropherogram is shown in Figure 2.7 (right). All the steroids, except for estriol, were separated in less than 5 minutes. At pH 2.0, estriol (not shown) migrates very slowly because of its strong affinity to the cyclodextrin. As a result, estriol peak is very broad, yielding small signal-to-noise ratio and unsuitable for chromatographic

quantitation. However, by manipulating the SDS to HPCD ratio or the separation voltage it is possible to force estriol to migrate faster as a sharp peak at the expense of lowering the resolution for early migrating steroids. The effect of the capillary length to the detection window and SDS to HPCD ratio on the separation time, resolution and selectivity is discussed below.

#### 2.3.4. EVALUATION OF MEKC OF STEROIDS IN STRONGLY ACIDIC MEDIUM (PH 2)

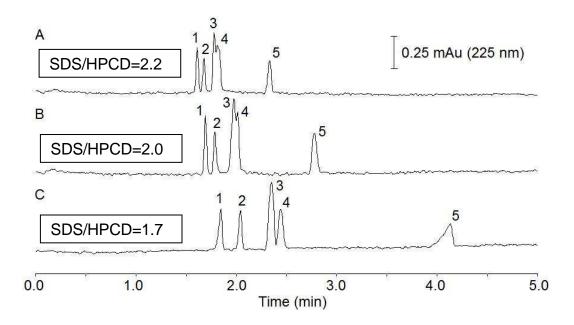
Separation in highly acidic media can be complicated since the analytes move through the capillary much slower under suppressed EOF. However, the separation time can be decreased by increasing the separation voltage. It can also be decreased by decreasing the length of the capillary, albeit at the expense of the resolution. Additionally, an increased capillary length leads to a decrease of the electric field strength at constant voltage (E = V/L, V – applied voltage, L – capillary length) and higher voltages have to be used for the separation. In CE, it is common to start a method development using a shorter capillary (typically 20 - 50 cm to the detection window) and then change the length of the capillary until the best resolution for all the analytes is achieved. The length of the separation capillary used for both types of CE analysis was 30 cm. In pH 10 method the detection window was positioned 19.8 cm from the injection point while the other end of the capillary was positioned 10.2 cm from the detection window. This method is referred to as the long end separation. In pH 2 method the effective length of the capillary was 10.2 cm with the detection window positioned closer to the inlet. This is the case of the short end separation.

Figure 2.8 shows the separation of steroids injected at short and long ends of the separation capillary. Even though the resolution improves, the detection signal decreases for slower migrating analytes such as ethynyl estradiol due to band broadening effects caused by longitudinal diffusion. Longitudinal diffusion is the diffusion of the solute molecules from the center of the analyte zone to the more dilute regions in front of and behind the zone. On the other hand it is evident that a short end separation under acidic conditions results in at least 60 % faster separation comparing to the long end separation and the separation efficiency (the narrow shape of the peak) for ethynyl estradiol improved dramatically which was predicted by the theory; however, the resolution suffered in the short end separation leading to the overlapping peaks for testosterone and androstenedione (Fig. 2.8-A).



**Figure 2.8.** Acidic MEKC of steroids injected hydrodynamically (0.5 psi, 4 sec) at short end (A) and long end (B). 350  $\mu$ M progesterone (1); 250  $\mu$ M hydroxyprogesterone (2); 500  $\mu$ M testosterone (3); 400  $\mu$ M androstenedione (4); 300  $\mu$ M ethynyl estradiol (5). BGE: 40 mM SDS, 20 mM HPCD, 20 % methanol in 10 mM phosphate buffered at pH 3.1. Separation: 16 kV and normal polarity.

The poorer resolution of testosterone and androstenedione peaks in the short-end analysis can be improved by changing the SDS/HPCD ratio of the running buffer as illustrated in Figure 2.9. As discussed earlier, the steroids with a higher affinity to SDS micelles migrate first under acidic conditions and reversed polarity. The migration order of steroids under acidic conditions (Fig.2.8-B) demonstrates that testosterone has higher affinity to SDS comparing to androstenedione which preferentially forms inclusion complexes with CD. If the ratio of SDS to HPCD is high the equilibrium will be shifted toward the inclusion of the steroids inside of SDS micelle cores and therefore steroids will spend more time travelling with the micelles thus migrating faster and producing worse resolution (Fig. 2.9-A). By increasing the amount of HPCD in the separation buffer the equilibrium is shifted toward the formation of the inclusion complexes of steroids with cyclodextrin cavities. Therefore, if the amount of HPCD in running buffer is increased androstenedione can be separated from testosterone. The representative electropherograms shown in Figure 2.9 were obtained using three running buffers of different ratios of SDS/HPCD: 2.2 (40 mM/18 mM), 2.0 (40 mM/20 mM), and 1.7 (30 mM /18 mM).



**Figure 2.9.** Short end separation of steroids in acidic MEKC. SDS/ HPCD is 2.2 (A), 2.0 (B), 1.7 (C). Sample injected hydrodynamically at 0.5 psi, 4 sec. 350  $\mu$ M progesterone (1); 250  $\mu$ M hydroxyprogesterone (2); 500  $\mu$ M testosterone (3); 400  $\mu$ M androstenedione (4); 300  $\mu$ M ethynyl estradiol (5). BGE is SDS/HPCD, 20% methanol in 10 mM phosphate buffered at pH 3.1. Separation at 16 kV and normal polarity.

The electropherogram in Figure 2.9-C demonstrates baseline resolution for all peaks obtained using SDS/HPCD = 1.7 although ethynyl estradiol peak is too broad if SDS/HPCD  $\leq$  1.7. The analysis time however is 40 % faster for the short end comparing to the long end separation and is beneficial for the fast screening of multiple steroid samples.

## **CONCLUSIONS**

The separation of selected steroid hormones by conventional MEKC has been conducted under both basic and acidic conditions. The study has shown that the migration order of steroids depends on their affinity to the hydrophobic cavities of SDS micelle or cyclodextrin molecule and can be manipulated by changing the pH of the running buffer and the polarity of the potential applied across the separation capillary. The resolution of the steroid peaks in the electropherogram depends on the ratio of SDS to cyclodextrin in the running buffer and therefore can be controlled. The electropherograms measured at basic conditions demonstrated the baseline separation of steroids in less than 3.5 min. The separation in acidic medium under suppressed EOF was achieved in less that 5 min. The latter was obtained using reversed polarity and a short end separation with the effective length of the capillary being 10.2 cm from the injection point to the detection window. Although the acidic method yields baseline resolved peaks and fast separation of steroids, the limits of detection for steroid analysis must still be compatible with the low levels of endogenous steroids in plasma. The development of a preconcentration technique for improving the steroid detection limits by MEKC-UV-visible absorbance detection is the subject of the next chapter.

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# <u>CHAPTER 3</u>

# STEROID PRECONCENTRATION

# **3.1. INTRODUCTION**

While the MEKC separation described earlier is fast and consumes low sample and reagent volumes, it has poor concentration limits of detection if measured by UV– visible absorption due to limited sample loading capacity and small path length for light absorbance. In CE, the inner diameter of the separation capillary has to be smaller than 100  $\mu$ m to minimize the solute dispersion caused by Joule heating and to obtain the high separation efficiency (*1*). A detection window in a silica capillary is prepared by burning off polyimide coating from a segment of the capillary. Due to a small diameter of the capillary, the path length for light absorbance is small (from Beer's law: A= $\epsilon$ bC, where  $\epsilon$  is the molar extinction coefficient of the sample, C is the sample molar concentration, and b is light path that equals to the capillary diameter) and therefore the detection limits are frequently poor and in the range of 10<sup>-5</sup> – 10<sup>-7</sup> M (2).

The capillary dimensions lead to injection volumes of a few nanoliters. If the injection volume is much larger than 1 % of the total length of the capillary, sample overloading occurs and the peaks broaden (3). Without improved detection limits CE cannot be used in bioanalytical studies such as analysis of very low levels of steroids in biological fluids. The approaches for the enhancement of the detection limits in CE include the use of laser-induced fluorescence (4, 5), electrochemical detection (6, 7), and mass spectrometry (8) as they allow the low detection limits comparing to UV-visible absorbance; chemical derivatization (9); extended length cells in UV-visible absorbance detectors (z-cells, bubble cells) (10, 11) and sample enrichment by means of the liquid-liquid extraction (12), selective membrane extraction (13), and solid phase extraction (14),

which are carried out before or after the capillary electrophoresis analysis. However, these methods are not directly applicable to the goal of this dissertation. For example, steroid molecules are neutral and require the use of MEKC separation which is not directly compatible with a mass spectrometer detector due to the non-volatile surfactant molecules that contaminate the ionization chamber, reducing the ionization efficiency and deteriorating the limits of detection (*15*). Steroids also lack of chromophores, required for LIF analysis, and would therefore need to be chemically modified.

An in-capillary sample preconcentration method can be a good alternative for steroid preconcentration without the use of expensive detectors and other modification of the instrument or time-consuming derivatization sample pretreatment. The current incapillary sample preconcentration techniques are based on electrokinetic injection of large sample volumes. These techniques combine the sample injection and separation in a single capillary and are based on the changes in the electrophoretic velocity of the analytes at the boundary between the sample and the running buffer zones. This allows narrowing the analyte bandwidth and increasing the amount of the sample that can be loaded into capillary leading to a better detection.

A series of preconcentration techniques used in combination with CE have been developed and include: field amplified sample stacking, sweeping, isotachophoresis, pH-stacking, or chromatographic preconcentration (16, 17). Field amplified sample stacking involves ions electrophoretically migrating through a low-conductivity buffer solution into a high conductivity buffer solution. The migrating ions slow down dramatically at the boundary of the two buffers and preconcentrate (18, 19). Isotachophoresis is performed with a discontinuous buffer in which the sample zone is between the background 69

electrolyte of higher (leading electrolyte) and lower (terminal electrolyte) electrophoretic mobilities (20). The ionic species are preconcentrated according to the difference in mobility by applying an electric field. The preconcentration by pH-stacking occurs at the boundary of two buffer solutions of high and low pH (21). Sweeping is based on the ability of non-polar analyte molecules to partition into a pseudo-stationary phase (22). Finally, chromatographic preconcentration uses silica beads modified by immobilized ligand which captures and preconcentrates a target analyte in-capillary (23).

The present chapter discusses the design and performance of two preconcentration techniques that were developed for steroid analysis: (1) a chromatographic preconcentration by means of a modified silica cartridge integrated in-capillary, and (2) a pH-stacking using functionalized cyclodextrins. Both methods can improve the selectivity and concentration limits of detection for steroid analysis and enable efficient steroid separation analysis in complex mixtures such as blood plasma. Investigation of the influence of several parameters that are used to obtain more efficient and more reproducible chromatographic separations is also discussed.

# **3.2. EXPERIMENTAL**

### 3.2.1. CHEMICALS AND MATERIALS

Carboxymethyl-β-CD, SDS, 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), 11-ketotestosterone, and testosterone were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPCD was purchased from VWR (West Chester, PA, USA). Steroids, 17βestradiol, estrone, 17-hydroxyprogesterone, and progesterone were purchased from Steraloids, Inc (Newport, RI, USA). 17α-ethynyl estradiol was purchased from Cayman Chemical (Ann Arbor, MI, USA). Sodium phosphate monobasic salt was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methanol was purchased from EMD Chemicals (San Diego, CA, USA). Deionized water was purified using ELGA Purelab Ultra (Lowell, MA, USA). Buffered solutions and standard stock solution of each sex steroid hormone were prepared as described in Section 2.2.1. The running buffer and the samples to be analyzed were prepared fresh every day prior to use.

## **3.2.2.** INSTRUMENTATION

All CE measurements were carried out as described in Section 2.2.2. Briefly, the electropherograms were collected at the wavelengths of UV-light where the analytes showed strong absorption (200 nm for estrogens and 254 nm for androgens and progesterone). All the separations were performed using 25  $\mu$ m id, 360  $\mu$ m od, 30 cm long fused silica separation capillary (Polymicro Technologies, LLC, Phoenix, AZ, USA) with the detection window positioned 10.2 cm from the cathodic reservoir located at the sample inlet. The capillary cartridge and sample trays inside the instrument were maintained at 25  $\pm$  1 °C while the trays holding the vials filled with the separation buffer were maintained at the room temperature which varied from 20 to 24 °C. All vials containing running buffers and samples were treated before analysis as described in Section 2.2.2.

# 3.2.3. CAPILLARY PACKING PROCEDURE

This type of preconcentration design uses silica beads with an immobilized ligand as a preconcentration plug held inside of the separation capillary by a porous frit. The 71

stationary phase captures and preconcentrates the targeted analytes. Silica beads modified with hydrophobic alkyl groups are widely used in reversed phase liquid chromatography as a packing material for chromatography columns. This material is ideal for strong retention of hydrophobic compounds, including steroids, and therefore was utilized in this project.

A pressure packing technique called slurry packing was used in-house to fabricate a capillary with the built-in stationary phase preconcentration cartridge similar to a previous work by Hoyt and colleagues (24). The polyimide coating was first removed from one end of a bare fused silica capillary (26 µm id) using a heating element. To make a temporary frit a small amount of silica was then introduced into this end of the capillary by gently tapping the end of the capillary into a vial containing the silica beads. The temporary frit was formed with a heating element. Slurry comprised of stable bond chromatography packing material Zorbax SB-C8 with the particle size of 5 micron (Agilent Technologies Inc., Santa Clara, CA, USA) and isopropyl alcohol was placed into a closed pressurized stainless steel container with two connectors. One of the connectors was attached to a high pressure LC system pump while the other one was connected to the fritless end of the capillary. After a high pressure had been applied to the capillary, the slurry was introduced into the capillary where it was held by the temporary frit while only the liquid component passed through. The packed capillary was then successively flushed with methanol and water at  $14 \times 10^3$  kPa ( $2 \times 10^3$  psi). While the capillary was flushed with water the stationary phase was gently sintered using commercially available Innova capillary fitting device. This procedure resulted in the formation of another frit, which held the stationary phase in place. The temporary frit with the excess of the silica phase was then removed by trimming the cartridge to  $\sim 1$  mm length and was used for the further CE experiment.

#### 3.2.4. CHROMATOGRAPHIC PRECONCENTRATION MEKC PROCEDURE

Prior to each analysis, the capillary with the chromatographic cartridge was sequentially flushed with acetonitrile: water (50: 50, v/v), water, 0.1 N hydrochloric acid, and the separation buffer which was 10 mM SDS in 10 mM phosphate buffered at pH 2.0. Acidic conditions were preferred due to the stability of the stationary phase at pH < 6 (25). Standard solution of estradiol in methanol was diluted in the mixture of methanol: separation buffer (20: 80, v/v) to 60  $\mu$ M concentration and injected at 69 kPa (10 psi) for 15 sec onto the activated preconcentrator. The retained steroid molecules were eluted from the stationary phase by flushing it with acetonitrile:10 mM phosphate buffered at pH 2.0 (70:30, v/v) at 17 kPa (2.5 psi) for 1 min and then with the separation buffer only at 6.9 kPa (1.0 psi) for 1 min. After elution the sample was analyzed in-capillary by MEKC at 16 kV, reversed polarity, and the detection wavelength of 200 nm.

#### **3.2.5. PH-STACKING MEKC PROCEDURE**

# 3.2.5.1. The Role of Cyclodextrins

Several books (26, 27) and a number of articles (28-31) are dedicated to CD hostguest chemistry. An important characteristic of CDs making them useful is their unique ability to solubilize lipophilic molecules in their molecular cavities forming inclusion complexes. This is accomplished without formation of chemical bonds and changing the guest molecules structure. The selectivity of this interaction is governed by the size, geometry, and physicochemical properties of both analyte and CD. Intramolecular interactions occur as the secondary hydroxyl groups at C2 and C3 (see Figure 2.4) can form hydrogen bonds. This restricts the free movement of their relative positions, maintaining the freedom of motion of the hydroxyl groups (27, 32). The cavity of the cyclodextrin is moderately hydrophobic, making it amenable to a wide variety of organic guests. Van der Waals and hydrophobic interactions are believed to be the main factors involved in complexation (33-36). However, steric effects and hydrogen bonding play a role as well (37, 38). The size of the cavity and the CD ring structure is very important. A tight spatial fit of the guest is necessary to maximize the interaction energy due to noncovalent bonding or allow complexation. Selectivity is determined by the size, structure, and physiochemical properties of both the guest and CD. In chromatographic applications, the formation of an inclusion complex is beneficial for nonpolar analytes increasing their solubility in water. Many groups can be introduced into the CD structure to increase the solubility of CDs (39). In this dissertation neutral and charged CD derivatives are used for the preconcentration and separation of steroid hormones.

Carboxymethyl- $\beta$ -CD was chosen as a preconcentration agent. This compound is commercially available and readily soluble in common CE running buffers. Sulfobutylether-CD is a popular anionic CD and has high aqueous solubility. However, it is relatively unaffected by pH due to its pK<sub>a</sub> being too low for stacking at pH 2.5 (40). Additionally, this material is more expensive and multiple labeled sulfobutylether- $\beta$ -CD can lead to peak broadening since the electrophoretic mobility changes with the degree of substitution. CMCD molecules form inclusion complexes with steroids in aqueous solutions (41, 42). Kano and co-workers (43) reported an apparent pK<sub>a</sub> of ~ 5 for 2,3-di-*O*-carboxymethyl- $\beta$ -CD (25 °C, 0.1 M NaCl). The pK<sub>a</sub> of CMCD is suitable for stacking a sample prepared in a basic medium and injected in a capillary filled with acidic running electrolyte. In the basic solution carboxylic groups surrounding CMCD molecule will become deprotonated thus making the entire molecule be negatively charged which allows electrokinetic injection into the separation capillary. Alternatively, while in the solution with low pH the carboxylic groups will be protonated back producing neutral CMCD molecule (Figure 3.1). Some physical and chemical properties of CDs used in the research are summarized in Table 3.1.

