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Method Development For A Chemical And Biological Analysis Of Glucocorticoids In Wastewater

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METHOD DEVELOPMENT FOR A CHEMICAL AND BIOLOGICAL ANALYSIS OF GLUCOCORTICOIDS IN WASTEWATER

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Dedication

To my loving family

METHOD DEVELOPMENT FOR A CHEMICAL AND BIOLOGICAL ANALYSIS OF GLUCOCORTICOIDS IN WASTEWATER

By

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Abstract

Glucocorticoids are a class of steroid hormones that can either be produced synthetically or naturally by the adrenal glands. The synthetic glucocorticoids are highly prescribed in the United States for their anti-inflammatory and immunosuppressive properties to treat a variety of ailments and diseases; however, these have been implicated in a number of adverse human conditions such as obesity, metabolic syndrome, immune-suppression, delayed puberty, adrenal insufficiency and Cushing Syndrome, among others. Ongoing research has shown that synthetic and natural hormones are transported to aquatic environments via mammalian excretion or wastewater effluent, and as a result, the release of glucocorticoids into the environment is potentially creating reproductive stress and mutations on aquatic vertebrates. The occurrence of glucocorticoids in environmental samples has only been reported worldwide in limited articles; nonetheless the studies have led to growing awareness of their potential environmental and health impacts at low concentrations ng/L. Their effects in the environment are unknown; however, the aforementioned compounds in water resources are of increasing concern due to their ability to act as endocrine disruptors.

Given that synthetic and natural estrogens have been previously found in wastewater influent and effluent for the Paso Del Norte Region, we hypothesized that glucocorticoids would be detected at significant concentrations. The objective of this research was to develop and optimize a chemical and biological method for the detection of glucocorticoids using solid phase extraction (SPE) followed by high performance liquid chromatography mass spectrometry (HPLC-MS)—in addition to a biological analysis using a 4-hour yeast assay.

Four glucocorticoids were included in this study and they are cortisone, hydrocortisone, prednisone and prednisolone. The average glucocorticoid concentrations in influent and effluent ranged from 4.62 (prednisolone/cortisone) to 15.56 (prednisone) ng/L, and from 3.43 (prednisolone/cortisone)

to 12.57 (prednisone) ng/L, respectively. Prednisone had the highest levels among the four glucocorticoids in all wastewater samples potentially reflecting a high consumption of prednisone for asthma and allergy treatments in our region. Wastewater samples were collected at different time of the day, 8:00 a.m., 12:00 p.m., and 6:00 p.m. The averages of the total glucocorticoids (i.e. sum of the four compounds) were 33.25 (±6.37), 24.20 (±3.54), 20.87 (±0.77) ng/L and effluent: 20.09 (±1.63) ng/L 24.23 (±6.38) ng/L, and 19.46 (±0.99) ng/L for 8:00a.m, 12:00p.m., and 6:00p.m. samples, respectively. For the influent samples, the finding showed that the levels of glucocorticoid in the morning hour samples were higher than that in samples collected from the rest of the day. As for the effluent samples, glucocorticoids are higher in the 12 pm samples. A 17.9 (±9.7) % estimated removal for the Northwest Wastewater Treatment Plant was calculated. To date, research has not identified a specific technology or wastewater treatment process that has the potential to completely remove glucocorticoids from wastewater effluent however anaerobic treatment is highly recommended.

Two previously hGR transfected yeast cells were tested for detecting glucocorticoids in wastewater: one with a higher promoter activity (MCY-212) in comparison to a lower promoter activity yeast cell (DSY-1345). The half maximal effective concentrations, $EC_{50,}$ for the glucocorticoids of interest were found to be in the μM range which is equivalent to high mg/L concentration. The results indicated that the biological analysis is not a suitable method for analyzing glucocorticoid activity in wastewater since the sensitivity of the assay was not sufficient for samples with concentrations of ng/L levels.

Even though this research was focused on the Paso Del Norte region, the presence of glucocorticoids in the environment is a worldwide problem. Semi-arid and arid regions represent 30% of the world's continental area. Limited water resources have the potential to be highly impacted by anthropogenic activities. It is important to target the removal of glucocorticoids and other environmental endocrine disruptors during wastewater treatment processes to avoid further dispersal of these

contaminants of emerging concern to freshwater systems and protect the water resources for the safety of public health.

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Chapter 1: Introduction

In the past century, population growth and industrial development have assisted in the creation of anthropogenic substances that could be harmful to human health and environment. A group of these compounds, —also known as endocrine disrupting compounds (EDCs), natural and man-made, can interfere with the function of the endocrine system potentially inducing adverse effects in humans and wildlife. Most of these compounds are found in everyday products—such as pharmaceuticals, personal care products, plastics, and food products containing residuals of agricultural chemicals. Much attention has been given to synthetic and natural steroid hormones, which even are found at small concentrations in the environment, but can consequently mimic endogenous hormones by binding to the receptor and blocking, altering, or mimicking the response. Steroid hormones are transported through the environment from human excretion and veterinary application in farming fields potentially leach into groundwater and sewage treatment effluents (Figure 1) (1) (2) (3). Wastewater from municipal sources is recovered and treated while veterinary waste is introduced into the environment directly through urine and manure. Urine is the primary source for environmental steroids while feces carry negligible concentrations (4) (5).

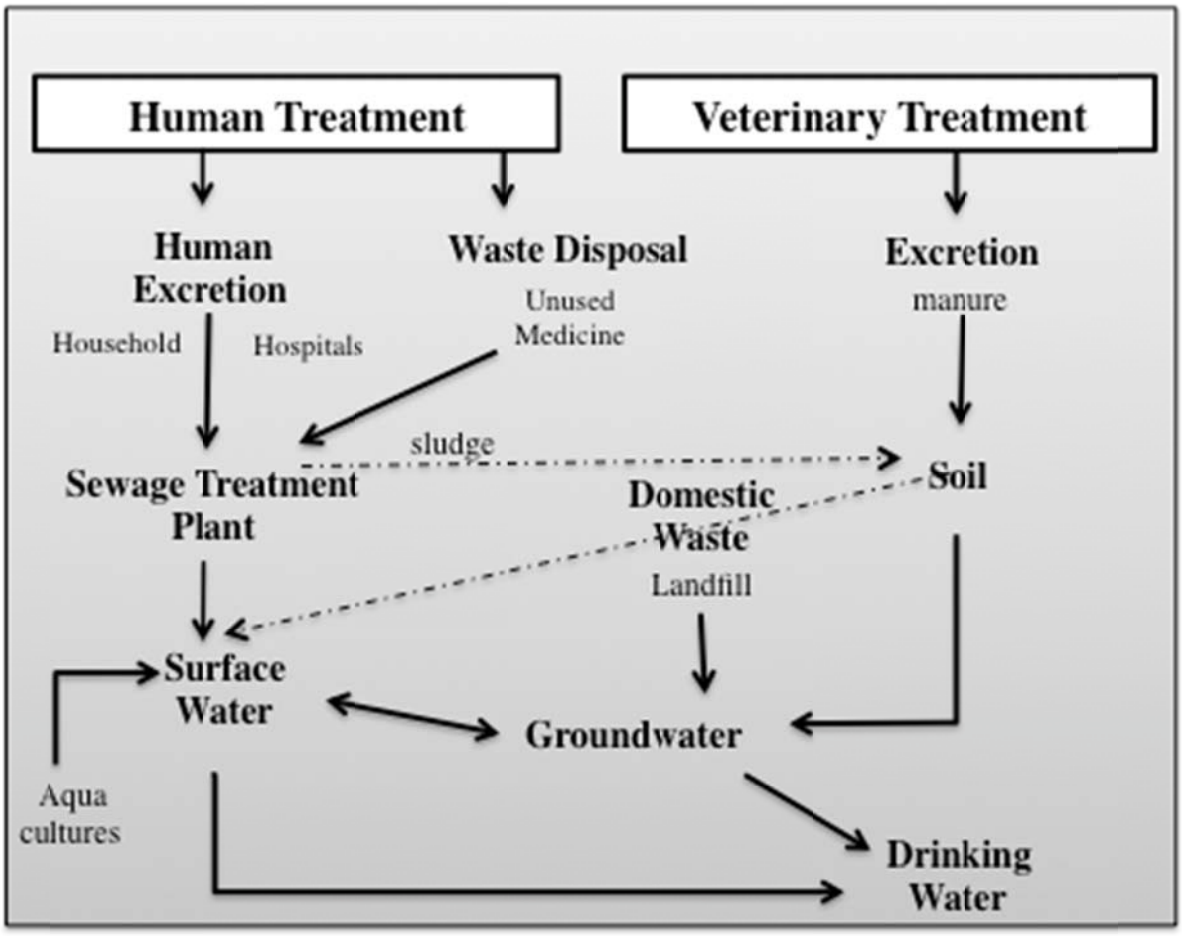


Figure 1 Scheme chart for environmental micropollutants and their transport through the environment(6)

There is a limited amount of data on the effects of anthropogenic compounds i.e. pharmaceuticals that exist in the environment as conjugates and metabolites and their potential effects to non-target species. The Food and Drug Administration (FDA) requires an environmental impact evaluation if a pharmaceutical is considered to significantly affect human health and the environment; (6) however, many FDA approved antibiotics, prescription drugs and non-prescription drugs have been detected at trace level concentrations for 80% of U.S stream samples; and, further, these concentrations were found within drinking water and aquatic life standards (7). Most federal regulations focus on nutrient monitoring, i.e. nitrogen and phosphorus, and their concentrations in freshwater systems but do

not apply to the detection of EDCs in the environment nor the removal of EDCs from wastewater treatment processes (8).

Studies have shown that the ecotoxicological effects of EDCs on vertebrates include disruptions in metabolism, reproductive, endocrine, and immune system henceforth affecting development (9). Exposures to estrogens in clinical and toxicological studies have shown altering the behavior in terms of fitness, survival and population level outcome for various types of fish (10) (11) (12). Alongside estrogens, androgenic and anti-androgenic compounds also have a strong presence in aquatic systems and they have been shown biological activity in the micromolar range for rivers in Italy (13). The synthetic androgen, 17β-trenbolone (TB), used in livestock as an anabolic steroid, has been found in receiving waters from livestock effluent—posing an ecological risk and potentially leading to masculinization of fish in that area (14). Androgens have shown to be the dominant endocrine disruptors in comparison to progestogens and estrogens in wastewater effluent in China (15). While the majority of studies for EDCs focus on sex steroid hormones such as estrogen and androgen, the dangers posed by another class of steroid hormones, glucocorticoids, have not been fully understood or studied. Little is known about new emerging compounds and their chemical transformation as they travel through the environment—posing a threat to wildlife, human health and the integrity of water quality. The scientific community has mainly focused on identifying their existence in the environment by tracing their occurrence, fate, and transport in the environment and assessing the physiological effects they pose on vertebrates in aquatic ecosystems.

1.1 Glucocorticoids

As mentioned before, most research regarding steroid hormones mainly focus on sex steroids, specifically: 17β-trenbolone (TB), 17β-estradiol (E2), estrone (E1) and estriol (E3) and contraceptive 17α-ethinylestradiol (EE2) (14) (16). Therefore, knowledge on a class of steroid hormones, known as glucocorticoids, in environmental matrices is limited. While estrogens and androgens induce changes in sex organs, glucocorticoids have the ability to affect many physiologic, metabolic and immunologic processes due to their ability to express in every cell in the body. Glucocorticoids are produced by the adrenal glands and bind to the glucocorticoid receptor (GR) forming the activated GR complex, and .are essential in regulation and maintenance of physiological processes such as cardiovascular, metabolic, immunologic and homeostasis development.

Cortisone and cortisol are steroid hormones naturally produced by the body while prednisone, hydrocortisone, prednisolone, dexamethasone, budesonide, and triamcinolone acetonide are synthetic drugs that are used to treat ailments such as asthma, allergies, osteoporosis, inflammatory diseases and veterinary purposes. In Great Britain, the glucocorticoid prescriptions have increased by 34% in the last two decades (17). For the U.S, several corticosteroid prescription drugs have made it to the 200 top RX drug list (Table 1) (18). Not all glucocorticoid require a prescription and are commonly found in drug stores to treat inflammatory ailments. Popular non-prescription glucocorticoids include hydrocortisone cream (Eumovate™ Cream) used for temporary relief of skin irritation, eczema, insect bites among other skin ailments. It should be noted that despite their therapeutic properties, glucocorticoids have been implicated in the advent of various health conditions, both mental/psychological and a variety of metabolic disorders (19).

Table 1 Corticosteroids found in Top 200 Prescribed list for 2009 in the United States (18)

Drug	Properties	Compound	Disease
Nasonex (64)	Anti-Inflammatory	Mometasone furoate monohydrate	Hay fever
Deltasone (50)	Antineoplastic Agents, Hormonal Anti-Inflammatory Immunosuppressant	Prednisone	Allergic reaction disorders
Advair Diskus (76)	Anti-Inflammatory	Fluticasone propionate	Asthma (COPD)
Medrol (107)	Anti-Inflammatory	Methyl Prednisolone	
Tobradex	Anti-Inflammatory Immunosuppressant	Dexamethasone	Eye Infection
Ciprodex Otic	Anti-Inflammatory Immunosuppressant	Dexamethasone	Ear Infection

1.2 Analytical Methods for Steroid Hormones

With the high consumption of pharmaceuticals and high production of commercial chemicals it is important to determine the transport and fate of these compounds in the environment with the help of chemical and biological analytical methods. The science behind analytical methods provides the experimental design and framework from sample collection to sample analysis where identification and quantification of synthetic and natural chemical components can be achieved. Biological based methods are promising in a way that a holistic effect can be measured through its toxicological potential in a biological system, while analytical identification exhibits sensitivity and precision regarding the compound specificity in an environmental sample. Analytical methods that measure the occurrence and concentration of steroid hormones in environmental samples consist of purification and analyte isolation by extraction and concentration, followed by chromatographic separation where identification and quantitation is achieved through mass spectrometric analysis. This research is focused on analytical and biological methods for detection of pharmaceuticals in wastewater.

1.2.1 Current Chemical Methods

The detection of steroid hormones can be performed with gas chromatography/mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) or fluorescence (20) (21) (22). Sample preparation for steroid hormones using GC-MS is time-consuming due to a derivatization step; the preferred method for these compounds is LC which combines solid phase extraction (SPE) for sample cleanup and extraction (2) (15). Mass spectrometric analysis is the most common method used to detect, identify and quantitate molecules. Many times, the combination of HPLC and MS is not sufficient to provide enough resolution thus the coupling of two or more mass analyzers, i.e. tandem mass spectrometers are used in complex matrices.

In theory, the separation of steroid hormones is based on the physicochemical characteristics and functional groups within a molecule and the proper combination of mobile and stationary phase affecting the separation based on polarity (Figure 2). In SPE, the analytes of interest and the unwanted matrix are filtered through hydrophobic and hydrophilic interactions with the end product of a concentrate of target analytes, making the chemical analysis more efficient, less time consuming, and overall successful.

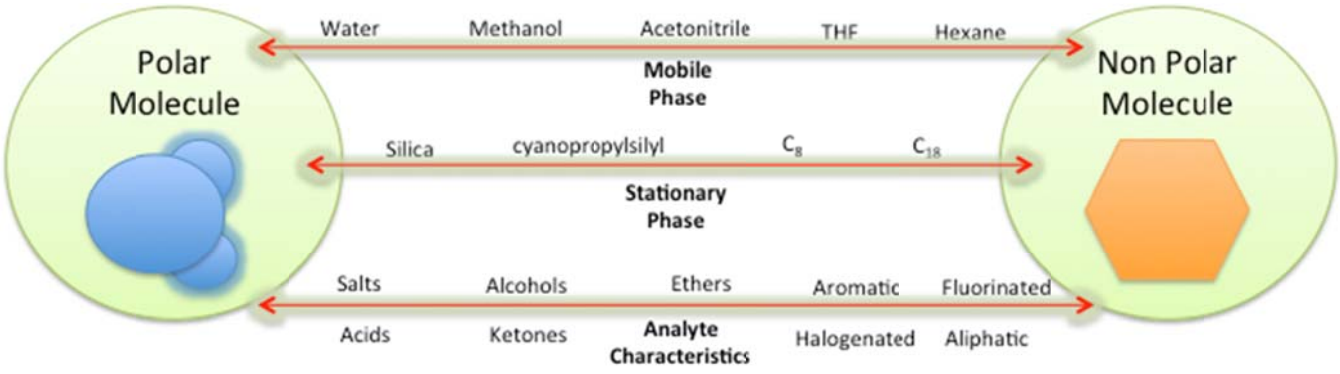


Figure 2 Polarity spectrum of analytes and the mobile and stationary phases in liquid chromatography

HPLC can be coupled to electron spray ionization (ESI) or atmospheric pressure chemical ionization (APCI) mass spectrometry. The ESI ionization is a soft method that allows less fragmentation of compound and is best suited for highly polar, non-volatile compounds. The analytes in the eluting solvent are introduced into the MS after a HPLC unit; high voltage is applied to create ultra-fine charged droplets; and as they disperse, the solvent evaporates increasing the electric field of the droplet. These droplets generate a protonated or deprotonated molecule that is then detected by the mass spectrometer. In comparison to ESI, APCI is suitable for low and medium polarity compounds. In this study, a reverse-phase SPE and HPLC-ESI-MS using Agilent 1100 coupled to the AccuTOF JMS-T100LC was used to perform the analysis of glucocorticoids in wastewater samples collected from local wastewater treatment facilities.

