FH University of Applied Sciences TECHNIKUM WIEN

MASTER'S THESIS

Thesis submitted in fulfillment of the requirements for the degree of Master of Science in Engineering at the University of Applied Sciences Technikum Wien - Degree Program Master Ecotoxicology and Environmental Management

Measuring cortisol in wastewater to complement community health surveillance

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Vienna, 22.10.2024.

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Kurzfassung

Das Ziel dieser Studie ist die Entwicklung einer Methode zur Extraktion und Quantifizierung von Cortisol und Cortison aus Abwasserproben (Zulauf, Ablauf) mittels Ultrahochleistungsflüssigchromatographie mit Tandem-Massenspektrometrie (UHPLC-MS/MS). Abwasserproben werden gesammelt und zur Analyse durch Filtration und Festphasenextraktion (SPE) vorbereitet. Das Cortisol und Cortison werden aus den Proben eluiert und durch UHPLC-MS/MS analysiert. Um eine genaue Detektion und Analyse durch das Instrument zu gewährleisten, muss eine instrumentspezifische Methode entwickelt und validiert werden. Dieser Prozess besteht aus mehreren Schritten, die die Anpassung der Flüssigchromatographie- (LC) und der Massenspektrometrie- (MS) Parameter umfassen, damit das Instrument präzise und zuverlässige Ergebnisse liefert. Mehrere mit Cortisol und Cortison angereicherte Abwassermatrizen können anschließend durch das Instrument geleitet werden, um die Extraktionseffizienz festzustellen. Wichtige Parameter für die Methodenentwicklung umfassen die Retentionszeit, die Ionisierungsquelle, Kalibrierkurven sowie die Festlegung der Nachweisgrenze (LOD) und der Quantifizierungsgrenze (LOQ). Nach einer erfolgreichen Pilotstudie, in der mehrere Abwassermatrizen vorbereitet und analysiert werden, kann diese Methode in einer umfassenden Studie zur Quantifizierung von Cortisol und Cortison in verschiedenen Abwasserproben eingesetzt werden, um zeitliche Schwankungen zu analysieren.

Schlagwörter: LCMS, Cortisol, Abwasserproben, Nachweisgrenze, Quantifizierung

Abstract

The objective of this study is to develop a method for the extraction and quantification of cortisol and cortisone from wastewater samples (influent, effluent) using ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS). Wastewater samples are collected and prepared for analysis by filtration and solid-phase extraction (SPE). The cortisol and cortisone are eluted out of the samples and run through UHPLC-MS/MS. To achieve accurate detection and analysis by the instrument, an instrument-specific method must be developed and validated. This process consists of several steps involving the adjustment of the liquid chromatography (LC) and the mass spectrometry (MS) parameters so that the instrument is best placed to deliver accurate and reliable results. Several spiked wastewater matrices can subsequently be run through the instrument in order to establish the extraction efficiency. Key parameters for the method development include the retention time, the source of ionization, calibration curves and the establishment of the limit of detection (LOD) and limit of quantification (LOQ). Following a successful pilot study where several wastewater matrices are prepared and analyzed, a full study can apply this method for the purposes of quantifying cortisol and cortisone in wastewater samples of different kinds and thereby analyze variation over time.

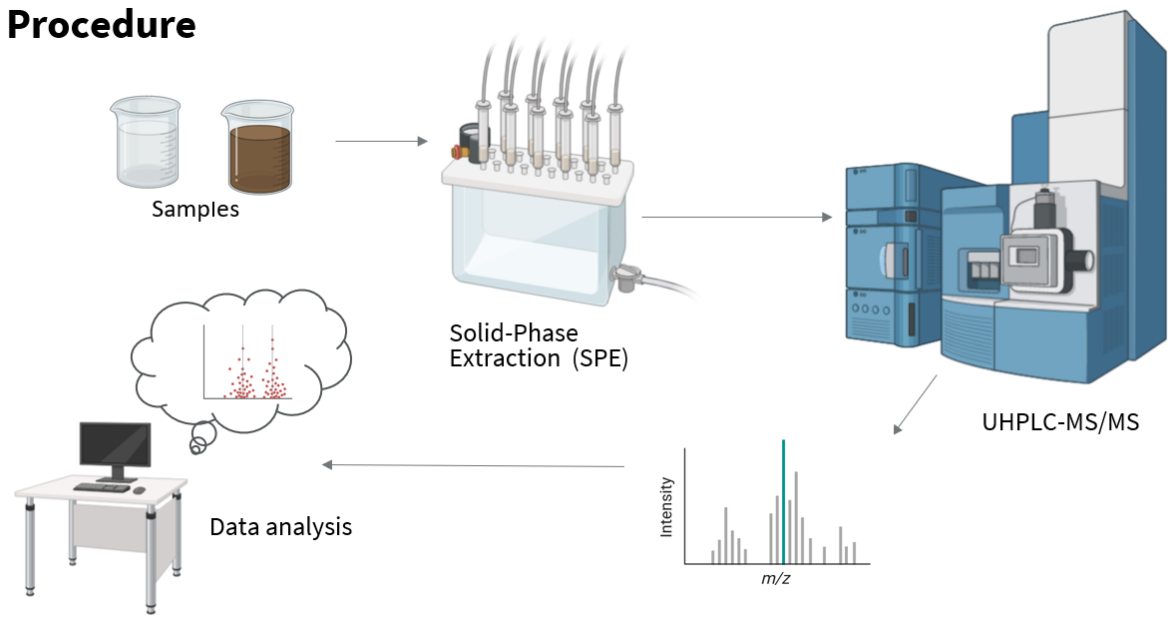

Figure 1: Overview of the procedure for this project. (Image created by Elena Tiis using icons from Biorender.com)

Keywords: LCMS, Cortisol, Wastewater Samples, Limit of Detection, Quantification

Acknowledgements

This research was made possible thanks to the individuals who supported, and inspired me before and during my stay at University of Carolina at Chapel Hill.

I am greatly indebted to Prof. Joe Brown at UNC for committing to my highly speculative email and for helping me to form it into an actionable project. His support with the practicalities of the stay were absolutely invaluable.

Thank you so much for Dr. Megan Lott, for being my go-to person in so so many cases, and Abby Boyer for being part of my core team at the Brown lab.

I am very appreciative my first supervisor Dr. Romana Hornek-Gausterer for her thorough commentary and support with the writing process. Her expertise in wastewater monitoring and ecotoxicology were the necessary ingredients to ground this thesis.

Next, I extend my sincere thanks to the conversations and camaraderie with Ian Choi, Gouthami Rao, Naomi Yang, Kevin Zhu, Jo Buehrer, Olivia Harmon and Mercy Neal, which were the fertile background that shaped this research.

Special thanks also go to Brandie M. Ehrmann, Cristina Arciniega and Lazaro Toledo at the UNC Chemistry Mass Spectrometry Core Laboratory for their counsel concerning lab practicalities and LC-MS method development.

I would also like to acknowledge my sponsors, the Marshall Plan Scholarship Foundation, and the support of the International Office at UAS Technikum Vienna for making the research stay a reality.

Finally, I would like to thank my family their love and support throughout my studies, for helping me maintain the drive as the going gets difficult.

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1 Introduction: Why Measure Cortisol?

Cortisol and cortisone are glucocorticoid hormones (GCs) which are naturally produced in the adrenal cortex along the hypothalamic-pituitary-adrenal (HPA) axis (Figure 2). They are part of a range of physiological processes specific to humans and other vertebrates. By contract, invertebrates, non-chordate animals, plants, fungi, and prokaryotes lack cortisol and use different hormonal or biochemical systems to regulate stress and metabolic processes.

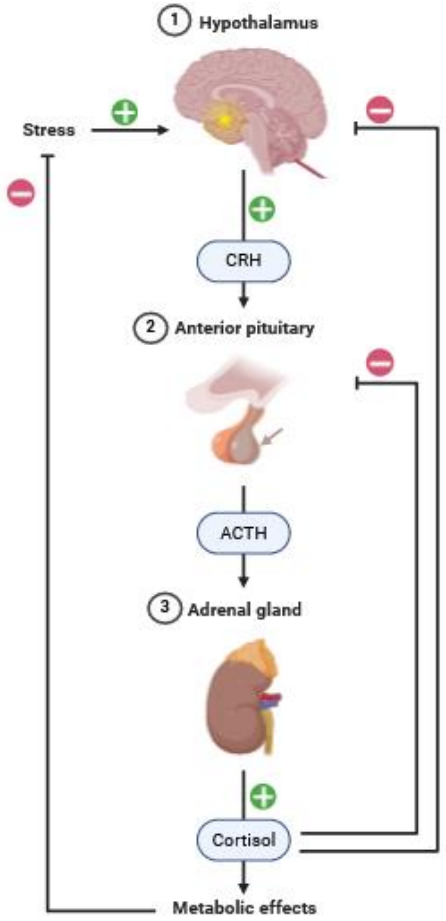

Figure 2: Cortisol production along the HPA-axis (Fontana, 2024)

GC hormones are colloquially called “stress hormones” because they serve an important role in the stress response for vertebrates. They signal the need to upregulate or downregulate physiological systems, importantly the immune system by suppressing inflammation, and the metabolic system with higher levels associated with higher metabolic rates (Haase et al., 2016). Since most bodily cells have cortisol receptors, cortisol is part of wide range of regulatory activities across cardiovascular, metabolic, homeostatic, cellular health, and central nervous systems (Fontana, 2024; Jones & Gwenin, 2021).

Cortisol dysregulation (i.e. chronically elevated or depressed levels) has significant effects for the human body. Extreme conditions such as Cushing's syndrome (hypercortisolism) and Addison's disease (primary adrenal insufficiency) aside, studies have looked at the biological link between chronic stress, depression, and type 2 diabetes mellitus (Joseph & Golden, 2017; Strehl et al., 2019), pro-inflammatory consequences and contribution to chronic conditions especially in the case of non-communicable diseases such as cardiovascular disease and autoimmune conditions (Jones & Gwenin, 2021; Strehl et al., 2019). Studies have looked at how chronic stress decreases motivation and reward processing by impairing dopamine transmission in mice (Holloway et al., 2023) and how it is also implicated in neurodegenerative disease and psychological disorders (Knezevic et al., 2023). In sum, chronic stress is associated with the progression of various types of illness due to the interplay between cortisol and various diseases at the level of the immune, nervous, and endocrine systems (Knezevic et al., 2023; Strehl et al., 2019). Cortisol production is thus linked to the development of chronic

conditions such as coronary heart disease, stroke, high blood pressure, metabolic diseases such as diabetes, and mental illness (Jones & Gwenin, 2021).

Synthetic versions of GC hormones are also produced and find uses in various clinical applications – for example, as treatment for rheumatoid arthritis, asthma and Chron's disease (Scherholz et al., 2019). It is therefore unsurprising that residues, whether due to human excretion or pharmaceutical industry, have been found to be present in wastewater samples (Wu et al., 2019). This draws the question of whether the presence of these hormones can be monitored and measured in order to draw conclusions about population health or environmental impact. Wastewater based epidemiology (WBE) is a field which specializes in monitoring wastewater for the presence of infectious diseases, most commonly viral nucleic acids (DNA or RNA). The ability to measure cortisol in wastewater samples would make it possible to explore whether this type of monitoring can yield complementary insights into community wellbeing.

Wastewater monitoring is a non-invasive tool which can offer insights into community stress levels, community health and possibly mental health, and medication and drug usage. While it does not require direct sampling from individuals, data obtained in this way can indirectly cause harm to communities (this aspect is further discussed in section 1.7.1). There are also some methodological concerns and difficulties for establishing correlations between cortisol levels in wastewater and health indicators associated with this approach. One of the main issues is that cortisol levels in wastewater can also be affected by factors such as diet, health, and lifestyle (Stachowicz & Lebiedzińska, 2016), which can cause fluctuations in cortisol levels that are not related to health/mental health concerns.

When communal and pharmaceutical wastewaters are both treated by the same facility, it can be difficult to distinguish the source of GC residue and draw reasonable correlations. Since the GC residues that can be measured in wastewater are a sum parameter, it means that the conclusions that can be drawn by default integrate various (positive or negative) health and epidemiological conditions. WBE's usefulness as a tool can come into play when comparing GC levels from various locations and on a temporal scale (for example, 24hr or seasonal trends).

Any sociological or medical interpretation of the cortisol levels depends on where the samples originate, which means that the local context will need to be carefully taken into account. Furthermore, it is unclear how accurately cortisol levels in wastewater correlate with (mental) health in a community and therefore how reliable a population biomarker cortisol can be. Any absolute values gleaned from measuring any specific variable in wastewater are difficult to interpret conclusively, however their relative change over time (longitudinal monitoring) has the capacity to offer most insight. Stressful events that impact populations (war, disease

outbreaks) may be indicated as elevated GC residues, but this kind of approach needs further research.

1.1 Chemical Structure

Chemically cortisol belongs to a class of steroids and is characterized by a four-ring carbon structure with hydroxyl and ketone functional groups. Cortisone is closely related to cortisol and can be converted to cortisol in the body, serving as precursor or metabolite. Cortisone differs from cortisol by being the biologically inactive form of the hormone, which is converted into the active form cortisol as and when needed. Chemically they are similar, though cortisone has a ketone group at the 11th carbon position whereas cortisol has a hydroxyl group at the position. They are both not very large molecules with mass-to-charge ratios (m/z) of 363-369 (Figure 3).

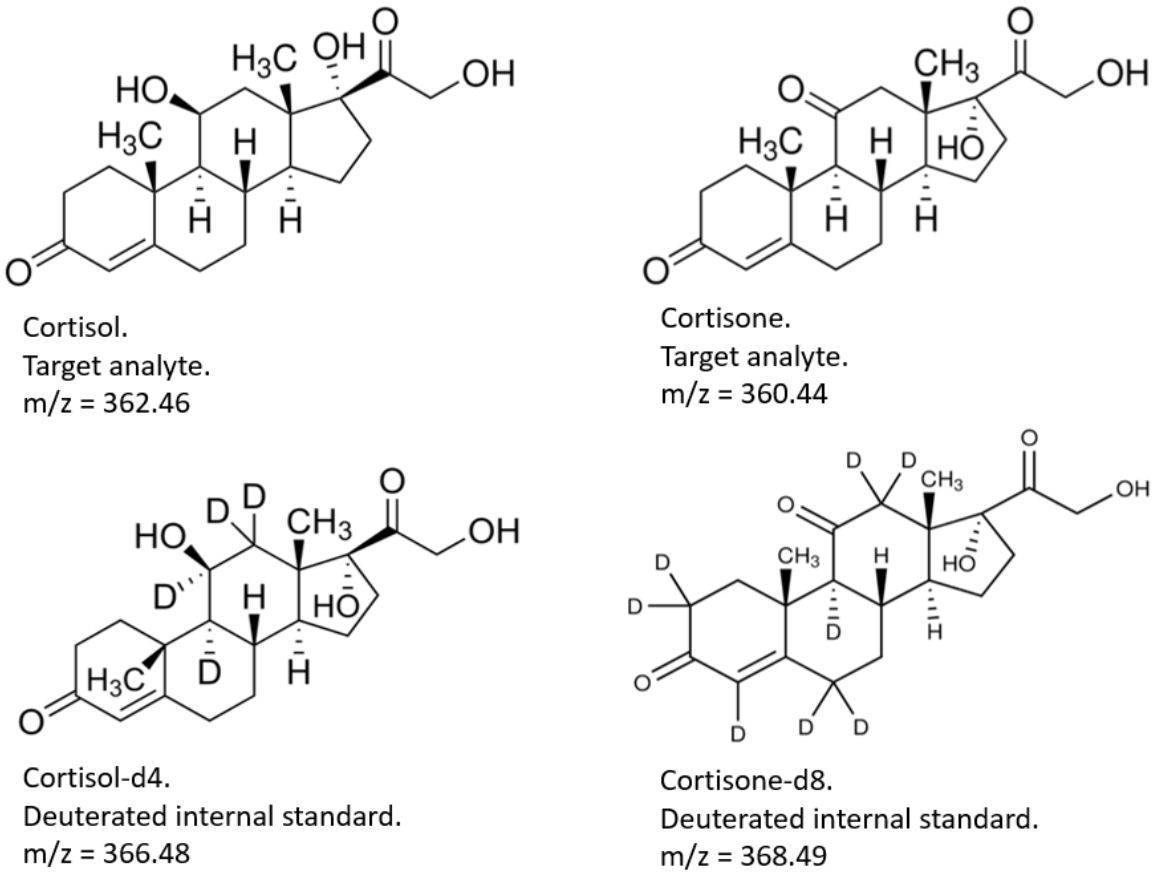

Figure 3: Target analytes and internal standards for this study. Deuterated versions where the heavier deuterium substitutes hydrogen are used as internal standards.