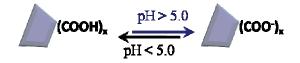


Figure 3.1. Dissociation of CMCD molecule depends on pH of the solution.

CD	Solubility in water, g/100 mL	MW <sub>AVE</sub>	-R
β-CD	1.8	1135	-H
Carboxymethyl-β-CD	5.0	1541	-CH <sub>2</sub> COOH
Hydroxypropyl-β-CD	33	1483	-CH <sub>2</sub> CH(CH <sub>3</sub> )OH

# 3.2.5.2. Stacking Mechanism

Steroid standards prepared in methanol were diluted to desired concentrations with an aqueous solution comprised of 1 mM CMCD in 50 mM CAPS buffered at pH 10. The concentration of methanol in the final sample was 5 % to aid the dissolution of a hydrophobic steroid. The running buffer comprised of 30 mM SDS, 13 mM HPCD in 200 mM phosphate buffered to pH 2.5. No methanol was added to the running buffer.

The capillary was first filled with the running buffer followed by the injection of a deionized water plug at 13.8 kPa (2 psi) for 6 sec. The steroid sample was then injected electrokinetically upon application of 10 kV high voltage at reversed polarity for 60 sec. The neutral steroid molecules carried by the CMCDs which are fully deprotonated (negatively charged) at high pH were driven into the separation capillary. Once in contact with the acidic running buffer, the neutral steroid-CD complex concentrated in a narrow zone at the pH junction. The negatively charged SDS micelles migrated as a function of electrophoretic mobility towards the anode, which was located past the detection window. The velocity of the neutral HPCD was insignificant as the EOF was suppressed (see Fig. 3.2-A). Following stacking, the reverse polarity run at 16 kV was started and the separation was initiated by replacing the sample vial with a cathodic reservoir containing 30 mM SDS, 13 mM HPCD in 200 mM phosphate buffered at pH 2.5. (Figure 3.2). The steroids partitioned or associated competitively with the SDS and CDs and were separated as illustrated in Figure 3.2-B,C,D.

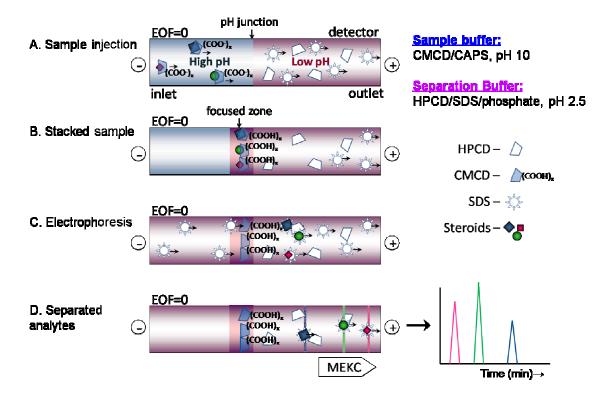


Figure 3.2. Schematic representation of pH-stacking using functionalized cyclodextrin.

# 3.2.5.3. Capillary Flushes

A special preconditioning and washing sequence was performed on each capillary to ensure more reproducible separation conditions. At the beginning of each day the capillary was conditioned with several timed flushes at 172 kPa (25 psi). The sequence of these flushes was 5 min methanol, 5 min water, 25 min 0.1 N hydrochloric acid, 5 min water, 25 min running electrolyte comprised of 30 mM SDS 13 mM HPCD, 200 mM phosphate buffered at pH 2.5. In between runs the capillary was flushed at 172 kPa (25 psi) for 2 min with the running electrolyte. The methanol was used to rinse the capillary interior after steroid stacking analysis. To suppress the ionization of the silica surface silanol groups and as a result the EOF 0.1 N hydrochloric acid was used. The water was used to remove any excess of the Cl ions and finally the capillary was filled with the running electrolyte used for the separation.

### **3.3. RESULTS AND DISCUSSION**

## 3.3.1. CHROMATOGRAPHIC PRECONCENTRATION OF STEROIDS

The chromatographic preconcentration by means of reversed-phase silica gel beads forming an in-capillary preconcentration plug was investigated and demonstrated good concentration enhancement despite several procedural limitations. In the following experiment the acidic separation buffer was used and the steroid separation was optimized at acidic conditions under suppressed EOF due to the preferential use of silica packing material stable at low pH for the fabrication of the preconcentration cartridge. The preconcentrator used in this study is schematically illustrated in Figure 3.3.

Figure 3.4 shows electropherograms obtained for the MEKC separation of the 60  $\mu$ M 17 $\beta$ -estradiol with and without the in-capillary chromatographic preconcentration step. The represented data confirms that the use of the solid phase preconcentrator produces higher estradiol peak while keeping its width small. The injection of the same sample at the same conditions without the preconcentrator results in a broad and short sample peak, significantly degrading the separation efficiency. The injected sample volume using the plug was approximately 75 times larger comparing to the volume of the sample injected using conventional MEKC under acidic conditions. This number was estimated by comparing the injection time and pressure used for acidic MEKC and chromatographic preconcentration MEKC in this work. However, while conducting this study it was noticed

that after just a few days of use the preconcentrator plug could be easily pushed out of its place and out of the separation capillary with the applied pressure. The biggest technical issue of this technique was the sensitivity of the preconcentrator to the storage conditions and incompatibility with the solvents used. Because of these technical difficulties, pH-stacking was tested as an alternative preconcentration method which could be integrated into a CE instrument without interfering with the automation (44-46).

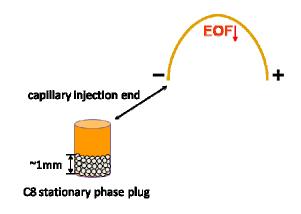


Figure 3.3. Schematic representation of the stationary phase preconcentration cartridge

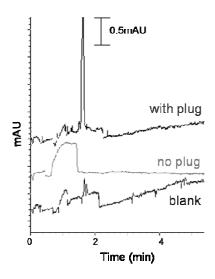


Figure 3.4. Electropherograms of a blank sample and  $60 \mu M 17\beta$ -estradiol with and without chromatographic preconcentration.

# 3.3.2. PH - STACKING OF STEROIDS

Stacking can be used for charged and neutral compounds, such as steroid hormones. The effective mobility of weak acids and bases in the capillary is a function of pH. This mode of stacking utilizes a pH junction formed at the boundary of two buffer zones, when the buffer solutions of low and high pH values are introduced into a separation capillary. The analyte preconcentration at the pH junction is referred to as pH-stacking (Fig. 3.5). The efficiency of pH-stacking depends on the pH of the solutions, the concentration of the sample matrix, as well as the  $pK_a$  of the stacked molecule.

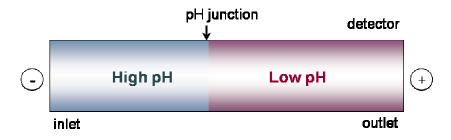


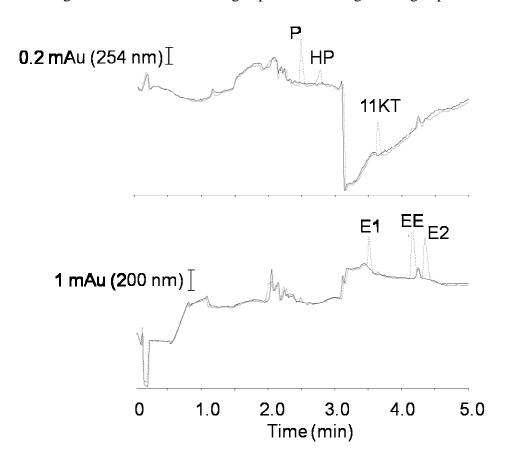
Figure 3.5. Formation of pH-junction at the interface of two, basic and acidic, buffer solutions.

Preconcentration by pH-stacking can be carried out using modified cyclodextrins. Only a few papers reported stacking preconcentration prior to MEKC separation of neutral steroids. Quirino and colleagues (47) demonstrated up to a 280-fold stacking enhancement for steroid determination using on-line sample preconcentration that was accomplished by using stacking and sweeping under pH-suppressed EOF in the presence of sulfated  $\beta$ -cyclodextrin. Urban and co-workers (48) studied the separation of three anabolic steroids (methyltestosterone, methandrostenolone and testosterone) using charged CD derivatives and online reverse migrating sample stacking with large-volume injection followed by

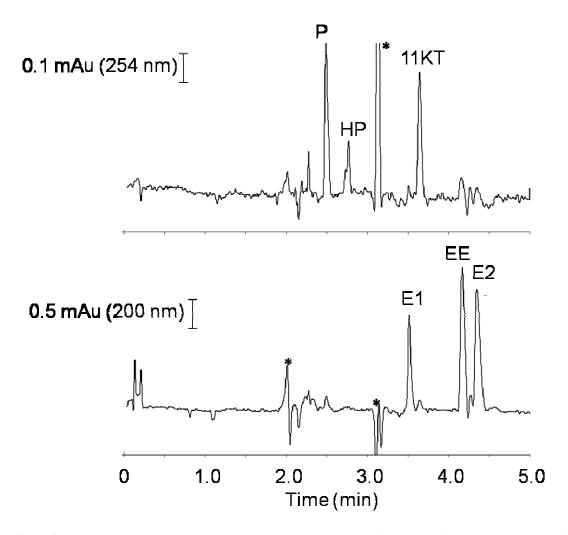
MEKC with UV-visible absorption. The detection limits obtained for these compounds were approximately  $2.8 \times 10^{-7} - 5 \times 10^{-5}$  M while the sensitivity enhancement factors were in the range from 100 - 190. In another report by Munro and co-workers (40) sweeping afforded detection of approximately 430 nM estrone and estradiol (S/N 55) using a 99 s hydrodynamic injection of steroids in sulfated  $\beta$ -CD followed by separation using an aqueous separation buffer comprising borate, phosphate, cholate, and acetonitrile. Britz-McKibbin and colleagues (49) demonstrated over a 100-fold enhancement using a dynamic pH junction-sweeping preconcentration with borate and  $\gamma$ -CD. In the present work, pH-mediated stacking of anionic steroid-CD complexes followed by MEKC provides at least a 500-fold enhancement in detection limit over hydrodynamic steroids. The separation targets introduction of native were progesterone, hydroxyprogesterone, 11-ketotestosterone, estrone, estradiol, and ethynyl estradiol. These analytes vary in CD affinity and therefore served as a model sample set to demonstrate the possibility of quantitative analysis following sample stacking by means of CD inclusion for molecules with different molecular characteristics.

The selectivity toward the targeted steroids requires a proper balance between the SDS and CD. Under reversed polarity, the androgens and progesterone used in this study migrate faster than estrogens as they have higher affinity to SDS micelles. Changes in the CD concentration affected the separation time and resolution. For example, using 30 mM SDS and 18 mM HPCD, progesterone, hydroxyprogesterone, and 11-ketotestosterone were baseline resolved in less than 5 minutes, but the migration times of the remaining estrogens increased and the peaks broadened substantially. Running electrolyte comprised of 30 mM

SDS and 10 mM HPCD degraded the resolution of progesterone, hydroxyprogesterone, and 11-ketotestosterone, but resulted in the baseline resolution of estrone, estradiol, ethynyl estradiol in under 5 minutes. The electropherogram shown in Figure 3.6 was obtained using running buffer comprised of 30 mM SDS and 13 mM HPCD. The run was finished in less than 5 minutes. Sample stacking with the procedure described in Section 3.2.5 was not affected by varying degree of carboxymethyl substitution, as there was no change in the overall charge for CD under the loading at pH 10 no during stacking at pH 2.5.



**Figure 3.6** displays superimposed electropherograms resulting from stacking and separating the blank (solid) and sample containing 6 steroids (dot). Data was collected at 254 nm and 200 nm. The analytes are labeled as follows: **P** – progesterone, **HP** – 17-hydroxyprogesterone, **E1** – estrone, **11KT** – 11-ketotestosterone, **EE** – 17 $\alpha$ -ethynyl estradiol, **E2** – 17 $\beta$ -estradiol. Separation conditions are summarized in text. Reproduced with permission from (*58*).



**Figure 3.7** displays the background subtracted electropherograms of 6 steroids following analyte stacking. The electropherograms were collected simultaneously using PDA detection. The analytes are labeled as follows: **P** – progesterone, **HP** – 17-hydroxyprogesterone, **E1** – estrone, **11KT** – 11-ketotestosterone, **HP** – 17 $\alpha$ -ethynyl estradiol, **E2** – 17 $\beta$ -estradiol. Peaks labeled \* are an artifact resulting from the background subtraction. Separation conditions are outlined in text. Reproduced with permission from (*58*).

## 3.3.2.1. STACKING EFFECTED BY CONDUCTIVITY OF THE WATER PLUG

A CE or MEKC analysis comprises three steps: (1) flushing the capillary with the separation buffer of a certain pH; (2) the sample injection, and (3) the electrophoresis of the analytes. In CE the electric potential applied across the separation capillary causes the ions in the solution to migrate generating the electric current flow. The ability of an aqueous solution to conduct the electric current depends on the concentration of the salts dissolved in water and is called a conductivity. Highly concentrated solutions are more conductive than those with the lower contents of free ions and therefore less resistant. Conductivity of very dilute solutions can be described by Kohlraush law (*50*) which sums the conductivity contributions of all ions in the solution:

$$\Lambda = \rho \cdot \Sigma(\lambda_i \cdot C_i) \tag{3.1}$$

where  $\Lambda$  is conductivity,  $\rho$  is density of water,  $\lambda_i$  and  $c_i$  are equivalent ionic conductance and concentration of an ion '*i*' respectively. On the other hand, the conductivity of a solution is inversely proportional to the electric filed (Equation 3.2) and to resistivity (Equation 3.3):

$$J = \boldsymbol{\sigma} \cdot \boldsymbol{E} \tag{3.2}$$

where J is the current density and E is the electric field strength.

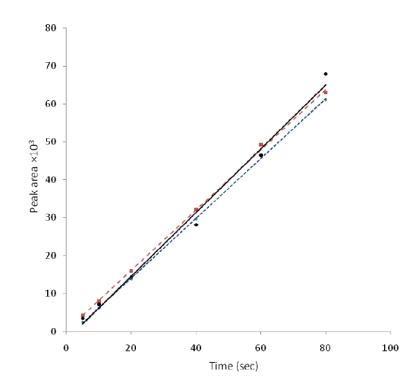
$$\sigma = \frac{1}{\rho} \tag{3.3}$$

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In order to accomplish better stacking in the present method, a water plug was introduced into the separation capillary immediately after filling it with the phosphate buffer and right before the sample injection. As a result, the low conductivity should provide an ion-vacant region to concentrate negative ions deeper into the capillary away from the injection point as well as ensure proper enhancement of the electric field at the injection point. The higher electric field, in turn, increases the electrophoretic velocity of the anionic CMCD molecules (Equation 2.1) which carry the neutral steroid analytes injected electrokinetically into the capillary water zone toward the pH-junction preconcentration boundary.

In this experiment, in the beginning of each CE run the capillary was rinsed at 172 kPa (25 psi) for 2 min with the running buffer. Then a water plug was hydrodynamically injected by 14kPa (2 psi) pressure for 13.5 sec, 6 sec, and 0 sec right before the sample injection performed for durations of time: 5, 10, 20, 40, 60, and 80 sec.

According to Fig. 3.8, stacking was not affected by the conductivity of the water plug in contrast to previous works (51, 52). In fact, the effectiveness of the sample stacking did not depend on the presence or the absence of the water plug in the capillary. However, to provide a small ion-free region for the stacked sample a water plug injected for 6 sec was used in the further studies. The length of the water plug injected for 6 sec was measured to be 5.5 mm or 5.4 % of the effective capillary length. This measurement was conducted by injecting the water at 2 psi into the capillary containing the running buffer and measuring the migration time of the water plug to the detection window.



**Figure 3.8.** The effect of water plug on sample stacking. Plot of the peak area versus sample injection time. Sample was injected electrokinetically at 8 kV and was 400  $\mu$ M ethynyl estradiol in 10 mM CMCD, 5% methanol, 50 mM CAPS buffered at pH 10. BGE was 30 mM SDS, 10 mM HPCD in 50mM phosphate buffered at pH 2.5. Separation: at 16kV and normal polarity. Water plug injection: 2 psi 13.5 sec (....), 2 psi 6 sec (--), no plug (--)

#### 3.3.2.2. STACKING TIME OPTIMIZATION

CE separation is affected by the length of the injection plug. This is because the longer the sample plug the broader the peak in the resulted electropherogram. Sample preconcentration by pH-mediated stacking provides a way to concentrate sample molecules in a narrow zone at the boundary of two buffer solutions of different pH values allowing the increase of the injection time without sacrificing the efficiency. Using electrokinetic injection, charged ions can be easily introduced into the capillary as a result of their electrophoretic migration (*53, 54*).