1.2.2 Current Biological Methods

In a biological method, the total biological effect is assessed. The principle of a bioassay is to compare biological activity produced a sample to a pharmaceutical standard, ultimately providing a quantitative estimation of the pharmaceutical's equivalent effects on living organisms. Depending on the analyte or biologic reaction of interest, the transfection of a responsive gene can be manipulated into yeast, mammalian, bacterial and plant cells. Well-known methods for the detection of estrogenic compounds based on the yeast-cell and mammalian cell model have been used for the detection of estrogenic, androgenic, and glucocorticoid activity in environmental samples (23) (24). In the yeast cell, *S. cerevisiae,* expression plasmids, receptor construct and reporter construct of specific hormone are introduced (25) (26). Expression plasmids include human estrogen receptor alpha (hERα), human estrogen receptor beta (hERβ), human androgen receptor (hAR), human progesterone receptor beta (hPRB) and human glucocorticoid receptor alpha (hGRα) coinciding with the correct ligand and receptor activating transcription in DNA (27). Reporter genes are also transfected into the yeast genome to measure the activity of the transfected gene i.e. hER, in the yeast population. Common reporter proteins

include β-Galactosidase, green fluorescent proteins and luciferase (28) (29). Recombinant yeast assay are robust, specific and suited to test characteristics of pure compounds (30). The limit of detection range for yeast assays are likely to be found at the low micromolar range while some can be detected in the picomolar range. In comparison, mammalian assays incorporate human cells with a transfected reporter gene but do not require plasmid transfection. In this type of assay, the cell culture preparation can take up to three days. After cell culture preparation, the cells are placed in 96-well in a steroid-free medium with the sample for an exposure period ranging from 24 hours to 5 days. Known estrogenic mammalian assays include ER-CALUX, MELN, T47D-KBlunc and E-Screen which incorporates T47D breast cancer cell, MCF-7 breast cancer cell, breast cancer T47D, and breast cancer cells MCF-7BOS, respectively (23). All mammalian assays follow a well-known protocol with a difference in sample exposure period ranging from 16 hours to 5 days. Mammalian assays are broadly used for biologic studies and are known to be highly sensitive and versatile; however, as aforementioned, they are also highly complex. Constraints include high costs, intense labor, time consuming and sterilization of cells prior to use and high risk for contamination. Mammalian cell based assays are recommended as initial screening tools to detect estrogenicity with an LOD in the picomolar to low nanomolar range (31).

In this project, the yeast estrogen screen (YES) method was used. The yeast cell *S. cerevisiae* is transfected with hGR and a reporter gene, i.e. lac-Z-gene, into the cell's genome. The glucocorticoid presence activates hGR inducing expression of the lac-Z gene. Substrates are added to produce β-Galactosidase, which is then measured by a spectrometer.

1.3 Wastewater Treatment Processes

A wastewater treatment plant (WWTP) constitutes a collection of structures designed to treat biological and chemical impurities in water promoting the re-use and recycling of wastewater. However, in the past 20 years, studies have reported that removal of pollutants in wastewater has not been completely accomplished by conventional water treatment (32) (33) (34). Studies carried out in Austria, Brazil, Canada, Croatia, England, Germany, Greece, Italy, Spain, Switzerland, The Netherlands and the U.S., have shown more than 80 compounds ranging from pharmaceuticals to drug metabolites detected in the aquatic environment (35).

The conventional design for wastewater treatment encompasses preliminary treatment, primary treatment, secondary treatment, and tertiary treatment in addition to residual management. Each WWTP is unique in the way that it is designed to treat certain volumes while combining different technologies for secondary and tertiary treatments, which are determined, based on industrial and agricultural processes occurring in the area. The Environmental Protection Agency (EPA) establishes water quality guidelines and regulations for surface and drinking water. These regulations prohibit certain pollutants from reaching WWTP or surface waters for more than 50 different industrial industries. These laws ultimately regulate point sources; however, they do not address regulatory effluents for emerging compounds in wastewater effluent. As the name implies, “emerging compounds” do not have established regulatory status and could potentially impact the environment and human health (36).

In this study, wastewater samples were collected from Northwest Wastewater treatment plant located in the west side of El Paso, Texas. The Northwest Wastewater Treatment plant treats 17.5 Million Gallons per Day (MGD) of industrial and residential wastewater and releases its effluent into the Rio Grande (37). The treatment process and sampling locations for this study are illustrated in Figure 3.

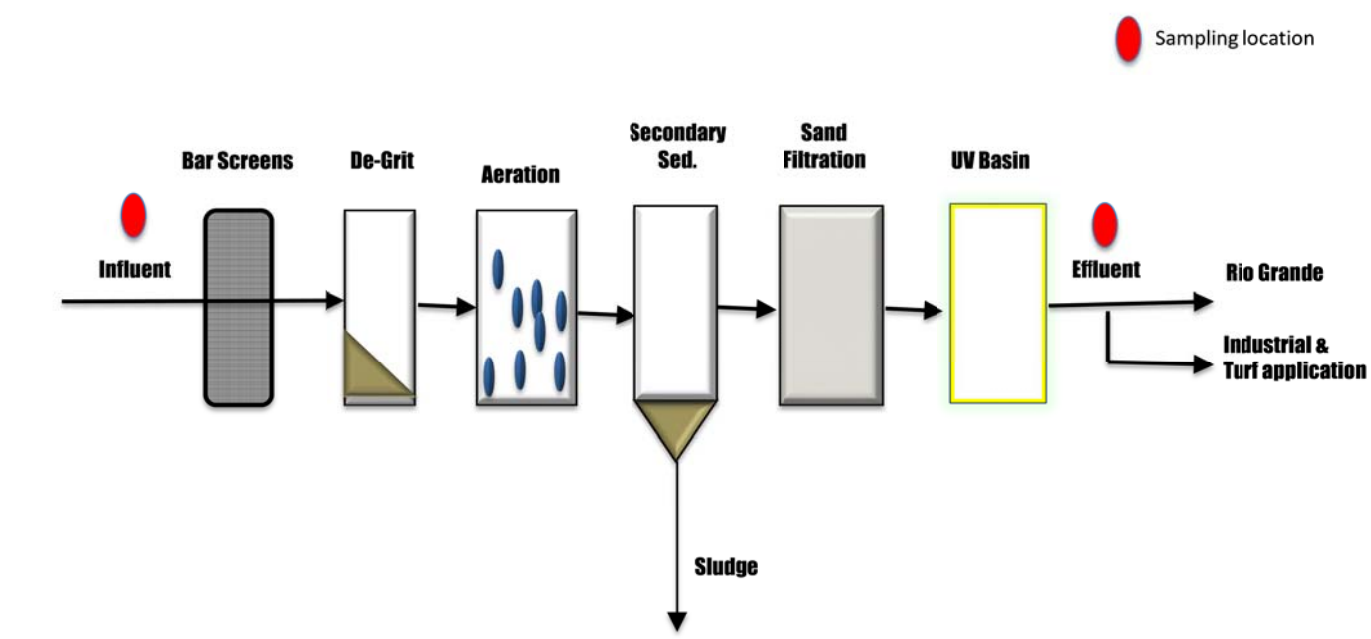


Figure 3 Northwest Wastewater Treatment Plant

In the Northwest Wastewater Treatment facility, the preliminary treatment encompasses bar screens and de-grit units that mechanically remove large material from incoming influent wastewater. The influent wastewater flow is adjusted to allow particles such as sand, gravel, or silt to settle in the de-grit unit. This grit material is treated with lime to kill pathogenic organisms. The wastewater is then sent to the aeration unit, also known as primary treatment, where aeration units provide oxygen for bacteria to thrive and enhance biological growth. In the secondary sedimentation unit, bacteria from the aeration unit settle out as flocs. Part of the sludge is sent back to aeration and the rest is dewatered and lime stabilized. In many cases, sludge is applied as fertilizer. The water follows sand filtration to remove solids that were too light to settle reducing turbidity. The water then is treated in the disinfection unit by ultra violet light further improving the quality of water. The water is then discharged to the Rio Grande or used for industrial and turf applications.

1.4 Research Objectives

The objectives of this research were to:

(1) Optimize a chemical and biological method for the detection of glucocorticoids using solid phase extraction (SPE) followed by liquid chromatography mass spectrometry (LC-MS)—in addition to a 4-hour yeast assay.

(2) Apply these methods to detect the occurrence, concentrations and bioactivity of glucocorticoids in wastewater.

The presence of glucocorticoids in the environment is a worldwide problem. This study will serve to highlight the potential problems in water management in areas with limited water resources. The Paso Del Norte region along the U.S.-Mexico border shares resources as well as environmental problems. Recycling and reusing treated water is becoming a necessary practice in areas with limited surface and ground water resources; maintaining the integrity of treated water is critical for protecting human health and the environment. This research is the first study conducted for the Paso Del Norte region--an international border, to address the presence of glucocorticoids in wastewater treatment plants and their impact on the environment.

Chapter 2: Methodology

Glucocorticoid standards (cortisone, hydrocortisone, prednisone and prednisolone (purity ≥ 98%)) were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Fisher Scientific (Waltham, MA, USA). Standards were prepared at a concentration of 1000 mg/L in 100% methanol and stored at -20 °C to minimize evaporation and degradation.

The manufacturers and part number of the material used in this research is as follows: OASIS HBL SPE cartridges (Part No. 186000117) by Waters (Milford, MA, USA); Atlantis® T3 3µm 2.1x150mm Column (Part No. 186003719) and corresponding guard column Symmetry® C18 5µm 3.9x20mm (Part No. WAT054225) (Milford, MA, USA), GF/F Whatman Glass Microfibre filter (70 mm) from Voigt Global Distribution Incorporated (Lawrence, Kansas, USA) (Part No. 1825070).

2.1 HPLC-MS Parameters and Operating Conditions

The natural and synthetic glucocorticoids chosen for this study were chosen based of their occurrence in environmental samples from various worldwide studies (

	Cortisone (Natural)	**Hydrocortisone** (Synthetic)	**Prednisolone** (Synthetic)	**Prednisone** (Synthetic)
Chemical Formula	C21H28O5	C21H30O5	C21H28O5	C21H26O5
Molecular Weight	360.44 g/mol	362.46 g/mol	360.44 g/mol	358.42 g/mol

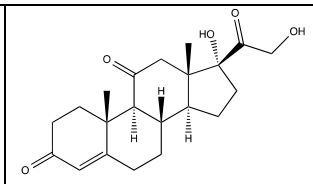
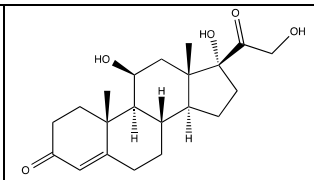
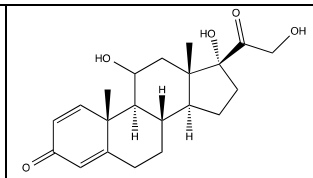
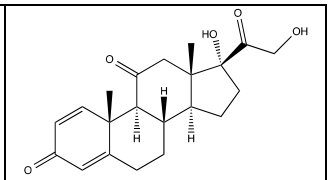
Molecule				

Table 2) (38) (39). The EPA Method 1694: Pharmaceutical and Personal Care Products in Water, Soil, Sediment and Biosolids by HPLC-MS/MS was used as a starting foundation for the development of the chemical analysis for glucocorticoids in environmental samples. For the HPLC method, EPA method involves reverse-phase chromatography utilizing a C_{18} HPLC column and a gradient method using mobile phases (A) LC-MS Grade Water and (B) 100% LC-MS grade Acetonitrile with 0.1% Formic Acid. In this study, several isocratic HPLC methods were tested using 0%B, 50%B and 100% B from the EPA Method, in addition to Methanol with 0.1% Formic Acid (

Table 3). The aim of the HPLC method is to achieve complete separation and distinguish distinct ion signatures (m/z) for cortisone and prednisolone in addition to an efficient elution time.

Table 2 Natural and synthetic glucocorticoids for this study

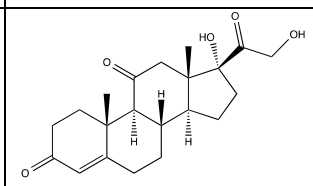
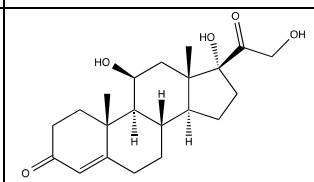
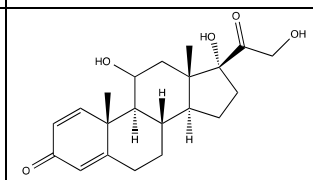
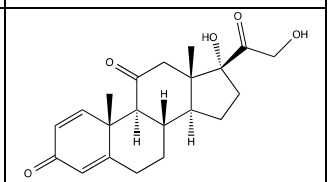
	Cortisone (Natural)	**Hydrocortisone** (Synthetic)	**Prednisolone** (Synthetic)	**Prednisone** (Synthetic)
Chemical Formula	$C_{21}H_{28}O_5$	$C_{21}H_{30}O_5$	$C_{21}H_{28}O_5$	$C_{21}H_{26}O_5$
Molecular Weight	360.44 g/mol	362.46 g/mol	360.44 g/mol	358.42 g/mol
Molecule				

Table 3 HPLC isocratic methods attempted for the detection of glucocorticoids in wastewater

Attempt:	A) 0.1% Ammonium Formate in LC-MS grade water	B) 0.1% Formic Acid in LC-MS grade Acetonitrile	C) 0.1% Formic Acid in LC-MS grade Methanol
1	100%	0%	--
2	50%	50%	--
3	0%	100%	--
4			100%

2.1.1 HPLC-MS Run

Target compounds were analyzed by Agilent 1100 (Agilent Technologies) equipped with a degasser, a binary pump and auto sampler. The chromatographic separation was completed by Atlantis® T3 3µm 2.1x150mm column with corresponding guard column SENTRY® C18 3µm 2.1x20mm. The column oven temperature was at ambient temperature and the injection volume was 15 uL. An isocratic elution using HPLC Methanol with 0.1% Formic Acid was applied at a flow rate of 0.150 ml/min. Prior to analysis, the column and corresponding guard cartridge were conditioned and equilibrated with 5mL of the mobile phase solution.

Mass spectrometry analysis was performed using AccuTOF JMS-T100LC with ESI+ mode. The MS operating conditions were set to as follows: MS Acquisition Method was set as:
gas temperature, 250 °C; Orifice1 80 °C; Needle Voltage, 2000V; Orifice1 Current, 223nA; Rings lens voltage, 10V; Orifice 1 Voltage, 70V; and Orifice 2 Voltage, 5V.