Synthetic analogues of cortisol include prednisolone, prednisone, and dexamethasone, which are commonly used in medicine to treat inflammatory and autoimmune conditions. Prednisolone and prednisone are structurally similar, with prednisone being converted to prednisolone in the liver. Synthetic glucocorticoids are usually more potent and longer-acting than cortisol, making them particularly useful in treating severe inflammatory conditions. These synthetic derivatives are designed to mimic cortisol's effects while offering enhanced potency and stability for therapeutic use, with the downside being that they do not degrade as easily and end up in the environment (Cantalupi et al., 2020; Weizel et al., 2020; Wu et al., 2019). For discussion of the environmental fate and degradation of cortisol and cortisone refer to section 1.3.

Our study uses simply the two naturally produced GCs cortisol and cortisone as target analytes and their internal standards. The deuterated or “labelled” versions of cortisol and cortisone are used as internal standards to improve measurement accuracy. Internal standards are used for the correction of effects, such as instrument drift or variable sample injection volume, or for internal calibration of the measurements (Sargent, 2013). Deuteration means that the hydrogen atoms in the molecule are replaced with the heavier deuterium (Figure 3). The physical and chemical properties of the analytes are collected in the following table (Table 1).

Table 1: Physical and chemical properties of cortisol and cortisone

Compound	**Cortisol (Hydrocortisone)**	**Cortisone**
CAS Number	50-23-7	53-06-5
Molecular Formula	$C_{21}H_{30}O_5$	$C_{21}H_{28}O_5$
Physical Description	Solid, odorless, colorless	Solid, odorless, colorless
PARTITION COEFFICIENT		
Log Kow	1.61 (National Center for Biotechnology Information, 2024a) = slightly lipophilic	1.47 (National Center for Biotechnology Information, 2024b) = slightly lipophilic
SOLUBILITY IN WATER		
	0.32 mg/mL at 25 °C (National Center for Biotechnology Information, 2024a) = soluble	0.28 mg/mL at 25 °C (National Center for Biotechnology Information, 2024b) = soluble
MELTING POINT		
	220 °C (National Center for Biotechnology Information, 2024a)	222 °C (National Center for Biotechnology Information, 2024b)

1.2 Cortisol as a Biomarker

Cortisol measurements have been used as a stress marker in animal behavior studies, so methods for its measurement in wastewater samples have been established (Chang et al., 2007; Schriks et al., 2010). Previous studies have analyzed cortisol as a stress indicator in farmed fish (Lemos et al., 2023) and during the academic year in college students (Driver et al., 2022).

Cortisol can be detected in direct human samples (saliva, urine, feces, blood plasma, milk, hair) in order to gather information on the physiological state of the person (Cook, 2012). Samples provide time-integrated measures that range from <1 h for urine and milk, several hours for feces and the timeframe of weeks for hair (Cook, 2012) with hair cortisol measurements being the most reliable biomarker for chronic stress in individuals (Noushad et al., 2021). Shorter timeframes are rather episodic (prone to variation). Population level information, by contrast, can be gathered through wastewater or environmental media (surface water). Immunoassays or bioassays can be used when the samples are biological fluids (de la Rosa et al., 2021). Apart from direct biological samples, chromatographic and mass spectrometry techniques can also be used for cortisol extracted from wastewater and environmental media (subject to appropriate sample preparation through solid-phase or liquid-liquid extraction) as these techniques are very sensitive to very low amounts.

However, cortisol as a population biomarker is relatively imprecise. It is quite generic and the causes behind elevated levels can be quite complex. First, it is subject to the diurnal cycle (levels normally peak in the early morning and decline throughout the day) (Dowd et al., 2009) (Figure 4). The early peak is known as the cortisol awakening response (CAR), and the values decline thereafter (Dowd et al., 2009; Jones & Gwenin, 2021). The influence of the diurnal cycle can be corrected for by collecting the samples at the same time point. Yet, lengthy exposure to stressors can lead to the overstimulation of the HPA axis resulting in fluctuating cortisol levels.

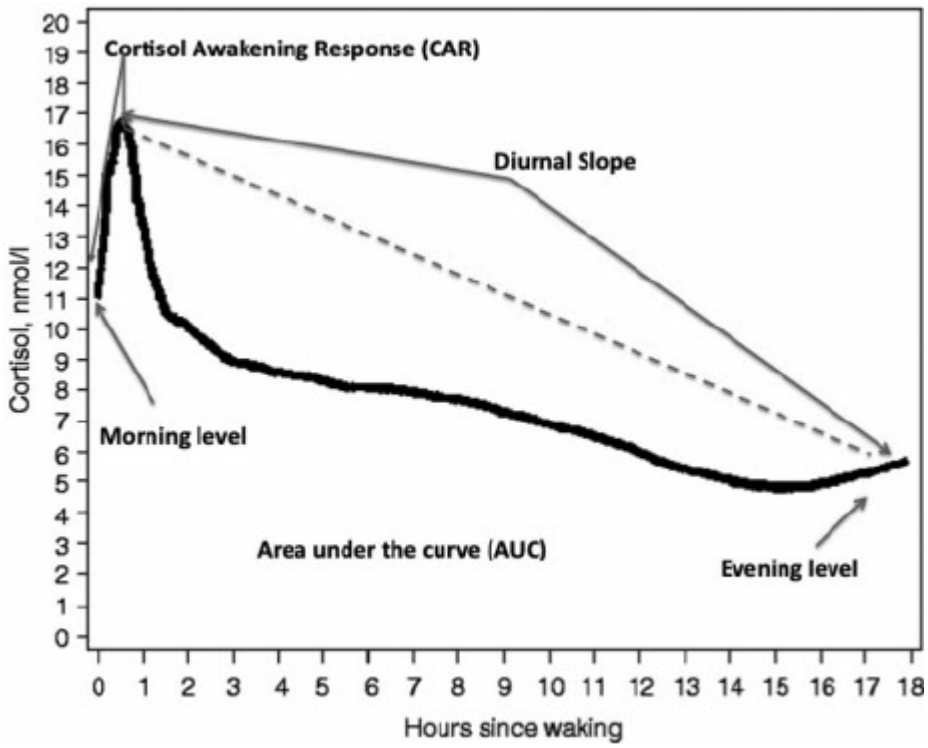

Figure 4: Example of diurnal pattern of cortisol secretion (Dowd et al., 2009)

Secondly, it is difficult to distinguish between chronic conditions and acute stress caused by various external factors such as lifestyle (movement, sleep, food and substances consumption patterns). Added to this, individual variability such as genetic differences and health conditions and contextual factors make the determination of a baseline difficult. Jones & Gwenin call it a "somewhat complex biomarker" (Jones & Gwenin, 2021) due to the number of chronic conditions it can refer to.

As concerns the responsiveness of cortisol as a biomarker, responses can be short-term or long term. It has a role in reflecting both immediate and sustained physiological states. While short-term cortisol fluctuations tend to provide real-time insights into an individual's acute stress response and overall HPA axis function, long-term cortisol patterns (such as those observed via hair analysis or chronic alterations in diurnal rhythm) may serve as markers of prolonged stress exposure (Noushad et al., 2021).

There are specific patterns associated with cortisol secretion across different timescales (Figure 5). Short-term response or acute stress response (minutes to hours) shows a rapid cortisol spike in response to acute stress which peaks around 20 minutes and declines to baseline within 1 to 2 hours. This response would typically occur in response to a physical or psychological stressor such as exercise or injury or emotional upheaval. Blood or salivatory

cortisol measurements are better placed in capturing this type of fluctuation (Noushad et al., 2021). Diurnal rhythm (24 hours) is the natural daily cycle of cortisol which peaks around 30-45 minutes after waking (cortisol awakening response) and tapers off throughout the day. This rhythm parallels the sleep-wake cycle and can be disrupted due to shift work or poor sleep quality. Measurements pertaining specifically to tracking the diurnal pattern would be best placed to take blood or saliva samples over 24 hours. Long-term response (weeks to months) highlights two extremes: the gradual increase in baseline cortisol due to chronic stress (hypercortisolism) and the gradual decrease in cortisol levels associated with prolonged stress or adrenal fatigue (hypocortisolism). Aside from these, chronic stress response may also lead to the "flattening" of the diurnal rhythm (i.e., less pronounced variation between morning and evening levels). Chronic cortisol level tracking in individuals is best accomplished through hair, which provides an integrated assessment of long-term cortisol exposure over weeks to months (Noushad et al., 2021).

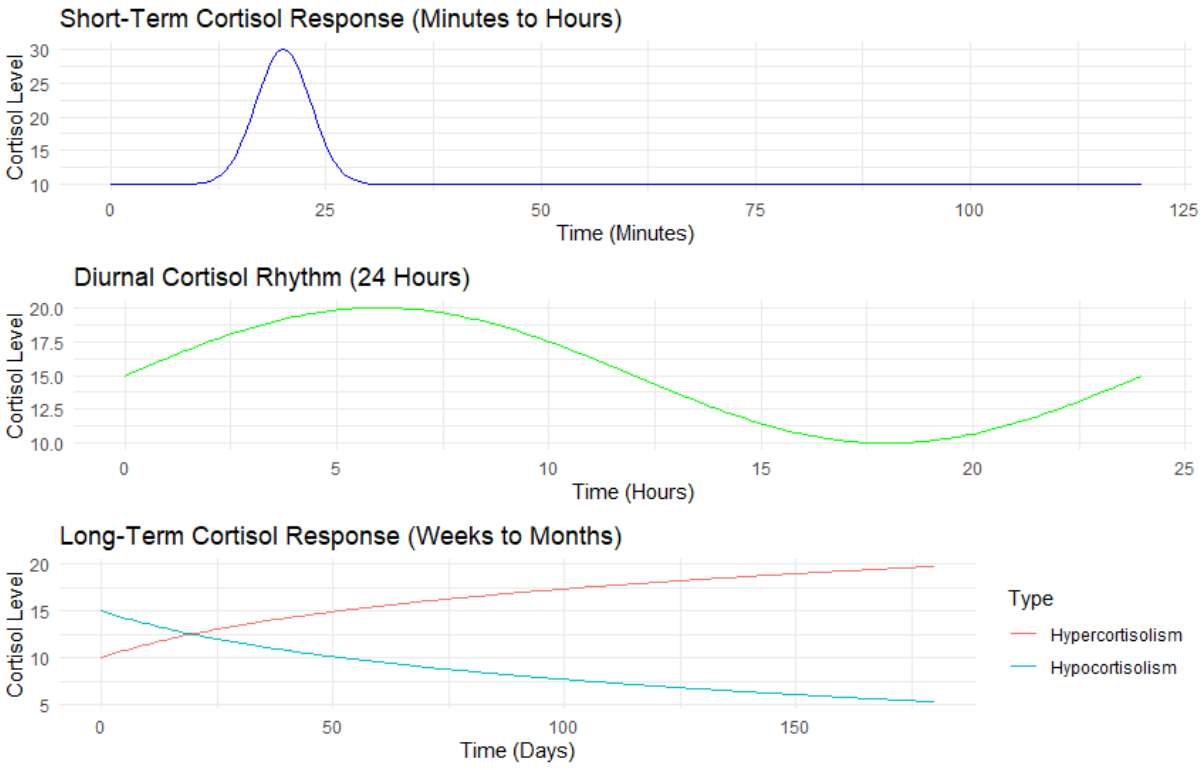

Figure 5: Example cortisol response curves across short-term and long-term timescales

The complexity of wastewater adds to the challenge of using cortisol as a biomarker for population health. Wastewater contains a broad range of substances including pharmaceutical and industrial chemicals and biological materials, which can impact the detection and cause matrix effects (Tisler et al., 2021; Zhang et al., 2011). This means that the analytical signal is altered and the accuracy and precision of measurement is lessened. Furthermore, cortisol is present in wastewater at very low concentrations, which means that analytical methods must

be highly sensitive and pre-concentration may be necessary. Influent may contain higher concentrations than effluent due to the degradation during the treatment stages in the wastewater treatment plant (WWTP).

The extraction of cortisol from wastewater is challenging, as it is a procedure with multiple steps that increases the possibility of error or contamination. Due to its chemical consistency and slightly lipophilic nature (see Table 1), it is conceivable that cortisol could be found in the solid fraction of sewage sludge. Extraction of cortisol from this type of material has been achieved with pressurized liquid extraction (PLE) followed by solid-phase extraction (SPE) (Herrero et al., 2013).

Cortisol can degrade due to environmental factors and over time which can affect its stability during collection, storage and analysis (for information on half-lives, refer to Table 2). It degrades in wastewater with the rate of decay increasing with temperatures higher than 15 °C (Kelkar et al., 2023). The presence of biofilms in sewer systems speeds up the degradation (Thai et al., 2019), making it unclear whether older, downstream samples or samples that have been stored a longer period of time can be analyzed reliably. This undescores the importance that sampling strategy and sample preservation have in achieving reliable results.

1.3 Cortisol Degradation and Presence in the Environment

Some of the most common sources for the presence of pollutants in the environmental waters are hospital wastewater, household sewage, agricultural and industrial wastewater, and possibly animal husbandry wastewater, and usually inadequate removal in many wastewater treatment plants (WWTPs) (Yazdan et al., 2021).

Although GCs are produced in small quantities and therefore exhibit low environmental concentrations, ecotoxicologically these substances especially when synthetic may pose a high-risk potential due to potent endocrine activity (Dierkes et al., 2021). Studies on the fate of GCs and synthetic GCs in the environment indicate that WWTPs or STPs remove about 90% of GCs, with best removal rates for naturally occurring GCs (Chang et al., 2007; Schriks et al., 2010; van der Linden et al., 2008). Dierkes et al. have investigated the degradation behavior of synthetic steroid hormones in activated sludge and have observed a range from extremely fast to relative stability depending on the chemical structure (Dierkes et al., 2021). In contrast, natural steroids are considered as biodegradable (Dierkes et al., 2021; Weizel et al., 2020; Wu et al., 2019).

The study by Chang et al. (Chang et al., 2007) investigated the occurrence of six natural and synthetic glucocorticoids in sewage treatment plants (STPs) and receiving waters, specifically

the Tonghui and Qing Rivers in Beijing, China. The detection of these compounds was performed using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The limit of quantification (LOQ) for glucocorticoids in river water samples ranged from 0.01 to 0.02 ng/L, while the STP effluent samples had LOQs between 0.02 and 0.04 ng/L(Chang et al., 2007). Results indicated that cortisol and cortisone were dominant in influents, with concentrations ranging from 9.2 to 120 ng/L and 4.6 to 86 ng/L (Chang et al., 2007). Although STPs removed about 90% of glucocorticoids, effluents still contained detectable levels of cortisol and cortisone with concentrations ranging from 0.25 to 1.9 ng/L and 0.13 to 0.58 ng/L and depending on particular STPs (Chang et al., 2007). Despite unclear removal mechanisms in STPs, Chang et al suggest that both biodegradation and sorption to sludge play a role, although sorption alone may not fully account for lower removal rates (Chang et al., 2007).

Numerical values concerning the half-lives and degradation pathways in the environment of cortisol and cortisone are scarce (seeing as most experiments are performed to figure out the fate of synthetic GCs) and are collected in the following table (Table 2).