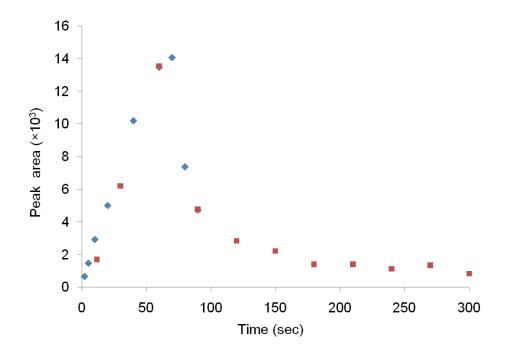
As mentioned earlier all CE measurements were conducted using a Beckman Coulter P/ACE MDQ capillary electrophoresis instrument (Beckman coulter, Fullerton, CA, USA). This instrument is fully automated and managed using 32 Karat<sup>™</sup> v7.0 Software. The software provides numerous control and analysis functions allowing programming various capillary electrophoresis runs. The programming option of the instrumental software allows injecting sample with the voltage of up to 10 kV for no longer than 99.9 sec. Both options allow controlling the duration to reach the injection voltage, so called "ramp time". However, while in the separation option a desirable ramp time has to be set, the injection option compensates the ramp time automatically, therefore the precise injection time is known. In this work the injection voltage was selected to be 10 kV as it's the highest possible voltage that can be applied by the instrument for the electrokinetic injection. However, in order to determine the optimal injection duration that would provide the best sample signal, the limited timing conditions needed to be exceeded. To increase the injection time limits the separation option available in method programming was used. With this option a sample can be injected for the infinite duration of time.

The following set of experiments uses only one steroid, ethynyl estradiol, a potential endocrine disruption compound and a component of birth control pills. The concentration of ethynyl estradiol in 1 mM CMCD sample was 10  $\mu$ M. The data obtained with the use of the injection option correlates with the data measured using the separation option for sample injection which is demonstrated in Figure 3.9. A 60 sec injection was chosen to be the optimal duration despite the maximum sample stacking being obtained with the injection at 10 kV for 70 sec. The further increase in the duration time of the

sample injection resulted in lower sample stacking. This can be due to the fact that longer injection durations can lead to the EOF formation in the water region of the capillary which will slowly drag the CMCD anions toward the cathode located at the inlet and therefore away from the pH-junction and even out of the capillary back to the sample vial. The 70 sec injection however, resulted in the splitting of ethynyl estradiol peak due to the sample overloading. Injection plug lengths longer than the diffusion controlled zone width will proportionally broaden peak width and in extreme cases distorted peak shapes, peak broadening and peak splitting is observed (55). The appearance of the peak splitting can be due to sample overloading, or electromigration dispersion, that can occur due to the differences in the electric field between the sample and the separation buffer if the plug is too wide (56). In this study a water plug was introduced in the capillary before the sample was injected. This created a capillary region with high electric field comparing to the region of the capillary filed with the separation buffer containing SDS and HPCD. As mentioned earlier upon application of the injection voltage both anionic CMCD carrying steroids and SDS micelles present in the capillary would start moving toward the anode. However, because of the high electric filed in the water filled region, CMCD anions will migrate much faster toward the pH-junction were they will be slowed down and preconcentrated. At the same time some steroids can exchange into micellar phase located on the other side of the pH-junction and start moving toward the outlet. If the injection duration is short, the SDS micelles entering the capillary upon application of the separation voltage will move faster toward the stacking zone where they will catch up the micelles which have already moved toward the outlet with steroids reached the junction during the first seconds of the stacking injection. If the injection time is too long, the 88

micelles entering the capillary will be separated by a longer distance from the first micellar band carrying the steroids. Therefore a split peak will appear in the resulting electropherogram.

The peak areas were used for signal quantitation as they provided longer dynamic ranges comparing to the peak heights. The increases in peak heights were not linear as the sample concentration increased due to the band broadening effects.



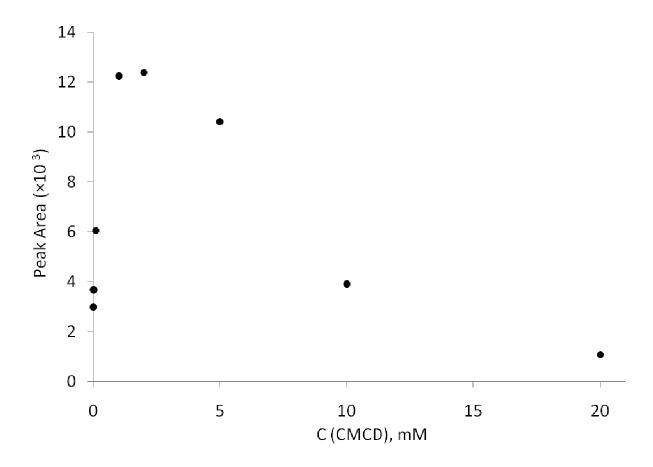
**Figure 3.9.** Stacking enhancement versus injection time. Sample injected electrokinetically at 10 kV by injection (blue) and separation (red) options in the instrument method programming. Sample is 10  $\mu$ M ethynyl estradiol in 1 mM CMCD, 5% methanol, 50 mM CAPS buffered at pH 10. BGE is 30 mM SDS, 13 mM HPCD in 50mM phosphate buffered at pH 2.5. Separation: at 16kV and normal polarity.

#### 3.3.2.3. The Effect of CMCD Concentration on Stacking Efficiency

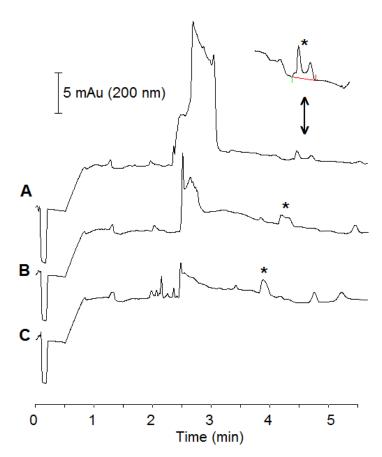
The use of charged CMCD for steroid stacking is attractive as it requires no chemical derivatization of steroids. The effect of the addition of CMCD to the sample solution and its effect on the stacking enhancement are demonstrated in Figure 3.10, that plots the estradiol peak area versus CMCD concentration in the sample buffer. The studies confirmed that the maximum stacking was obtained with the use of 1 mM carboxymethyl- $\beta$ -CD. The further increase of CMCD concentration decreased the signal area as seen in the Figure 3.11.

There are a few reasons for lower stacking at the higher CMCD concentration. 1) As the concentration of CMCD increases, there is higher chance of injecting empty CMCDs into the capillary due to their higher electrophoretic mobility comparing to those CDs carrying steroids. Therefore fewer steroids will be preconcentrated at the pH-junction. 2) It is known from the literature that CD-steroid complex is mostly 1:1 ratio, although 2:1 complex formation can take place as well (*57*). If this were the case, one steroid molecule would be "locked up" by two CD molecules and this would affect the interaction with SDS micelles which serve to remove steroids from the stacking zone and carry them toward the detection window. 3) Finally, as the concentration of CD increases compared to the concentration of SDS in the running buffer, the equilibrium will be shifted toward the formation of the steroid-CD complex as discussed in Section 2.3.4 and away from incorporation into micellar phase, therefore decreasing the steroid detection response. The steroid concentration in the sample does not determine the optimal

concentration of CMCD if increased from 500 nM to 10  $\mu$ M that still provides the best stacking efficiency using 1 mM CMCD. Based on these observations 1mM CMCD was chosen as the optimal concentration for better stacking efficiency.



**Figure 3.10.** Stacking enhancement versus CMCD concentration in the sample buffer. Sample is 1  $\mu$ M estradiol in CMCD, 5% methanol in 50 mM CAPS, pH 10. Running buffer: 30 mM SDS, 13 mM HPCD in phosphate, pH 2.5

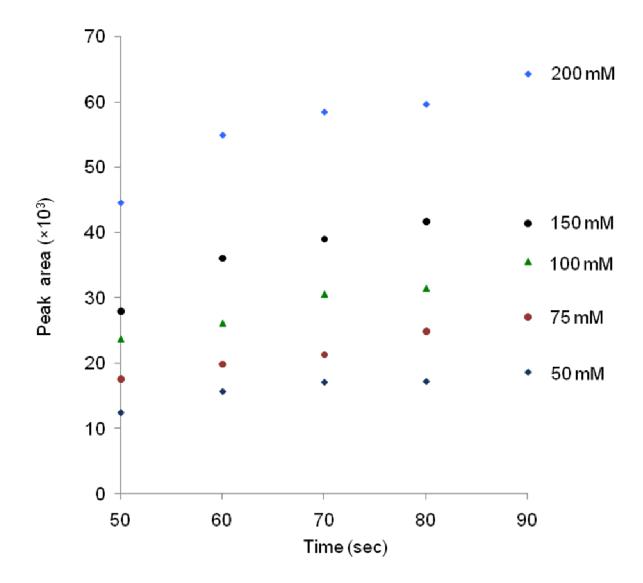


**Figure 3.11.** Representative electropherograms of the stacking enhancement versus CMCD concentration in the sample buffer. CMCD in the sample: A - 5 mM, B - 2 mM, C - 1 mM, peak labeled as \* - 1  $\mu$ M estradiol,

# 3.3.2.4. Stacking versus Phosphate Concentration

Figure 3.12 shows the importance of adjusting the concentration of phosphate buffer for improving of the stacking efficiency. Since phosphate was used for the preparation of the running buffer, it was important to maintain not only a particular and constant pH but also a proper buffer capacity for the reproducible separation. Buffer concentration has a significant effect on the EOF because it changes the  $\zeta$ -potential on the capillary wall. As the concentration of the buffer increases, the ionic strength increases as well, thus lowering  $\zeta$ -potential and EOF. Moreover the higher the ionic strength of the 92 buffer in use the more chance of getting Joule heating effect in the capillary due to high separation currents. In the present case, EOF was suppressed because the surface silanol groups were protonated at the low pH of the separation buffer. However, it was necessary to find out how large a phosphate concentration could be used without causing excessive Joule heating.

The separations were performed in the presence of 50, 75, 100, 150, and 200 mM phosphate buffered at pH 2.5 containing 30 mM SDS and 13 mM HPCD. The sample was 10  $\mu$ M ethynyl estradiol injected at 10 kV for different durations of time 50, 60, 70, 80, and 90 sec. The use of 200 mM phosphate produced the best stacking effect measured in the peak area as shown in the figure below. The 60 sec sample injection was still the most advantageous since the longer sample injections produced distorted electropherograms due to peak broadening.

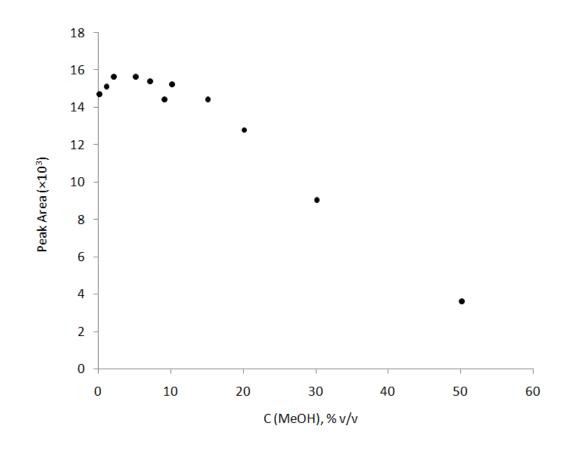


**Figure 3.12.** Stacking enhancement versus concentration of phosphate buffer and sample injection time. Sample injected electrokinetically at 10kV. Sample is 10  $\mu$ M ethynyl estradiol in 1 mM CMCD, 5% methanol, 50 mM CAPS buffered at pH 10. BGE is 30 mM SDS, 13 mM HPCD in phosphate buffered at pH 2.5. Separation: at 16kV and normal polarity.

# 3.3.2.5. Stacking versus Methanol Concentration in Sample Matrix

The effect of added methanol on stacking enhancement was investigated by increasing the volume fraction of methanol (0.1, 1, 2, 5, 7, 9, 10, 15, 20, 30, 50 %, v/v) in the sample containing 1  $\mu$ M estradiol in 1 mM CMCD, 50 mM CAPS at pH 10. The plot of

the peak area measured for preconcentrated estradiol versus the percentage of methanol in the sample is illustrated in Figure 3.13. It is evident that the formation of steroid-CMCD inclusion complex takes place even in the absence of methanol in the sample. The addition of 5 % methanol increases the peak area by 6 % versus the area for the sample without methanol. The results show that the further increase of the methanol amount in the sample shifts the equilibrium away from the formation of CMCD-steroid inclusion complex, thus, lowering the stacking efficiency.



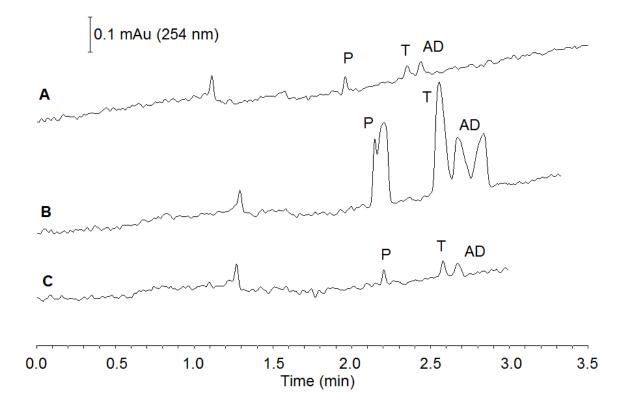
**Figure 3.13.** Stacking enhancement versus methanol content in the sample buffer. Sample is 1  $\mu$ M estradiol in 1 mM CMCD, methanol, 50 mM CAPS, pH 10. Running buffer: 30 mM SDS, 13 mM HOCD in 200 mM phosphate, pH 2.5. Injection performed electrokinetically at 10 kV for 60 sec. Separation performed at 16 kV and normal polarity.

# 3.3.2.6. Stacking versus pH of the Sample Buffer

An effective preconcentration by means of pH-stacking depends on the pH of the buffer solutions used to create the pH-junction and more importantly on the  $pK_a$  of the analyte to be preconcentrated. As mentioned earlier, CMCD used for stacking preconcentration has  $pK_a \sim 5$ . From the Henderson–Hasselbalch equation:

$$pH = pKa + \log\frac{[A^-]}{[HA]} \tag{3.4}$$

CMCD is expected to be fully (100%) deprotonated at pH = 7, and fully protonated at pH = 3. To completely suppress EOF, the pH of the running buffer must be less than 3. Only phosphate ( $pK_a \sim 2.1$ ) was suitable for being used at pH < 3. The sample containing steroids was prepared in 50 mM MOPS buffer at pH 7 and subject to stacking procedure as described in Section 3.2.5. The results demonstrated stacking enhancement for the steroids as expected (see Fig. 3.14-A). However a 10 times better stacking enhancement was achieved using pH 10 CAPS. Figure 3.14-B, C.

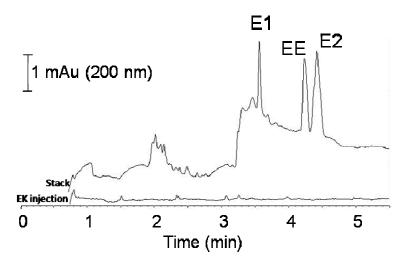


**Figure 3.14.** Stacking enhancement versus pH of the sample buffer. Sample contains 10  $\mu$ M progesterone, testosterone and androstenedione in 5 % methanol, 1 mM CMCD, and A – 50 mM MOPS, pH 7.0; B – 50 mM CAPS, pH 10. C – measured for 1  $\mu$ M progesterone, testosterone and androstenedione in 5% methanol, 1 mM CMCD in 50 mM CAPS, pH 10. Injection performed for 60 sec at 20 kV.

# 3.3.2.7. Stacking Enhancement

In capillary electrophoresis, sample injection at high injection parameters (voltage and pressure) typically leads to band broadening (2). The developed stacking method allows to use longer injection time without sacrificing separation efficiency. The advantage of the developed stacking method is demonstrated in Figure 3.15 by comparing the upper trace (stacked for 60 seconds) with the lower trace (injected electrokinetically for 2 seconds). Enhancement in limits of detection for each steroid was calculated by dividing the LOD obtained by means of stacking-CE by the LOD obtained with the use conventional MEKC and hydrodynamic injection.

For stacking-CE analysis the limits of detection were measured at a signal-to-noise ratio of 3. The signal and noise were determined as the peak height and the standard deviation of the baseline respectively. LODs were measured at 200 nM progesterone (n=9), 200 nM hydroxyprogesterone (n=6), and 500 nM ketotestosterone (n=7), 50 nM estradiol (n = 9), 70 nM estrone (n=10) and 70 nM ethynyl estradiol (n=7). Each sample containing only one steroid was prepared in 1 mM CMCD, 50 mM CAPS, pH 10 and was analyzed by stacking-MEKC as described in Section 3.2.5. The limits of detection for conventional MEKC analysis of steroids were calculated by the same principle. A series of samples containing 50  $\mu$ M concentration of each steroid were prepared in the running buffer (30 mM SDS, 13 mM HPCD in phosphate, pH 2.5, 5% methanol) and injected at 0.5 psi for 3 sec. The peak areas were measured and used for stacking enhancement evaluation. The calculated enhancements in limits of detection are represented in Table 3.2. The stacking-MEKC method demonstrated at least 350-fold up to 1100-fold improvement in LODs over the conventional hydrodynamic injection.