2.2 Solid Phase Extraction (SPE)

All one-liter wastewater samples were filtered through GF/F Whatman Glass Microfibre filter (70 mm) and pH adjusted with hydrochloric acid to 2.5. The OASIS cartridges were placed onto the SPE apparatus (Supelco Visiprep Large Volume Sampler: 57275) connected to a vacuum and filtering flask (EYELA Aspirator) and filtering flask (2000 mL Kimax Filtering Flask), which allowed the sample to filter through the OASIS cartridge (Oasis HBL, 6mL and 500mg each) at a flow of 5-10mL/min. Each OASIS cartridge was pre-conditioned with 10mL of MeOH, followed by 10mL of HPLC grade water never allowing the cartridge to reach a dry state. The wastewater samples were then introduced at the same rate onto the OASIS cartridge followed by a rinse with 50 mL of 5% (v/v) methanol in HPLC grade water and dried for 2 hours under vacuum. The target compounds were eluted using 12 mL of ethyl acetate and dried under a nitrogen flow. The dried samples were reconstituted to 100 uL of methanol for instrumental analysis.

The sample cleanup and HPLC conditions for this study were initially selected according to the EPA Method. The initial attempt involved EPA Method 1694 reverse phase chromatography; however, no response was obtained leading to further modification of the elution solvent (Table 4). According to literature review for environmental glucocorticoids, the elution solvent typically used for the extraction of glucocorticoids in environmental samples is ethyl acetate (2) (15). In the second SPE attempt, methanol was substituted with ethyl acetate and same volume was maintained.

Table 4 SPE methods attempted in this research for a glucocorticoid recovery

Method	(1) Cartridge	(2) Sample Pre-Treatment	(3) Conditioning Solvent	(4) Wash	(5) Elution	(6) Eluate-Post Treatment	Recovery
EPA 1694	WATERS	-Filtration	20 ml MeOH	6ml reagent	12 ml	Concentration	NA

	HBL OASIS	-pH =2.0 -EDTA	+ 6ml DI water	water + 5 min dry	methanol	with N_2 Flow	
EPA 1694 (Modified)	WATERS HBL OASIS	-Filtration -pH =2.0 -EDTA	10 ml MeOH + 6ml DI water	6ml reagent water + 5 min dry	12 ml ethyl acetate	Concentration with N_2 Flow	NA
Liu et al. Method	WATERS HBL OASIS	-Filtration -pH =2.0	10 ml MeOH + 10 ml HPLC water	50 ml of 5% MeOH in HPLC grade water + 2 hour dry	12 ml ethyl acetate	Concentration with N_2 Flow	71+/- 0.98%

NA= Not available

The main problem encountered for the first two SPE methods were cartridge clogging and inability to completely process the 1-liter sample. This method was then compared to other SPE glucocorticoid methods where ethyl acetate was used as the elution solvent followed by a second SPE process for each sample which led to <90% recoveries (Figure 4) (15) (38).

In the third attempt, the method was optimized following literature SPE methods for extraction of glucocorticoids in wastewater; however, eliminated the secondary SPE method. In comparison to the EPA: 1694 method, literature methods excluded the addition of ethylenediaminetetraacetic acid (EDTA), a chelating agent, and included a 50mL of HPLC grade wash and 2-hour cartridge drying under vacuum. The SPE test was completed using literature method Liu et al., specific for 1-liter wastewater samples, while excluding the secondary SPE process (Figure 4) (38).

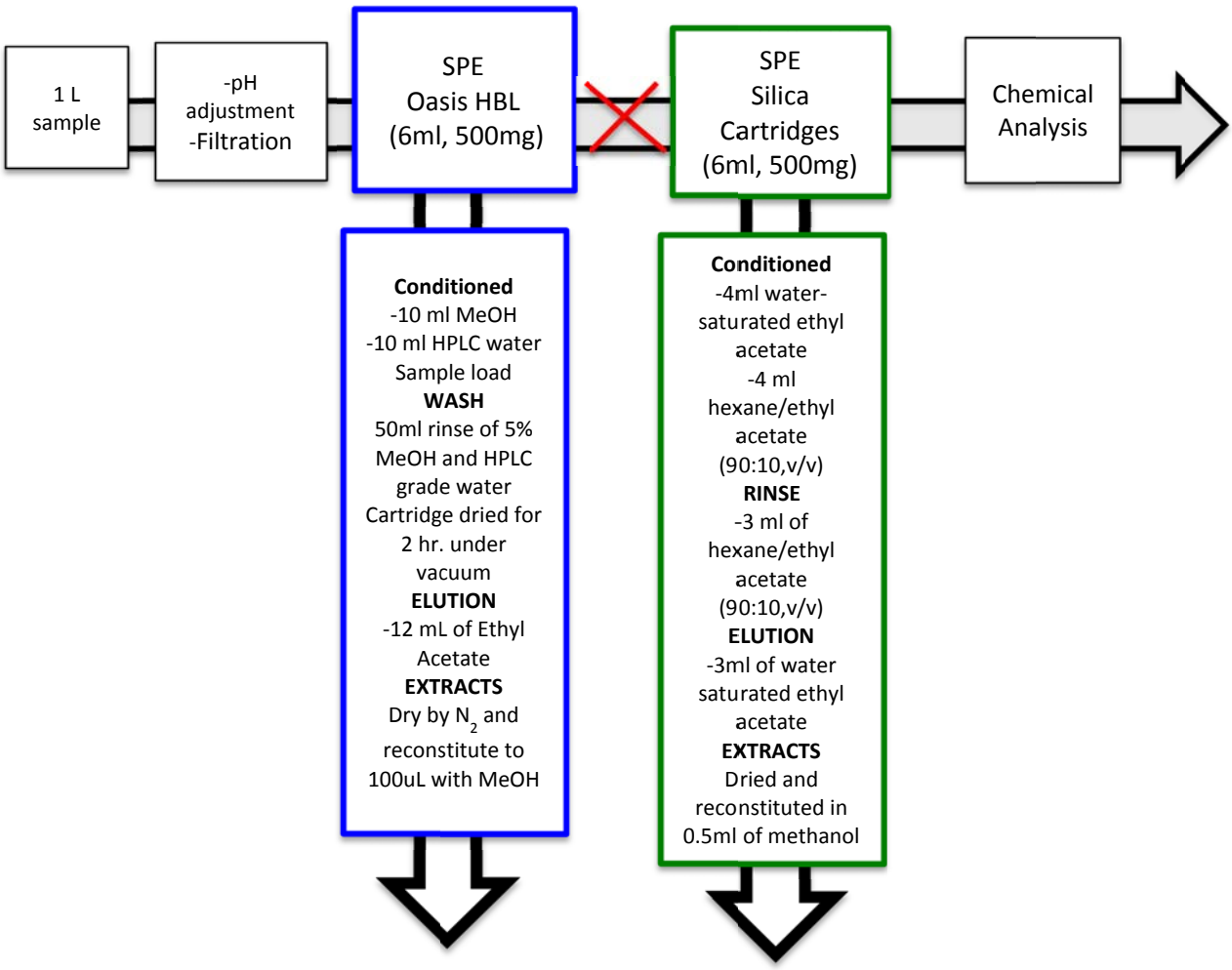


Figure 4 SPE Method used in this research (blue) and suggested SPE method for glucocorticoid extraction in wastewater samples (blue + green) SPE method used in this research (blue) and suggested SPE method for glucocorticoid extraction in wastewater samples (blue + green)

2.2.1 Recovery Test

A recovery test was used to determine the ability for the SPE method to reduce the matrix effect and measure the loss during of target analytes during the sample preparation process from a wastewater sample. Some of these processes that could have induced analyte loss include adsorption to glassware, filter paper, or evaporation from solution-sample transfer. A one-liter sample blank with Milli-Q water

was spiked with known cocktail of glucocorticoid concentrations and put under the same condition and processes as an actual sample using the Liu et al. 2011 SPE extraction method.

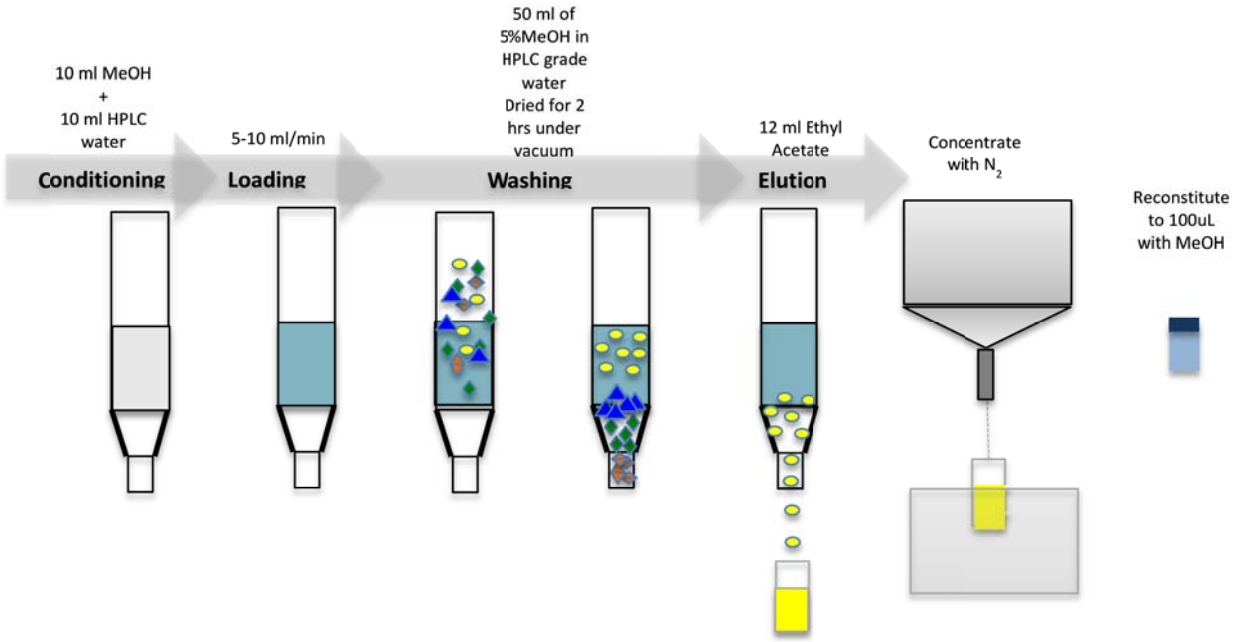


Figure 5 Basic steps in the SPE method used in this study

In the recovery test, the expected final sample concentration and volume was 1 mg/L in 100 uL volume (Table 5). The recovery test was performed in triplicates followed by chemical analysis. This test evaluates the ability for the sorbent to extract the analyte of interest while reducing the matrix effect for an enhanced signal during instrumental analysis.

Table 5 Concentration of glucocorticoid cocktail solution and the amount spiked to the blank for SPE recovery test to achieve a final volume of 1 mg/L

Solution	Stock	Spiked volume in 1000	Final	Extract

	Standard (mg/L)	**mL blank (µL)**	**Concentration** (mg/L)	**volume (µL)**
Glucocorticoid cocktail	5	20	1	100

2.3 Bioassay

In this research, a previously established four-hour yeast assay for estrogenic activity was implemented and optimized for glucocorticoid receptor activity. Two previously hGR transfected yeast cells were used; compared for optimization, one with a higher promoter activity (MCY-212) while in comparison to a yeast cell (DSY-1345) exhibiting lower promoter activity.

Standard solutions of the individual glucocorticoids were prepared in ethanol (Table 6). All steroid hormones were prepared as a 10mM stock solution in ethanol and the positive control,

deoxycorticosterone (DOC), was prepared as a 20mM stock solution in ethanol. Initial and final concentrations are represented in a 1:100 dilution. All stock solutions were stored at a -4°C.

Table 6 Standard dilutions for glucocorticoids ranging from 50nM to 10mM (1:100 dilution)

Initial	Final	Log Units (Final)
0	0	01.00 E-10 (*arbitrary)*
50 nM	0.5 nM	5.00E-10
100 nM	1 nM	1.00E-09
500 nM	5nM	5.00E-09
1 mM	0.01 M	1.00E-08
5 mM	0.05 M	5.00E-08
10 mM	0.1mM	1.00E-07
50 mM	0.5mM	5.00E-07
100 mM	1 mM	1.00E-06
500 mM	5 mM	5.00E-06
1 mM	0.01 mM	1.00E-05
5 mM	0.05 mM	5.00E-05
10 mM	0.1 mM	1.00E-04

2.3.1 Four-Hour Yeast Assay Protocol

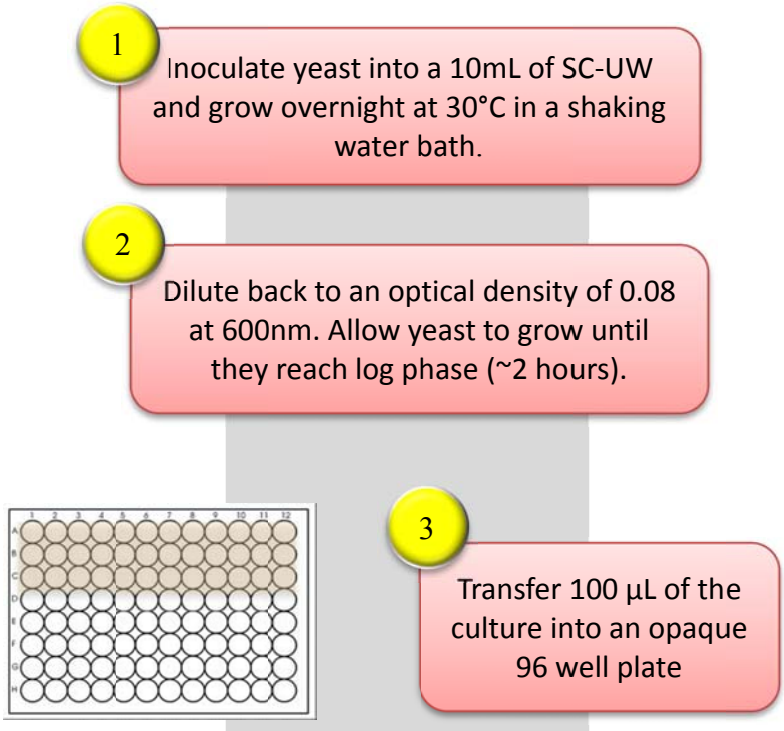
The yeast were grown in freshly prepared media containing histidine and lucine amino acids and lacking uracil and tryptophan (SC-UW), at 30 °C in a shaking water bath. The liquid media was prepared using a pre-prepared 1-yeast media soluble 4X-concentrated SW-UW solution, with 375mL Milli-Q autoclaved water, in addition to 1mL of each: Histidine and Lucine. Culture tube is incubated overnight in a 30°C incubated shaker, leaving the lid open allowing air exchange.

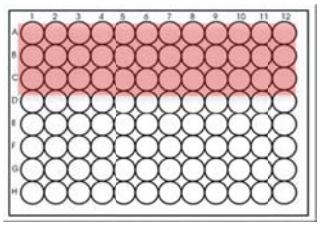
The next day, the spectrometer warmed up for at least 20 minutes at 600nm. Using the appropriate cuvettes, a blank using fresh liquid media SC-UW was used to calibrate the spectrometer,

followed by optical density (O.D) measurement. Air bubbles formed in the cuvettes from fresh media or liquid culture must be removed by tapping the cuvette and outer surface of cuvette must be wiped clean before a reading.

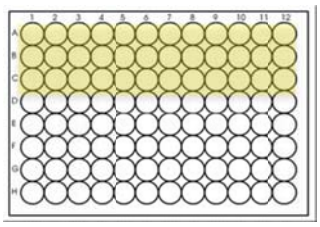
Colonies for each yeast strain were grown separately in an SC-UW plate for 3 days in an incubator. Once developed, the yeast culture was transferred with a sterile swab to liquid medium and incubated in a 30 C water-shaking bath and monitored until yeast reached exponential growth, which would typically take 1.5 hrs. This was measured by the spectrometer with an OD_{600} reading of 0.08.

The induction phase follows by preparing a labeled 96-well plate and adding 100 µL of the yeast culture into each well. One microliter of each of the stock hormone dilutions are introduced into each well from the highest to lowest concentration, to the corresponding well containing 100µL of yeast culture. The experimental design was set up to be twelve doses and three replicates for each making a total of 36 wells. The 96-well plate is then incubated for 2 hours at 30°C. After the two-hour incubation period, one hundred µL of Tropix- B-Gal is added to each well followed by a tape cover and allowing air exchange. The 96-well plate is set at room temperature for 2 hours, away from disturbances. Chemiluminescense intensity is then measured by microplate luminometer. The raw data was normalized to its control (ethanol) and Graph Prism was used to make dose-response curves and EC-50 detection. Schematic of the 4-hour yeast assay is shown in Figure 6.