Table 2: Half-lives and degradation pathways of cortisone and cortisol

Compound	Cortisol (Hydrocortisone)	Cortisone
HALF-LIFE DT50 [d]		
Water	Readily biodegradable (ECHA, 2024)	x
Sediment	x	x
Soil	x	x
Activated sludge	<0.02 (Dierkes et al., 2021)	<0.02 (Dierkes et al., 2021)
DEGRADATION PATHWAY		
Hydrolysis	Stable in the pH region 4 to 9 at 25°C (ECHA, 2024)	x
Photolysis	0.0033 per min per 50 µg/L in river water (Cantalupi et al., 2020b)	0.00128 per min per 50 µg/L in river water (Cantalupi et al., 2020b)
Abiotic degradation	x	x
Biotic degradation	Readily biodegradable (ECHA, 2024)	x

Synthetic cortisol (hydrocortisone) and cortisone (cortisone acetate) are used in clinical settings to treat a variety of conditions, due to their anti-inflammatory and immunosuppressive properties. Treated conditions include adrenal insufficiency, autoimmune diseases, and skin conditions. Hydrocortisone, prednisone, methylprednisolone, and dexamethasone are used for

various veterinary purposes in emergency and critical care (ECC) settings. These include anaphylaxis, acute lung injury/acute respiratory distress syndrome, septic shock, and spinal cord injury, though routine use has also been associated with adverse effects such as hyperglycemia, pneumonia, urinary tract infection and gastrointestinal ulceration (Aharon et al., 2017).

Understanding the concentrations and behavior of these hormones in wastewater can help assess their potential impact on aquatic ecosystems. Research into the environmental fate of cortisol and its analogues is still rather new. Bethamethasone, a synthetic GC, has been found to have endocrine disruptive potential in fish models (Vestel et al., 2017). Other studies have reported adverse effects of synthetic GCs on fish even at lower concentrations of ng/L (Yazdan et al., 2021).

1.4 Selection of Analytical Method

Identifying glucocorticoid (GC) compounds in wastewater requires the careful selection of analytical methods that can accurately detect and quantify these compounds within the complex matrix of wastewater. Analytical techniques commonly used include liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS), both of which offer high sensitivity and specificity. Another analytical avenue might employ bio- or immunoassays.

The analysis is only as accurate as the method and the instrument. GC-MS is less specific for non-volatile and thermally stable compounds. LC-MS can offer precise quantification at low concentrations, even in complex mixtures like wastewater, by separating compounds based on their chemical properties and identifying them by their unique mass-to-charge ratios (m/z). However, it requires the careful optimization of parameters to ensure accurate detection. To this end a method must be developed and validated and calibration standards must be prepared.

Bioassays measure the biological activity of a substance by observing its effects on living cells, making them useful for detecting overall activity, especially in cases where the target compound is unknown. They generally lack substance specificity and measure an “overall” agonistic and antagonistic activity based on equivalents of a standard. Immunoassays (like ELISA) rely on the specific binding between an antigen and an antibody, making them highly specific and quantitative, though they may suffer from cross-reactivity with structurally similar compounds (de la Rosa et al., 2021).

Athough there are highly responsive bioassays that can quantify GCs in serum samples (Poulsen et al., 2024), there is limited evidence of using such bioassays to quantify GCs

extracted from wastewater. While bioassays are useful for measuring biological activity, they are generally less reliable for precise quantification and distinguishing between different compounds in environmental samples like wastewater. This, however, does not exclude the possibility that more precise bioassays can be developed specifically for the analysis of wastewater samples in the future. Jonkers et al used effect-directed analysis (EDA), which combines bioassay testing with high-resolution mass spectrometry (HRMS), to detect antibiotics and glucocorticoid activity in wastewater treatment plant effluents (Jonkers et al., 2023). Although Jonkers at al were unable to explain the GC activity, the study highlighted the sensitivity gap between biological and chemical analyses, where differences in detection limits between methods may hinder the identification of certain compounds (Jonkers et al., 2023). The study points out that the combination of bioassays (to detect the toxicity of unidentified bioactive substances) with chemical analysis (identify and quantify the contaminants present) can be a valuable approach for environmental risk assessments (Jonkers et al., 2023).

In the study by van der Linden et al. (van der Linden et al., 2008), human cell-derived CALUX (Chemically Activated LUciferase gene eXpression) reporter gene bioassays were employed to assess the estrogen (ER), androgen (AR), progesterone (PR), and glucocorticoid (GR) receptor-mediated activity of various wastewater effluents (industrial, hospital, municipal) ad surface water. CALUX bioassays use genetically modified cells to detect hormone receptor activation by measuring the light emitted through a luciferase enzyme. The highest GR CALUX activity was observed in industrial effluents, with a level of 243 ng dexamethasone equivalents per liter (Dex-equiv/L) and untreated hospital effluents similarly showed high levels of glucocorticoid activity (96 ng Dex-equiv/L), likely due to the mix of natural excretion and pharmaceuticals (van der Linden et al., 2008). Treated municipal wastewater exhibited glucocorticoid activity levels of 38 and 11 ng Dex-equiv/L, which likely contribute to surface water contamination, where activity levels ranged from 0.39 to 1.3 ng Dex-equiv/L(van der Linden et al., 2008). Since these results are reported in dexamethasone equivalents (12 times stronger than cortisol), the estimated glucocorticoid-like activity reached up to the surprising 2900 ng cortisol-equiv/L for the industrial samples (van der Linden et al., 2008). This kind of result points out that perhaps just scaling up from Dex-equiv/L to achieve the cortisol equivalent is too simplistic to come up with reliable amounts.

De la Rosa et al developed a breast cancer-derived glucocorticoid receptor (GR) bioassay, 231GRE, to specifically measure GR ligands, providing a sensitive and cost-effective method for detecting total glucocorticogenic activity (TGA) in human serum (de la Rosa et al., 2021). It would be interesting, if this type of bioassay could be adapted to screen wastewater for glucocorticoid activity. Since it is less specific than LC-MS for distinguishing individual compounds, it might be more useful when the objective is to quickly and cheaply establish a “background level” of glucocorticoid activity.

Quantifying glucocorticoids extracted from wastewater using LC-MS rather than bioassays offers several key advantages, particularly in terms of specificity, sensitivity, and data accuracy. The main reasons are summarized in the following table (Table 3):

Table 3: Comparison of LC-MS and bioassays as a method for quantification

Quantification method	LC-MS	Bioassay
Specificity & selectivity		
	separates compounds based on their chemical properties and then identifies them by their unique mass-to-charge ratio	biological responses (e.g., receptor binding, enzyme activity) that may not distinguish between different glucocorticoids or may cross-react with similar
Quantitation		
	measures the amounts of individual compounds, even in complex mixtures like wastewater	semi-quantitative and depend on a biological response that may not have a direct or linear correlation to the concentration of glucocorticoids
Sensitivity		
	trace amounts detection in ng and pg ranges	lower sensitivity, false negatives
Matrix		
	chromatographic separation removes or reduces the effect of interfering substances	complex matrix containing various organic and inorganic substances can cause non-specific binding or unrelated biological responses
Dynamic range		
	can measure several orders of magnitude (calibration to determine interval)	limited range of concentrations beyond which results may plateau or become unreliable
Metabolite screening		
	can identify	cannot differentiate

Reproducibility		
	generally, more reproducible, standardization, instrument setup	may fluctuate based on cell line conditions, reagent quality, or operator technique

1.5 Study Setup

This research project aims to develop a method to detect cortisol and cortisone in diverse kinds of wastewater matrices and possibly sludge, which contains more solids and is typically thicker than wastewater. To this end, an extraction protocol was set up for the cortisol/cortisone and the samples were prepare for analysis with ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS). Extraction, preparation, and analysis are each lengthy processes, each involving several steps. The UPLC-MS/MS instrument needs to be calibrated, and the limits or quantification (LOQ) and the detection (LOD) need to be established. Furthermore, the working process of the instrument can be formally validated as well to ensure accurate and reliable detection of the target analytes. The mass spectrometry fragmentation pattern helps to confirm the identity of cortisol and understand its structural components. The peaks observed in the spectrum correspond to various fragments produced during the ionization process.

There are several challenges associated with this process. Regarding the sample collection and storage, where, when, how and under what conditions the collection and storage took place can have an effect on the degradation rate of cortisol since cortisol degrades rapidly in wastewater in the presence of biofilms (Thai et al., 2019).

As this is primarily a method development study, significant time will be afforded to the troubleshooting the various steps in the extraction and the LC-MS calibration and validation processes. The sampling process in an adjacent but not the focus of this procedure (Figure 6). There is a feedback loop between the detection, quantification, quality assurance or quality control (QA/QC), and data analysis at method development phase in which each aspect is adjusted in reference to the others. In the case of QA/QC for example, “known unknowns” or samples with known concentration will be queued into the instrument as unknowns in order to check how well the instrument performs.

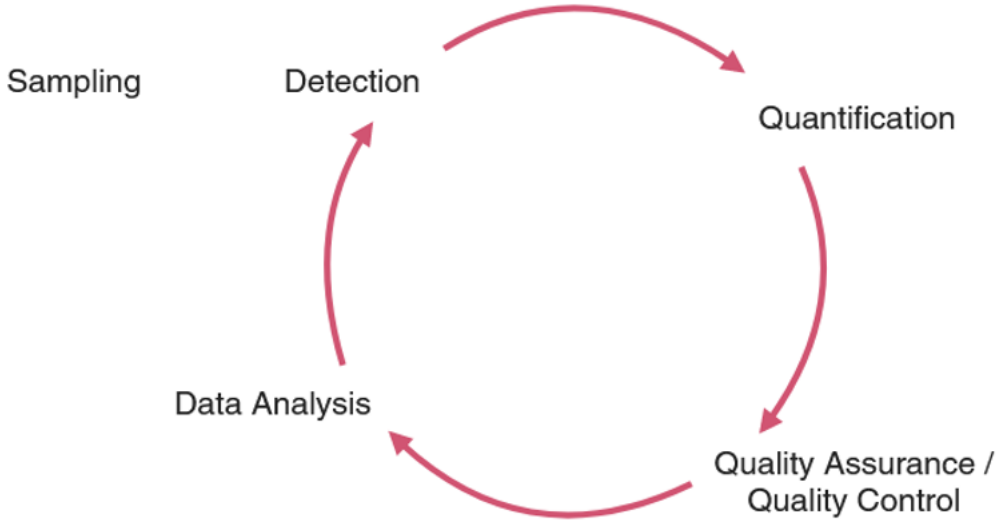

Figure 6: Feedback loop of the study.

1.6 LC-MS

Method development for liquid chromatography-mass spectrometry (LC-MS) is an iterative process that takes months to accomplish. The institutional setup and the available expertise are crucial. LC-MS is the combination of two techniques that allows the analytes to be isolated and measured from highly complex mixtures and matrices (Sargent, 2013).

Mass spectrometry (MS) is an analytical technique used to measure the mass-to-charge ratio of ions. Liquid chromatography (LC) differentiates compounds by their physico-chemical properties. This dual selectivity makes LC-MS a powerful tool that is used in various fields, including chemistry, biology, and environmental science, for identifying and quantifying molecules in a sample, determining structural information, and studying the dynamics of chemical reactions.

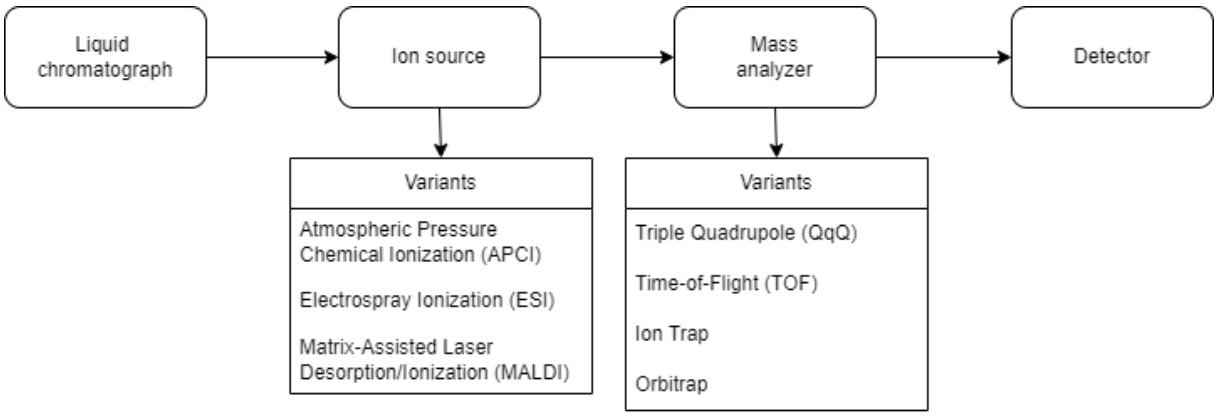

Figure 7: Basic components of an LC-MS system.

In LC, the sample mixture is dissolved in a liquid solvent (the mobile phase) and then passed through a column packed with a solid material (the stationary phase). As the mixture flows through the column, the various components interact differently with the stationary phase. Components that have a stronger affinity for the stationary phase will move more slowly, while those with a weaker affinity will move more quickly. This differential movement causes the components to separate as they travel through the column. Finally, the separated components exit the column at different times (retention times) and can be detected and quantified. The choice of stationary and mobile phases, as well as the conditions under which the chromatography is performed, determines the efficiency and specificity of the separation.

In MS, the sample is ionized to produce charged particles (ions). Common ionization techniques include atmospheric pressure chemical ionization (APCI) which uses gas-phase to ionize the sample, electrospray ionization (ESI) which produces ions by applying a high voltage to a liquid to create an aerosol and matrix-assisted laser desorption/ionization (MALDI) which uses a laser to ionize large biomolecules in a matrix. From thereon, the ionized particles travel to the mass analyzer where the ions are separated based on their mass-to-charge ratio (m/z). Different types of mass analyzers include quadrupole which uses oscillating electric fields to filter ions of specific m/z (triple quadrupoles or “tandem” mass analyzers are especially common). Others include time-of-flight (TOF) which measures the time it takes for ions to travel a known distance, the ion trap which captures ions in a three-dimensional electric field and orbitrap which uses an electrostatic field to trap ions in an orbit and thus measures their frequencies. In the detector the abundances of the separated ions are measured. The detector converts the ion signal into an electrical signal that can be recorded. For our project, electrospray ionization (ESI) was used.

LC-MS continues to evolve with technological advancements, expanding its applications and enhancing its capabilities for scientific research and industrial applications. The primary

difference between ultra(-high)-performance liquid chromatography (UPLC/UHPLC) and high-performance liquid chromatography (HPLC) lies in the particle size of the stationary phase and the operating pressure, which together influence the resolution, speed, and sensitivity of the chromatographic process. Overall, UPLC is an advanced form of liquid chromatography that offers faster, more sensitive, and higher-resolution separations compared to HPLC, but it requires more sophisticated and expensive equipment.

Matrix effects or ion suppression can occur when the ionization mechanism is disturbed by other components in the sample which co-elute with the analyte of interest and can lead to poor precision data (Sargent, 2013). Liquid chromatography (LC) has been employed for decades to analyze a variety of compounds in wastewater samples. It is well-suited for the identification, quantification, and separation of components in these complex matrices, offering a cost-effective and reliable method of analysis. When using LC, key methodological considerations include sample preparation, chromatographic conditions, sample injection, and data analysis. Sample preparation for LC is particularly important for reducing matrix effects. This preparation typically involves the removal of solid particles and other contaminants to minimize interference during analysis. Chromatographic conditions, such as the choice of stationary phase, mobile phase, and column temperature, are critical in optimizing separation and further reducing matrix effects. Sample injection, or the process of introducing the sample into the LC system, must be done carefully to ensure consistency and precision.

The accuracy of results obtained using LC is influenced by several factors, including the type of detector used, the sample preparation procedure, and the chromatographic conditions, all of which play a role in mitigating matrix effects. The reliability of LC results depends on the number of replicates analyzed, the accuracy of the calibration curves, and the quality of data analysis, with particular attention to controlling for matrix effects to ensure precise and accurate measurements.