**Figure 3.15.** Sample containing 500 nM estrone (E1), ethynyl estradiol (EE), and estradiol (E2) is injected at 10 kV, for 60 s (upper trace) or 2s (lower trace) in 1 mM carboxymethyl- $\beta$ -CD diluted in 50 mM CAPS buffered at pH 10. The electropherogram is obtained using 30 mM SDS, 13 mM HPCD, 200 mM phosphate buffered at pH 2.5. Reproduced with permission from (*58*).

	Steroid	LOD <sub>ave</sub>	LOD	LOD
		(STACK)	(MEKC)	ENH
		nM	mM	
1	progesterone	7.1	5.3	746
2	hydroxyprogesterone	15	5.3	353
3	dihydroxyprogesterone	11	4.7	427
4	testosterone	4	3.5	875
5	estrone	3.1	2.1	677
6	11-ketotestosterone	14	5.9	421
7	ethynyl estradiol	4.7	2.2	468
8	estradiol	2.9	3.2	1103

**Table 3.2.** Enhancement in limits of detection for steroids measured by stacking-CE injected electrokinetically versus conventional MEKC with hydrodynamic injection.

For experimental conditions see text.

# 3.3.2.8. Stacking Figures of Merit

This section outlines the figures of merit that demonstrate the effectiveness of the developed and optimized stacking approach. Figures of merit (Table 3.3) were obtained using a 10 kV 60 second stacking injection and a separation buffer containing 13 mM HPCD, 30 mM SDS in 200 mM phosphate buffered at pH 2.5. The baseline in the electropherograms that results from sample stacking had some distortion (see for example Figure 3.7). The data shown in Figure 3.7 were obtained from successive runs of a sample and blank and subsequently processed using a spreadsheet program.

For all steroids, the within-day and day-to-day reproducibility in migration time was  $\leq 1$  % RSD and  $\leq 2$  % RSD, respectively. The within-day and day-to-day reproducibility in peak area was  $\leq 9$  % and  $\leq 22$  % RSD, respectively. The day-to-day reproducibility was affected by the variations in room temperature from 20 to 24 °C, which changes the solubility of the steroid in carboxymethyl- $\beta$ -CD. Although a dedicated thermostat was not available at the time of measurements, the use of calibration curves within-day almost completely circumvents this issue due to reasonable temperature stability during the day.

Linear ranges were determined from nine calibration curves (3 per day for 3 days) and measured at 0.50  $\mu$ M hydroxyprogesterone, estrone, ethynyl estradiol and estradiol, 0.75  $\mu$ M progesterone, 11-ketotestosterone. The curves were measured simultaneously for 6 analytes. Detection was performed by UV-visible absorbance at detection wavelength of 254 nm for progesterone (R<sup>2</sup>  $\geq$  0.98), hydroxyprogesterone (R<sup>2</sup>  $\geq$  0.97), and ketotestosterone (R<sup>2</sup>  $\geq$  0.99), or 200 nm for estradiol (R<sup>2</sup>  $\geq$  0.99), estrone (R<sup>2</sup>  $\geq$  0.98), and 100 ethynyl estradiol ( $R^2 \ge 0.99$ ). The data from individual curve fitting are shown in Table 3.3. Under the experimental conditions, calibration curves for steroids with high  $\beta$ -CD affinity (estradiol, ethynyl estradiol, and estrone) are easily obtained in the range from 250 -10,000 nM. Progesterone, 11-ketotestosterone, and hydroxyprogesterone were measured at 254 nm to maximize absorbance. By using a photodiode array, all steroids were detected within a single run. The different detection wavelength and lower  $\beta$ -CD affinity of progesterone, 11-ketotestosterone, and hydroxyprogesterone resulted in higher limits of detection and smaller concentration range of linear response. Finally, a hydroxyprogesterone formed a split peak, especially at higher concentrations. This splitting of hydroxyprogesterone may result from stereoisomers or from multiple complexation equilibria with carboxymethyl- $\beta$ -CD.

	LOD (nM)	Linear Range (µM)	Reproducibility			
Steroid			Within day (n=10)		Day-to-day (n= 3)	
			Time (min)	Area (x10 <sup>3</sup> )	Time (min)	Area (x10 <sup>3</sup> )
progesterone	$7.1\pm0.7$	0.25 - 1.0	$2.47\pm0.01$	$1.3_8\pm0.0_7$	$2.49\pm0.02$	$1.3_6\pm0.0_5$
hydroxy- progesterone	$15\pm1$	0.25 - 0.75	$2.74\pm0.02$	$0.81\pm0.05$	$2.77\pm0.04$	$0.78\pm0.03$
estrone	$3.1\pm0.8$	0.25 - 5.0	$3.55\pm0.03$	$5.2\pm0.5$	$3.61\pm0.06$	$5.5\pm0.9$
11-keto testosterone	$14 \pm 3$	0.50 - 10.	$3.68\pm0.03$	$1.5 \pm 0.1$	$3.74\pm0.07$	$1.2 \pm 0.3$
ethynyl estradiol	$4.7\pm0.4$	0.25 – 10.	$4.21\pm0.03$	$7.6\pm0.5$	$4.30\pm0.08$	$7.9\pm0.4$
17β-estradiol	$2.9\pm0.6$	0.25 - 5.0	$4.39\pm0.04$	$8.9\pm0.5$	$4.5\pm0.1$	$8.8\pm0.2$

Table 3.3. Stacking - MEKC Figures of Merit

#### **CONCLUSIONS**

A preconcentration technique using the chromatographic preconcentrator positioned in-capillary for enhancement of the detection limits for steroid analysis by CE has been demonstrated. The benefit of this preconcentration approach is the possibility to inject a much larger sample volume opposed to conventional CE or MEKC while maintaining a high efficiency separation. However, the technical limitations of this method, the resulting low reproducibility and very sophisticated packing procedure preclude its application in systematic and high throughput screening of steroid hormones required to detect endocrine disruption.

In contrast, a newly developed pH-mediated stacking MEKC method for the preconcentration of selected sex steroid hormones demonstrated outstanding performance and stacking efficiency exceeding two orders of magnitude with targeted steroids. The analysis of parameter space revealed that the increased amount of CMCD and methanol in the sample buffer result in lower stacking efficiency. However, the high concentration of the running buffer improves the stacking. The within-day and day-to-day reproducibility of this method, and the low limits of detection justify its promise for the determination of steroids in fish plasma samples which will be presented in chapter 4.

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# <u>CHAPTER 4</u>

# MODIFICATION OF THE METHOD FOR ANALYSIS OF FISH PLASMA

#### 4.1. INTRODUCTION

In the previous chapter, a newly developed pH-mediated stacking MEKC method for analysis of sex steroid hormones was evaluated using aqueous solutions prepared from steroid standards, and demonstrated reproducible performance and low limits of detection. To fully demonstrate the applicability and efficiency of the method in endocrine disruption studies, it was required to apply the method to real-life samples such as wild fish blood or plasma. Two major challenges in this analysis are the small volume of the fish plasma and the presence of interfering compounds, which obstruct the CE separation.

The fish have long been used as biosentinel models of water quality in biological studies, mainly because of the concerns associated with the increased production, use, and disposal of chemicals in the environment (*1-3*). Both, the wild fish and the fish exposed to pollutants in a laboratory can provide valuable information for studying the effects of endocrine disruption. The fish acquires EDCs via the gills, skin, and the diet, including plants and smaller invertebrates, which in turn accumulate pollutants from the water or sediments during their lifecycle. The physiological system of the fish is similar to mammals, including humans, and many of the pollutant-induced disorders are likely to have similar effects. The reproductive endocrine system is particularly similar within the vertebrates (*4*). Furthermore, the accumulation of pollutants in the muscle of the edible fish has clear implication for the human health.

Recent effort has focused on the "environmental estrogens" – industrial chemicals that may mimic natural estrogens. EDCs affect the estrogen receptor in the fish, as well as the metabolism or catabolism of sex steroids, thus primarily disrupting the fertility of the fish. Suppressed reproduction and signs of feminization in the male fish, as well as sexual *dimorphism* (differences in the body appearance based on sex), are mainly due to estrogenic hormones such as  $17\beta$ -estradiol, estrone, and ethynyl estradiol, or hormone mimics (5, 6). The concentration of estrogenic steroids in the aquatic environment ranges from 0.010 ng/L to 7.0 ng/L (7, 8). Steroid estrogens can be harmful for the fish at a concentration as low as 2.0 ng/L (9).

Several factors must be considered when using fish to test for effects of EDCs. The temperature, photoperiod, diet, availability of spawning substrates, proximity and reproductive readiness of the potential mates, and the water quality all affect the reproductive status, ovulation, and spawning in fish (2). Seasonal and daily changes in sex hormone levels must be considered in sampling schemes because there can also be a significant contribution of extrinsic factors, such as the stress from capture, handling and confinement which have a rapid effect on the sex hormone levels in the fish blood plasma (10). The fish species desirable for EDC analysis should be easy to breed and handle in the laboratory, readily available year round, widespread, and economically feasible. Finally, it is important to choose species with the well-studied reproductive biology.

Endocrine disruption in fish can be monitored using physical and chemical biomarkers. Disrupted fertility of fish can be evaluated by the number and viability of the eggs, fertilization, the survival of the offspring, the physiological abnormalities in size and growth, and histology (the anatomical study of the microscopic structure of animal tissue). Vitellogenin has been used as a biomarker for exposure to anti-estrogenic compounds in several fish studies (*11-16*). Vitellogenesis is the process in which the vitellogenin, a

female egg protein, is synthesized by the liver in response to endogenous estrogen and then absorbed by growing oocytes, where it provides nutrition for the developing embryos (17). Plasma vitellogenin concentrations increase during sexual maturation of female fish (18), and can reach tens of milligrams per milliliter in some species. In contrast, the concentration of the endogenous estrogen in male fish is too low to trigger the expression of the vitellogenin gene therefore very little vitellogenin can be detected in male fish (19). However, vitellogenesis can be induced if the male fish is exposed to various concentrations of natural and synthetic estrogens present in water (20). For example, 0.1 ng/L of  $17\alpha$ -ethinylestradiol can cause a significant increase in the plasma vitellogenin concentration after a relatively brief exposure (21, 22). Although the high levels of vitellogenin can be related to endocrine disruption, it is essential to control the levels of sex steroid hormones in plasma. This can determine whether the high concentrations of vitellogenin in male or immature female fish were caused by exogenous EDCs and not by the endogenous secretion of  $17\beta$ -estradiol, and whether the presence of estrogenic EDCs had a negative effect on endogenous steroid secretion (23).

It has been mentioned earlier that the reproduction in teleost, as in vertebrates, is controlled by the brain-pituitary-gonodal axis (Section 1.1.4.). In male teleost, 11ketotestosterone and testosterone are the most important androgens that control a variety of reproductive processes (24-27). Estradiol, in turn, is the primary female estrogen in charge of gonodal development while  $\alpha$ , $\beta$ -dihydroxyprogesterone is responsible for final maturation of teleost oocytes (28, 29). The four steroids were therefore selected to help identify and study the occurrence of intersex triggered by endocrine disruption and to monitor reproduction in the fish.

In the present chapter, the complex composition of the plasma samples required the development of a sample purification procedure for steroid extraction prior to the stacking preconcentration and CE separation. Four different fish species: catfish (*Ictalurus punctatus*), smallmouth bass (*Micropterus dolomieu*), largemouth bass (*Micropterus salmoides*), and yellow perch (*Perca flavescens*) were used in order to ensure that method can handle the differences in the blood samples from different fish. An improved three-step extraction procedure, introduced here for the first time, allows for the separation of the endogenous steroids from fatty acids and highly lipophilic interferences such as cholesterol prior to CE-analysis. The method is applicable for volume-limited plasma samples.

Finally, the optimized sample preparation procedure followed by pH-mediated stacking MEKC was applied to the analysis of endogenous steroids in plasma of the yellow perch (*Perca flavescens*) captured from natural aquatic environments. The results were obtained with an external standard calibration curve and compared to the results measured by means of the standard addition method. The developed method was validated by measuring the recoveries of the standard steroids added to fish plasma before extraction. The steroid levels quantified in yellow perch plasma were also measured and compared to radioimmunoassay method as a reference method.

#### 4.2. EXPERIMENTAL

#### 4.2.1. CHEMICALS AND MATERIALS

3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), CMCD. SDS. 11ketotestosterone, and testosterone were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPCD was purchased from VWR (West Chester, PA, USA). Steroids, a,adihydroxyprogesterone,  $\alpha,\beta$ -dihydroxyprogesterone, 17 $\beta$ -estradiol were purchased from Steraloids, Inc (Newport, RI, USA). Sodium phosphate monobasic salt was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methanol was purchased from EMD Chemicals (San Diego, CA, USA). Ethyl acetate was purchased from Mallinckrodt (ACS grade, Phillipsburg, NJ, USA). MOPS was purchased from Alfa Aesar (Ward Hill, MA, USA). Deionized water was purified using ELGA Purelab Ultra (Lowell, MA, USA). Buffered solutions were prepared as described in Section 3.2.1. Standard stock solution of each sex steroid hormone was prepared and stored as described in Section 3.2.1. Stock samples were diluted to a desired concentration each day prior to use with the proper buffer. The running electrolyte and the samples to be analyzed were prepared fresh every day prior to use. The sample buffer comprised of 1 mM CMCD, 5% methanol in 50 mM CAPS, pH 10. The running buffer comprised of 30 mM SDS, 13 mM HPCD in 200 mM phosphate, pH 2.5.

# 4.2.2. INSTRUMENTATION

All CE measurements were carried out using a Beckman Coulter P/ACE MDQ capillary electrophoresis instrument (Beckman coulter, Fullerton, CA, USA) equipped with 113

a photodiode array UV-visible absorbance detection system as described in Section 3.2.2. The separations were performed in a 25  $\mu$ m id, 360  $\mu$ m od, 30 cm long fused silica separation capillary (Polymicro Technologies, LLC, Phoenix, AZ, USA) with the detection window positioned 10.2 cm from the cathodic reservoir located at the sample inlet. The separations were conducted at 25 ± 1 °C. To prevent the disruption in the separation current during the electrophoresis all vials containing freshly made running buffers and samples were subject to vacuum degassing for short periods of time: 1 min for 1 mL vial or 15 sec for 250  $\mu$ L vials. Ultrasonication was also used for this purpose when it was necessary.

#### 4.2.3. CE SEPARATION

CE separations preceded by pH-stacking preconcentration were performed as described in Chapter 3, Section 3.2.5. Briefly, steroid standards prepared in 1 mM CMCD, 5% methanol in 50 mM CAPS buffered at pH 10 were injected electrokinetically at 10 kV for 60 sec in silica fused capillary filled with the running buffer and containing the deionized water plug injected before the sample introduction at 13.8 kPa (2 psi) for 6 sec. The running buffer comprised of 30 mM SDS, 13 mM HPCD in 200 mM phosphate buffered to pH 2.5. Following the sample loading, the reverse polarity run at 16 kV was initiated to accomplish the separation of the steroids.

# 4.2.4. FISH BLOOD/PLASMA SAMPLES

All blood and plasma samples of the fish used in this work were generously provided by our colleagues Christy Foran, and Jennifer Stueckle from WVU Department of Biology; Kristine Willett of the University of Mississippi; Luke R. Iwanowicz and 114

Vicki S. Blazer from U.S. Geological Survey Leetown Science Center, Kearneysville, WV. Whole blood is a bodily fluid that flows through the heart, arteries, veins, and capillaries carrying nourishment, electrolytes, hormones, vitamins, antibodies, heat, and oxygen to the tissues of an organism. Blood contains red blood cells, white blood cells, and platelets suspended in fluid called *plasma*. Plasma is obtained by separating the liquid portion of blood from the cells and constitutes about 55 % of blood volume. Plasma contains 90 % of 10 % water. the rest constitute albumin (the major protein constituent), fibrinogen (responsible for the clotting of blood), globulins, glucose, mineral ions, hormones and carbon dioxide. Frozen plasma is plasma frozen within hours after being collected in order to preserve clotting factors, stored for one to seven years, and thawed before used for research or medical purposes (30).

#### 4.2.5. PLASMA COLLECTION BY USGS

Plasma collection was performed by Luke R. Iwanowicz of USGS, and is described briefly here. During early March 2008 yellow perch were collected from the Choptank or Severn Rivers (U.S. state of Maryland) during their spring spawning run. Fyke nets were deployed for fish capture and checked daily. Yellow perch were removed from the nets and transported to the US Fish & Wildlife Service, Chesapeake Bay Field Office (Annapolis, MD) for processing. Fish were maintained at the USGS laboratory in aerated water from the collection site for transport and prior to plasma sample collection. The fish were euthanized with a lethal dose of tricaine methane sulphonate (Finquel MS-222) (Argent Labs, Redmond, WA). The blood was drawn from the caudal vessels from each fish with a heparinized syringe, transferred to a heparinized vacutainer, and stored 115 on wet ice. The plasma was collected by centrifuging the vacutainers at  $1000 \times g$  for 20 min at 4 °C. The plasma was removed and stored at – 80 °C in cryovials. While vitellogenin (an egg-yolk precursor protein) concentrations were not measured in 2008 female fish, the fish collected during the same time in 2007 had measurable vitellogenin concentrations ranging from 2 – 10 mg/mL.