4 Add 1 µL of stock hormone dilutions. Incubate at 30°C for 2 hours.



5 Add 100 µL of Tropic Gal-Screen reagent. Incubate at room temperature for 2 hours.

6 Luminometer

2.4 Wastewater Analysis

Studies have shown environmental glucocorticoids are most likely to be found in influent samples; (15) (38) therefore, the sampling plan of this research is aimed towards the analysis of incoming wastewater. The daily wastewater flow for influent and effluent for the Northwest Wastewater Treatment Facility is shown in Figure 7. It is observed influent flow begins to increase in the morning (8:00 a.m.), peaks at noon (12:00pm) and is continuous with an average flow in the evening (6:00p.m.). These specific times were chosen to analyze the occurrence of glucocorticoids in wastewater in order to evaluate their occurrence throughout the day.

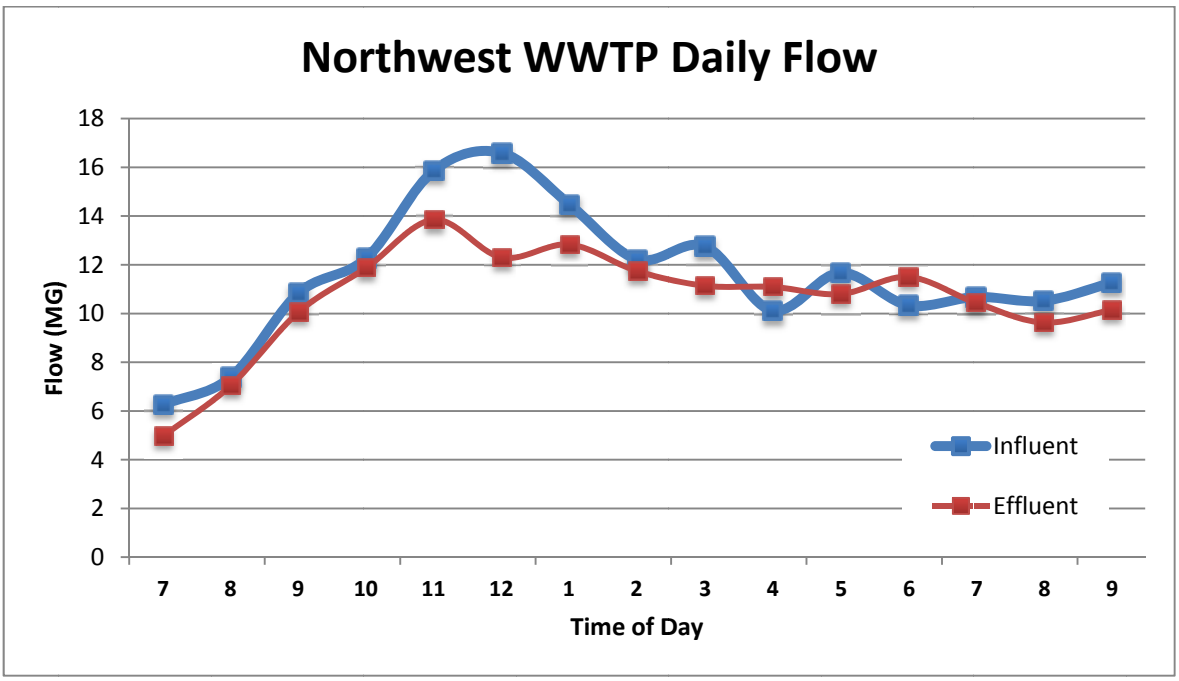


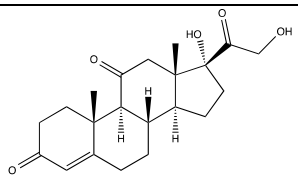
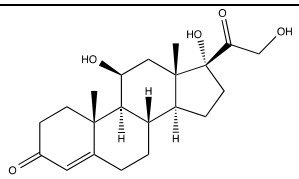
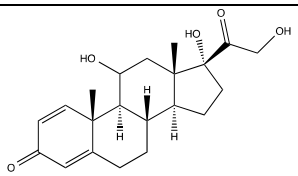
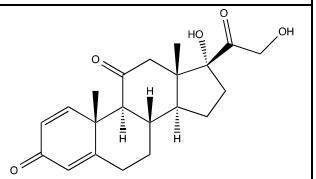
Figure 7 Wastewater influent and effluent flow for the Northwest Wastewater Facility (Million gallons of incoming/outgoing waters. Time of day--Oct. 24, 2013)

Chapter 3: Results and Discussion

3.1.1 Chemical Analysis: HPLC Methods

The main goal of the chemical analysis was to create a method that identifies the signature of the analytes based on retention time and mass-to-charge ratio (m/z) signature. The most difficult challenge for the analysis of glucocorticoids is the similar molecular signature of the analytes, specifically for prednisolone and cortisone which share the exact molecular weight, have similar structure

Table 2) and retention times in HPLC-MS identification. As shown in Table 7, the retention times for mobile phase A, B, C and D do not show significant separation with a difference in retention

	Cortisone (Natural)	**Hydrocortisone** (Synthetic)	**Prednisolone** (Synthetic)	**Prednisone** (Synthetic)
Chemical Formula	C21H28O5	C21H30O5	C21H28O5	C21H26O5
Molecular Weight	360.44 g/mol	362.46 g/mol	360.44 g/mol	358.42 g/mol
Molecule				

time ranging from 0-0.4 minutes and an average +/-0.2 minute separation. These tests demonstrated that alternating the composition of the mobile phase will not improve the separation of glucocorticoids of interest and therefore isocratic solvent system (i.e. constant composition) was used in this study. Mobile phase D showed the highest elution strength and was chosen for this study based on the shorter acquisition time and clean spectra response, in comparison to other methods.

Table 7 Retention times for glucocorticoids using four different isocratic mobile phases

Analyte	**Mobile Phase**			
	A	**B**	**C**	**D**
	100% LC-MS Grade Water, 0.1% Formic Acid	**(1:1, v/v)** **(A)** LC-MS Grade Water, 0.1% Formic Acid **(B)** 100% ACN LC-MS Grade, 0.1% Formic Acid	**100%** ACN LC-MS Grade, 0.1% Formic Acid	**100%** MeOH LC-MS Grade, 0.1% Formic Acid
	RT (min)	RT (min)	RT (min)	RT (min)

Cortisone	4.4	4.7	5.7	3.6
Hydrocortisone	4.6	4.9	5.7	3.6
Prednisone	4.4	4.7	5.5	3.3
Prednisolone	4.8	4.6	5.7	3.7

The retention times for methods A, B, C and D are in agreement with literature research for the analysis of environmental glucocorticoids (Table 8). All studies utilize a C_{18} column as a stationary phase and a polar solvent as mobile phase. While most studies utilize LC-MS grade water and acetonitrile as mobile phases, in this study, methanol was determined to be the strongest eluent with shorter retention times. It should be noted that the separation of these glucocorticoids was poor in all studies.

Table 8 HPLC literature methods for glucocorticoids

Analytes Of interest	**Column Dimensions**	**Mobile Phase**	**LC-MS system**	**Retention Time (min)**	**Reference:**
CR CL PRL PRN + 18 others	ACQUITY BEH-C18 (50mm* 2.1mm, 1.7µm)	Gradient A-Water +0.1%FA B-ACN +0.1%FA	UPLC-MS	CR- 2.9 CL-2.8 PRL-2.8 PRN-2.8	(40)
CR CL PRL PRN + 2 others	Zorbac Eclipse XDB-C18 (50mm X 4.6mm, 1.8µm)	Gradient A- (Water/ACN) 78:22 +0.1%FA B- (MeOH/ACN) 78:22 +0.1%FA	LC-MS/MS	CR- 5.5 CL-5.4 PRL-5.0 PRN-4.9	(41)
CR CL PRL PRN + 3 others	UPLC BEH C18 column (100X 2.1mm, 1.7µm)	Gradient A- Water +0.1%FA B- MeOH	LC-MS/MS	CR- 4.9 CL-5.4 PRL-5.4 PRN-4.7	(15)
CL PRL + 1 other	RP Gemini C18 column (50mm X 4.6mm, 5µm)	Isocratic (3:1) ACN/ Water + 0.1% FA	LC-MS/MS	CL-4.0 PRL-3.9	(65)
CL PRN + 1 other	Supelco Supelcosil ABZ + PLS (3 mm×150 mm, 3 µm)	Gradient A- Ammonium Acetate B-	LC-MS/MS	6-9.5min	(43)

		MeOH			
CR CL PRL PRN	Atlantis T3 Column (2.1 X 150 mm, 3µm)	Isocratic MeOH + 0.1% FA	LC-MS	CR- 3.6 CL-3.6 PRL-3.7 PRN-3.3	This research

CR=Cortisone; CL=Hydrocortisone; PRL=Prednisolone; PRN=Prednisone; ND=no data

3.1.2 Chemical Analysis of Glucocorticoids: HPLC-MS Identification

The analysis and confirmation for selected glucocorticoids were defined using ESI positive mode which led to the addition of a protonated molecule [H^+] to each target analyte during mass spectrometry analysis (Figure 8). All samples (calibration standard, SPE recovery test, and wastewater sample analysis) were analyzed at a specific retention time range (3.3-4.3 minutes) and specific analyte ions signature based on their mass-to-charge ratio (m/z). Selected Ion Monitoring mode (SIM) was used for mass spectra identification where a specific m/z ratio for each glucocorticoid (Table 9) was monitored. SIM is recognized as a sensitive analysis method as opposed to SCAN Mode in which every m/z signature in the sample injection is plotted.

Table 9 Identification and confirmation criteria of analytes in mass spectra

Compound	**Retention Time (min)**	**ESI + Mode Signature (m/z)**
Cortisone	3.6	361.16- 361.50
Hydrocortisone	3.6	363. 16- 363.50
Prednisone	3.3	359.16- 359.50
Prednisolone	3.7	361.16- 361.50

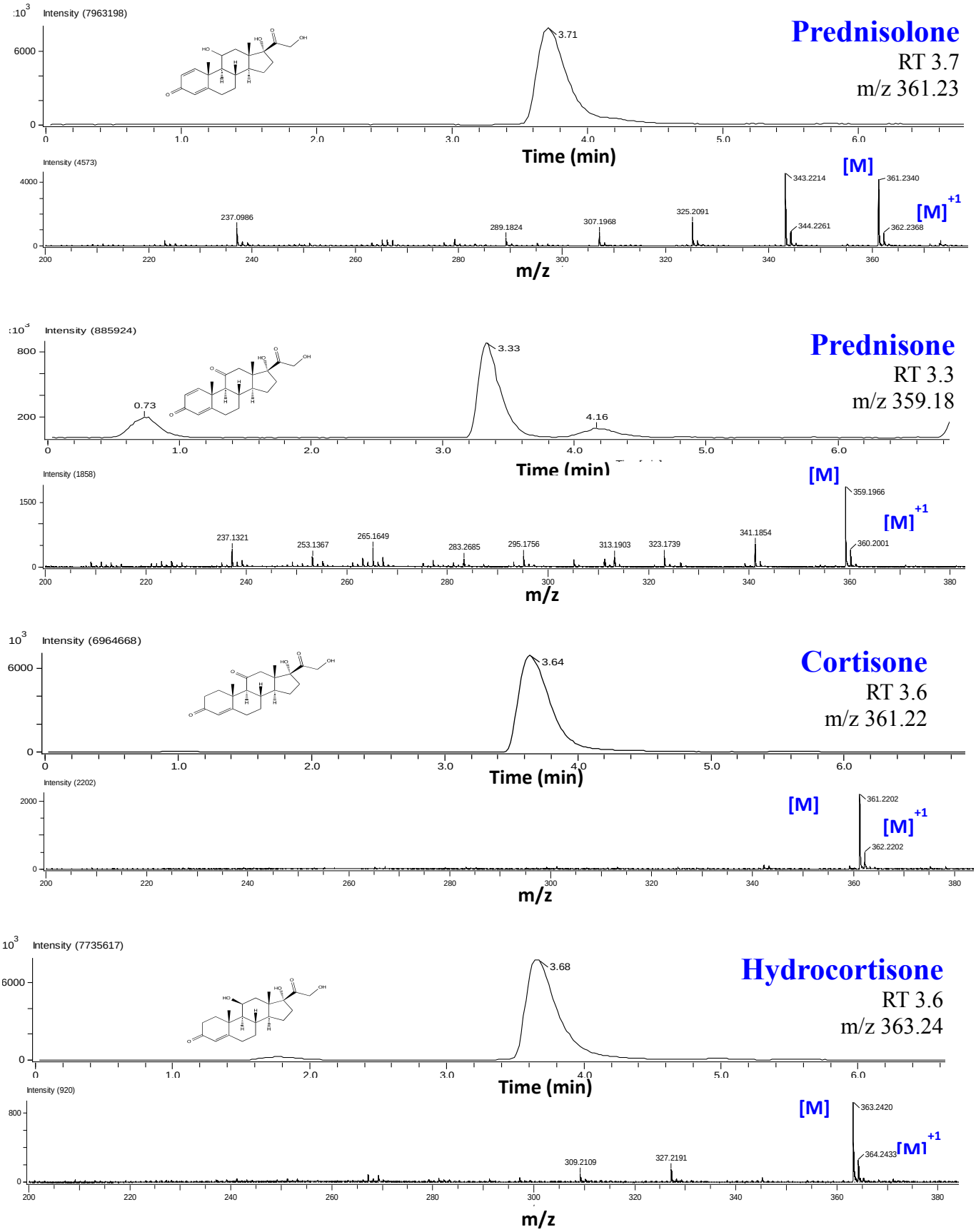


Figure 8 Chromatogram and mass spectra of target analytes in 1 mg/L glucocorticoid standard solution

3.1.3 Quantitative Analysis

In this study, glucocorticoids were quantified individually except for cortisone and prednisolone which two compounds share the exact molecular signature and similar retention time. Standard calibration curves were created for individual prednisone and hydrocortisone, combined prednisolone and cortisone; in addition to a cocktail (i.e. mixture of all four glucocorticoids) solution. Prior to constructing the calibration curves, a preliminary study was conducted where the cocktail and individual solutions were injected through the HPLC-MS to determine the lowest concentration that the mass spectrometer could detect in the sample. This preliminary work led to the construction of calibration curves with concentrations ranging from 0.1 to 1 mg/L, (standard concentrations 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mg/L) where concentration was plotted against corresponding peak area for each analyte. The standard calibration curves were used to determine the linear range of detection by the time-of-flight mass spectrometer for quantification purposes. All HPLC-MS runs were completed in triplicates with a 10-minute acquisition time and a 2-minute column equilibration time. Linearity for individual calibration curves for hydrocortisone, prednisolone and cortisone, prednisone and cocktail solution reached correlation coefficients of 0.96, 0.99, 0.99, and 0.98 respectively, as shown in Figure 9. Individual calibration curves for individual compounds are listed in Appendix A.