1.7 Community Health Surveillance

Whether the presence of cortisol in wastewater alone can tell us much about the health of a community is unclear, but efforts to measure it in various samples can provide interesting insights and may complement other health surveillance efforts. In the United States and in many other countries, (mental) health care does not always reach the populations that need it most. Passive surveillance methods that do not depend on access to clinical care can therefore fill an important gap in what is known about community stress. Ideally, such data could inform and target public health interventions where they are needed.

1.7.1 **Ethical Aspects of Wastewater Sampling**

Sampling from a population involves ethical considerations (Figure 8), which can fail for several reasons, for example due to flaws in the research design, implementation, or oversight. These failures can lead to biases, misleading claims and potentially harmful outcomes for participants and society at large. In the following, I will outline the broad consequences of lapses in ethical consideration.

Firstly, the scale at which sampling is undertaken is significant. Sampling at a wastewater treatment plant serving a city population of 1 million people is different, ethically, than sampling an apartment building where a handful of families live. The latter is far less anonymous. However, since individual variables also tend to smooth out in large sewer sheds, sampling from areas with a large population may prove less "noisy" and skewed.

There are concerns that WBE might be quietly becoming mainstream without adequate oversight, which means that much as any other surveillance technology (facial recognition and Internet tracking) its adoption is a “slow creep” that reinforces existing inequalities (Rinde, 2023). There is a tendency for new surveillance and policing technologies to affect marginalized communities the most (Rinde, 2023). For example, health insurers might like to use predatory tactics and claim that “objective” wastewater-based data is sufficient ground to raise premiums in poorer neighborhoods (Rinde, 2023).

If informed consent is lacking because the consent form is overly complicated or lacks a clear explanation of purpose, risks and benefits, participants may not fully understand what they are consenting to. This is further complicated by the fact that for WBE, tracking back “active participants” might be completely impractical. Consent may also be not quite voluntary due to pressures to participate due to power dynamics or financial incentives. If opting out of the study is not easy or withdrawal results in negative consequences, the participation is not truly voluntary.

Sampling maybe (un)intentionally not representative of the population and achieves only skewed results due to convenience sampling (only sampling easily accessible participants) (Russell, 2020). Failure to include diverse groups may lead to results that do not apply to these groups and may thereby perpetuate inequalities. Vulnerable groups can be exploited because of convenience or cost-saving purposes without providing fair benefits or protections. Added to this, research benefits may be distributed unequally, benefiting some while posing disproportionate burdens on others. In addition, ignorance of cultural contexts may result in overlooking norms that can result in harm or loss of trust in certain communities. Failures to minimize harm include causing unwanted consequences to participants’ lives, especially if outcomes and implications are not clearly communicated beforehand.

As regards to the handling of data, failures to securely store data or anonymize it may lead to confidentiality breaches, exposing participants to risks (e.g. social stigma or identity theft). How the data is used, who will have access to it and how long it is stored are all potential sources of misuse. Not disclosing sources of funding or conflicts of interest may lead to biased findings and unethical practices. The non-disclosure of methods may obscure failures in sample selection, data collection or how guidelines were followed. Furthermore, data may be fabricated, selectively reported, or manipulated to fit a desired outcome which undermines the integrity of the research (Russell, 2020).

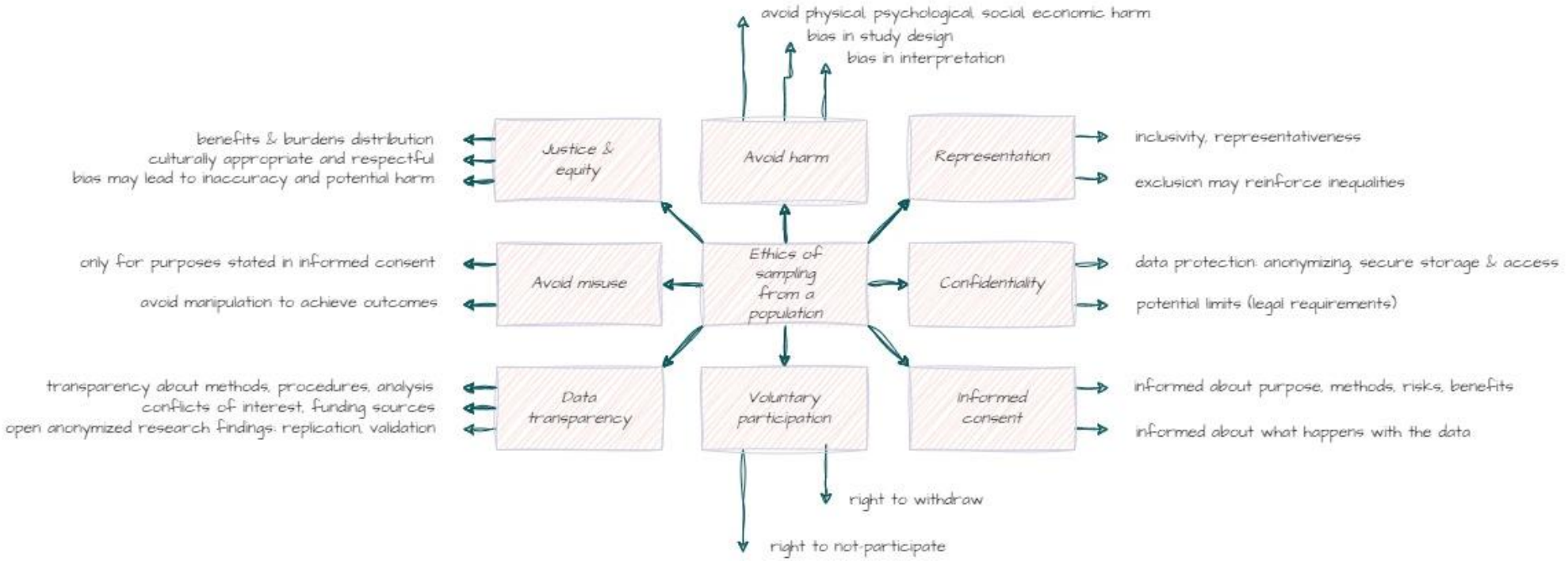

Figure 8: General ethical considerations that come into play when sampling for a population. The lesser the size of the population, the more likely that distortions might affect individuals directly. (Figure by Elena Tiis)

1.7.2 Ethical Considerations of Human Microbiome Research, Wastewater-Based Epidemiology and Community Health Surveillance

There is a number of ethical considerations raised when wastewater-based epidemiology (WBE) methods are applied to track human hormones. These concerns parallel those recognized in genetic and biobank-related research as well as human microbiome research (Hawkins & O'Doherty, 2011). These fields offer valuable insights into public health, disease prevalence, and community health trends, as well as the consumption of drugs and pharmaceuticals yet their methods are involved with ethical concerns related to privacy, discrimination, informed consent, cultural sensitivity, ownership of biological material, the return of research results, and potential misuse of data. Thus, wastewater research can be considered from a data ethics perspective (Doorn, 2022). In the following, the ethical considerations concerning genetic and microbiome research, as discussed by Hawkins & O'Doherty in their aptly named paper "Who owns your poop?" (Hawkins & O'Doherty, 2011), are discussed in relation to WBE. Finally, how this specifically relates to our sampling project is discussed.

1.7.2.1 On Privacy and Personal Identification

One of the main ethical concerns in microbiome research is the potential for individuals to be identified by their unique biological signatures, such as their microbial "fingerprint" (Hawkins & O'Doherty, 2011). This is similar to how genetic data can reveal identity and sensitive health information. Microbial data, like genetic information, can potentially expose an individual's predisposition to certain medical conditions or possibly even reveal aspects of a person's socioeconomic background (Hawkins & O'Doherty, 2011). Specifically, when microbiome data is combined with genetic information, this could provide a more detailed picture of an individual's health than genetic data alone (Hawkins & O'Doherty, 2011). Simply the technical possibility of arriving at such detailed personal data coupled with "grey areas" in data-protection mandates might raise significant privacy concerns.

Although in WBE data collection typically occurs at the community level and the source of data is considered "waste", there still might remain a risk of re-identification, especially in smaller communities or when the sampling area is narrow (Hawkins & O'Doherty, 2011). Compiled or combined data may also reveal sensitive information about specific groups, potentially leading to discrimination (Pujol & Machanavajjhala, 2021). In the case of this project, tracking the amounts of cortisol and cortisone in wastewater does not include genetic analysis.

1.7.2.2 On Potential for Discrimination and Stigmatization

For microbiome data, there are concerns that employers or insurance companies could misuse such information to discriminate against individuals based on life expectancy, job prospects, or health risks (Hawkins & O'Doherty, 2011). Additionally, microbiome-based discrimination could extend beyond health-related biases to include socioeconomic status, geographical origins, or even travel history (Hawkins & O'Doherty, 2011). Similarly, studies using the methods of WBE might disproportionately target certain neighborhoods or communities to monitor specific health conditions or behaviors, especially if the purpose is to monitor illegal drugs and their metabolites and couple this with enforcement (Bowes et al., 2023; Rinde, 2023). This can lead to the stigmatization of these communities. Misinterpretation of the findings by the public, media, or policymakers could also lead to misinformed actions targeting specific groups or communities (Hawkins & O'Doherty, 2011). Although cortisol and cortisone are mainly found in urine, the broad considerations when using microbiome data apply here as the source of sampling is wastewater which contains both feces and urine.

1.7.2.3 On Obtaining Informed Consent

When biological specimens are stored in research banks indefinitely and may be used for unspecified future research, it raises the question whether participants can make fully informed decisions (Hawkins & O'Doherty, 2011). In the case of WBE, there is sometimes even no opportunity for individuals to provide informed consent, as the method involves sampling entire communities without any opt-in process or awareness because obtaining it is impractical, such as sampling from a municipal wastewater facility that services a sizeable population. Simply the lack of mechanisms for community consent raises ethical concerns, as such data may be used for surveillance. Seeing as most institutional review boards (IRBs) and ethics committees that oversee research require that consent must be obtained when personal data is collected, which wastewater-based data by default is not, then explicit procedures for collecting and using such data have been until recently mostly absent. Bowes et al have compiled a structured ethical review for wastewater-based testing (Bowes et al., 2023) which attempts to bridge this oversight. This structured ethical review provides a set of questions that help researchers to provide a score (the higher the score the greater the ethical concern) when considering an application of WBE (Bowes et al., 2023).

1.7.2.4 On Microbial Data

There is ongoing scientific research into the stability of an individual's microbiome over time and how much it is influenced by their genetic makeup and environmental factors (Fassarella et al., 2021). However, microbiome research may still be considered more invasive or culturally

unacceptable by some groups, particularly when it involves collecting sensitive biological samples, such as stool (Hawkins & O'Doherty, 2011). If this results in the exclusion of certain cultural or ethnic groups, the findings are only partially applicable.

1.7.2.5 On Ownership

The question of ownership is particularly complicated in microbiome research. The issue of "who owns your poop?" features in research on the gastrointestinal microbiome, which relies heavily on fecal samples (Hawkins & O'Doherty, 2011). Although mainly considered "waste", feces can now hold valuable data about a person's health and identity, complicating the notion of ownership (Handsley-Davis et al., 2023; Hawkins & O'Doherty, 2011). The implications of ownership are significant not just in terms of personal dignity and cultural identity, but also in relation to potential financial benefits derived from commercial applications of the research, such as drug development or biotechological innovations (Handsley-Davis et al., 2023).

1.7.2.6 On Data Use, Misuse, and Governance

Both microbiome research and WBE raise concerns about the potential misuse of data and the lack of clear governance frameworks. It is possible that data collected for one purpose could be used for other purposes without proper oversight or consent. Similarly, WBE data, if not carefully managed, could be used beyond its initial public health intentions, such as for law enforcement or commercial purposes. The absence of clear guidelines on data handling, storage, and sharing creates risks for data breaches and misuse (Bowes et al., 2023). The development of a data management plans is therefore instrumental (Bowes et al., 2023).

1.7.2.7 On Return of Research Results

The return of research results is a controversial issue in both genetic and microbiome research. While some argue that participants have a right to know their genetic or microbial profiles, others argue that there is a right not to know, especially if the information has limited clinical utility or could cause undue harm (Hawkins & O'Doherty, 2011). There is some concern that the obligation to return results could make studies unsustainable and that premature disclosure could lead to misinterpretation or harm (Hawkins & O'Doherty, 2011). Conversely, if a microbial profile indicates a potentially harmful condition that is easily treatable, the implications of disclosing this information could be beneficial and impactful. For this project in cortisol/cortisone sampling, detailed information of health conditions is not expected and the tracking information is shared with the school board of the school that is being sampled.

1.7.2.8 On Equity, Justice, and Access to Benefits

Concerns about equity and justice are central to both microbiome research and WBE. As mentioned earlier, there is a risk that the benefits derived from the research, such as the development of probiotic interventions, will only be availvable to those who can afford them. The financial rewards of such research could disproportionately flow to researchers and developers rather than to participants who contributed their time, samples, and exposed themselves to potential risks (Handsley-Davis et al., 2023; Hawkins & O'Doherty, 2011).

For WBE, the benefits and risks may not be equally distributed. For example, if vulnerable communities are more likely to be targeted for monitoring, it may lead to a disproportionate focus on certain populations while neglecting others. Furthermore, in the case of WBE identifying a public health issue, there may be inequitable access to the resources needed to address it.

1.7.2.9 On Transparency, Accountability, and Public Policy

The lack of transparency and accountability is another shared ethical concern. In microbiome research, communities and participants need to be informed about how their data will be used and protected. If WBE data is used to justify policies that overreach personal freedoms or disproportionately affect certain populations, it could result in significant ethical dilemmas. Furthermore, unclear accountability for acting on the findings of WBE data may undermine public trust in public health efforts and research practices.

1.7.2.10 In Relation to This Project

In order to incorporate data on cortisol and cortisone variability over a longer time-period, samples are taken directly from a school. Access to the samples is provided top-down, from the school principal and the county and the sampled population (i.e. minors) did not explicitly consent. The results, when published, are anonymized and their purpose is mainly to gather enough data to see general patterns and variations and to advance research in this field, without attempting to make categorizations about this subset of a population. It is conceivable that this type of data could be used to support policy interventions, in which case an ethical review (for example as outline in Bowes et al (Bowes et al., 2023)) might be appropriate to see what kind of impact this might have, although it is difficult to say at this early stage.

2 Method Development

The aim of this study is to develop and validate a method for extracting and quantifying cortisol and cortisone from wastewater influent samples (and sewage sludge) using ultra-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS). The practical part of the project was done at University of North Carolina (UNC) at Chapel Hill as a team effort, which is what I am alluding to whenever there is a mention of “we” or “our”.

Wastewater samples are collected and prepared for analysis by filtration and solid-phase extraction (SPE). Cortisol and cortisone are eluted from the samples and run through UHPLC-MS/MS. To achieve accurate detection and analysis by the instrument, an instrument-specific method must be developed and validated. This process consists of several steps that involve the adjustment of the liquid chromatography (LC) and the mass spectrometry (MS) parameters so that the instrument is best placed to deliver accurate and reliable results. Several spiked wastewater matrices were subsequently run through the instrument in order to establish the extraction efficiency. Key parameters for the method development include the retention time, the source of ionization, calibration curves and the establishment of the limit of detection (LOD) and limit of quantification (LOQ). Following a successful pilot study in which several wastewater matrices were prepared and analyzed, this method can be used for quantification of cortisol and cortisone in various wastewater samples.

The step-by-step optimization of the LC-MS method to achieve adequate sensitivity and selectivity is a somewhat circular process with several co-dependencies (Figure 9). Different parts of this system interplay in the optimization process.