#### 4.2.6. PLASMA PRETREATMENT PROTOCOL # 1

Ethyl acetate was employed to extract free steroid hormones prior to CE separation as the first step of the plasma sample pretreatment. For this experiment the whole blood sample of the female wild catfish (*Ictalurus punctatus*) frozen for 6 years was used. The steroids were extracted from the blood using ethyl acetate similar to a procedure described in (22). Briefly, 250  $\mu$ L (0.28 g) of thawed blood was extracted three times with 750  $\mu$ L of ethyl acetate. This was accomplished by agitating the sample using a vortex mixer for 5 sec, then spinning it for 5 min at 4,000 rpm and 4 °C followed by the removal of the top organic fraction. The three ethyl acetate fractions were pooled, evaporated to dryness under the stream of nitrogen gas, and reconstituted in the sample buffer containing 1 mM CMCD, 5 % methanol in 50 mM CAPS buffered at pH 10. A flow chart of the extraction protocol with ethyl acetate is illustrated in Figure 4.1. The reconstituted extract was then preconcentrated using pH-stacking and analyzed by CE-UV-visible absorbance detection analysis as described above Section 3.2.5.

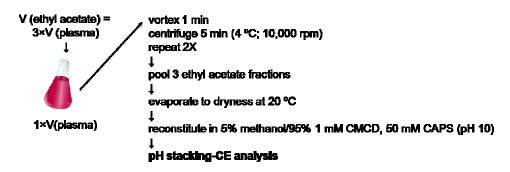


Figure 4.1. Flow chart of the plasma extraction protocol #1.

#### 4.2.7. PLASMA PRETREATMENT PROTOCOL #2

The ethyl acetate extracts of fish plasma samples were further purified by strong anion exchange extraction cartridges. In the experiment, a plasma sample from a single catfish was split into two fractions each at 412  $\mu$ L in volume. Only one of the two fractions was spiked with 14 ng estradiol (50. pmol). Both fractions were then extracted three times with ethyl acetate (as described in Section 4.2.6). For each sample the three ethyl acetate fractions were pooled, evaporated to dryness under the stream of nitrogen gas, and reconstituted in a solution comprised of 5% methanol, 95% aqueous 5mM MOPS buffered at pH 7.0. To facilitate the removal of the fatty acids collected in the extract, the solution was then loaded on a disposable quaternary amine  $(N^+)$  strong anion exchange (SAX) cartridge (BAKERBOND speTM Quaternary Amine disposable extraction columns, J.T. Baker, Phillipsburg, NJ, USA) activated twice by consecutive washes with 6 mL of methanol, 6 mL of water and 6 mL of 5 mM MOPS buffered at pH 7.0 (see Fig. 4.2). A quaternary amine is a strong base and exists as a positively-charged cation that exchanges or sequesters anionic species in the solution. It can therefore be used to isolate and discard them. The  $pK_a$  of a bonded quaternary amine functional group is very high (>

14) and leaving it charged at all pHs when in contact with an aqueous solution. For an anionic compound of interest, the pH of the matrix must be 2 pH units above its  $pK_a$  for it to be charged. Therefore, to extract fatty acids ( $pK_a \sim 5$ ) a buffer solution of pH 7 was used. Following introduction of the sample, the column was rinsed with 1 mL of solution comprised of 5 % methanol, 95 % aqueous 5 mM MOPS buffered at pH 7.0. The eluted fraction was evaporated to dryness using a rotary evaporator and reconstituted in 50 µL of the solution comprised of 5 % methanol, 23 mM CMCD, 50 mM CAPS buffered at pH 10. The sample was preconcentrated and analyzed by CE as described in Section 3.2.5.

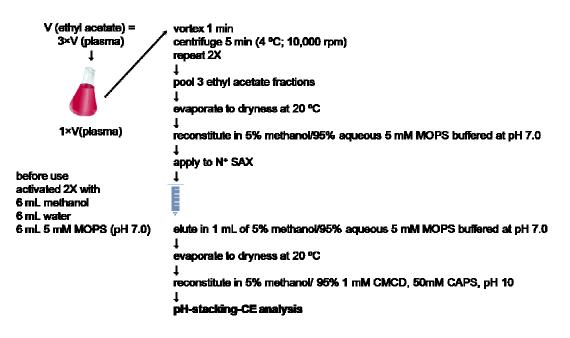


Figure 4.2. Flow chart of the plasma extraction protocol #2.

# 4.2.8. PLASMA PRETREATMENT PROTOCOL #3

For this study plasma samples from bass and yellow perch were used. An aliquot of a thawed plasma sample was split in half. Only one half was spiked with a 118

gravimetrically determined amount of steroids, 2.7 ng of estradiol and 14 ng of testosterone. Each fraction was transferred into a separate borosilicate glass vial and extracted with the volume of ethyl acetate that is three times larger than the sample volume. After adding ethyl acetate, the solution was mixed for 1 min using a vortex mixer and then centrifuged for 5 min at 10,000 rpm and 4 °C. The organic fraction was then removed. Extraction was repeated two more times, and three ethyl acetate extracts were pooled together and evaporated to dryness at room temperature using a Savant SpeedVac® concentrator. Each dry extract, spiked and unspiked, was reconstituted in the solution comprised of methanol: 5mM aqueous MOPS buffered at pH 7.0 (20:80, v/v). The solution was loaded on a quaternary amine disposable extraction column (BAKERBOND spe<sup>™</sup> Quaternary Amine disposable extraction columns, J.T. Baker, Inc., Phillipsburg, NJ, USA) to remove fatty acids extracted with ethyl acetate. Before use, the ion exchange column was activated twice by consecutive washes with 6 mL of methanol, 6 mL of water and 6 mL of 5 mM MOPS buffered at pH 7.0. The column with the loaded sample was washed with 1 mL of methanol: 5 mM aqueous MOPS buffered at pH 7.0 (20:80, v/v). The eluted solution was then loaded onto a C18 extraction column (Supelco Discovery DSC-18 SPE tube bed, Sigma Aldrich, St. Louis, MO, USA), activated in advance twice with 5 mL of methanol and 5 mL of water. The column was then washed with 1 mL of water, and the retained steroids were eluted with 1 mL of methanol: water (75:25, v/v) solution. The eluted fraction was evaporated to dryness at room temperature using the SpeedVac® concentrator. The dry sample was finally reconstituted into 30 microliters of solution comprising 5 % methanol, 1 mM CMCD in

50 mM CAPS buffered at pH 10, and finally analyzed by pH-stacking MEKC (see Fig.

4.3).

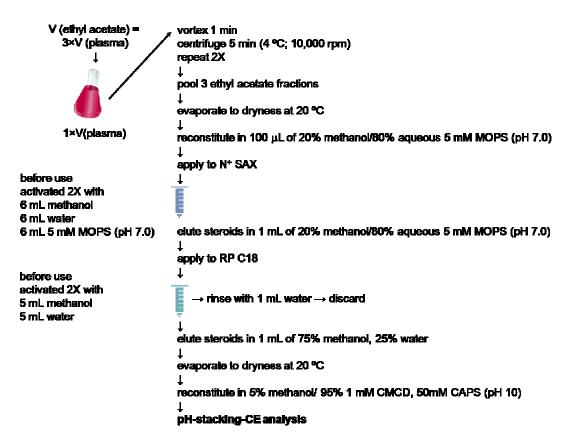


Figure 4.3. Flow chart of the plasma extraction protocol #3

# 4.2.9. RADIOIMMUNOASSAY PROCEDURE BY USGS

Plasma samples from yellow perch were analyzed for the sex steroids 17β-estradiol (E2) and testosterone (T). All samples were quantified via radioimmunoassay (RIA) by Luke R. Iwanowicz of USGS. Estradiol antibodies (#244 anti-estradiol-6-BSA) were purchased from Gordon Niswender (Colorado State University, Fort Collins, CO). Testosterone antibodies (polyclonal anti-testosterone R156/7) were purchased from Coralie Munroe (UC School of Veterinary Medicine, Davis, CA). Extraction efficiencies were

determined for the sex steroids, and all assays were validated and optimized for yellow perch.

Plasma samples were extracted twice in a ten-fold excess of diethyl ether prior to RIA. Plasma estradiol concentrations were determined using the RIA method of Sower and Schreck (31) with slight modifications. The samples and estradiol standards were solubilized in 200 µL of room temperature phosphate buffered saline gelatin (PG) buffer (0.1% knox gelatin in Dulbecco's phosphate buffered saline). 100  $\mu$ L of anti-estradiol antiserum was added to the samples, vortexed and incubated at room temperature for 30 min. The same volume of PG buffer was added to tubes designated to determine nonspecific background and total counts per minute (CPM). Following incubation 100  $\mu$ L of tritiated 17 $\beta$ -estradiol (5000 CPM in PG buffer) was added to all tubes, vortexed and incubated at room temperature for 60 min. Samples were immediately cooled in an ice bath for 30 min and 500 µL of ice-cold charcoal-dextran solution (0.63 % alkaline charcoal and 0.4 % dextran in PG buffer) was added. Samples were vortexed, incubated on ice for 15 min and centrifuged at  $2200 \times g$  for 20 min at 4°C. Supernatant was then decanted into scintillation vials containing 4 mL of OptiPhase HiSafe 2 scintillation fluid (Perkin Elmer, Waltham, MA, USA) and mixed well. Sample CPM were measured using a Tri Carb Liquid Scintilation Counter (Perkin Elmer, Waltham, MA, USA) and mean sample CPM was determined over an 8 minute integration time. All samples were run in duplicate and plasma estradiol values were interpolated from a standard curve. Plasma hormone concentrations were interpolated from the standard curve using curve fitting software (Prism 4.0, GraphPad Software Inc.). The same procedure was repeated on fresh plasma samples for the determination of testosterone using its specific antibody.

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# 4.3. RESULTS AND DISCUSSION

#### 4.3.1. Four Steroids Targeted for Analysis

This study focuses on four steroids referred to as "classical reproductive steroids" considered to be important members of the endocrine system functioning in teleost fish: estradiol,  $\alpha$ , $\beta$ -dihydroxyprogesterone, testosterone, and 11-ketotestosterone (Fig. 1.1). These four steroids were selected as targets for the analysis as their control can assist in the identification and study of the occurrence of intersex triggered by endocrine disruption and to monitor reproduction in fish species (*32*).

The endogenous levels of 11-ketotestosterone, unlike those of testosterone, in the blood plasma of teleost fish are reported to be higher in males than in females (*33-37*). Although sexual development and behavior of the male species is commonly due to testosterone, the dominant androgen in male teleost is 11-ketotestosterone (*26*). Testosterone is also secreted by both sexes; however, testosterone levels in the female fish plasma are elevated during the breading season and its concentration in most female samples is found to be comparable or higher than that in the male fish plasma (*35, 38*). The concentrations of the female hormone estradiol are higher in female fish plasma. Estradiol is produced in the follicle of the developing oocyte while  $\alpha$ , $\beta$ -dihydroxyprogesterone is one of the key mediators for the final oocyte maturation (*39*). The molecular properties of the targeted steroids are summarized in Table 2.2.

#### 4.3.2. Optimized Capillary Flushes

After the developed CE-method was first applied to the fish plasma extracts, the migration times of steroids became irreproducible from run to run and their detection signals decreased dramatically even in the standard samples. This stimulated a need in a new capillary flush protocol development. The poor reproducibility of the analysis of plasma samples was traced to nonspecific surface adsorption of the impurities extracted from the blood/plasma samples, and introduced into the separation capillary during the sample injection. One way to overcome this problem was to change the separation capillary daily. However, the frequent change of the capillary is not only costly and time consuming but also has a potential of introducing systematic measurement errors. Therefore the flushing protocol used to regenerate the inner surface of the separation capillary has been modified by replacing the methanol wash used in the morning described in Chapter 3 with 1 N sodium hydroxide. Conditioning the separation capillary with a strong base actively regenerates the inner capillary surface by dissolving the silica substrate and therefore helps remove any compounds remaining in the capillary after the blood/plasma sample analysis (40).

The recent work was conducted under the conditions of suppressed EOF when the charges of the silica surface were eliminated by the suppression of the dissociation of the surface silanol groups. The new capillary flush procedure, however, introduced a slight change in the steroid migration times. The new protocol for capillary flushes used at the beginning of each day included sequential timed flushes at 172 kPa (25 psi) for 15 min with 1N sodium hydroxide, 5 min water, 25 min 0.1 N hydrochloric acid, 5 min water, 25

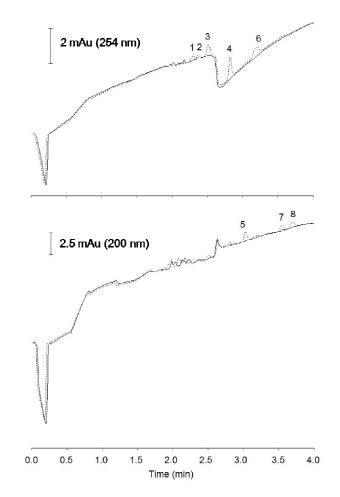
min with running buffer. Running electrolyte was comprised of 30 mM SDS, 13 mM HPCD in 200 mM phosphate buffered at pH 2.5. In between runs, the capillary was flushed at 172 kPa (25 psi) for 2 min with the running electrolyte. Due to the change in the migration times for two of the previously analyzed steroids, estradiol and 11-ketotestosterone, the linear range, the within-day and day-to-day reproducibility in peak area and migration time were determined with the new flush set. Two newly selected steroids,  $\alpha$ , $\beta$ -dihydroxyprogesterone and testosterone, were included in the method evaluation studies.

#### 4.3.3. METHOD SELECTIVITY

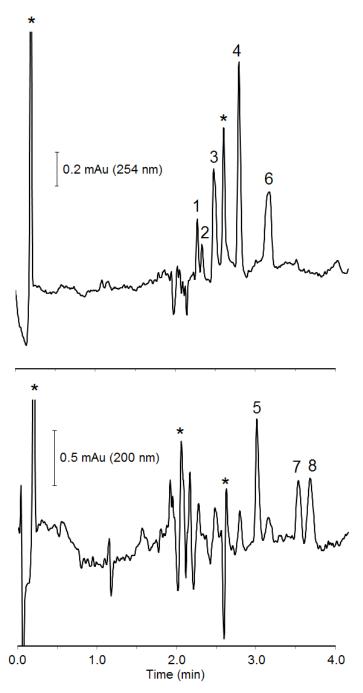
The selectivity of the method for the four targeted steroids was assessed using a set of eight steroids mixed in a single sample. These eight steroids:  $\alpha$ , $\beta$ -dihydroxyprogesterone, 17 $\beta$ -estradiol, estrone, ethynyl estradiol, hydroxyprogesterone, 11-ketotestosterone, progesterone, and testosterone, were analyzed using stacking-MEKC. All eight steroids were baseline separated and the resulting electropherograms are represented in Figure 4.4. The representative background subtracted electropherograms are shown in Figure 4.5. The background subtraction was not used for the peak quantitation as it does not affect the reproducibility of steroid analysis.

Interference from androstenedione was also studied. Androstenedione is a precursor of testosterone in the steroid biosynthesis pathway. A sample containing both steroids was analyzed at the stacking conditions. The resolution for both steroids was

calculated as described in Section 2.1.3 (Equation 2.5) and was  $\geq$  0.91 at the experimental conditions.

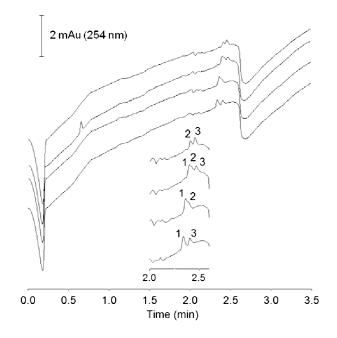


**Figure 4.4** illustrates the raw electropherograms obtained for 8 steroids simultaneously present in the analyzed sample. Data collected at 254 nm (top): (1) progesterone (500 nM); (2)  $\alpha$ , $\beta$ -dihydroxyprogesterone (500 nM); (3) hydroxyprogesterone (2  $\mu$ M); (4) testosterone (1  $\mu$ M); (6) 11-ketotestosterone (2  $\mu$ M).Data collected at 200 nm (bottom): (5) estrone (500 nM); (7) ethynyl estradiol (250 nM); and (8) 17 $\beta$ -estradiol (250 nM).



**Figure 4.5.** Background subtracted electropherograms obtained for 8 steroids simultaneously present in the analyzed sample. Data collected at 254 nm (top): (1) progesterone (500 nM); (2)  $\alpha$ , $\beta$ -dihydroxyprogesterone (500 nM); (3) hydroxyprogesterone (2  $\mu$ M); (4) testosterone (1  $\mu$ M); (6) 11-ketotestosterone (2  $\mu$ M). (B) Data collected at 200 nm (bottom): (5) estrone (500 nM); (7) ethynyl estradiol (250 nM); and (8) 17 $\beta$ -estradiol (250 nM). The separation conditions are summarized in text.