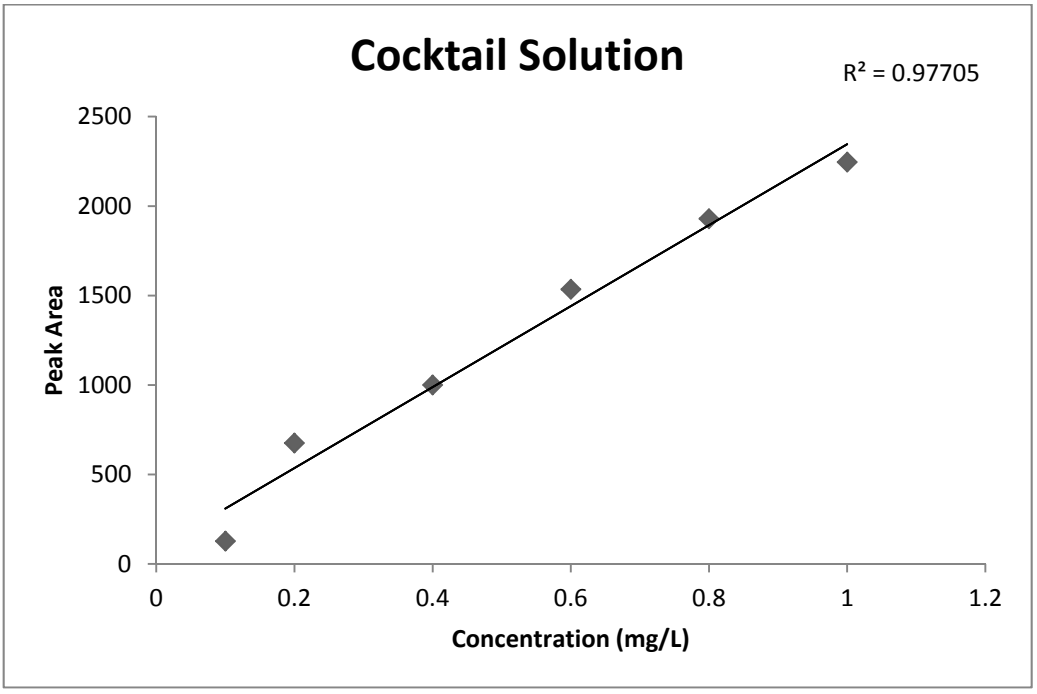


Figure 9 Calibration curve for glucocorticoid cocktail solution (Peak area (detection response) vs. Concentration)

3.1.4 Chemical Analysis: HPLC-MS Validation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on the signal-to-noise ratio (SNR). The LOD and LOQ are defined as the smallest measure that can be detected and quantified with reasonable certainty by an instrument of by using a specific method. LOD and LOQ values for standard calibration curves were calculated using the EXCEL function STEYX and slope, as shown in Equation 1 and Figure 10. The function STEYX measures the standard error of the predicted Y-value for each X in a regression. The LOD and LOQ were calculated according to standard curves for all analytes. The quantification method for the analysis of target analytes is summarized in Table 10.

Equation 1 Equations used to calculate LOD and LOQ for glucocorticoids using calibration curve, slope and EXCEL software.

$$(1) \qquad LOD = (STEYX / Slope) \; X \; 3.3$$

$$(2) \qquad LOQ = (STEYX / Slope) \; X \; 10$$

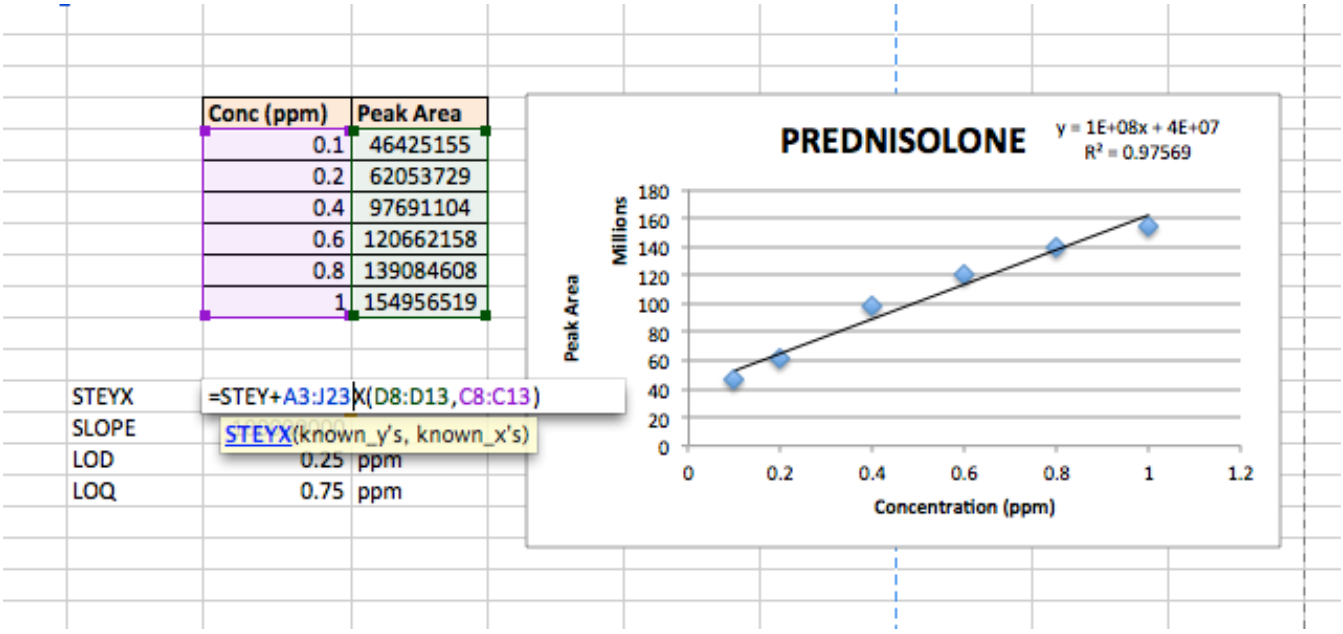


Figure 10 Example of LOD and LOQ calculation using EXCEL

Table 10 Summary for chemical validation and quantification methods

Compound	R^2	LOD (mg/L)	LOQ (mg/L)	Equation
Hydrocortisone	0.96	0.28	0.87	y= 581324x +18862
Prednisone	0.99	0.22	0.67	y= 586788X -15947
Prednisolone+ Cortisone	0.99	0.16	0.50	y=1186859x -8338
Cocktail	0.98	0.19	0.59	y= 2E+06x + 83517

3.2 SPE Recovery Test

The recovery test was completed under the established methods in this study as shown in Figure 11. Using the standard curve for cocktail solution, cocktail solution was spiked to 1 L of DI water to reach a final concentration of 1 mg/L in the final 100 µL extract. The extract was analyzed for glucocorticoids based on m/z identification and retention time of 3.3-4.3 minute range using SIM mode. Table 12 shows the average recovery for total glucocorticoid, i.e. the sum of all four analytes. The SPE recovery for a 1 mg/L final extract from in a 1-liter sample resulted in a 67 ±0.01% recovery.

Table 11 SPE recovery test results

	Recovery %
Peak Area for SPE Test 1	0.67
Peak Area for SPE Test 2	0.68
Peak Area for SPE Test 3	0.67
Average Recovery	**67 ±0.01%**

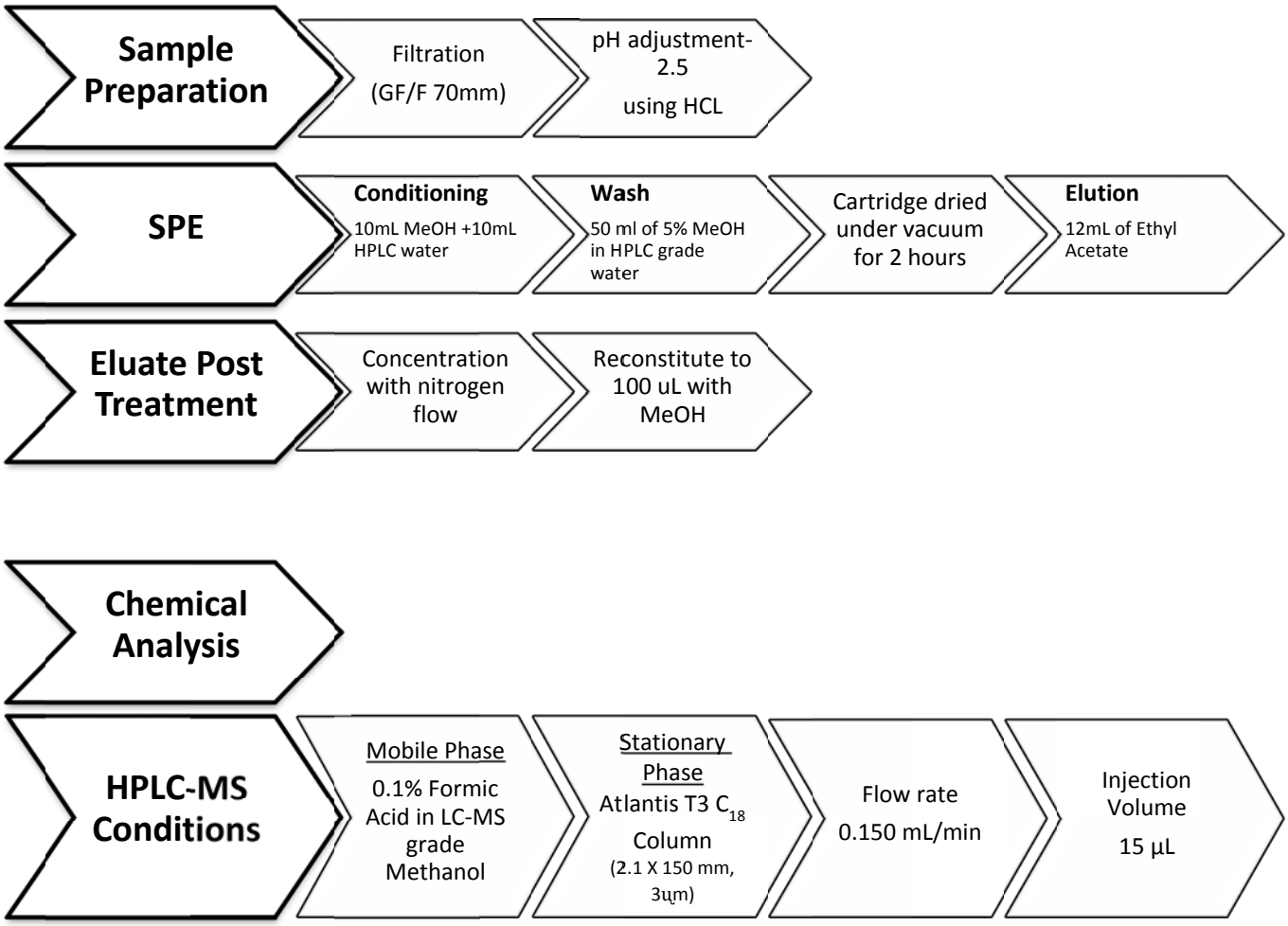


Figure 11 Optimized chemical method for the analysis of glucocorticoids in wastewater

3.3 Wastewater Analysis

The purpose of the wastewater analysis is to detect the occurrence and variability of glucocorticoids throughout the day in influent and effluent wastewater. A 1-liter influent and a 1-liter effluent sample were collected at 8:00 a.m., 12:00 p.m., and 6:00 p.m. for 3 sampling days from the Northwest Wastewater Treatment Plant facility making a total of 9 influent and 9 effluent samples.

3.3.1 Environmental Concentrations

Concentrations of four glucocorticoids, prednisone, hydrocortisone, prednisolone and cortisone in wastewater were determined by HPLC/MS. Prednisolone and cortisone were combined due to their identical m/z value and retention time. All target analytes were present in all the influent and effluent samples (Table 12 and Table 13). Individual glucocorticoid concentrations in the influent ranged from 2.3 to 19.0 ng/L while glucocorticoid concentrations in effluent ranged from 2.7 to 14.4 ng/L. The most prominent glucocorticoids in influent and effluent samples were as follows: prednisone> hydrocortisone> prednisolone/cortisone. These natural and synthetic glucocorticoids can be metabolized and excreted through urine or washed off from the skin at different rates. The higher level of prednisone in wastewater influent samples could be a result of it being a highly prescribed medication. Prednisone has biological half-life of several hours and 1-hour metabolism rate from the body (44). Its metabolite is prednisolone which is also found in the wastewater samples. Prednisone is commonly used for asthma, rheumatic disorders, allergic disorders, and among other diseases. In this study, prednisone concentrations in wastewater samples remain pretty constant in the influent samples collected from different time. The samples were collected during the month of October, a month known where many seasonal changes occur and this might suggest the high use of prednisone to treat seasonal allergies. Cortisone, prednisolone and hydrocortisone concentration in influent trends also did not vary much throughout the day. Prescribed prednisolone medications require a follow up of several dosages and these results might correlate with continued dosages. Cortisone is naturally produced in the body and the highest levels are produced in naturally in the morning. One constraint in this study was that our method is not able to differentiate between natural cortisol and synthetic prednisolone and therefore not allowing us to hypothesize about the potential sources. Hydrocortisone is easily obtained over the counter therefore introducing higher variability in concentrations as shown in sampling day 2 with a

15.41 ng/L when compared to sampling day 1 (9.84 ng/L) and sampling day 3 (4.74 ng/L) morning samples.

The total concentrations of the four glucocorticoids in the influent samples show a range from 20 to 38 ng/L while glucocorticoid effluent concentrations range from 18 to 31 ng/L. Though it is not statistically significant, the glucocorticoid concentrations in the influent show a trend in which concentrations are at its highest in the morning and decrease as the day continues (Table 14). This observation supports the renal excretion of glucocorticoids and it might suggest that most of the glucocorticoid pharmaceuticals are mainly consumed in the morning. No particular trend of glucocorticoid level was noticed in the effluent.

Table 12 Glucocorticoids Concentrations (ng/L) in Wastewater Influent Samples

Influent	Sampling Time	Hydrocortisone	Prednisolone/Cortisone	Prednisone	Total
Day 1	8:00 a.m.	9.84	6.80	19.06	35.70
	12:00 p.m.	4.97	5.78	15.92	26.67
	6:00 p.m.	2.33	4.01	14.00	20.34
Day 2	8:00 a.m.	15.41	4.15	18.47	38.03
	12:00 p.m.	5.61	4.84	15.32	25.78
	6:00 p.m.	3.15	3.79	13.58	20.52
Day 3	8:00 a.m.	4.74	5.42	15.86	26.01
	12:00 p.m.	2.90	3.36	13.88	20.15
	6:00 p.m.	4.39	3.42	13.95	21.76

Table 13 Glucocorticoids Concentrations (ng/L) in Wastewater Effluent Samples

Effluent	Sampling Time	Hydrocortisone	Prednisolone/Cortisone	Prednisone	Total
Day 1	8:00 a.m.	2.74	3.52	12.32	18.58
	12:00 p.m.	14.39	3.70	13.50	31.59
	6:00 p.m.	3.30	3.29	11.76	18.35
Day 2	8:00 a.m.	3.35	3.16	13.37	19.88
	12:00 p.m.	4.72	3.38	12.50	20.60
	6:00 p.m.	4.86	3.33	12.05	20.24
Day 3	8:00 a.m.	4.91	3.88	13.03	21.81
	12:00 p.m.	4.34	3.41	12.73	20.49
	6:00 p.m.	4.69	3.23	11.88	19.80

Table 14 Average Concentration of Glucocorticoids (ng/L) in Influent and Effluent Samples by the time of the day

	Sampling Time	Hydrocortisone	Prednisolone/Cortisone	Prednisone	Total
Influent	8:00 a.m.	10.00	5.46	17.80	33.25
	12:00 p.m.	4.49	4.66	15.04	24.20
	6:00 p.m.	3.29	3.74	13.84	20.87
Effluent	8:00 a.m.	3.67	3.52	12.90	20.09
	12:00 p.m.	7.81	3.50	12.91	24.23
	6:00 p.m.	4.29	3.28	11.89	19.46

Table 15 Total Average Concentrations (ng/L) of Glucocorticoids. Data are presented as Average ± standard deviation

	Hydrocortisone	Prednisolone/Cortisone	Prednisone
Influent n=9	5.93 ± 4.18	4.62± 1.18	15.56± 2.02
Effluent n=9	5.26 ± 3.52	3.43± 0.23	12.57± 0.64

Glucocorticoid concentrations found in this study were within the range of ppt or ng/L as published by other studies (Table 16). The glucocorticoid influent concentrations in this study were comparable to those in China of which were found to be within 0.5 to 40 ng/L range.

All three studies conducted in China and the study in Spain show that glucocorticoid concentration in influent wastewater range from 1 to 190 ng/L for cortisone and hydrocortisone while prednisone and prednisolone range from 1.7 to 25 ng/L. The effluent glucocorticoid concentrations for the studies in China show that cortisone, hydrocortisone and prednisone were still found at low ng/L concentrations in effluent water. In this study, the glucocorticoid effluent concentrations are higher than those reported in literature with average concentrations ranging from 3.43 to 12.57 ng/L.

The studies conducted in the Netherlands and Spain show higher glucocorticoid concentrations in influent in comparison to this study and China's study. This comparison reflect that the levels of glucocorticoid in the environment could be depending on surrounding industrial sources which could cause the concentrations of glucocorticoids to be as high as in the µg/L range (45). To date, there is no literature that addresses the occurrence of environmental throughout the day. This is the first study that addresses environmental glucocorticoid occurrence in influent and effluent wastewater samples at different times of a sampling day.