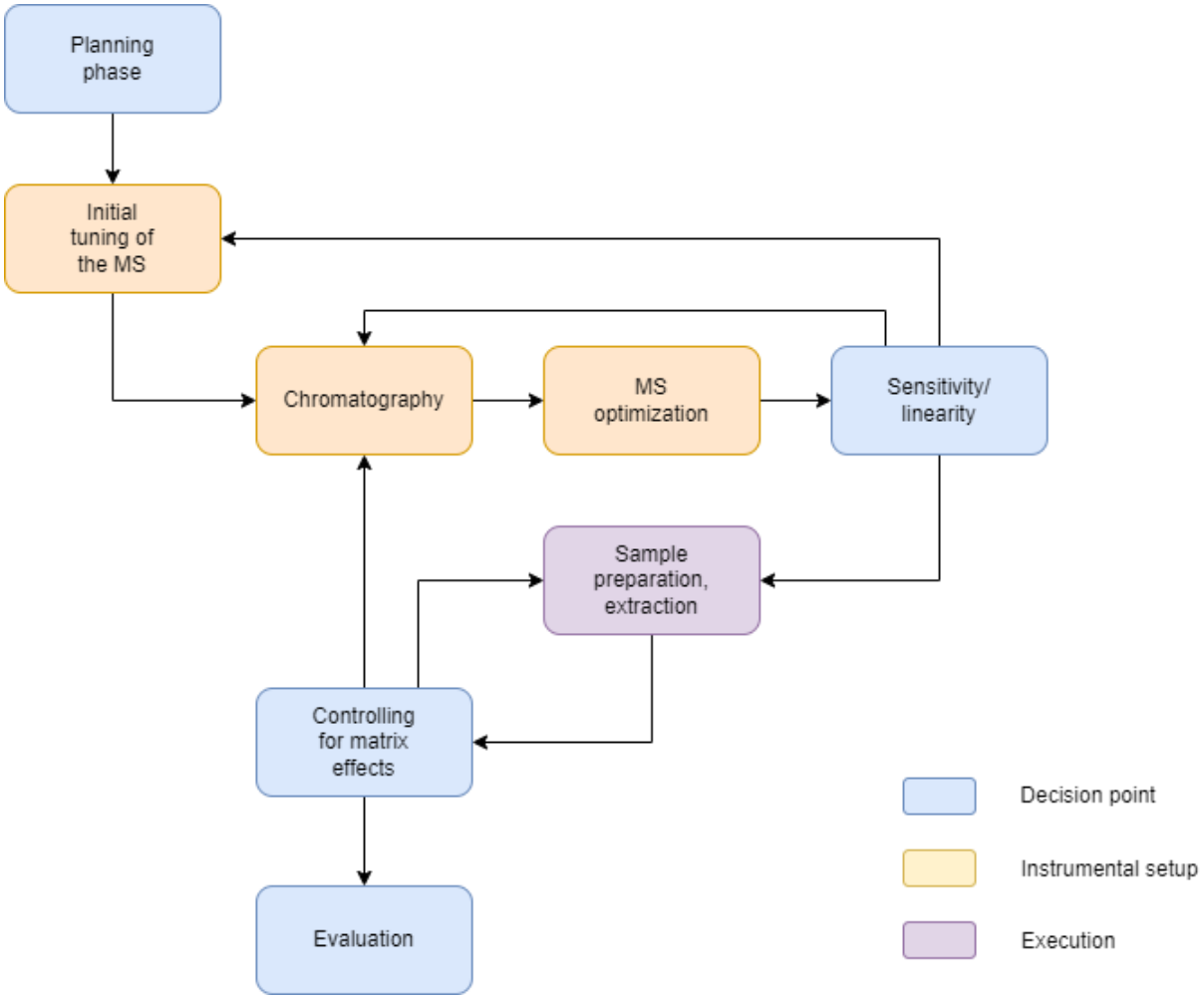

Figure 9: Optimization of the LC-MS method (Elena Tiis after Sargent, 2013)

At the planning phase, there are information requirements. These include the chemical structure of the analytes being measured, the type and condition of the samples, references to previous efforts and the appropriate guidelines (Sargent, 2013). The initial tuning of the MS is made in order to determine whether the analytes will ionize in the mass spectrometer. This step introduces a stream of analyte into the ions source using a syringe pump. In this process, the MS parameters (gas flow, ionization voltage etc.) will be tuned to the analyte to allow for the best conditions for ionization and sensitivity. This is where, at the pilot stage, we produced the breakdown curves for the analytes and the deuterated standards (see Figure 13).

Some suitable starting conditions for the chromatographic method are identified in the planning stage. This includes the selection of the column. In the absence of previous method information, determining the logD of the analyte is a sensible starting point (Sargent, 2013). LogD is the measure of lipophilic quality at a given pH and is related to its retention on a reverse-phase column such as C18 and the more lipophilic the analyte the more it will tend to

retain (Sargent, 2013). Besides the column, other considerations include flow rate and the selection of mobile phases and additives (formic acid). In our case, there was previous literature to lean on to search for this type of information and the laboratory at UNC had previously developed a method for cortisol but not cortisone.

After the determining of the LC conditions the MS response is worth observing again, since the ion source parameters are dependent on the composition and the flow rate of the mobile phase (Sargent, 2013). Sensitivity is determined by the combination of MS response, LC condition and extraction efficiency (Sargent, 2013).

Sample preparation and extraction are some of the more technically challenging stages of the method development. Their aim is to remove the maximal amount of interferences while maximizing the recovery of the analytes. Where low concentrations are expected, it is desirable to introduce a concentration step. The chosen extraction technique for the wastewater matrix is solid-phase extraction (SPE). Part of the development process is to measure the effect of the matrix on the ionization process and analyte recovery. If there is not enough selectivity, it may be necessary to adjust the LC conditions or separate out any interferences.

Method development necessitates a feedback loop between the sample preparation procedure and the analysis with LC-MS. For this purpose, we conceptualized a pilot run which would tease out any difficulties and set up our method for analysis of real samples.

The pilot run to establish recovery efficiencies included banked grab influent samples provided by Orange Water and Sewer Authority (OWASA) which is the main drinking water and wastewater treatment authority in Orange county, North Carolina, serving Carrboro and Chapel Hill. These samples were spiked with a known quantity of deuterated standards. Additionally, deionized water was similarly spiked and run, in order to establish whether cortisol or cortisone residue remains in the cartridge or is being wasted as part of the initial conditioning of the cartridge.

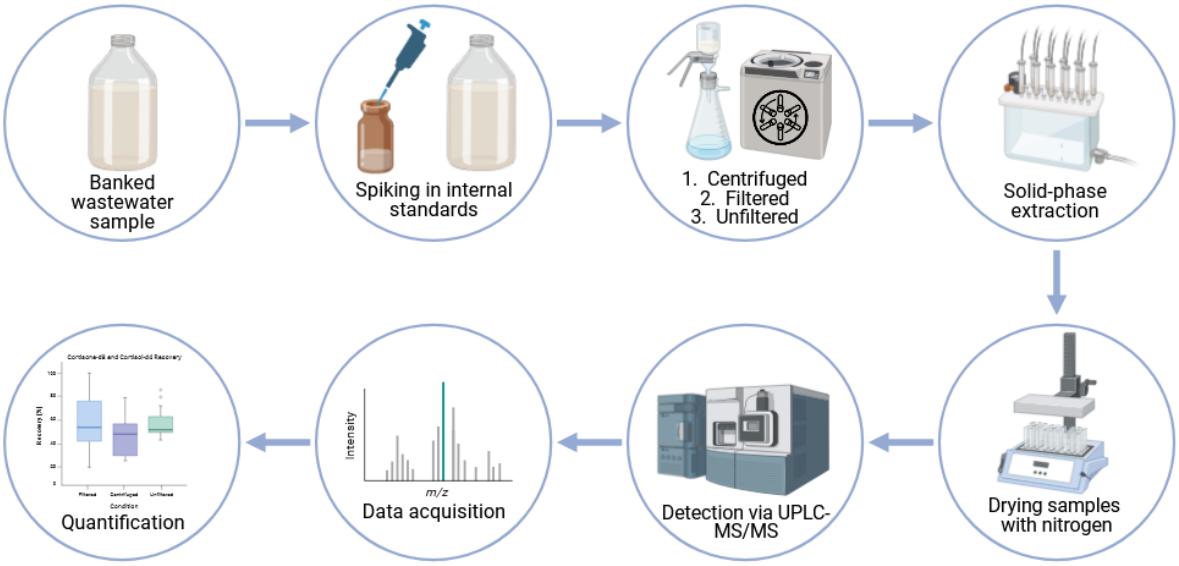

Figure 10: Pilot method (compiled by Elena Tiis with Biorender after Abby Boyer)

2.1 Chemicals and Reagents

Glucocorticoid standards cortisol (Cat No. C2755), cortisol-d4 (Cat No. 705594), cortisone (Cat No. H4001), and cortisone-d8 (Cat No. 900170) were purchased from Sigma Aldrich. LC-grade methanol (Cat No. 1.06035), acetone (Cat No. 270725), formic acid (Cat No. F0507) and acetonitrile (Cat No. 900667) were also acquired from Sigma Aldrich. Standards and standard dilutions were stored at –20°C in LC-grade methanol.

2.2 Sample Collection

Previously collected (September 2023) influent grab wastewater samples from the Orange County Water and Sewer Authority (OWASA) were used for the pilot sample preparation (cortisol and cortisone recovery experiments) and analysis. A grab sample is a volume of the wastewater (or aliquot) that is taken at one time, maximally in the duration of 15 minutes (International Labmate Limited, 2024). These samples were stored at 4°C until they were used for our experiment as our matrix. As this temperature does not inhibit bacterial degradation, the target analytes were already likely decayed.

Presently there is an ongoing project to collect wastewater from a school on Tuesdays, between 11:30AM and 1:30PM (lunchtime). Approximately 3 liters of wastewater are collected

directly from the sewer line access point on the campus of the school. The sample is time-composite and analyzed in two replicates, cortisol and cortisone are extracted with SPE and subsequently measured with LC-MS.

2.3 Sample Preparation and SPE

This section describes the extraction procedure including pre-treatment (e.g., filtration, pH adjustment), the solid-phase extraction (SPE) procedure and the optimization of extraction parameters (e.g., solvent selection, pH, extraction time).

Wastewater samples are a complex mixture where the target analytes are present in very low quantities and require clean-up before analysis with LC-MS. The aim is producing a sample extract that is compatible with the LC-MS system: a particulate-free liquid that contains the concentrated analytes (Sargent, 2013). Since the wastewater matrix for the pilot run was an older sample, we did not expect to find target analytes in it but used it primarily as a background to see what kind of sample preparation would yield the best recovery of a spiked internal standard. In other words, the environment and management of the sample was considered at the preparation stage and not the sample collection and storage stage. The standards to be spiked in were stored at -20 °C in amber glass vials to ensure their stability.

2.3.1 **Pilot (Extraction Experiments)**

The pilot run consisted of spike recovery experiments where we compare the recovery of the deuterated standards from wastewater that is either (1) centrifuged, (2) filtered, or (3) unfiltered prior to solid-phase extraction (SPE). SPE is a technique that uses a solid stationary-phase sorbent (contained in the cartridge) to clean-up and concentrate the components prior to analysis. No pH adjustment of the samples was performed.

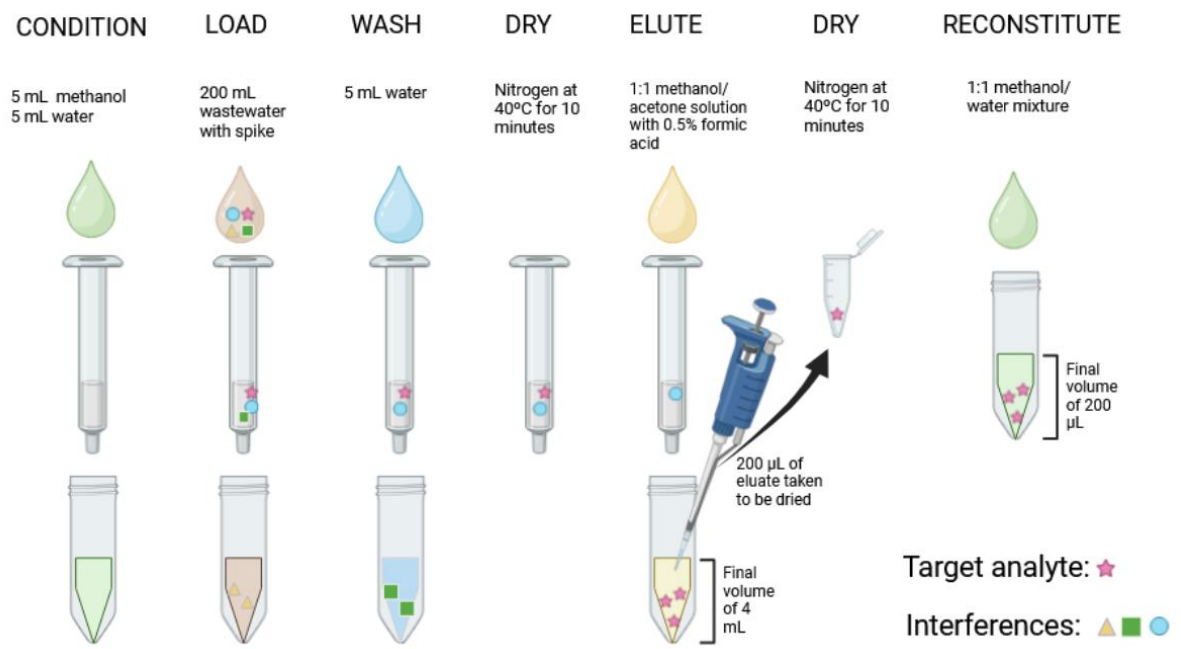

Figure 11: Solid-phase extraction procedure for the pilot run (compiled by Elena Tiis with Biorender after Abby Boyer)

We prepared the spiked wastewater samples from OWASA by combining 198 mL of raw, influent wastewater with 2 mL of deuterated cortisol-d4 and cortisone-d8 standards to a final concentration of 2 µg per L. We processed this 200 mL of spiked wastewater under each experimental condition in triplicate, alongside a technical duplicate. For filtered samples, we passed 200 mL of spiked wastewater through a grade 6 glass-fiber filter (Whatman, Cat No. 1037004) using vacuum filtration. The filtrate was collected for solid-phase extraction, and the filters were discarded. To remove solids via settlement, we centrifuged 200 mL of spiked wastewater at 1,500 RPM for 20 minutes at 23°C, collecting the supernatant and discarding the settled solids. For comparison, we processed 200 mL of spiked wastewater in duplicate without removing the solids.

We extracted cortisol-d4 and cortisone-d8 from the spiked wastewater samples using SPE with 6 mL HLB cartridges from OASIS, following a method by Chen, Venkatesan, and Halden (Chen et al., 2019) who used it for tracking human metabolites of alcohol and nicotine in raw sewage. The cartridges were conditioned with 5 mL of methanol, equilibrated with 5 mL of water, and loaded with 200 mL of spiked wastewater. After washing with 5 mL of water, the cartridges were dried under nitrogen using a TurboVap for 10 minutes at 40°C. Analytes were eluted with 4 mL of a 1:1 methanol and acetone solution containing 0.5% formic acid. From the eluate, 200 µL was collected, dried under nitrogen for 15 minutes at 40°C, and reconstituted in 200 µL of a 1:1 methanol and water solution for subsequent analysis LC-MS.

Each extraction included a negative control (200 mL deionized water) and a positive control (200 mL deionized water with cortisol-d4 and cortisone-d8 at 2 µg/L). The expected final concentration of the eluates was 100 ng/mL (or µg/L) of cortisol-d4 and cortisone-d8.

Since the initial setup of the SPE method, we changed the UHPLC-MS/MS instrument and with that the mobile phase B from 100% methanol with 0.1 % formic acid to 100% acetonitrile with 0.1 % formic acid. Acetonitrile is a stronger solvent. In future runs, it might be possible to change the solvents in the SPE to match those used in the LC-MS.

2.3.2 Further Experiments

At the time of writing, several other experiments are ongoing or being prepared at UNC. There is ongoing wastewater sampling from a school and the available data presents the beginning of a small longitudinal study. Further, matrix matching and sludge extraction experiments are planned. The data is not yet available to be incorporated into this thesis.

2.4 UHPLC-MS/MS

2.4.1 Instrumentation and Analytical Conditions

The method development and pilot run took place at the Department of Chemistry, Mass Spectrometry Core Laboratory at University of North Carolina at Chapel Hill (UNC-CH). This laboratory had previous experience with developing a method for cortisol analysis. The presets of the previous method were used as a starting point for the development of this method for both cortisol and cortisone. The instrumentation-specific parameters including column type and dimensions, mobile phase composition and gradient and the mass spectrometry settings are listed in Table 4 and Table 5.