A separate study was conducted in order to determine the migration time for  $\alpha, \alpha$ dihydroxyprogesterone, an isomer of  $\alpha,\beta$ - dihydroxyprogesterone that can possibly be present in plasma (*41*, *42*) and interfere with the separation. The results demonstrated that  $\alpha,\alpha$ - dihydroxyprogesterone co-migrated with progesterone during the analysis (Fig. 4.7). Baseline resolution of these two hormones can be achieved by adjusting the SDS/HPCD ratio in the background electrolyte. However, both of them were baseline separated from  $\alpha,\beta$ - dihydroxyprogesterone, which is typically present at much higher concentration levels in the fish plasma (*41*, *42*). The electropherogram in Figure 4.7 shows the separation of the three steroids. Progesterone and  $\alpha,\alpha$ - dihydroxyprogesterone were not analyzed in this study because of their expected low concentrations in the fish plasma.



**Figure 4.6.** Stacking electropherograms of (1) progesterone; (2)  $\alpha$ , $\alpha$ -dihydroxyprogesterone; and (3)  $\alpha$ , $\beta$ -dihydroxyprogesterone. Concentration of each is 500 nM. Data collected at 254 nm.

### 4.3.4. FIGURES OF MERIT OF MODIFIED METHOD

The linear regression parameters for the calibration curves and all the figures of merit in this chapter were derived from the samples with four targeted steroids. The linear range for each steroid was determined from nine calibration curves (3 per day for 3 days). Curves were measured simultaneously for 4 analytes. The detection was performed by UV-visible absorbance at detection wavelength of 254 nm for dihydroxyprogesterone ( $\mathbb{R}^2 \ge 0.990$ ), testosterone ( $\mathbb{R}^2 \ge 0.997$ ), and ketotestosterone ( $\mathbb{R}^2 \ge 0.995$ ), or 200 nm for estradiol ( $\mathbb{R}^2 \ge 0.996$ ). The within-day and day-to-day reproducibility were measured at 1.0  $\mu$ M dihydroxyprogesterone, testosterone, 11 ketotestosterone, and 0.50  $\mu$ M estradiol. The within-day and day-to-day reproducibility of the migration time was  $\le 1$  and  $\le 2$  % RSD respectively for all four steroids. The corresponding values of the peak area ranged from 1 to 20 % RSD. The data are summarized in Table 4.1.

Table 4.1. Stack	ing - MEKC l	Figures of Merit	Reproducibility					
			Within day (n=	=10)	Day-to-day (r	Day-to-day (n= 3)		
Steroid	LOD	Linear Range	Time	Time Area $(x10^3)$		Area $(x10^3)$		
	(nM)	(μM)	(min)		(min)			
α,β-Dihydroxy progesterone	11±3	0.20 - 5.0	$2.45\pm0.00_7$	$1.1_1\pm0.0_6$	$2.43\pm0.03$	$1.1_5\pm0.0_6$		
Testosterone	$4 \pm 1$	0.10 - 14.	$2.93\pm0.01$	$3.1_9\pm0.0_7$	$2.86\pm0.07$	$3.5\pm0.3$		
11-Keto testosterone	$14 \pm 3$	0.50 - 10.	$3.33\pm0.02$	$1.0_5\pm0.0_6$	$3.23 \pm 0.04$	$1.0 \pm 0.2$		
17β-Estradiol	$2.9\pm0.6$	0.050 - 5.0	$3.88\pm0.03$	$8.4\pm0.1$	$3.82\pm0.06$	$8.3 \pm 0.1$		

#### 4.3.5. Development of the Plasma Sample Purification Method

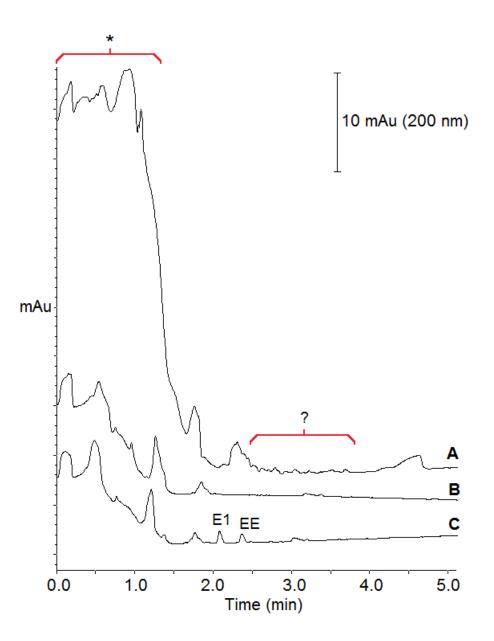
Blood plasma is a complex mixture and contains many compounds that can interfere with the analysis of steroids by stacking preconcentration. Therefore, the analysis of plasma samples by the developed method required a development of a thorough sample purification protocol. Many methods exist for biological samples purification, including the extraction with organic solvents and different combinations of solid phase extraction (43,44). Steroid hormones are present in plasma in the free (unconjugated) or conjugated form such as sulfated and glucuronidated conjugates. The free lipophilic steroids can be extracted from plasma using non-polar organic solvents. As mentioned earlier, immunoassay is widely used in biological laboratories to measure circulating steroid levels in fish. In immunoassay analysis the extraction of steroids from biological fluid samples is often accomplished using ethyl acetate or diethyl ether (22, 45). The organic solvent denatures carrier proteins releasing hormones such as estradiol and testosterone bound to SHBG with high affinity (46). Therefore the total concentration of the steroids, free and bound, can be determined while the hydrophilic sulfated and glucuronidated conjugates remain in the aqueous fraction (i.e. plasma) after ethyl acetate extraction has been performed.

One of the biggest concerns in this extraction method is the potential to concentrate trace impurities from the extraction solvent. Therefore the blank extractions of deionized water employing 4.5 mL of reagent grade ethyl acetate and ACS grade ethyl acetate (99.5% purity) were first performed. The results demonstrated reagent grade ethyl

acetate contained a large number of interfering peaks comparing to the ACS grade ethyl acetate (99.5% purity) which was therefore used in further studies.

Additionally, the other lipid constituents of blood, i.e. cholesterol and fatty acids, are also extracted in the organic phase. Cholesterol has very high affinity to CD cavities and competes with the steroids of interest forming strong inclusion complexes with CDs (47). The fatty acids in turn are anionic at the pH 10 owing to the presence of the carboxylic group and therefore are driven into the separation capillary along with the CMCD molecules carrying lipophilic compounds and therefore are preconcentrated at the pH-junction. Both types of compounds will interfere with the separation of the steroids by the developed stacking-MEKC and have to be eliminated.

The limitation of the sample pretreatment using only ethyl acetate for stacking-MEKC analysis is demonstrated in Figure 4.7. For this experiment the catfish fish plasma sample was pretreated with ethyl acetate as described in methods Section 4.2.6. As seen in the Figure 4.7-A the electropherogram contains several highly intensive co-migrating peaks in the migration time interval from 0 to 2 min. The nature of these interfering peaks is caused by the compounds extracted in ethyl acetate that either have very high affinity to the SDS micelles or are anionic and therefore migrate early driven by their anodic attraction. No steroids were detected in this sample. The electropherogram in Figure 4.7-B was measured for the same reconstituted plasma extract but ten times diluted with the sample buffer. The dilution decreased the concentration and as a result the signal of the interfering compounds, however, no steroids were detected in the sample as confirmed in the electropherogram C (Fig. 4.7) measured for the 10 times dilute extract spiked with 10  $\mu$ M ethynyl estradiol and estrone.



**Figure 4.7.** The electropherograms obtained for the catfish blood extract. A – ethyl acetate extract reonstituted in 1 mM CMCD, 5% methanol, 50 mM CAPS, pH10. (\* - unknown interference from blood, ? – steroid migration time window). B – extract, ten times dilute with the sample buffer; C – extract, ten times diluted with the sample buffer, spiked with 10  $\mu$ M ethynyl estradiol (EE) and estrone (E1). Stacking conditions are outlined in text.

In order to eliminate the presence of the charged fatty acids another blood sample was subject to extraction by anion exchange using disposable cartridges. The method was applied to the blood sample from male catfish frozen for 6 years. The plasma sample was pretreated prior to analysis as described in Section 4.2.7. The spiked portion of the plasma sample reconstituted in 1mM CMCD did not contain an analyte peak for estradiol in the electropherogram. This is likely due to the presence of neutral lipids, such as cholesterol, co-extracted in ethyl acetate, which solubilize the steroids so their association with CMCD is significantly reduced. This was prevented by increasing the concentration of CMCD in the sample to 23 mM, which provided more cyclodextrin available for capturing estradiol from the sample. However, standard solutions of steroid reconstituted in aqueous CMCD with the concentration greater than 1mM yield a lower detector response (Section 3.3.2.3). As a result, the determination of steroids in blood required the analyte quantification using a method of standard addition and increased concentration of CMCD. A three point standard addition curve was obtained for each sample ( $R^2 \ge 0.999$ ) by analyzing the reconstituted sample as well as the reconstituted sample spiked successively with 6.0 ng of estradiol. Using this method, a concentration of estradiol in the unspiked blood sample was  $0.38 \pm 0.04 \,\mu\text{M}$  that corresponds to  $5.6 \pm 0.6$  ng in the sample analyzed, or in other words  $14 \pm 1$  ng/mL blood. The sample spiked with an additional 14 ng estradiol prior to extraction contains a concentration of  $1.4 \pm 0.1 \,\mu\text{M}$ , which corresponds to  $21 \pm 2 \,\text{ng}$  in the sample analyzed, equivalent to 110 % extraction recovery. The advantage of the standard addition calibration method is a constant composition of the matrix for all the samples. However, in this study a three point external standard calibration curve would be a better

option as it decreases the total analysis time, which is beneficial for high throughput sample screening. In addition, to properly carry out the standard addition method the sample has to be split in 4 equal volume fractions. An increasing volume of the steroid standard is added to each portion of the sample and then diluted to the same final volume with the sample buffer. In this work, the total volume of the reconstituted plasma extract was 30 microliters and further spiking and dilution of such small volume would be an error prone, therefore the standard addition was performed by repeatedly spiking the sample with the standard. In this case, the response was normalized to account for the dilution factor, which was minimized by keeping the total volume change less than 5.5 %. However, in this case the same sample was analyzed subsequently for at least 4 times, after which it could not be used for further analysis if necessary. Therefore the critical steps in the sample preparation protocol needed to be changed in order to: (1) enhance overall steroid recovery while eliminating the interfering compounds extracted from plasma and (2) allow for a 3-point standard calibration curve instead of the standard addition. These led to the optimization of the sample preparation protocol by including a third and final extraction step in the extraction protocol. This extraction step utilizes a reversed phase C18 extraction column used to eliminate interference from neutral highly lipophilic substances, such as cholesterol, and was measured as described in Section 4.2.8. The steroids were eluted in the solvent containing 75% methanol and therefore could easily be dried out. The complete improved extraction scheme is outlined in Figure 4.3 and includes three stages: (1) extraction with organic solvent, ethyl acetate; (2) combination of (1) and a strong anion exchange extraction cartridge; (3) combination of (2) and a reversed phase C18 extraction cartridge.

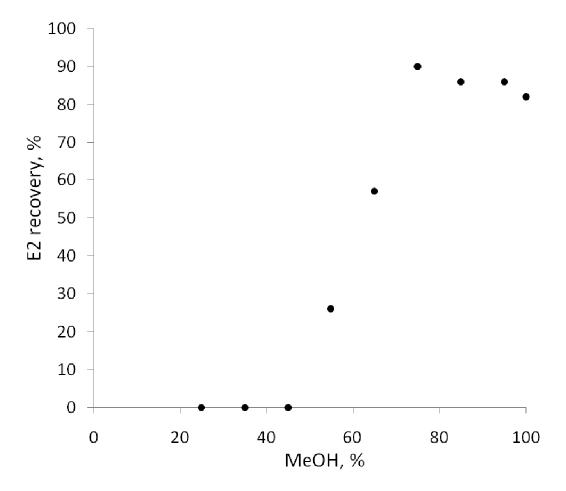
Most aquatic exposure studies require a reasonably large population for statistical significance, therefore there was the need for automated sample preparation and fast instrumental analysis to provide high sample throughput. For faster sample preparation in this study a vacuum box with twelve cartridge holders was used. Comparing to the gravity flow methods the use of the vacuum box offers more rapid flow rates by applying increased vacuum to each extraction cartridge holding a sample, therefore, the extraction proceeds with the same rate and twelve samples can be prepared simultaneously reducing the total analysis time. In commercial laboratories sample purification protocol can be accomplished using robotic systems which increase the speed of the sample preparation and the number of the samples purified up to 96 and more.

# 4.3.5.1. The Evaluation of the Steroid Elution from the SAX Cartridge

Ethyl acetate extracts require reconstitution in aqueous buffer before loading them onto a SAX cartridge. Because steroids are lipophilic compounds they cannot be dissolved in aqueous solutions, therefore the addition of small amount of methanol to the dried out extract assisted steroid dissolution. Different percent concentrations of methanol in 5 mM aqueous MOPS buffered at pH 7.0 used for reconstitution of the dried ethyl acetate extracts before loading them onto SAX cartridge were tested. The methanol content was minimized to avoid the possible loss of steroids out of the extraction column, during sample loading. The concentrations tested were methanol: 5 mM MOPS (15:85, 20:80, and 25:75, v/v). Aqueous samples containing 14 ng of testosterone were prepared and extracted as described in Section 4.2.7. The evaporated extracts were reconstituted in 30 µl of the sample buffer containing 1 mM CMCD and analyzed by the stacking-MEKC 134 as described in Section 3.2.5. The solution comprised of methanol: 5 mM MOPS (20:80, v/v) resulted in 64% testosterone recovery. The further increase of methanol concentration did not improve the recovery, therefore, methanol: 5 mM MOPS (20:80, v/v) was used in the further studies.

# 4.3.5.2. Recovery of Standard Steroids from C18 Cartridge

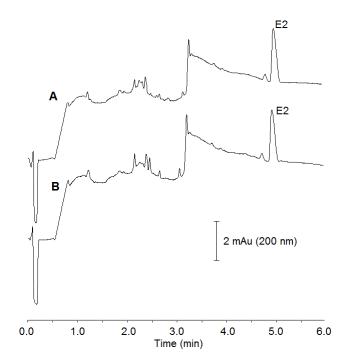
The purpose of the present experiment was to determine the amount of methanol required for elution of the targeted steroids from the C18 silica while leaving the highly lipophilic compounds such as cholesterol being retained by C18 cartridge. The experiment was conducted using estradiol due to its high hydrophobicity (log P = 4.1) comparing to the other targeted steroids (Table 2.2). The optimal amount of the methanol used to collect the most of the estradiol from the cartridge should be sufficient to elute the rest of the targeted steroids as they are less hydrophobic and require more polar solvent. Each steroid sample comprised of 2.7 ng of estradiol prepared in 1 mL of methanol: 5 mM MOPS (20:80, v/v) was loaded onto a pre-activated as described in Section 4.2.8 C18 cartridge and then eluted with 1 mL of elution solvent comprising deionized water and different volume percent concentrations of methanol (25, 35, 45, 55, 65, 75, 85, 95, 100, %v). The Figure 4.8 demonstrates the plot of estradiol percent recovery versus the amount of methanol used to elute the steroid from the C18 extraction cartridge. The elution solution containing methanol: water (75:25, v/v) was used in the further studies as it is just enough to give the highest recovery of estradiol (90 %) from the cartridge. The further increase in methanol content also resulted in a high recovery of estradiol. However, it can cause the elution of highly lipophilic cholesterol form the C18 cartridge and therefore was avoided.



**Figure 4.8.** Plot of the estradiol recovery (%) versus the percent concentration of methanol in water used to elute steroids retained by C18 extraction cartridge during the plasma sample pretreatment.

## 4.3.5.3. Stability Studies

Two estradiol standards were compared in order to study the stability of steroids over the storage period. Two sets of four solutions of 1  $\mu$ M estradiol samples prepared in the sample buffer were analyzed by stacking-CE. The first set was prepared using the freshly made estradiol stock in methanol prepared from a recently purchased powdered chemical. The second set was prepared out of the 1-year old solution of estradiol in methanol made using a solid estradiol stored for 6 years in dark at room temperature. The representative electropherograms are shown in Figure 4.9. The peak areas for the fresh estradiol set were  $(14.5 \pm 0.1) \times 10^3$  and for the old estradiol standard peak area was  $(14 \pm 1) \times 10^3$  (n=4).



**Figure 4.9.** Representative electropherograms for the steroid stability study. A – 1.0  $\mu$ M estradiol prepared from fresh standard (peak area =  $14_{.7} \times 10^3$ ), B – 1.0  $\mu$ M estradiol prepared from the aged standard (peak area =  $15_{.7} \times 10^3$ ). Samples were prepared in the sample buffer containing CMCD and subject to stacking-CE analysis as described in the text.

The signals measured for both estradiol sample sets demonstrated no statistical difference which suggests that if the steroid stock is stored properly (in case of the solid reagent – in a dark dry place at room temperature, and in case of the methanol stock solution – in a dark dry place at – 20 °C) degradation is less than 4 % during the storage period. However, it was important to eliminate the loss of the steroids due to photo- or thermal degradation during the sample preparation procedure, which consists of several steps including extraction, evaporation and sample reconstitution. The corresponding study is discusses in the following section.