Table 16 Literature review for chemical analysis of glucocorticoids in environmental matrices

Location	Sample Matrix	Method		Results (ng/L)				Ref:
				CRN	PRL	PRN	CRL	
China	Wastewater	SPE-LC-ESI-MS/MS	Influent	30.53	3.02	2.62	39.3	Chang et al. 2009
			Effluent	0.26	0.56	0.18	0.51	
China	Wastewater	SPE-LC-ESI-MS/MS	Influent	1.0	1.7	0.56	15.6	Chang et al. 2007
			Effluent	0.13	0.07	0.06	0.24	
Spain	Wastewater	SPE-UHPL-ESI-MS/MS	Influent	135	25	<20	190	Herrero et al. 2012
			Effluent	n.d	n.d	n.d	<10	
China	Wastewater	SPE-LC-ESI-MS/MS	Influent	14.5	--	--	12.7	Liu et al. 2011
			Effluent	n.d	--	--	n.d	
The Netherlands	Hospital wastewater	LC-MS/MS	Influent	381-472	315-1918	117-545	275-301	Schriks et al 2010
Hungary	River water	SPE-LC-API-MS/MS	A	--	--	--	<0.17-0.36	Tolgyesi et al. 2010
			B	--	0.04-0.58	--	0.32-2.67	

CRN=Cortisone; CRL=Hydrocortisone; PRL=Prednisolone; PRN=Prednisone; ND=no data

To date, there is limited information of the risks that environmental glucocorticoids pose to the ecosystem. Those studies mostly focused on the effects of fish to high dietary glucocorticoid exposures and have found that glucocorticoids could inhibit aggressive behavior, immunological response and locomotor activity in rainbow trout (46). Only one in-vivo study has evaluated environmentally relevant concentrations of synthetic glucocorticoids and their effects on aquatic organisms (47). In the study conducted by Kugathas et al., fish were exposed to synthetic glucocorticoids, prednisolone and beclomethasone dipropenate. The tests evaluated potential physiological changes by measuring plasma

glucose levels and leucocyte counts. The results revealed that glucose level concentrations for the exposure of both synthetic glucocorticoids significantly increased in comparison to the control group. Increased plasma glucose levels are associated with hyperglycemia which is known for creating problems in the eyes, kidneys, nerves and blood vessels. The results also revealed that the glucocorticoid exposed groups had a reduced total leucocyte count or white cell counts which are known as disease-fighting cells. In addition, the finding indicated that increased plasma glucose levels and the effects were dose-dependent ---greater at higher concentration (10 µg/L). Other studies have confirmed that cortisol administration to fish elevates the plasma glucose up to 3-fold depending on the concentration (48). These studies suggest that environmentally relevant concentrations can affect aquatic organisms and highlight the point that effects are concentration dependent.

The study by Kugathas et al. highlights the importance for future studies to address the effects of glucocorticoids as a whole rather than to consider their individual effects to aquatic organisms. In this study, the total glucocorticoid concentrations in the effluent were found to have an average of 21.3 ng/L (Figure 12). Such level could potentially induce ecotoxicological effects when present in aquatic ecosystems even though they would not be considered high enough to induce physiological changes as shown in Kugathas study.

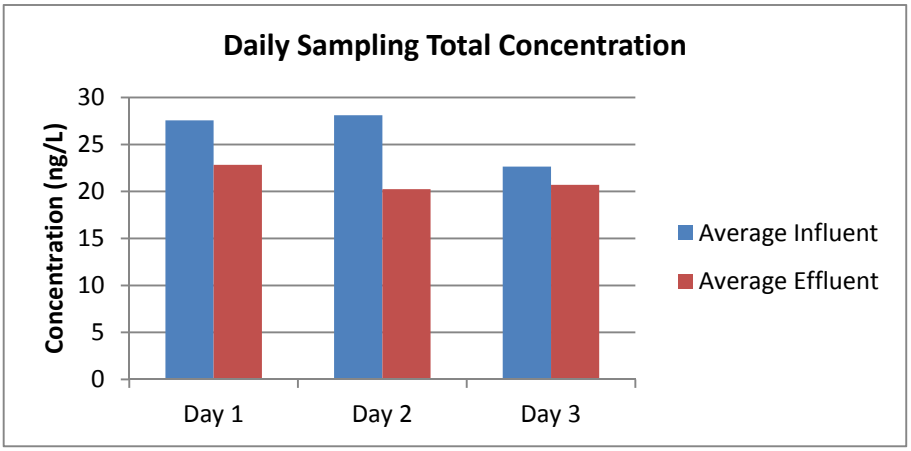


Figure 12 Total Glucocorticoid Concentrations (ng/L) in the Influent and Effluent from Different Sampling Dates.

3.3.2 Estimated Removal

It is evident that the degradation and elimination of glucocorticoids in the wastewater treatment process are incomplete (Table 15). The removal of glucocorticoids by the treatment processes is estimated using the differences between the average total glucocorticoid in the influent and effluent. The estimated removals of glucocorticoids for sampling day 1, 2 and 3 were 17.2%, 28% and 8.6%, respectively, as shown in Table 17. The total average estimated removal is 17.9 (± 9.7) %.

Table 17 Estimated Glucocorticoid Removal

	Day 1	Day 2	Day 3	Total Removed
Average Influent	27.57	28.11	22.64	
Average Effluent	22.84	20.24	20.70	
Estimated Removal %	17.2	28.0	8.6	**17.9 ± 9.7%**

The detection of glucocorticoids in effluent might be due to metabolism and transformation of glucocorticoids in the complex wastewater matrix. Compounds with similar structures to those of glucocorticoids have previously shown the ability to transform in wastewater and surface water, e.g. estradiol to estrone (49). Chemical transformation of environmental glucocorticoids is unknown as there are no existing databases that track their transformation in the environment (50). It is important to identify what wastewater treatment is needed to remove glucocorticoids and other EDCs from wastewater processes.

Studies in China have attempted to understand the biological degradation of estrogens, androgens, progestogens and glucocorticoids during wastewater treatment processes. The studies found that the highest degradation, which have shown glucocorticoid removals of 92-100%, occur during the secondary processes with a combined treatment of anaerobic-anoxic-aerobic units (15). Mass changes

of glucocorticoid through combined treatment of anaerobic-anoxic-aerobic units have shown to that anaerobic positively contributed to the degradation of hydrocortisone, cortisone and prednisone with a 87%, 70% and 74% removal, respectively (15).

Not all wastewater facilities, including the Northwest Wastewater Treatment facilities are equipped with the combination of these units. The Northwest facility treats wastewater with pressurized air in the aeration process and anoxic and anaerobic units are not present which could have led to the inability to completely remove glucocorticoids from effluent as shown in this study.

To date, research has not identified a specific technology or wastewater treatment process that has the potential to completely remove glucocorticoids from wastewater effluent however the anaerobic treatment is highly recommended. Alternate secondary treatment and processes are available, i.e. activated sludge and biofiltration, in wastewater treatment; however, some are more effective for reducing estrogenic compounds and do not eliminate completely (51) (52) (53). Other common secondary treatment processes include coagulation, flocculation, and precipitation and have shown to be ineffective for the removal of endocrine disrupting compounds (54) (55). Other studies focused on tertiary treatment technologies and their ability to degrade steroid hormones (56) (57). Tertiary processes are mainly used for disinfection purposes, i.e. UV-light disinfection, chlorination, ozone or activated carbon and it is unknown how they specifically affect glucocorticoid removal during wastewater treatment. Activated carbon has shown to remove a large percentage of organic micro pollutants; however, it is highly complex as it depends on carbon type, contact time, contaminant solubility, and greater affinity than natural organic matter in wastewater (58) (59). These units are not found in many wastewater treatment facilities consequently, resulting in residual EDC concentrations in drinking water, surface water, wastewater effluent and sediment (60) (61) (62) (63).

Even though this research is focused on the Paso Del Norte region, the presence of glucocorticoids in the environment is a worldwide problem. Semi-arid and arid regions represent 30% of

the world's continental area. The cities of El Paso, TX and Juarez, Mexico form the largest metropolitan area in the world with a population of 2.2 million in an area with limited surface and ground water resources. Water supply demands are critical and highly implicated by growing populations and anthropogenic activities. It is important to address the presence of glucocorticoids and other potential problems related to EDCs in water management in semi-arid areas like Paso Del Norte. There are six wastewater treatment facilities in the region and the treated water from these plants are released into irrigation canals or Rio Grande, a river that forms part of the Mexico – U.S. border. It is important to target the removal of glucocorticoids during wastewater treatment processes to avoid further dispersal to freshwater systems and protect the water resources for the safety of public health.

3.4 Biological Method

Two previously hGR transfected yeast cells (developed by Dr. Marc Cox) were compared for method optimization: one with a higher promoter activity (MCY-212) in comparison to a lower promoter activity yeast cell (DSY-1345). Sensitivity was determined based on the half maximal effective concentration $EC_{50,}$ which describes a specific concentration that elicits half of the maximal response. The comparison of dose response curves and EC 50 values for the two stains using a positive control deoxycorticosterone (DOC) is shown on Figure 13.

The yeast strain DSY-1345 in the dose response curve correlates with lower plasmid expression in comparison to yeast strain MCY-212 that was transfected with a higher hGR plasmid expression. EC_{50} values for both yeast strains rendered response in the micromolar range and are not significantly different; however, yeast strain DSY-1345 had better R-squared values and had more consistent performance; therefore it was chosen in the continuation of this study.

Dose response curves were completed in triplicates for cortisone, hydrocortisone, prednisone and prednisolone with strain DSY-1345 (Figure 14). Their EC_{50} values were found within the micromolar range, excluding hydrocortisone where the EC_{50} response exceeded the proposed concentration range

used in this study. Summary of EC_{50} values for all target analytes using DSY-1345 is shown in Table 13 and the dose response curves are included in Appendix B.

Table 18 EC-50 values for DOC using MCY-212 and DSY-1345

Yeast Strain	**EC_{50} (M)**	**R^2**
MCY-212	2.38E-06	0.62
	2.21E-06	0.64
	1.96E-06	0.62
DSY-1345	4.58E-06	0.70
	3.82E-06	0.73
	4.26E-06	0.72

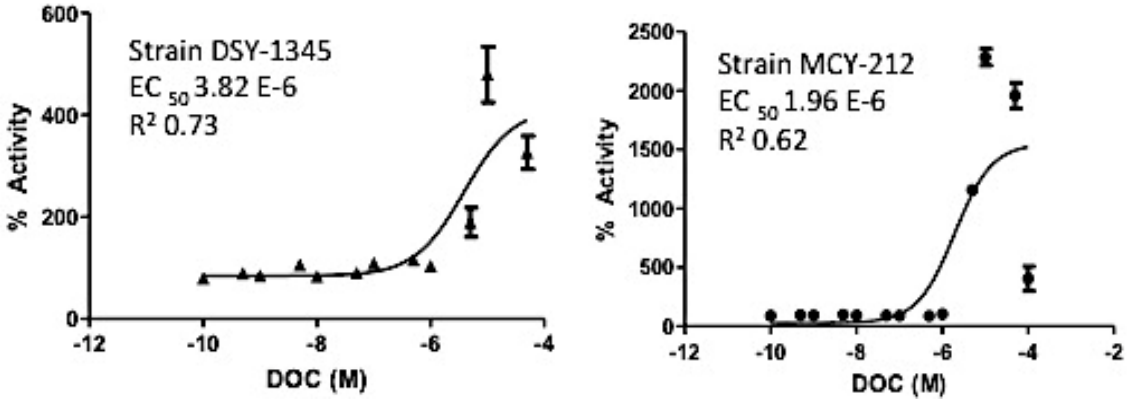


Figure 13 Dose-response curve comparisons for yeast strain DSY-1345 and MCY-212 using positive control DOC

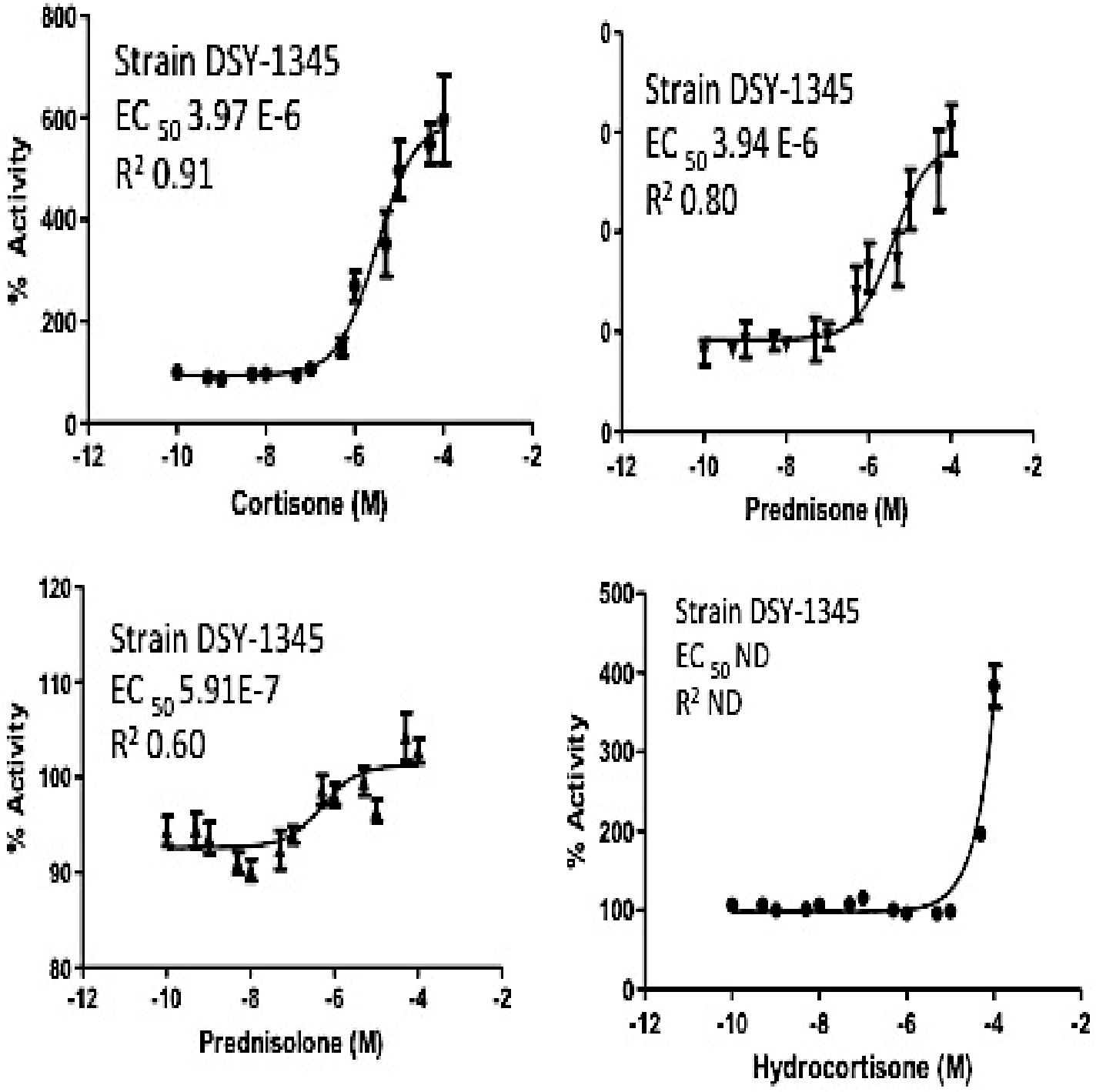


Figure 14 Dose-response curves using DSY-1345 for target analytes

Table 19 Summary of EC-50 values for target analytes using yeast strain DSY-1345.