As the instrumentation in use is UHPLC, this allows us to use a column with <2 um in particle size and smaller internal diameter. The length of the column allows to achieve increased peak capacity but higher back pressure. Since the samples will go through a clean-up process there is no need for a pre-column filter to act as a trap for particulates and strongly retained components.

The mobile phases used are water with 0.1% formic acid as the inorganic solvent and acetonitrile with 0.1% formic acid as the organic or “strong” solvent. The choice between using methanol and acetonitrile depends on their properties. Whereas methanol has weaker elution strength and a higher portion of solvent is needed, the result is more effective removal of solvent (desolvation) and this enhances the electrospray response (Sargent, 2013).

Acetonitrile by contrast will elute analytes earlier and reduce run times and it is less viscous than methanol and hence generate less back pressure (Sargent, 2013). The addition of a weak acid (formic acid in our case) is to increase conductivity and to facilitate the protonation of the analyte.

Table 4: Parameters for the UHPLC-MS/MS.

UHPLC-MS/MS Parameters.	
Injection Volume	10 µL
Column Type	Waters BEH C18 (2.1 x 100 mm,1.7 um)
Column Temperature	45 °C
Sample Temperature	10 °C
Eluent	Mobile Phase A (MPA): Water with 0.1% formic acid Mobile Phase B (MPB): Acetonitrile with 0.1% formic acid
Flow Rate	0,2 mL/min
LC Sample Ramping Method (Gradient)	Begin with 100% A decrease to 45% A over 6 min., hold for 1 min., then decrease to 20% A at 10 min., followed by decrease to 10% A at 10 mins. Hold 1 min. Return to starting condition at 14 minutes and hold for 4 minutes to re-equilibrate the column. (Total: 18 mins)
MS Analysis Mode	ESI positive mode
MS Scan Type	SRM

Liquid chromatography (LC) was used to establish retention times and mass spectrometry (MS) was used to establish the mass to charge (m/z) ratio of the selected product ions (Table 5).

Table 5: MS Transitions and Retention Times.

Compound	**Q1**	**Q3**	**CE**	**S-Lens**	**Retention Time (min)**
Cortisone	361.1	301.07/343.04	19/15	119	8.55
Cortisol	363.1	91.06/121.01	52/28	107	9.08
Cortisol-d_4	367.1	120.9/331.16	26/18	126	9.06
Cortisone- d_8	369.1	169.09/351.1	22/17	136	8.50

Notes: **Q1 = precursor ion (in 1st quadrupole), Q3 = selected product ions (in 3rd quadrupole), CE = collision energy, S-Lens = ion guide voltage setting**

To ensure the instrument can accurately analyze the compounds of interest, we generated breakdown curves to identify the optimal product ions (Q3) (Table 5). This was accomplished through the infusion of standard solution containing the analyte into the ionization source of the MS at a flow rate set by the instrument using a syringe pump. This provided a steady signal from analyte ions, the response of which was then monitored, and adjustments were made to the parameters using the instrument control software (Sargent, 2013). As we were testing only a few analytes, this was a reasonable approach although the solvent composition and the flow rate of the direct infusion did not match the actual LC elution conditions (Sargent, 2013). It was, however, necessary to establish the compound-dependent parameters such as collision energy (CE) and the ion guide voltage settings.

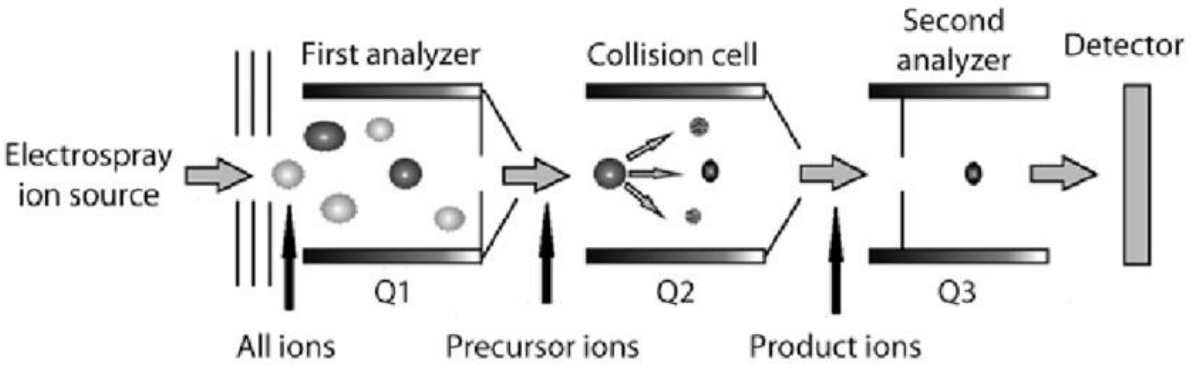

Figure 12: Schematic view of MS analysis with triple quadrupole (QqQ) (Faktor et al., 2012).

The precursor ions (Q1) fragment during MS transitions optimization and produce product ions (Q3) which are used for identifying and quantifying the compounds. This process is largely automated and driven by the operating system software and includes the following steps: recording the mass spectrum over a wide range, then selecting a suitable precursor ion (based on the analyte mass) and then selecting suitable product ions by increasing the collision energy until fragments are formed through collision induced fragmentation (CID). Technically this means that the analytes enter the nano spray where the electric field and thermal heat is applied over the analyte droplets. Thereafter the charged particles proceed into QqQ instrument where the first (Q1) and third quadrupole (Q3) serve as mass filters while the second one (Q2) works as a collision cell where fragmentation occurs through CID (Faktor et al., 2012). Product ions enter Q3 where only the product ions used for quantification are selected and sent to the detector.

The mass spectra showing the abundance of the various product ions are recorded as breakdown curves. During the initial testing phase, we observed that the cortisol peaks were not clearly visible, whereas the deuterated cortisol peaks (ion intensity) were distinct.

Therefore, for the subsequent testing phase, we refined the method by improving the breakdown curve to enhance the detection of cortisol.

Subsequently an acquisition method was developed including a gradient, injection volume and column and sample temperatures to train the instrument to acquire the required information from the samples correctly. Furthermore, a processing method was developed to then be able to process the data into a format that can be analyzed. These methods are specific to the instrument available.

2.4.2 **Calibration Curve**

Generating a reliable calibration curve for LC-MS is crucial for accurately quantifying analytes in unknown samples. We selected external calibration in which a series of separately prepared calibration solutions (in solvent solution) are measured in the same run as the samples are analyzed. The standards were stored at -20°C in between runs. The process of figuring out the right calibration concentrations and preparing the dilutions needed a few trial runs until we settled on factor 2 serial dilution in 13 steps with highest concentration at 1000 µg/L and lowest at 0,24 µg/L. The calibration series was run twice, before and after the samples, to account for any possible instrument drift.

The pilot run with OWASA samples were spiked with 100 µg/L of internal standards, and the internal standards were added to the calibrated dilutions to match that concentration. To make this easier we prepare the 50:50 methanol/H_2O solution and added them to that. This way each time we added solvent, we were adding the deuterated standards at the required amount without needing to adjust the concentration. The calibration curve dilution procedure is included in Appendix A.

2.4.3 **Sequence Setup and Quality Control**

Solvent blanks (50% methanol) were run at the beginning of the sequence, before and after the calibrants and after about every 10 samples. First blanks help to condition and equilibrate the system, ensuring a stable baseline and removing any potential contaminants or residual analytes from previous runs. Then, blanks are before and after the calibrants to check for cross-contamination and to verify that the calibration standards are free from carryover effects. The calibrants are queued in order of smallest to greatest concentration so as to not reduce sensitivity and saturate the column. Periodic blanks after every 10 samples provide ongoing monitoring of the system's cleanliness, and to help detect any buildup of contaminants that might affect the accuracy and precision of the subsequent measurements.

The practice of placing solvent blanks helps to maintain the integrity of the analytical process, ensuring that any detected peaks are truly from the samples and not due to residual compounds or solvent impurities. At the end of the run, the same calibration curve was run again to see whether there is drift in the ability of the instrument to quantify the analytes.

Table 6: Example sequence setup

Sequence	Compound	Purpose
1-3	blanks	condition, equilibrate
4-17	calibrants	reference
18-19	blanks	cleaning column
20-30	samples	quantification
31	blank	monitoring
32-42	samples	quantification
43-56	calibrants	reference
57-59	blanks	cleaning column

For the pilot (recovery experiments) run, no quality control (QC) samples or “known unknowns” were analyzed. The spiked samples with the known concentration were in principle fulfilling this role. In future runs, a known quantity of analyte will be run as an unknown sample in order to gauge how accurately the detection is working. For the longitudinal sampling experiment, QC samples were incorporated and are discussed in section 3.4.

2.5 Method Validation

Method validation is a formal process designed to establish that the method is capable of producing results that are adequate for the purpose and to provide an estimate of the measurement uncertainty associated with the results produced (Sargent, 2013). The performance parameters evaluated by a valuation study might include linearity, sensitivity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOD). The procedures for assessing each parameter would need to be fully documented. To date we have carried out a few experiments with the method. A formal validation procedure is beyond the scope of this project.

3 Results

3.1 Sample Preparation and SPE Optimization

The pilot run was primarily an optimization study for different extraction parameters. The first pilot run produced no noticeable spikes for all the samples except for the spike solution which was not run through a cartridge. To test where the cortisol and cortisone were lost, we devised another experiment only with deionized water (DI). This was an attempt to amend the experiment by try to also leach out any spike residue that was possibly “getting stuck” in the different stages of the SPE process. We also added a second stronger elution step with acetonitrile to ensure that the cortisol/cortisone were being retained in the column. To do this we had a final elution step of 4 ml of acetonitrile and collected 200 µL of that as well. The results influenced the sample preparation and SPE process in a circular way (refer to section 3.3).

After doing this, we found out that the fault was simpler than supposed. It was not the SPE process in itself, but the eluate amount of 200 µL was too low for the needle to reach them for analysis by LC-MS. To remedy this, the 200 µL was transferred into inserts in the vials so that the needle could reach them during the second round of the pilot study. This second part of pilot run thus included the OWASA samples again. The samples that were not found by the LC-MS the first time around could be analyzed after this correction. The experiment only with deionized water (DI) did not yield meaningful results, so cortisol/cortisone were not discarded as waste and did not get “stuck” in the cartridges in the course of the SPE process. For future runs, the eluate amount was increased to 500 µL.

Only the internal standards were spiked into the samples. Seeing as the compounds have the same chemical attributes and the internal standards are merely heavier and the calibration curves were only made for the analytical standards, we transposed the internal standards onto the calibration curve (Figure 15).

3.2 UHPLC-MS/MS Method

The scope of this LC-MS method development is instrument priming and optimization, calibration and analysis of pilot samples that are used to adjust the method. To date, we have outstanding experiments with analyte recovery from sludges and from live sampling from a school. In the following, the chromatographic separation quality, sensitivity, specificity and linearity as well as the recovery efficiency of the LC-MS part of the method will be presented.

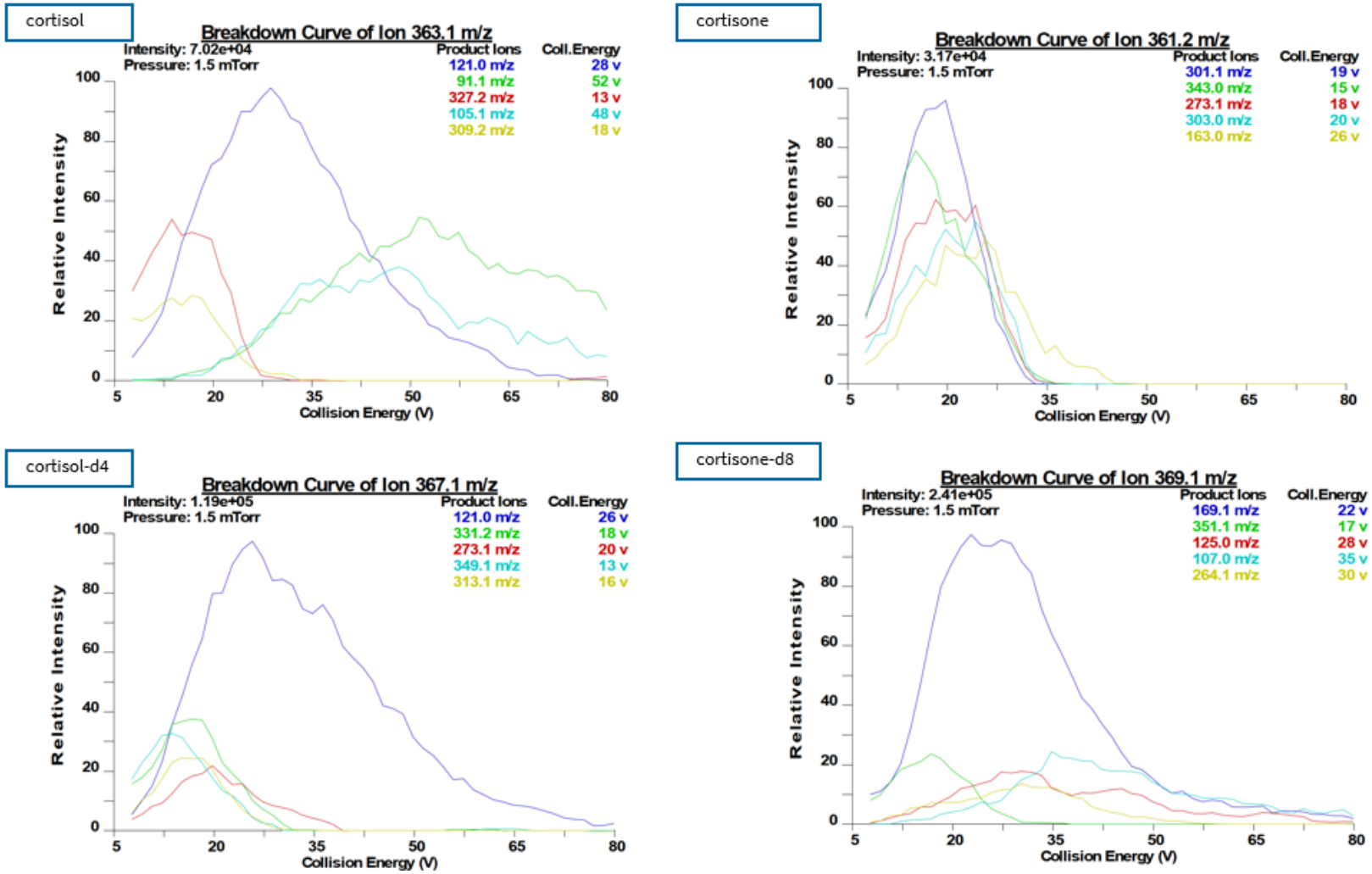

Figure 13: Transitions or breakdown curves of the analytes generated as part of the amended instrument tuning. The breakdown curve for cortisol was redone

LC-MS optimization strategy is the selection of acquisition parameters that provide the best signal for the analyte precursor and product ions (Sargent, 2013). Transitions are selected on the basis of them giving the best compromise of selectivity and sensitivity as measured by the signal-to-noise ratio which can be properly evaluated in the presence of real samples or “matrix” in the selected reaction monitoring (SRM) mode (Sargent, 2013). These transitions (or breakdown curves) are generated as part of the initial “instrument tuning” so that it is capable of recognizing the target analytes (Figure 13). The best two product ions are selected to characterize each analyte.

Concerning chromatographic separation quality, cortisone and cortisol elute close to each other but have noticeably separate peaks, as shown in (Figure 14). Cortisone is retained around the 8.71-75 mark while the labelled cortisol is retained a little later at the 9.27 mark. Pure cortisol is not registered sufficiently clearly. Following this result, we generated another breakdown curve for cortisol and subsequently the peak appeared clearly thereafter.