#### 4.3.5.4. Effect of Temperature During Dry Down on the Recoveries

Although the literature reports consider the photo-degradation to be the primary reason for the decomposition of testosterone (48-50) (the kinetics of the photo-degradation was not studied in this research), the experimental studies in this research demonstrated that testosterone is partially decomposed under the influence of the heat. In the early experiments for the steroid recoveries a rotary evaporator was used for the gentle removal of the solvents form the sample solutions before reconstituting them in the sample buffer containing CMCD. The rotary evaporator device includes a condenser, a temperature controlled water bath for the sample heating, and a vacuum system for the vapor being drawn off of the sample. The samples to be evaporator was covered with the layers of aluminum foil. To speed up the evaporation process of aqueous samples of > 2 mL the temperature of the water bath was increased to 50 °C. The highest percent recovery of testosterone prepared using this evaporation system was only 64 %. An alternative 138

experiment was conducted using a SpeedVac <sup>®</sup> concentrator device that evaporates samples containing water at room temperature. Testosterone samples covered to prevent their exposure to the ambient light were evaporated at 20 °C and the recovery of testosterone pretreated this way was 93 %. Based on this the dry down of the samples extracts was further carried out at the room temperature using SpeedVac® concentrator.

#### 4.3.5.5. Recovery of Standard Steroids from Aqueous Solutions

The sample extraction steps were studied using aqueous standards as well as fish plasma samples. Prior to estimating total recovery, the recovery of estradiol and testosterone at each step of the optimized extraction scheme (Fig. 4.3) were measured using aqueous solutions of both steroids. The recovery study was performed by taking a sample of deionized water, splitting it in half. Only one half was prepared by adding a desired amount of steroids (2.7 ng of estradiol and 14 ng of testosterone) to deionized water with 5% of methanol present to avoid steroid precipitation. Each sample was extracted with ethyl acetate as described above in Section 4.2.6, evaporated to dryness, reconstituted in CMCD matrix and subjected to CE separation. The recoveries of estradiol and testosterone, extracted in ethyl acetate were 106 % of each. Then the recoveries for testosterone and estradiol from extraction columns were measured. The aqueous sample of testosterone and estradiol was loaded on a quaternary amine SAX cartridge, followed by C18 cartridge application. Three elution fractions were collected, fraction 1 and 2 were 1 mL of methanol: water (75:25, v/v) each, and fraction 3 was 100 % methanol. The highest recovered concentration of steroids was measured only in the first fraction, 91 % recovery for estradiol and 84 % recovery for testosterone. No spiked 139

steroids were found in fractions 2 and 3. The results are summarized in Table 4.2. The advantage of these elution techniques is a high recovery of steroids from the extraction columns in a small volume of the solvent (1 mL), while leaving highly lipophilic substances, which require less polar solvent for elution, strongly retained on C18 material.

Table 4.2. Recovery of standard 17β-estradiol and testosterone from aqueous solutions.

Steroid	Spiked	Recover	red
	ng	ng	%
Ĩ17β-estradiol			
EA extraction	2.7	$2.9\pm0.6$	106
$(N^{+})$ SAX + RP C18 fraction 1	2.7	$2.5\pm0.2$	91
Testosterone			
EA extraction	14	$15 \pm 3$	106
$(N^+)$ SAX + RP C18 fraction 1	14	$12 \pm 2$	84

EA = ethyl acetate

SAX = strong anion exchange cartridge

RP C 18 = reversed phase C18 cartridge

# 4.3.5.6. Steroid Recovery from Fish Plasma

To determine whether the biological origin of the fish species can affect the steroid analysis using the optimized procedure, four different fish species: catfish (*Ictalurus punctatus*), smallmouth bass (*Micropterus dolomieu*), largemouth bass (*Micropterus salmoides*), and yellow perch (*Perca flavescens*) were used for investigation. Each blood or blood plasma sample was split in half. One half was unspiked and analyzed to measure the endogenous steroids. The other half of the plasma sample was spiked with 2.7 ng of estradiol and 14 ng of testosterone and was used to measure the recovery of steroids comparing to the unspiked sample. The sample pretreatment and sample analysis were performed similar to the method described in Section 4.2.8. Briefly, each sample was extracted with ethyl acetate, evaporated to dryness, reconstituted in MOPS, loaded on a quaternary amine SAX cartridge, followed by C18 cartridge purification. The first 1 mL of methanol: water (75:25, v/v) was collected, evaporated to dryness, reconstituted in CMCD buffer and analyzed by CE as described in 3.2.5. A schematic flow-chart for sample pretreatment is illustrated in Figure 4.10.

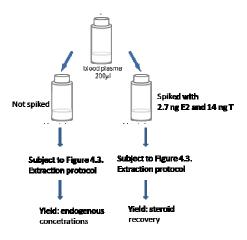


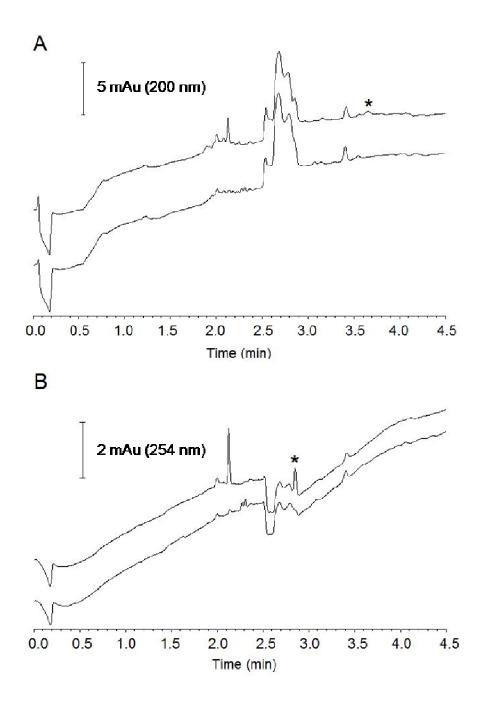
Figure 4.10. Steroid recovery from fish plasma. Flow chart of the plasma sample preparation.

The results are summarized in Table 4.3. All the concentrations were measured using a three-point external standard calibration curve. The estradiol recovery ranged from 74 % in catfish blood to 85 % in yellow perch plasma, and testosterone recovery ranged from 77 % in catfish blood to 102 % in largemouth bass ( $\mathbb{R}^2 \ge 0.98$ ). Figure 4.11 illustrates representative electropherograms of estradiol and testosterone recovery from a catfish blood sample. After running the sample by CE, it was spiked with additional amounts of targeted steroid and reanalyzed. The increase of the peak at a specific migration time for each added steroid confirmed the presence of the steroid in the sample.

Steroid	Sample volume	Spiked	Recovered	Recovered		Linear regression data parameters		
	μl	ng	ng	%	Slope	Intercept (×10 <sup>3</sup> )	R <sup>2</sup>	
17β-estradiol								
Catfish*	120		$2.0\pm0.2$	74	$19 \pm 1$	$-0.1\pm0.1$	0.998	
Smallmouth bass	100	2.7	$2.1\pm0.0_5$	78	$14\pm03$	$0.1\pm0.0_5$	1.000	
Yellow perch	100	2.7	$2.3\pm0.6$	85	$17 \pm 1$	$-0.2 \pm 0.3$	0.997	
Testosterone								
Catfish*	h* 120		$11 \pm 1$	77	$3.4\pm0.2$	$0.0_2\pm0.1$	0.997	
Smallmouth bass	100	14	$133\pm02$	94	$3.1\pm0.0_2$	$0.1\pm0.0_1$	1.000	
Largemouth bass	100	14	$14.5\pm0{2}$	102	$3.1\pm0.0_2$	$0.1\pm0.0_1$	1.000	
Yellow perch	100	14	$12 \pm 3$	84	$2.6\pm0.4$	$0.4   0.0_8 \pm 0.2   0.982$		

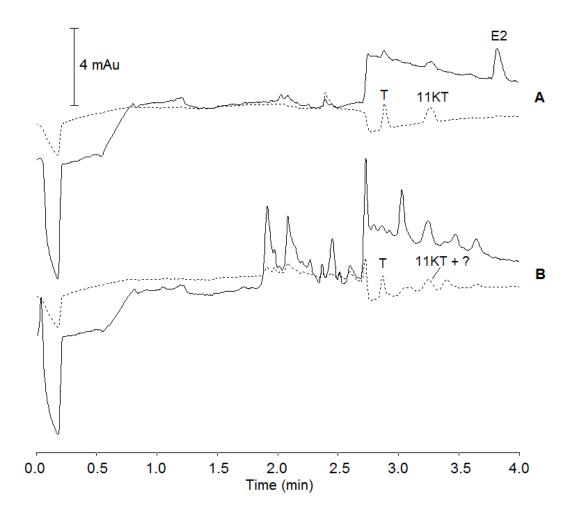
**Table 4.3.** Recovery of standard steroids from fish plasma.

\* - catfish blood stored at -80 °C for 7 years.



**Figure 4.11.** Steroid recovery data from a single catfish blood sample. Lower trace in A and B is the electropherogram obtained for the unspiked catfish blood extract. Top trace in A and B is catfish blood spiked with 14 ng of testosterone and 2.7 ng of  $17\beta$ -estradiol prior to extraction. In A, \* is recovered estradiol; in B, \* is recovered testosterone.

In teleost fish 11-ketotestosterone is important for sexual differentiation and male sexual development (51, 52) and is a mediator of altered cellular function and differentiation, which makes steroid availability susceptible to environmental signals. While performing the measurement of the steroids in the fish plasma it was noticed that the levels of 11-ketotestosterone were much higher comparing to the results reported in the literature. Although both testosterone and 11-ketotestosterone strongly absorb light at the 254 nm wavelength they also produce low detection response at 200 nm as demonstrated in Figure 4.12-A. The intensity of the peak corresponding to the migration time of testosterone in the reconstituted fish plasma did not deviate from the effect observed for a standard steroid mixture, however, the opposite was observed for the peak corresponding to the migration time of ketotestosterone (Fig. 4.12-B). The intensity of this peak was much higher at 200 nm than at 254 nm thus suggesting the presence of an interfering compound which migrates at the same time as 11-ketotetsosterone. 11ketotestosterone was not quantified in the following fish studies as it requires massspectrometry detection in order to evaluate the nature of the interference and eliminate its presence from the sample for the accurate 11ketotestosterone detection.



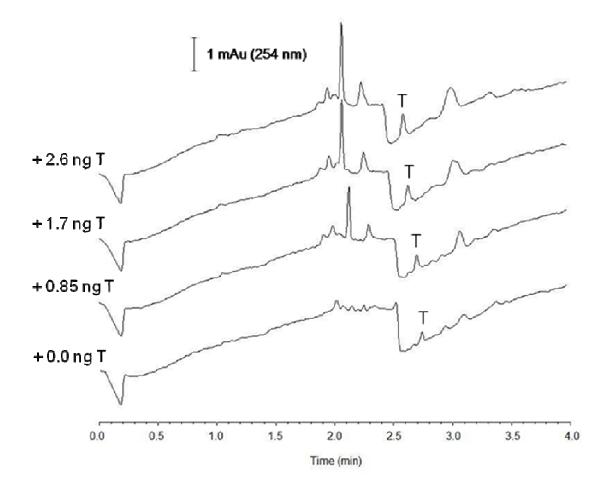
**Figure 4.12.** The electropherograms representing the analysis of a sample containing standard T, 11 KT, and E2 (A), and yellow perch plasma extract (B). Electropherograms shown in solid are measured at 200 nm, electropherograms represented in dotted lines are measured at 254 nm. Standard sample contained 250 nM of estradiol, 550 nM testosterone and  $2\mu$ M 11 ketotestosterone. Standard samples were prepared in 1 mM CMCD, 5% methanol in 50 mM CAPS buffered at pH 10. The blood extract was reconstituted in the same sample buffer. The separation conditions are outlined in the text.

### 4.3.5.7. Calibration Method and Effect of Plasma Volume on Stacking

The results obtained with the use of a three point external standard calibration curve were compared to the results measured with a three point standard addition method. The effect of the plasma sample volume on the analysis of steroids by the stackingmethod was studied as plasma volume can cause the variance in steroid recovery because the amount of extracted steroids as well as the interfering compounds increases with the increased plasma volume. A plasma sample from a single yellow perch fish was used for this experiment. A 100 µL plasma volume was subjected to extraction with ethyl acetate and two solid-phase extraction columns as described in Section 4.2.8. An endogenous concentration of testosterone measured using a three point standard calibration curve was equal to  $12.6 \pm 0.1$  ng/mL plasma ( $R^2 = 1.000$ ). By means of a standard addition method  $11 \pm 1$  ng of testosterone was measured per 1 mL of plasma ( $R^2 = 0.990$ ). As a standard addition curve the sample was sequentially spiked with 0.85 ng of testosterone. Figure 4.13 is a representative example of a three point standard addition method used for analysis a yellow perch blood plasma sample. The results obtained by both calibration methods demonstrated close values, based on what, a 3-point standard calibration curve was further used for all the measurements.

In order to investigate how the volume of plasma taken for analysis affects the recovery of steroids the higher amount of the same plasma sample was extracted. The endogenous concentration of testosterone analyzed in 200  $\mu$ L of the same yellow perch plasma sample was found to be 13.5 ± 0.3 ng/mL of plasma (R<sup>2</sup> = 1.000). Thus, the

extraction procedure combined with stacking-CE analysis was not limited by a plasma sample volume.



**Figure 4.13.** Illustrative stacking electropherograms obtained by the method of standard addition for a blood plasma sample from a single yellow perch fish. The lower trace is unspiked plasma sample. A three point standard addition curve ( $R^2 = 0.990$ ) was obtained by repeatedly spiking the plasma extract with 0.85 ng of testosterone. Peak labeled T is testosterone.

#### 4.3.6. METHOD APPLICATION TO THE STEROID ANALYSIS IN YELLOW PERCH

The stacking-MEKC method was applied to the analysis of a larger set of yellow perch fish provided by the researchers from USGS who were interested in studying the possibility of endocrine disruption in yellow perch. Yellow perch (*Perca flavescens*) are a valued resource in the Chesapeake Bay watershed and the Great Lakes as they are exploited commercially and recreationally. Historically, yellow perch in the Chesapeake Bay have supported a major commercial fishery, as well. However, the harvest declined from over one million pounds per year around 1900 to 66,000 pounds in 1990 (53). Landings rebounded to nearly 300,000 pounds by 2002, driven primarily by catches in the Head-of-Bay region (54). In 1989, these population declines led to commercial and recreational closures (54). While some rivers have reopened their recreational fishery, several suburbanized tributaries (Severn, Magothy, South, and West Rivers) have not. One operational hypothesis for the decline of yellow perch in these rivers was that urban growth and development near the watersheds reduced the water quality, thereby decreasing recruitment success (55). Poor yellow recruitment has been observed in the Severn River, while stocks remain rather health in the Choptank River.

In the method application study eleven fish plasma samples were analyzed by pHstacking MEKC. The fish samples were collected by the USGS researchers from two different sites: Severn River and Choptank River, sacrificed and processed for plasma samples as described in Section 4.2.5. The reproductive characteristics of the yellow perch fish used in this research have been studied by different fish biologists for several years (56-61). The plasma levels of estradiol, testosterone, 11-ketotestosterone and dihydroxyprogesterone in male and female yellow perch are summarized in Table 4.4. For female fish the concentration range for steroids in plasma are: 0.1 - 5 ng/mL estradiol; 0.1 - 30 ng/mL testosterone; 0.1 - 2 ng/mL dihydroxyprogesterone. In the male fish the reported levels of the steroids in plasma are: 0.2 - 8 ng/mL 11-ketotestosterone range; 0.1 - 8 ng/mL testosterone; 0.01 - 0.02 ng/mL estradiol. All the values reported in the literature were determined by radioimmunoassay.

Female yellow p	erch		Male yellow perch			
E2, ng/mL	T, ng/mL	DHP, ng/mL	11KT, ng/mL	T, ng/mL	E2, ng/mL	
$0.4 \pm 0.1$ to $3.0 \pm 0.3$ (fall); $0.2 \pm 0.1$ to $2.5 \pm 0.5$ (winter)	$2.0 \pm 0.5$ to $3.0 \pm 0.5$ (fall); $2.0 \pm 0.5$ to $8 \pm 3$ (winter)	Not reported	$2.0 \pm 0.5$ to $4.0 \pm 0.5$ (fall); $2.0 \pm 0.5$ to $7 \pm 2$ (winter)	2.5 $\pm$ 0.5 to 6.0 $\pm$ 0.5 (fall); 2.0 $\pm$ 0.2 to 8 $\pm$ 4 (winter)	Not reported	56
Not reported	Not reported	Not reported	$0.20 \pm 0.05$ to $0.6 \pm 0.3$	0.1 to 4.3	0.01 to 0.02	57
0.087 to 0.155	0.469 to 0.793	Not reported	0.523 to 0.881	0.856 to 1.035	Not reported	58
$0.08 \pm 0.05$ to $5.13 \pm 1.42$	0.1 to 30	Not reported	Not reported	Not reported	Not reported	59
0.1 to $3 \pm 1$	$0.1$ to $7 \pm 1$	$0.1$ to $2.0\pm0.5$	8.0 ± 1.2	$2.8 \pm 0.8$	Not reported	60

Table 4.4. Levels of plasma steroids in yellow perch fish measured by RIA reported in the literature.