	Cortisone		**Hydrocortisone**		**Prednisone**		**Prednisolone**	
	EC_{50} (M)	**R^2**	**EC_{50} (M)**	**R^2**	**EC_{50} (M)**	**R^2**	**EC_{50} (M)**	**R^2**
	4.61E-06	0.87	N.A.	N.A.	3.94E-06	0.8	7.37E-07	0.65
	3.74E-06	0.89	N.A.	N.A.	1.17E-06	0.87	5.19E-07	0.60
	3.97E-06	0.91	N.A.	N.A.	1.27E-06	0.78	9.02E-07	0.19
Average	**4.10E-06**		N.A.	N.A.	**2.13E-06**		**7.19E-07**	
mg/L	1.7		N.A.		0.77		0.26	

N.A. Not applicable

The average EC_{50} values for each compound were then translated into mg/L. Literature review has shown that environmental glucocorticoids are present in wastewater influent and possibly in effluent in the part per trillion ranges (ng/L) (38) (15) (27). Since environmental glucocorticoid concentrations are expected at low part per trillion ranges, the findings from this study lead to the conclusion that the biological method in this study is not sensitive enough to detect glucocorticoid activity in municipal wastewater samples. In addition, the coloration of the extracts was causing interference with the yeast assay and greatly reduced the application of the bioassay in wastewater analysis (Figure 15). If sampled wastewater were to be collected from hospital effluent, the yeast assay and mammalian assays would be a higher probability for biological activity as other studies have demonstrated (29).

Figure 15 The color in wastewater sample extract after SPE

As previously mentioned, mammalian and yeast cell assays are popular models for the biological analysis of steroid hormones in environmental samples. There are a limited number of studies that have reported biological effects of glucocorticoids in environmental matrices (Table 20). Literature shows that mammalian cell bioassays are the preferred choice for the detection of environmental glucocorticoids. However, it is still possible for yeast cell assays to biologically detect glucocorticoid when the concentrations were in high ng/L range as shown by Schriks et al. 2010.

Table 20 Literature review for biological analysis of glucocorticoids in environmental matrices

Type of Assay	**Matrix**	**Cell Type**	**Results**	**Ref:**
Recombinant yeast cell	Standard comparison	*Saccharomyces cerevisiae*, transfected with pCDNA3/hGRα plasmid containing a full-length cDNA of hGRα	**EC_{50} (µM)** CRL=123 CRN=not active PRL=643 PRN=not active	Bovee et al. 2011
GR-CALUX bioassay	Standard comparison	Human osteoplastic osteosarcoma U2-OS cells transfected with hGRα expression plasmid and a luciferase reporter construct	**EC_{50} (nM)** CRL=37 CRN=not active PRL=12 PRN=not active	Bovee et al. 2011
GR-CALUX bioassay	Wastewater	Human osteoplastic osteosarcoma U2-OS cells transfected with hGRα expression plasmid and a luciferase reporter construct	**ng dex EQs/L** 243 +/-32 (industry) 93+/-13 (hospital 1) 609 +/-79 (hospital 2) 38+/-13 (sewage effluent)	Schriks et al. 2010
CRL=Hydrocortisone; CRN=Cortisone; PRL=Prednisolone; PRN=Prednisone; ND= No data				

Green fluorescent protein-tagged nuclear receptor	Freshwater	Mammalian cell line, 36171	28% glucocorticoid activity in 105 samples for 14 states in the U.S	Stavreva et al. 2012
Recombinant yeast cell	Wastewater	*Saccharomyces cerevisiae*, transfected with hGRα plasmid	**EC $_{50}$ (µM)** CRL=ND CRN= ND PRL= ND PRN= ND	This research

In a study, five bioassays (yeast estrogen screen, ER-CALUX, MELN, T47D-KBlunc, and E-SCREEN) were compared and validated for the detection of estrogenic chemicals in wastewater, groundwater and freshwater. These biological methods involved yeast and mammalian cell assays and were compared based of sensitivity, robustness, MQL and reproducibility (Table 21). The mammalian cell assays were rated in the following order: MQL> sensitivity> robustness=reproducibility>low maintenance and cost. In comparison, the yeast assay is considered to be cost efficient and reproducible however, not sensitive, robust or have a good MQL. In addition, inability of interlaboratory reproducibility was observed by all assays except for yeast assay and E-SCREEN (64). It is apparent that the bioassays, such as yeast assay, still pose many constraints for the analysis of steroid hormones in environmental samples.

Table 21 Comparison in performance for the analysis of estrogenic compounds in wastewater between yeast and mammalian cell models

	YES	**ER-CALUX**	**MELN**	**KBlunc**	**E-SCREEN**

	(Yeast cell)	(Mammalian cell)	(Mammalian cell)	(Mammalian cell)	(Mammalian cell)
Sensitivity	-	+++	++	++	++
Robustness	-	++	++	++	++
Method quantification limit	_	+++	+++	+++	+++
Reproducibility	++	+++	+	++	++
Low maintenance and cost	+++	-	+	+	+

Chapter 4: Conclusion

An optimized chemical method using solid phase extraction (SPE) followed by liquid chromatography mass spectrometry (LC-MS), and a biological analysis using a 4-hour yeast assay were developed for detection of glucocorticoids in wastewater. The biological analysis used two previously hGR transfected yeast cells, MCY-212 and DSY-1345, to determine most sensitive strain. Half maximal effective concentrations for both strains were not significantly different and both strains exhibit a response in the micromolar range. The yeast strain DSY-1345 was selected for this study due to higher correlation coefficient for dose-response curves. The effective concentrations for cortisone, prednisone, and prednisolone resulted in the micromolar range concentrations that transfer to mg/L in environmental samples. The dose response for hydrocortisone was not completed due to its response exceeding the proposed concentration range and solubility problems that dealt with higher concentrations. The biological method in this study did not provide sufficient sensitivity for low glucocorticoid concentrations expected in the environment.

The optimization of the chemical method dealt with testing a variety of solvents for solid phase extraction and mobile phases for chemical analysis. With the chemical method developed, HPLC parameters and operating conditions were established. SPE recovery tests were completed with a total recovery of 67 ±0.01%. The glucocorticoids of interest were reported as a group and identified based on retention time and mass-to-charge ratio signature using selective ion monitoring (SIM).

The second objective was to apply the optimized methods to detect the occurrence, concentrations and bioactivity of glucocorticoids in wastewater. The chemical method of which involved sample preparation, extraction method and chemical analysis was applied to 9 influent and 9 effluent wastewater samples from the Northwest Wastewater Treatment Plant located in El Paso, TX. Glucocorticoid concentrations in the influent and effluent samples were determined for individual and total glucocorticoid compounds. The average glucocorticoid concentrations of individual target analytes in the influent samples ranged from 2.3 to 19 ng/L and glucocorticoid concentrations for individual target analytes in the effluent ranged from 2.7 to 14.3 ng/L. In this study, prednisone was the most prominent in influent and effluent wastewater samples. These findings might reflect that prednisone is highly consumed in the region.

The combined glucocorticoid concentrations of target analytes in influent wastewater ranged from 20 to 38 ng/L, while glucocorticoid concentrations in the effluent ranged from 18 to 31 ng/L. A trend was observed showing that glucocorticoid concentrations are at its highest in the morning and decrease as the day continues. This observation might further confirm that most of the glucocorticoid pharmaceuticals are consumed in the morning, in addition to higher natural production of cortisone in the morning. This is the first study that addresses the occurrence of glucocorticoids in wastewater at different times during a sampling day.

The influent glucocorticoid concentrations found in this study are in agreement with the levels found in other literature. However, the glucocorticoid effluent concentrations were higher than levels reported in the literature (0.06 to 0.5 ng/L in China and <10ng/L in Spain).

The estimated removal of glucocorticoids from wastewater treatment processes in the Northwest Wastewater Treatment facility is 17.9 ± 9.7%. The finding in this research suggests that effluent water from this facility could be a substantial source of glucocorticoid in the aquatic environment. It is important to target wastewater treatment processes to avoid aquatic organisms to be exposed to environmental glucocorticoids. In addition, it is recommended for wastewater treatment processes to include aerobic-anoxic secondary processes in wastewater treatment facilities. Due to high costs of implementing new units, the retention time during wastewater treatment processes is a parameter that could be further researched and modified to enhance further degradation of organic contaminants.

4.1 Future Work

This research serves as a preliminary study for the detection of glucocorticoids in wastewater. The biological response was not assessed in this study and it is recommended to implement a mammalian cell assay for the detection of environmental glucocorticoids. It is also important to implement analytical instrumentation that is suitable of detecting low levels of glucocorticoids as expected in environmental concentrations. Though HPLC/MS used in this study was able to obtain some results, further study may consider tandem MS for better identification of different glucocorticoid compounds in the environmental samples.

In this study, prednisone was found to be the most dominant glucocorticoids in the sample. The findings suggest that prednisone could be highly consumed in the west side of El Paso, Texas. Further

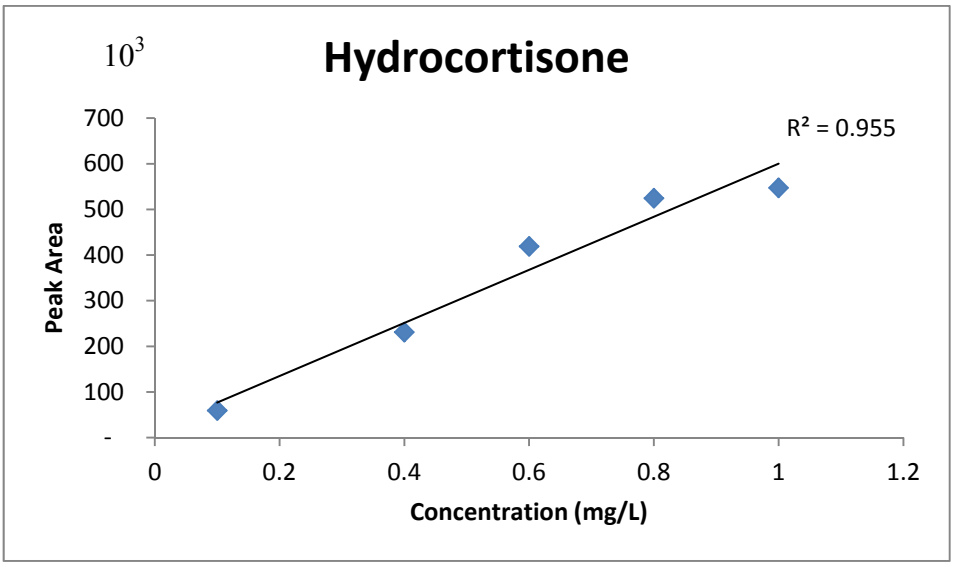
research could be done to determine specific conditions that lead to high consumption of prednisone in this area.

Another important highlight is that the Northwest Wastewater Treatment Plant is not capable of removing glucocorticoids from wastewater influent. Future studies can be conducted where the retention time of secondary and tertiary processes can be modified to determine a suitable time for the removal of EDCs at this specific facility. This could potentially save millions of dollars since no additional units would be necessary.

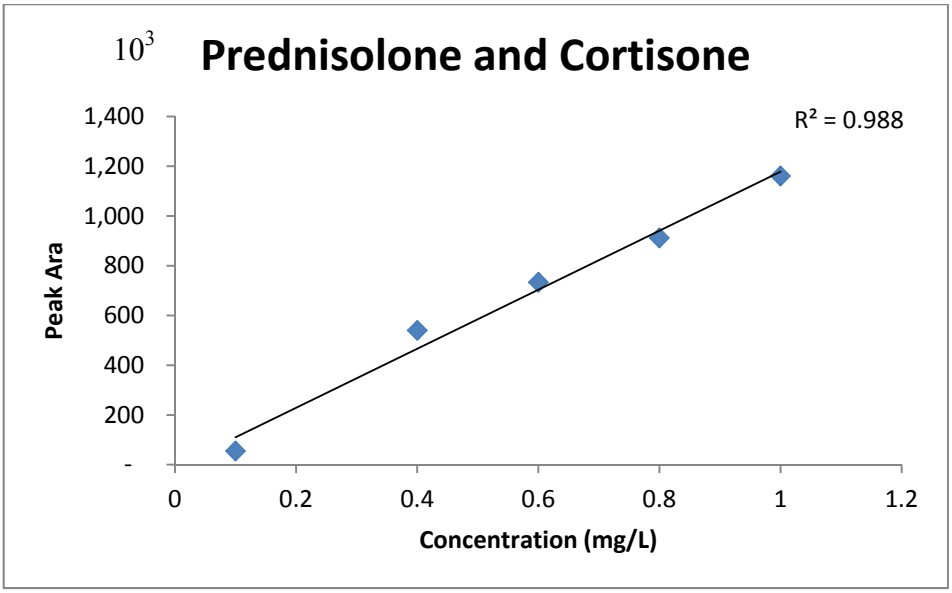
Further studies need to address the in-vitro and in-vivo total effects of glucocorticoids. There is a need for toxicity studies to determine no observable effect level concentrations (NOEL) so environmental regulation standards and monitoring can be established in environmental settings. In addition, it is important educate the public and wastewater treatment personnel on EDCs, their sources, effects, and daily exposures.

Appendix A

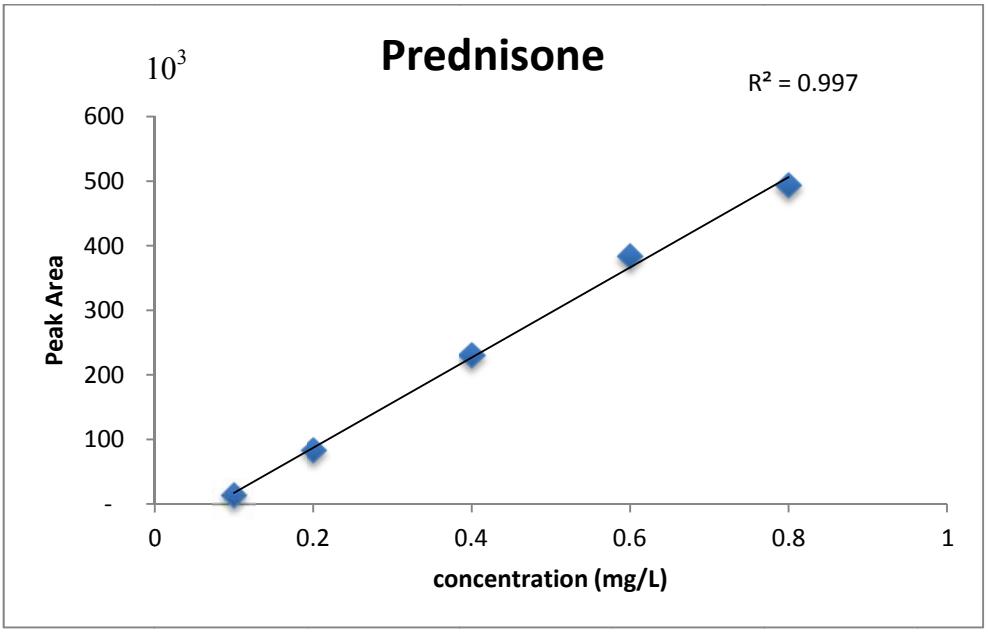
Calibration curves of the glucocorticoids in this study.