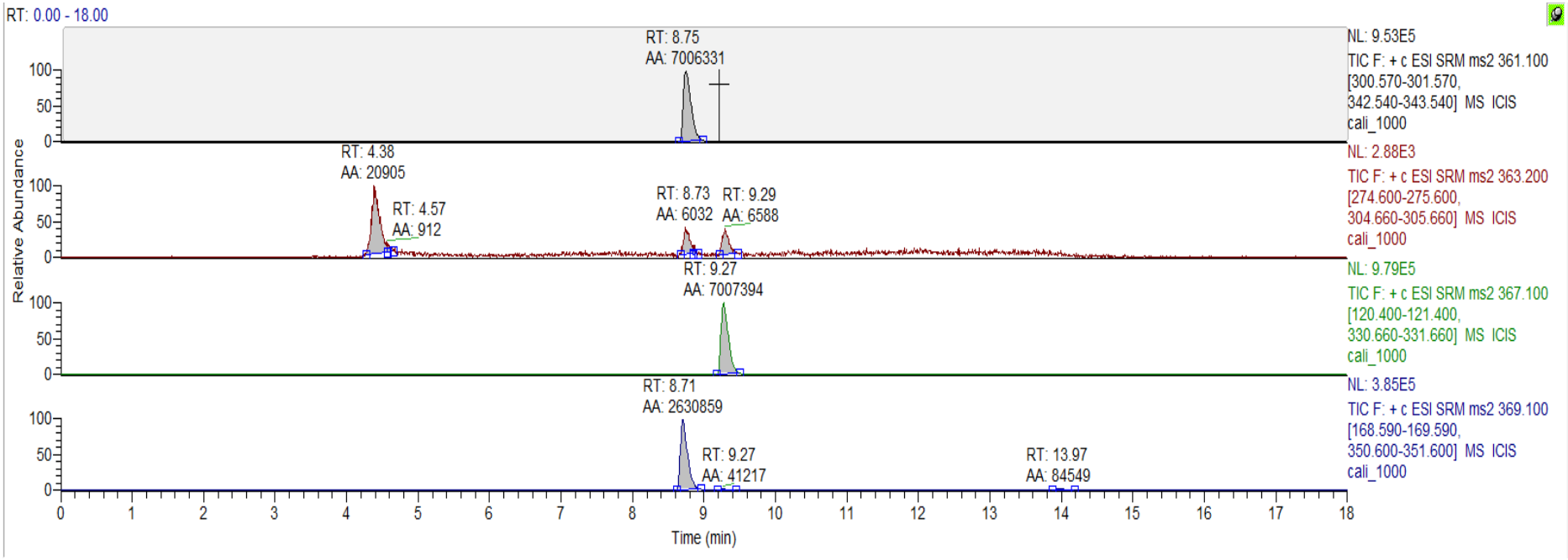

Figure 14: The retention peaks for the 4 analytes: cortisone (361.1), cortisol (363.2), cortisone-d8 (369.1), cortisol-d4 (367.1) as shown in TSQ Vantage Quant Browser software. The cortisol peak shows an anomaly: interference is making a greater signal than cortisol itself. Following this result, we generated a new breakdown curve for cortisol so that the instrument would be better able to recognize it

Sensitivity refers to the detection limits achieved by the instrument or the LOD and LOQ measures. The detection limit refers to the lowest concentration at which the analyte can be reliably observed while the quantitation limit refers to lowest concentration at which the analyte can be reliably measured. Following the analysis on the pilot data, the LOD for cortisol is 0.49 µg/L (ng/mL) and 0.98 µg/L (ng/mL) and the LOQ using the multiplier method would be at least 3 times the LOD.

For the analysis of real wastewater samples, the amounts will be very low. This means that the wastewater has to be concentrated. For the pilot, raw wastewater with spike was concentrated times 50. Selectivity is the ability of the procedure to measure the analyte of interest without the interference of other sample components. Specificity of cortisol detection was improved by remaking the breakdown curve for it.

The linearity and the working range (i.e. “calibration curve”) denoted the range over which the method will produce results with an acceptable uncertainty. The calibration curve was generated for the analytes by factor 2 serial dilution, ranging from 1000 µg/L down to 0.24 µg/L in 13 steps (Figure 15). Seeing as the calibration curves were generated only for the cortisol and cortisone and the spike contained the deuterated standards, the data from the analytical standards was transposed onto the deuterated standards. At the top of the dynamic range, a non-linear response may be observed, which is likely due to the saturation (Sargent, 2013). On the basis of calibration curves, concentrations around 100-200 µg/L is a great range up to around 1000 µg/L it is showing saturation.

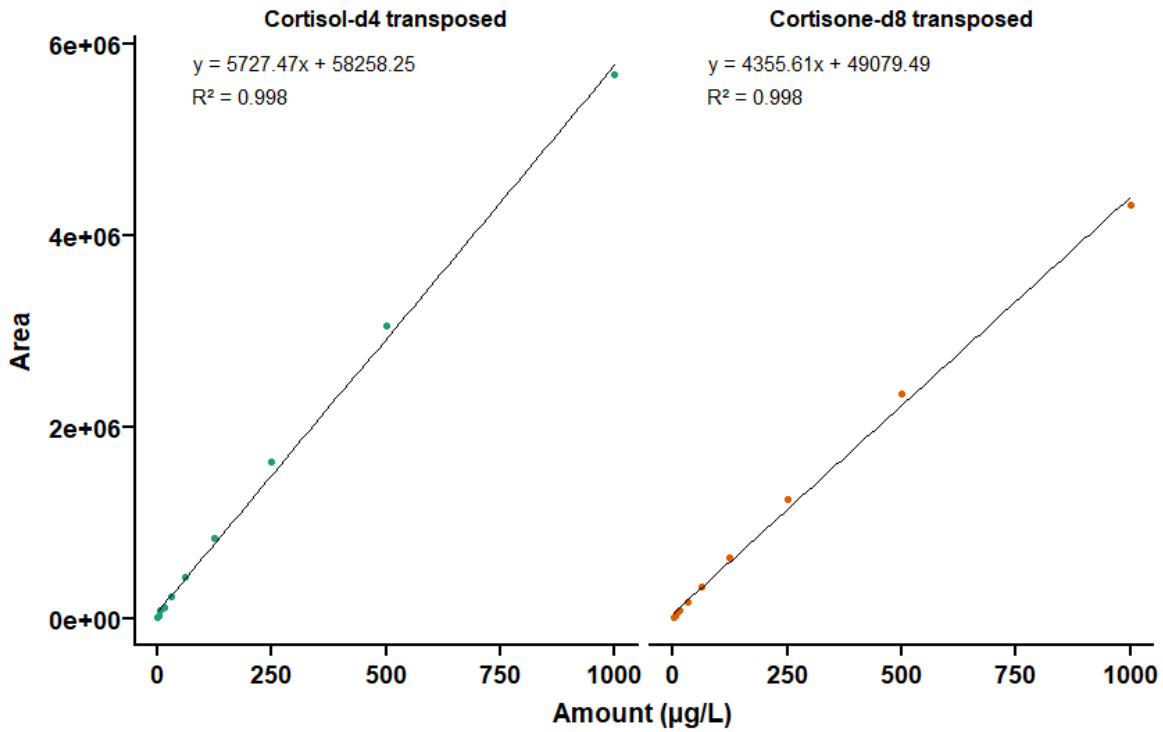

Figure 15: Calibration curves were prepared to the analytical standards (cortisol and cortisone), but since the spike solution contained only the internal standards, the values of the calibration curves needed to be transposed onto the results (in effect, “cortisol = cortisol-d4” and “cortisone = cortisone-d8”).

The recovery efficiency for cortisol and cortisone were tested by spiking raw wastewater (198 mL) with internal standard (2 mL at 100 µg/L) and subsequently comparing the recovery when the samples were either (1) centrifuged, (2) filtered, or (3) unfiltered prior to solid-phase extraction (SPE) (Figure 16). The unfiltered SPE resulted in the worst extraction efficiency because the solids were clogging the column and would not let the same volume of water to pass.

3.3 Pilot (Recovery Experiments)

The pilot experiments compared the recovery of the deuterated standards from wastewater that is either (1) centrifuged, (2) filtered, or (3) unfiltered prior to SPE extraction (Figure 16). The centrifuged samples showed the best extraction efficiency and least variance (Figure 18).

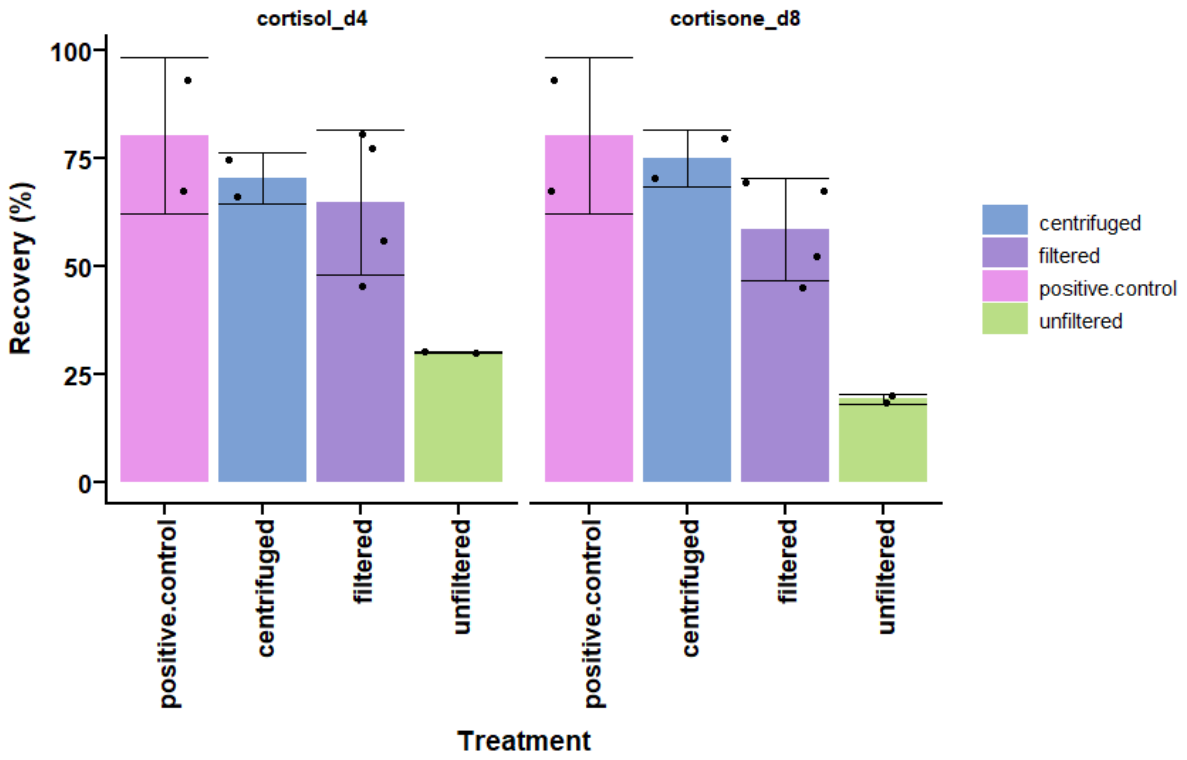

Figure 16: Results of the pilot recovery experiments for 3 treatments and positive control. The error bars denote deviation from the mean. The jittered points display individual recovery percentages.

In order to gauge whether the data is normally distributed, Shapiro-Wilk test was performed and elicited the p-value of 0.1678. As this is >0.05, it assumed that the recovery percentages are normally distributed. One-way ANOVA was performed on the filtered dataset and then Tukey's HSD post-hoc test was conducted to analyze pairwise comparisons between treatments to see whether there are meaningfully different results (Figure 17). The figure shows the median recovery percentages for each treatment, providing an overview of the variability within treatments. The “centrifuged” treatment shows significantly higher median recovery percentages compared to the other two treatments (filtering and not filtering at all), which suggests that this treatment is more effective in improving recovery rates. Conversely, “unfiltered” treatment shows a significantly lower median, an indicates that it is much less effective. Tukey's test indicates significant differences between centrifuged and both unprocessed and filtered treatments. The standard deviation of the recovery percentages for each treatment was calculated to find the method with the lowest variability (Figure 18): Following this experiment, the centrifugation was selected as the treatment of choice in future experiments.

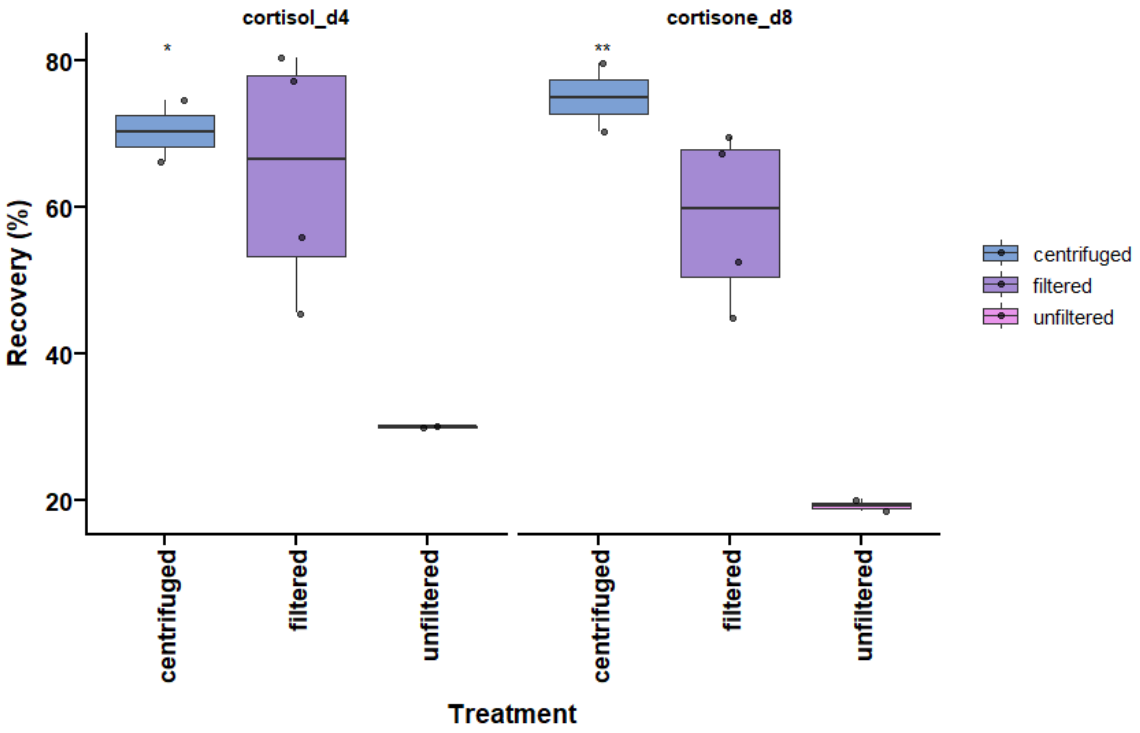

Figure 17: Results from the analysis of recovery percentages, excluding the positive control. For both cortisol and cortisol, centrifugation as a treatment method shows the most statistical significance (Tukey's post-hoc test).