If 200  $\mu$ L of blood is collected from each fish, extracted and reconstituted in a sample volume suitable for automated CE measurements (30  $\mu$ L) the endogenous concentrations of steroids in this sample are expected to be for the female fish: 2.5 - 120

nM estradiol; 2.3 – 690 nM testosterone; 2.0 - 40 nM dihydroxyprogesterone. In male fish samples these concentrations will be: 4.4 - 180 nM 11-ketotestosterone; 2.3 – 190 nM testosterone; 0.25 - 0.50 nM estradiol. The limits of detection of the developed method are reported in Table 4.1 and are  $2.9 \pm 0.6$  nM estradiol,  $4 \pm 1$  nM testosterone,  $11 \pm 3$  nM dihydroxyprogesterone and  $14 \pm 3$  nM 11-ketotestosterone. The linear range of the method is  $0.050 - 5.0 \mu$ L estradiol,  $0.10 - 14 \mu$ L testosterone,  $0.20 - 5.0 \mu$ L dihydroxyprogesterone, and  $0.50 - 10 \mu$ L 11-ketotestosterone. Due to the low concentrations of the endogenous steroids in fish only major steroids were quantified in this work. The results for steroids measured in yellow perch by stacking-CE are summarized in Table 4.5. The results are obtained from a three point external calibration curve. Because of the limited plasma volumes available for this study, the obtained values were based on a single analysis for each yellow perch sample. Each analyzed plasma volume was 200 µl except for SE 21, which was 250 µl. As seen from the data the concentrations of steroids measured by the developed method were at the higher range of the expected levels reported in the literature.

		Testosterone	Testoste	erone	Estradiol	Estradio	bl	Dihydroxy- progesterone
Plasma	Fish	Stacking-CE	RIA		Stacking-CE	RIA		Stacking-CE
sample	sex	(ng/mL	(ng/mL	plasma)	(ng/mL	(ng/mL	plasma)	(ng/mL
code <sup>b</sup>		plasma)			plasma)			plasma)
			1	2		1	2	
SE 21	F	$28{28}\pm0{04}$	25.16	26.67	$1.5 \pm 0.4$	1.35	1.21	-
SE 24	F	$135\pm03$	10.91	11.73	-	0.97	0.93	$16\pm7$
SE 25	М	$30.5\pm07$	29.28	26.64	-	0.264	0.203	$16 \pm 6$
SE 26	F	$439\pm09$	41.59	39.6	-	0.997	0.924	5 ± 7
SE 28	F	$1.3 \pm 0.9$	2.68	1.91	-	0.688	0.725	$34 \pm 6$
SE 29	М	$9.3\pm0.8$	7.34	7.82	-	0.277	0.31	$11 \pm 7$
SE 32	М	$15.5\pm0.5$	13.55	12.94	$1.3 \pm 0.3$	0.248	0.281	-
CH 23	F	0.9 ± 0.6	0.986	1.0	$1.7 \pm 0.8$	1.44	1.62	-
CH 24	М	$4.8\pm0.6$	4.69	4.74	-	0.294	0.221	-
CH 27	F	$20\pm 2$	27.22	28.38	-	0.993	1.07	$19\pm8$
CH 36	М	ND	30.52	27.8	$1.2_8\pm0.0_5$	0.281	0.392	-

Table 4.5. Analysis of endogenous steroids in blood plasma of yellow perch<sup>a</sup>

<sup>a</sup> Results are obtained using a 3 point external calibration curve (n = 1). Each analyzed plasma volume was 200  $\mu$ l except for SE 21 which was 250  $\mu$ l. <sup>b</sup> SE - Severn River; CH - Choptank River; ND – not determined; and (–) – below lowest standard, not quantified.

#### 4.3.7. METHOD COMPARISON TO COMMERCIALLY AVAILABLE TECHNIQUES

There are two ways to evaluate the accuracy of the developed method: (1) by performing the recovery studies of the steroid-free plasma that is spiked with gravimetrically determined amounts of a steroid and (2) by comparison to the measurements obtained by an independent method. In biomedical laboratories steroid hormones are frequently quantified by immunoassay technique (Section 1.2.3). Most of the immunoassays used are commercially available kits, which contain all the reagents required to perform an instrumental measurement of steroids in biological samples. However, a few publications reported a need for more thorough validation of the commercial immunoassay kits prior to use (62-65). The lack of specificity and the need of a different assay for each steroid are the major problems associated with immunoassays for the steroids. Taieb and co-workers (66) conducted a study in which they compared ten different commercially available immunoassay kits with isotope-dilution GC-MS for analysis of testosterone in sera of 116 men, women and children. They concluded that none of the immunoassays tested were fully reliable for analysis of testosterone. Seven immunoassays demonstrated systematic 46% overestimation and the other three showed systematic 12% underestimation of the testosterone levels compared to the results obtained by the isotope dilution GC-MS. The possible reasons for the poor agreement between the results obtained by the two methods were: (1) matrix effect; (2) crossreactivity of the antibody with structurally similar steroids and metabolites; (3) the limit of detection and sensitivity of the immunoassay. Other reports suggested the measurement of plasma testosterone by current commercially available immunoassay is also a matter of concern for similar reasons (67, 68).

Isotope-dilution GC-MS, another reference method (Section 1.2.2) requires expensive isotope standards and in order to be applied for method comparison in our lab, the method had to be developed and optimized with respect to the fish plasma sample matrix. In order to save time an attempt to find contract laboratory groups who had validated the isotope-dilution GC-MS method in-house and would be interested to analyze a few fish plasma samples prepared by our method for the presence of targeted steroids and method comparison. Due to difficulty locating any commercial laboratories capable of isotope-dilution GC-MS analyses of steroids a few laboratories which analyzed steroids by isotope-dilution LC-MS/MS were contacted. It was shown previously that isotope-dilution LC-MS/MS demonstrates the same accuracy for steroid analysis as isotope dilution GC-MS (69-71) and is more popular in the clinical laboratories. Several contract laboratories have been contacted (72, personal communication by L. Bykova); however, most indicated concern that their methods have not been validated for the fish plasma matrices. Therefore, the developed method was evaluated by performing the recovery studies and by comparison to immunoassay which was available from the USGS collaboration group.

As expected some steroids were below the limit of detection, some fell into the expected concentration range, while some steroids were detected at higher concentrations than expected. The results were compared to radioimmunoassay as described in Section 4.2.9. The 80 % of the results measured by stacking-CE were close to and deviated by no more than 20 % from the results measured by radioimmunoassay. Testosterone was not detected by the stacking method in the sample CH 36 due to a shift in the baseline of the 153

electropherogram caused by unknown reasons that interfered with the detection of the testosterone peak. The levels of estradiol in SE 32 and CH 36 exceeded the results reported by radioimmunoassay by > 74% and could be due to the fact that the peak areas for estradiol measured by stacking-CE corresponded to the concentrations at the very low region of the calibration curve where larger standard deviations were observed. The concentration of estradiol in samples SE 24-26, 28, 29 and CH 24, 27 was not quantified because the peak areas for estradiol corresponded to the concentrations below the lowest steroid standard in the calibration curve. Dihydroxyprogesterone, in turn, was not measured by radioimmunoassay and, therefore, could not be compared to stacking-CE. However, the presence of dihydroxyprogesterone was determined in the female plasma samples SE 24, 26, 28, and CH 27 as well as in two male plasma samples SE 25, and 29 by stacking-CE.

### 4.3.8. FISH INSIGHT

While in males the levels of 11-ketotestosterone and testosterone remain constant throughout the year, the high levels of testosterone in female fish are due to the fact that no estradiol needs to be produced after the vitellogenesis (formation of the yolk of an egg) in the females (*17*). Because the yellow perch in this study were captured during their spawning which occurs after the vitellogenesis the high levels of testosterone in the female fish (see Table 4.5) are not surprising. The presence of dihydroxyprogesterone in female samples SE 24, 26, 28, and CH 27 suggests the presence of the developing oocytes in these fish species, while the presence of dihydroxyprogesterone in two male plasma samples, SE 25 and 29, is alarming because it can be associated with the presence of the developing eggs in the males. Both, SE 25 and 29, samples were collected from the Severn River 154

where the recruitment of the yellow perch population has been decreasing over the past few years due to the water pollution caused by the rapid urbanization of the surrounding areas (*55*). Ethynyl estradiol, a component of the birth control pills and a potential EDC, was not found in the fish plasma samples. The data for 11-ketotestosterone is not included (Table 4.5) as it co-migrates with an unknown interference and thus requires a mass-spectrometry analysis in order to determine it.

# **CONCLUSIONS AND FUTURE DIRECTIONS**

Chapter 4 demonstrated the development of a three step extraction protocol for the plasma pretreatment prior to the stacking-MEKC analysis which was validated using the aqueous steroid samples and the extracts of the plasma from four different fish species. The optimized extraction protocol followed by pH-stacking-MEKC method was applied to the analysis of steroids in the plasma of yellow perch and the obtained results were validated by immunoassay. The method showed good promise for the analysis of steroids in plasma samples.

In future studies, the method can be applied to other fish species, wild or lab maintained. The lab maintained fish, for example Japanese medaka, is widely used in biological studies due to their ability to reproduce in the lab and the short period of the development of sexual differentiation, which is extremely important for monitoring endocrine disruption. The small size of the fish allows for the statistical description of a large fish population. Using robotic systems available in many pharmaceutical laboratories, it is possible to multiplex the set of the samples prepared for analysis simultaneously to a minimum of 96 samples. Therefore the total sample preparation time will be considerably decreased.

Additional studies may include the analysis of the steroid conjugates circulating in the plasma, glucuronides and sulfates. Plasma samples can be treated by enzymes for hydrolysis of the steroid conjugates. Simultaneous analysis of all the sample constituents is important because it is desirable to determine not only the targeted steroids but also any interfering compounds or unexpected steroid metabolites which may appear as a result of the fish exposure to the EDCs. CE-MS can be used for structural elucidation of those steroids and conjugated steroids for which no standards are available.

The method can be incorporated on a microchip to create a portable device that can be used for the field studies. This device will eliminate the need of collecting fish plasma samples and sending them over to chemistry laboratories for profiling steroids in fish samples.

Finally the method can be applied for analysis of steroids in human plasma. In this dissertation research the developed method was applied to the analysis of a human serum certified reference material with a known content of estradiol. Human serum (17 $\beta$ -estradiol, high level) certified reference material was purchased from RT-Corp (Laramie, WY). The sample was certified by BCR (Community Bureau of Reference, the former reference materials program of the European Commission). A certified concentration of 17 $\beta$ -estradiol present in human serum standard was 1.34 ± 0.07 nmol/L measured by isotope dilution GC-MS. The method used different isotopic labeled internal standards and chromatographic columns, following extraction with CH<sub>2</sub>Cl<sub>2</sub> and clean-up by solid-phase

extraction. The standard was reconstituted following instructions in the certificate of analysis. However, the obtained results for the estradiol concentration measured in human serum standard were inconclusive because of the sample matrix effect. This was expected to occur as the composition of human serum differs from the composition of fish plasma and the levels of endogenous steroids in fish are higher than those in humans. Therefore the analysis of human biological fluids requires a reconsideration of the plasma/serum pretreatment scheme prior to the use of the stacking-CE analysis.

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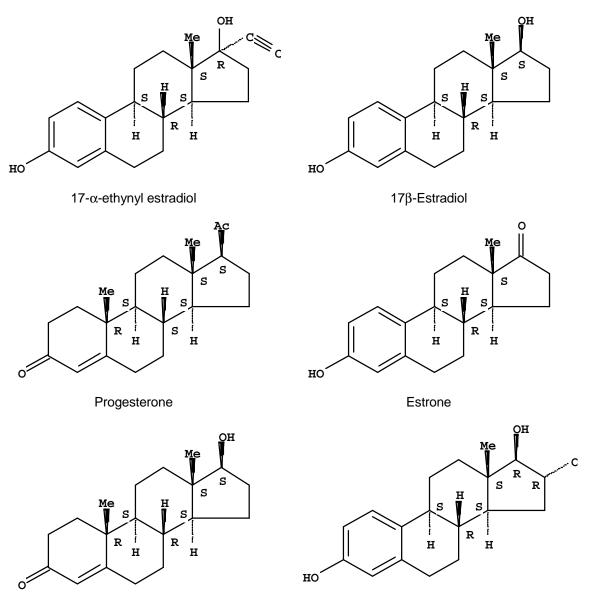
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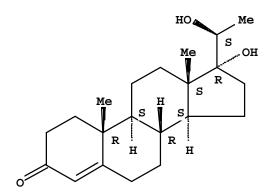
# APPENDIX

Table A.1. Chemical structures of sex steroid hormones employed in research.

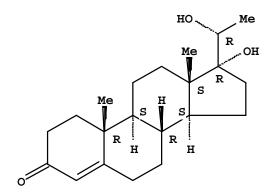


Testosterone

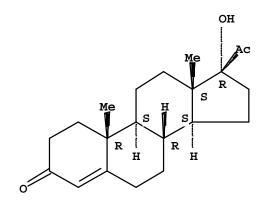
Estriol



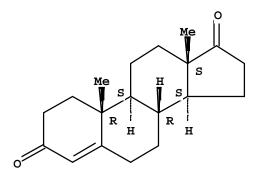
 $\alpha\alpha$ -Dihydroxyprogesterone



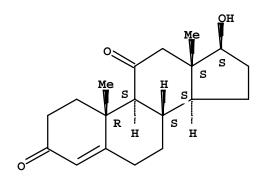
 $\alpha\beta$ -Dihydroxyprogesterone



17-hydroxy-progesterone



Andorstenedione



11-Ketotestosterone

# **CURRICULUM VITAE**

# **EDUCATION**

2004 – 2009: Ph.D. Student – Analytical Chemistry, Chemistry Department, West
Virginia University, Morgantown, WV, USA
Graduation: May 2009
Advisor: Dr. Lisa A. Holland
2001 – 2002: Specialist of Science – Environmental Chemistry, Department of

- 2001 2002: Specialist of Science Environmental Chemistry, Department of Chemistry, National Taras Shevchenko University of Kyiv, Kyiv, <u>UKRAINE</u> Advisor: Prof. Olga A. Zaporozhets
- <u>1997 2001: Bachelor of Science Environmental Chemistry, Department of Chemistry,</u> <u>National Taras Shevchenko University of Kyiv, Kyiv, UKRAINE</u> Advisor: Prof. Olga A. Zaporozhets

# **Research Experience**

Separation chemistry (capillary electrophoresis, micellar electrokinetic chromatography, high performance liquid chromatography, gas chromatography); development of preconcentration techniques for sensitivity enhancement in separation methods; analysis of endocrine disruption compounds.

# **TEACHING EXPERIENCE**

2004 – 2008: Teaching assistant for general chemistry laboratory sessions (CHEM 110,112, 115, 116).

2008 (spring): Developed and introduced into practice a new laboratory procedure for CHEM 112 under the supervision of Dr. Betsy Ratcliff.

# **AWARDS**

WVU Dissertation Fellowship 2008-2009

Forrest Ferrell Award 2008 (outstanding teaching assistant award)

# **CONFERENCE PRESENTATIONS**

1. pH-Stacking CE Analysis of Steroids in Fish. <u>Liliya Bykova</u>; Bollinger, C. ; White, C.; Holland, L. A. 60th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, McCormick Place, Chicago, IL, USA, March 2009

- 2. Analysis of Sex Steroid Hormones by Sample Stacking CE. <u>Liliya Bykova</u> and Lisa A. Holland *Eberly College of Arts & Sciences' Research Horizons Poster Day*, Morgantown, WV, USA, March 2008
- Sample Stacking MEKC Approach for Profiling Endogenous Sex Steroid Hormones for Monitoring Environmental Contamination. <u>Liliya Bykova</u> and Lisa A. Holland 59th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Morial Convention Center, New Orleans, LA, USA, March 2008
- 4. MEKC Approach for Analysis of Sex Steroid Hormones. <u>Liliya Bykova</u> and Lisa A. Holland *Eberly College of Arts & Sciences' Research Horizons Poster Day*, Morgantown, WV, USA, April 2007
- Capillary Electrophoresis Approach for Sex Steroid Hormones. <u>Liliya Bykova</u> and Lisa A. Holland 58th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, McCormick Place, Chicago, IL, USA, March 2007
- Rapid Separation-Based Analysis of Sex Steroid Hormones. <u>Liliya Bykova</u>; C. White,
   L. A. Holland 57th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Orange County Convention Center, Orlando, FL, USA, March 2006
- 7. Microanalytical Approaches for Concentration and Separation of Sex Steroid Hormones. <u>Liliya Bykova</u>; C. White; L. A. Holland *ECAS Research Poster Session*, Morgantown, WV, USA, October 2005
- 8. Micellar Electrokinetic Capillary Chromatography Approach of Sex Steroid Hormones. Liliya Bykova; C. White; L. A. Holland 2005 Conference on the Application of Systems Biology Methodologies to Environmental Research, Morgantown, WV, USA, August 2005

# **Peer Review Journal Publications**

- 1. <u>L. Bykova</u>, L. R. Iwanowicz, V. Blazer, L. A. Holland, "Steroid analysis in fish plasma by pH-mediated Stacking MEKC", to be submitted in Environmental Toxicology and Chemistry.
- 2. <u>L. Bykova</u>, L. A. Holland, "Stacking enhanced determination of steroids by CE", Electrophoresis 2008, 29, 3794–3800.