Calibration curve for glucocorticoid hydrocortisone (Peak area (detection response) vs. Concentration)

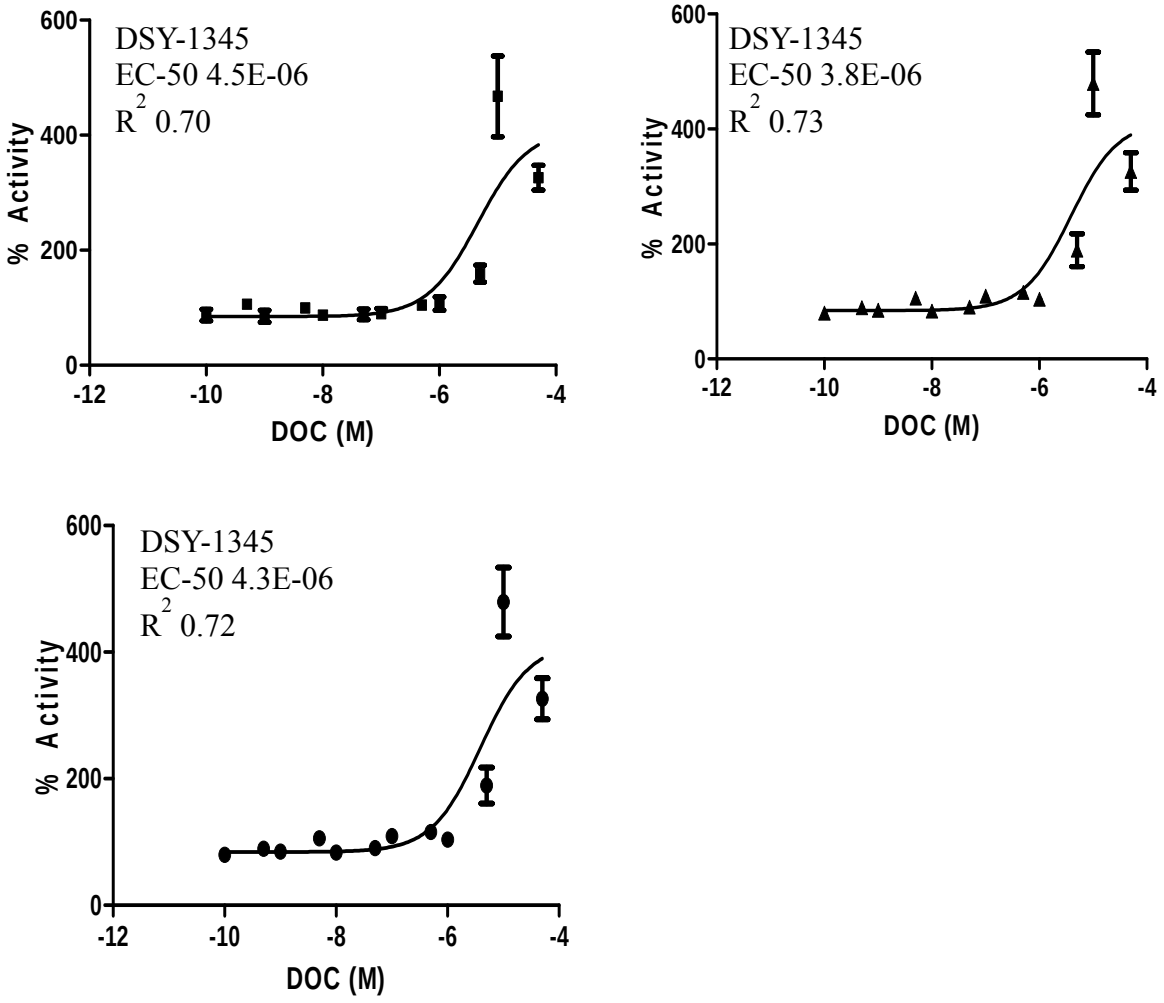


Calibration curve for glucocorticoid prednisolone and cortisone (Peak area (detection response) vs. Concentration)

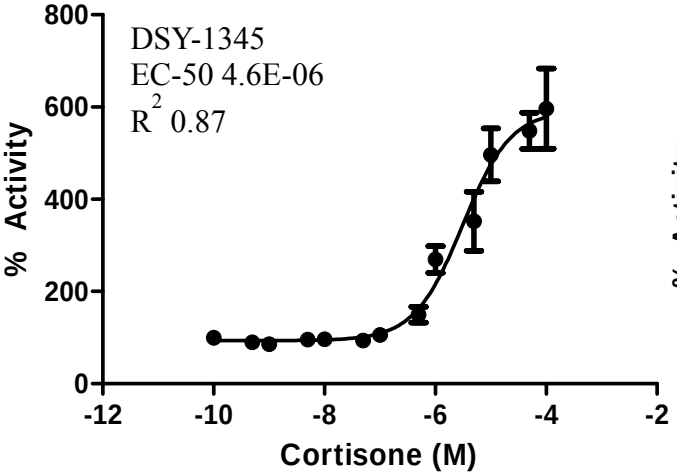


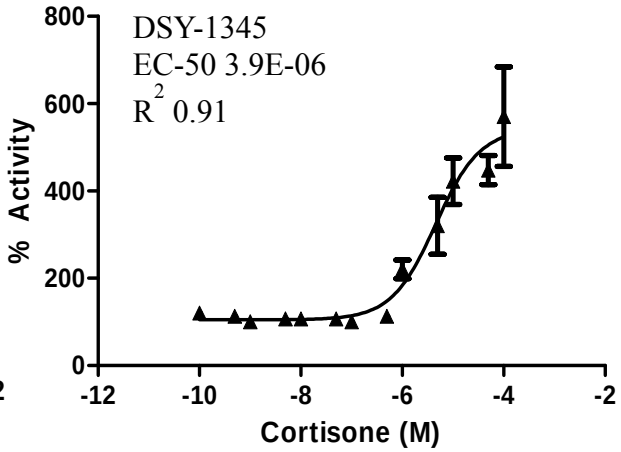
Calibration curve for glucocorticoid prednisone (Peak area (detection response) vs. Concentration)

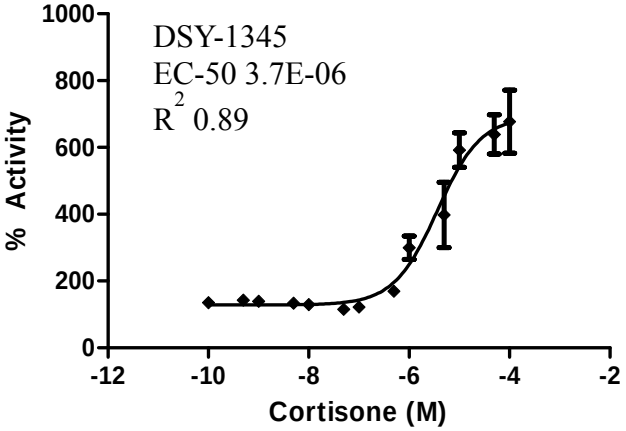
Appendix B



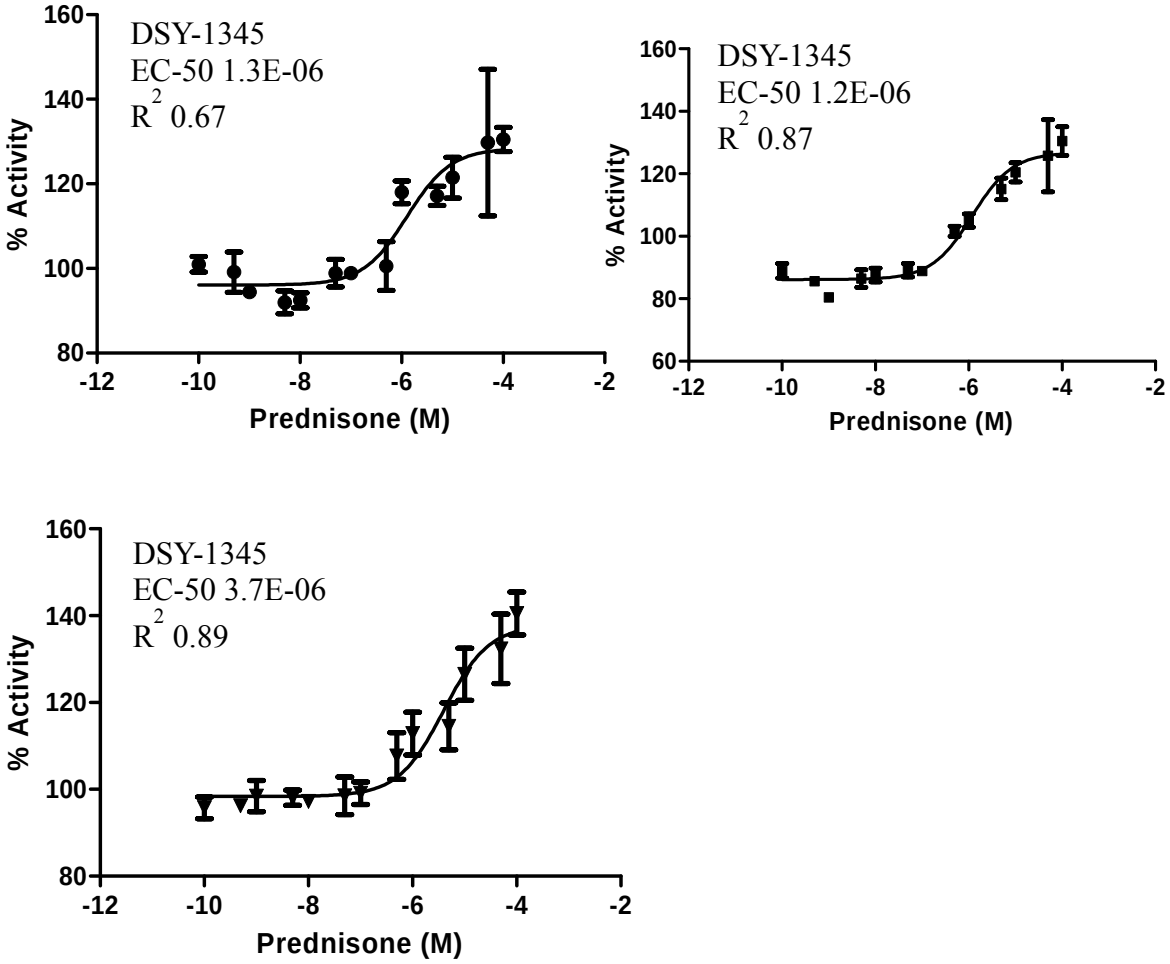
Dose response curves for positive control deoxycorticosterone (DOC) using yeast strain DSY-1345



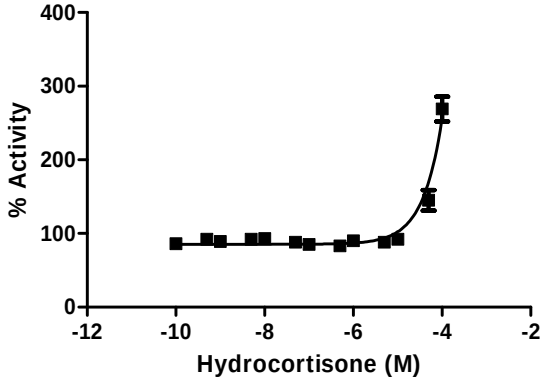


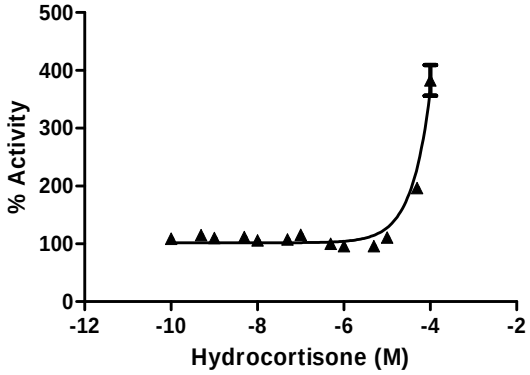


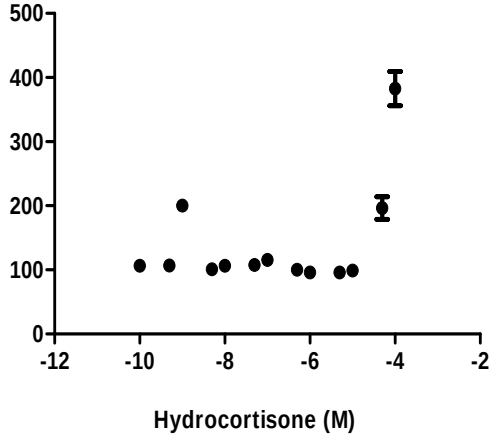
Dose response curves cortisone using yeast strain DSY-1345



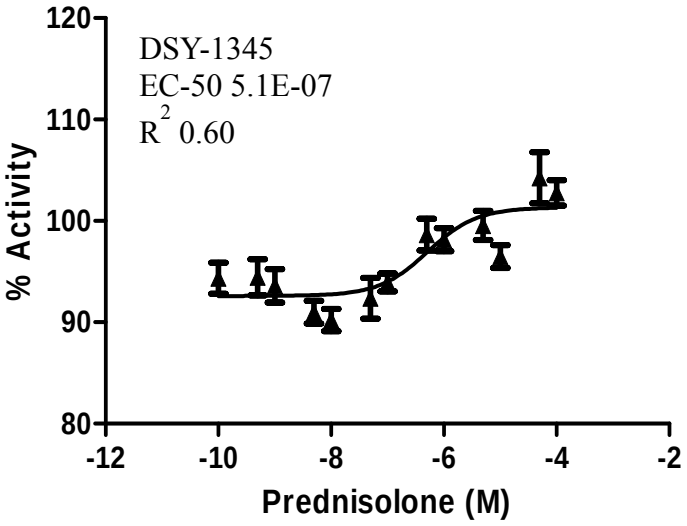
Dose response curves prednisone using yeast strain DSY-1345

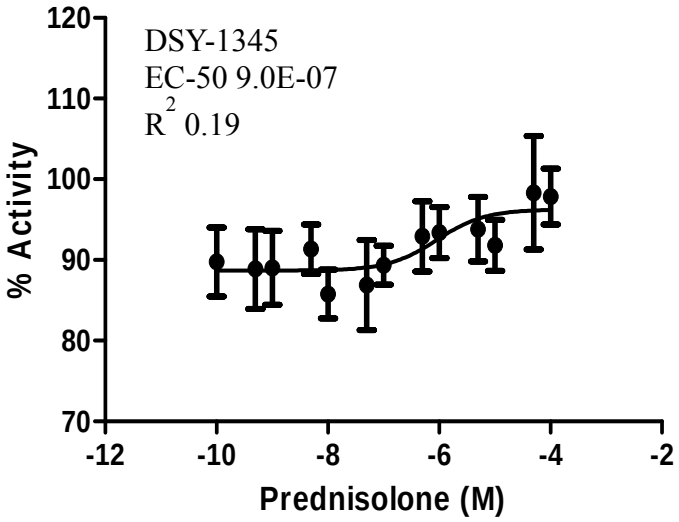


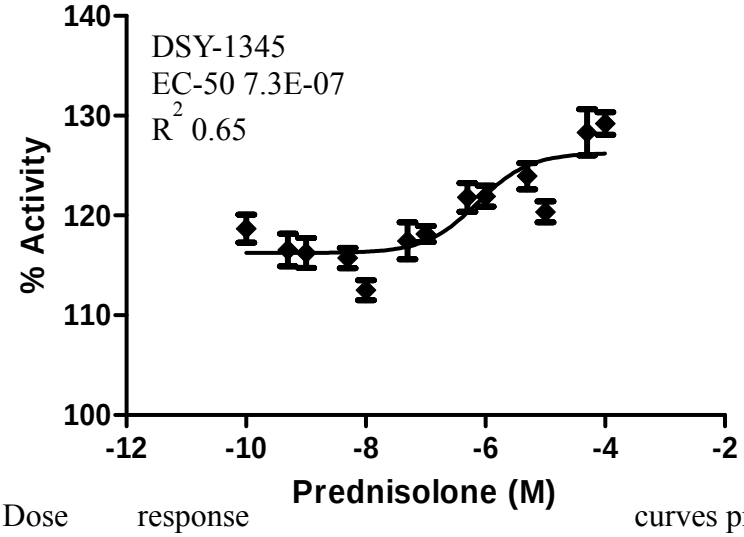




Dose response curves hydrocortisone using yeast strain DSY-1345







Dose response curves prednisolone using yeast strain DSY-1345

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Curriculum Vitae

Maria Del Carmen Lozano was born in Las Cruces, NM from Bertha and Jose Lozano. She graduated from Santa Teresa High School, Santa Teresa, New Mexico in 2005. After graduation, she attended New Mexico State University where she became a recipient of a Bachelors of Science degree in Environmental Sciences in 2011. During her undergraduate studies, she served as an intern for the Bureau of Land Management where she identified physical hazards from abandoned mines for the state of New Mexico to ensure public safety. After graduation in 2011, she became part of a research team in La Rochelle, France where she carried research in international business. She pursued her Masters Degree at the University of Texas at El Paso where she conducted research under the mentorship of Dr. Wen-Yee Lee. Her project involved the development of a chemical and biological method for the detection of glucocorticoids in wastewater by High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) and a 4-hour yeast assay. She presented her research at the Society of Environmental Toxicology and Chemistry (SETAC) national conference and the University of Texas at El Paso Graduate Expo. In addition, she was recognized by the Computing Alliance of Hispanic-Serving Institution (CAHSI) for student of the month. While in the program, served as a teaching for Environmental Science labs. She became the recipient of the National Science Foundation Louis Stoke Alliance for Minority Participation (LSAMP) fellowship and the Dobson Research Grant that funded her graduate studies.

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