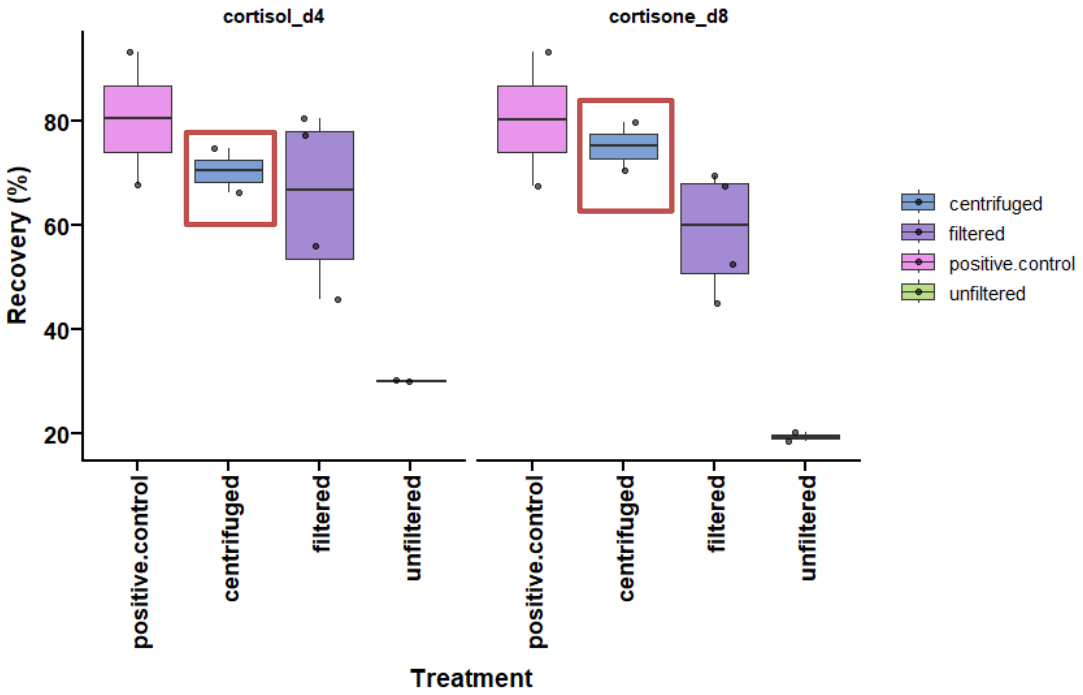

Figure 18: The standard deviation calculation for extraction experiments shows that the centrifuged samples are least variable.

3.4 Longitudinal Sampling Experiment and QC

The precision and accuracy of a method can be best evaluated on the basis of results from intra- and inter-day studies over a period of time.

At the time of writing, the only data that is available from the longitudinal sampling at a school is 2 datapoints over 4 days (Figure 19). The raw LC-MS results show the concentrations in the eluate, which it the result of the wastewater sample having gone through SPE and concentrated 50-fold. In order to get the levels of the original, the levels had to be back calculated to match.

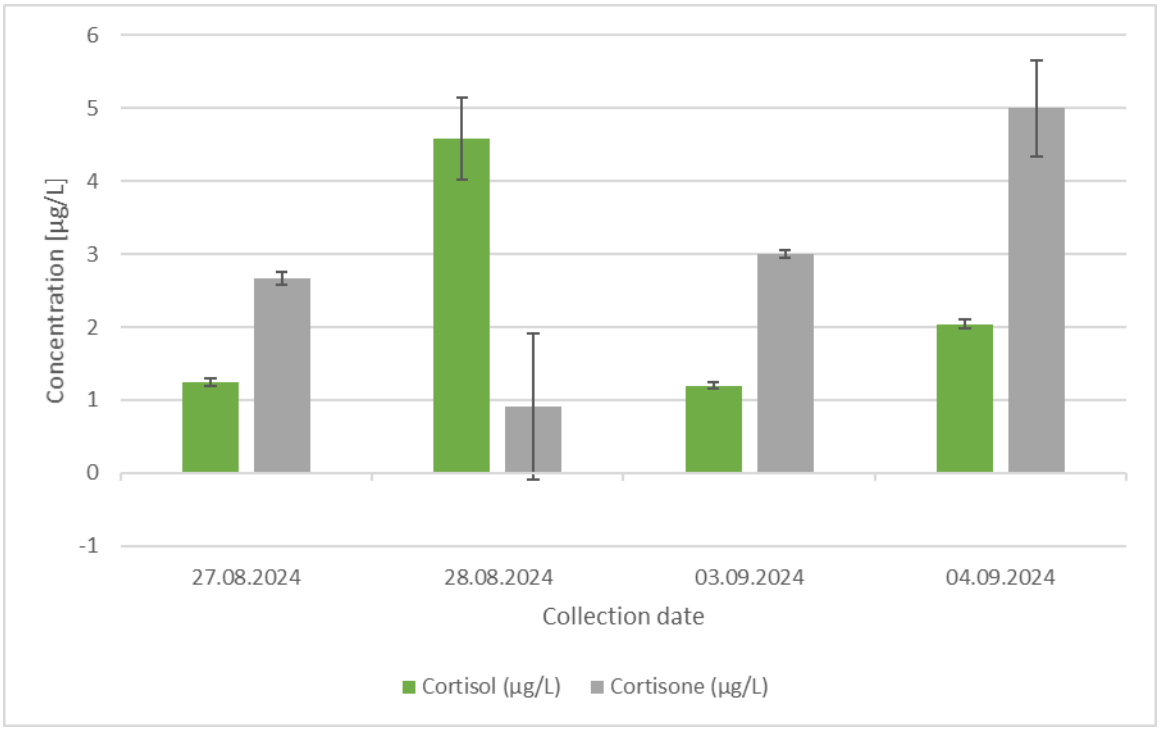

Figure 19: First data from longitudinal sampling experiment, where cortisol data was collected from the wastewater of a school over a period of two weeks. Cortisol ranges from about 1-4,5 µg/L (ng/mL) and cortisone ranges from about 0,9-5,2 µg/L (ng/mL).

Along with the wastewater samples, quality control (QC) samples or “known unknowns” were also prepared, run through SPE and analyzed with LCMS. Cortisol was spiked in at 10, 50, 100 and 200 µg/L and cortisone at 10 and 50 µg/L to see how recovery varies across a range of input values. The result shows that there is a level of variability in the extraction process, particularly in regards to cortisone (Figure 20). This should be accounted for in future steps.

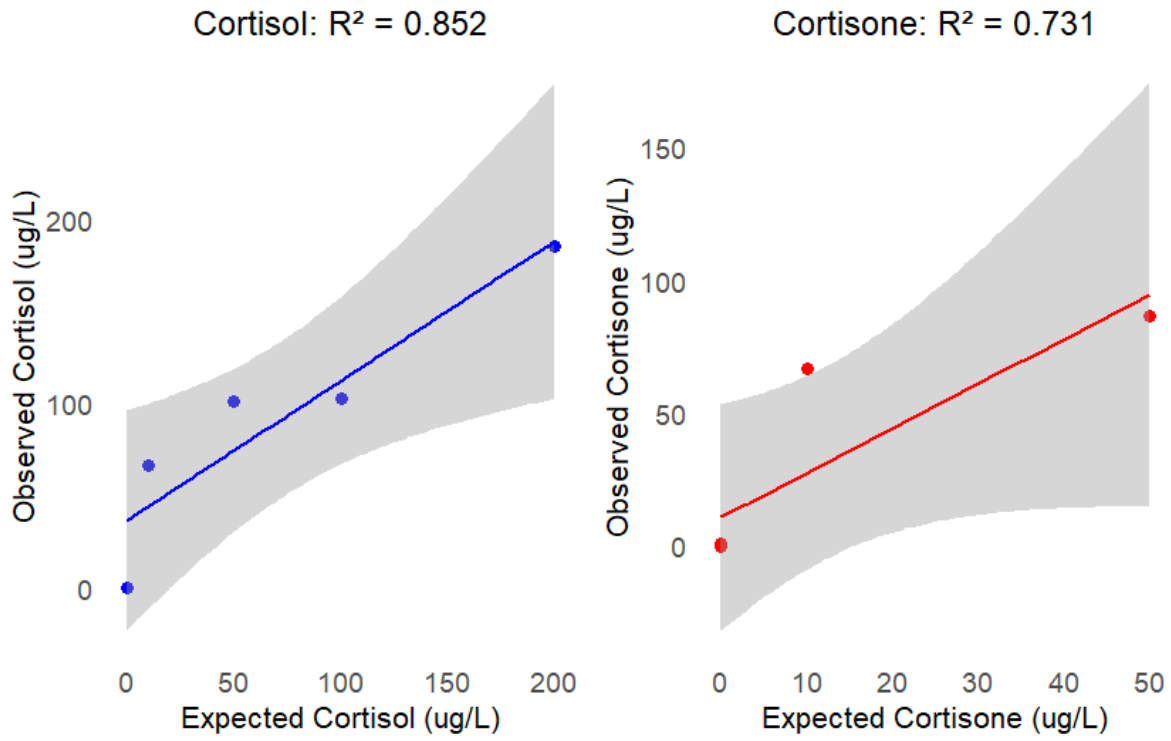

Figure 20: Quality control of the SPE process expressed as a linear regression between expected values and observed values. It shows that cortisone extraction exhibits a stronger variability than is desirable (R^2>0.8).

3.5 Pilot Sample Data Analysis

3.5.1 Recovery Percentage Calculation

The recovery percentage calculation for the pilot data was accomplished by first calculating the observed concentration from the sample's area using the standard curve equation, then adjusting this concentration based on the volumes of eluate and processed sample, then finding out the expected concentration based on the spike solution and volumes and finally calculating the recovery percentage to determine how much of the analyte was recovered relative to the expected amount.

1. The observed concentration is determined using the standard curve:

For each sample, the standard curve equation (derived from linear regression) was used to estimate the concentration of the analyte based on the measured area.

$$observed\ concentration = \frac{area - intercept}{slope}$$

2. The sample volume is adjusted by converting to final concentration:

The observed concentration was used to account for the volume of the eluate and the volume of the processed sample.

$$adjusted\ observed\ concentration = \frac{observed\ concentration\ x\ volume\ of\ eluate}{volume\ of\ processed\ sample}$$

3. The expected concentration was determined on the basis of the spike solutions:

For each sample, the spike solution concentrations were used to estimate the expected concentration.

$$expected\ concentration = spike\ solution\ concentration\ x\ (\frac{volume\ of\ eluate}{volume\ of\ processed\ sample})$$

4. The recovery percentage was calculated by comparing the observed to the expected concentration:

$$recovery\ percentage = \left(\frac{adjusted\ observed\ concentration}{expected\ concentration}\right) x\ 100$$

Following these steps, we arrived at the percentage of the analyte that was recovered in the sample compared to what was expected based on the spike.

3.5.2 ANOVA (Analysis of Variance)

ANOVA (Analysis of Variance) is used to compare means across multiple groups. It is used to determine whether the differences between group means are statistically significant. As it is specifically designed to analyze the variability within and between treatment groups, it's the appropriate choice to compare the means of recovery percentages across multiple treatment methods.

The total variability (SST or Total Sum of Squares) is broken up into two parts:

$$SST = \sum(each\ data\ point - overall\ mean)^2$$

1. Between-group variability (SSB or Sum of Squares Between) i.e. variability of recovery percentage means between each treatment group.

$$SSB = \sum(treatment\ mean - overall\ mean)^2\ x\ observations\ per\ treatment$$

2. Within-group variability (SSW or Sum of Squares Within) i.e. variability of the individual data point within the treatment group.

$$SSW = \sum(data\ points - group\ mean)^2$$

3. Degrees of Freedom (*df*):

$$dfbetween = number\ of\ treatments - 1$$

$$dfwithin = number\ of\ data\ points - number\ of\ treatments$$

4. Mean Squares (MSB or Mean Squares Between) and (MSW or Mean Squares Within):

$$MSB = \frac{SSB}{dfbetween}$$

$$MSW = \frac{SSW}{dfwithin}$$

5. F-ratio (i.e. is the between-treatment variability significantly larger than within-treatment variability):

$$F = \frac{MSB}{MSW}$$

6. P-value is used to determine whether the differences between treatment groups are statistically significant and can be calculated from the F-ratio:

$$p = P\ (F\ dfbetween, dfwithin\ \geq Fcalculated)$$

If the p-value is ≤ chose significance level (0.05), the null hypothesis (H_0) (all treatment means are equal) is rejected and at least one treatment is significantly different.

3.5.3 **Tukey HSD Test**

The Tukey HSD (Honestly Significant Difference) is a post-hoc test used after ANOVA to find out which specific treatments have significant differences. While ANOVA determines that there is a difference, but it doesn't specify which treatments differ.

$$HSD = q\ x\ \sqrt{\frac{MSW}{number\ of\ data\ points\ per\ treatment}}$$

The q or Tukey's Q distribution depends on the number of treatments and degrees of freedom.

4 Discussion

4.1 Applying the Method to Real Samples

The results of the analysis of real wastewater samples are still coming in, so the following discussion on the levels of cortisol and cortisone found is still very preliminary. The wastewater samples in our analysis come from a middle school in North Carolina with about 600 students. For ethical considerations, the anonymity of the students is maintained by keeping the school's name anonymous. Comparisons with previous studies or expected values are at present challenging as the scale of most other studies is not comparable to ours, for example Chen et al (Chen et al., 2019) and supplementals in Driver (Driver et al., 2022). As our sampling is directly from the premises and concerns a "captive population", i.e. the wastewater is sampled before it is piped to the treatment plant, the study by Driver et al (Driver et al., 2022) comes closest to our study in terms of scale and design.

As our method has been only applied to a handful of samples, more sampling and analysis is needed to begin to see real results. The data collected during this pilot study will be important for establishing future interpretation of the results of cortisol and cortisone levels in wastewater. Since wastewater data is a way of tracking health indicators over time, looking at trends with the aid of longitudinal sampling will be most valuable.

4.2 Challenges and Solutions

Method development is an iterative process and relies on a feedback loop between calibration and sample preparation. The results obtained so far are based on experiments that aimed to (1) establish the extraction procedure and (2) begin to see longitudinal trends by sampling over a longer period of time. Experiments 2 is underway and there are plans to conduct (3) matrix

matching and (4) extractions from sludge to see if sorption to solids influences cortisol/cortisone levels. These experiments result in feedback loops that improve the method. Additional quality controls and uncertainty reduction would lead to further improvements.

Future research could include matrix-matched calibration in order to reduce uncertainty. Given the possibility of matrix effects when dealing with wastewater, in general matrix matching would be preferable to solvent standards for preparing the calibration curves (Sargent, 2013). The wastewater may be approximated as “blank matrix” which will most likely not have quite the same composition as the sample but is still the best compromise achievable (Sargent, 2013).

There are many sources of uncertainty in developing and improving methods, and evaluating these sources can help to identify the parameters which are prone to increase uncertainty (Sargent, 2013). Sources of measurement uncertainty can be found in the sampling process (random variation in composition and bias) and how the sample composition (homogeneity, stability, interferences) as well as sample pre-treatment may impede the extraction of the target analytes (Sargent, 2013). Laboratory conditions, the equipment used and the LC-MS instrumentation conditions can be further sources of uncertainty (Sargent, 2013). Finally, the analyst applying the method, computational as well as random effects due to any type of interference that cause an increase in variation can also contribute to measurement uncertainty (Sargent, 2013).

At present, variation due to the SPE process is quite high in our method development and needs to be further streamlined. Some of these are simple fixes such as the increase of eluate volume from 200 µL to 500 µL for analysis as discussed in section 3.1, which saves time and reduces the possibility of “results-not-found”.

Examples of options that may iron out some extant variations include the choice of measurement (volumetric, gravimetric) or size of sub-sample (smaller is more variable), dilutions and their steps and type (for information on calibrants see Appendix A), and the number of replicates (Sargent, 2013).

4.3 Concluding Remarks

In general, fine-tuning (variance reduction) and application on a broader range of samples is needed. The wider range would help in assessing the effectiveness of the developed method. Further experiments to assess the longitudinal effects, matrix effects and wastewater matrix consistency are underway.

The detection and quantification of cortisol and cortisone in wastewater is a significant analytical challenge. The nature of wastewater matrices means that the extraction and

analytical methods must be both sensitive and robust. This study aims to establish reliable techniques for the measurement of these hormones using UHPLC-MS/MS, and thereby contributing to the fields of environmental monitoring and public health. Through our enhanced ability to track these hormones in wastewater, we can better understand their environmental impact, assess the effectiveness of wastewater treatment processes, and support WBE initiatives aimed at early detection of health issues at the population level.

This research has the potential to address health and environmental concerns using the tools of analytical chemistry and data ethics. We aim to provide a detailed methodological framework and demonstrate its application to actual sampling practice, paving the way for future WBE-based monitoring programs and interdisciplinary research efforts. Although it is difficult to say what an improved health outcome would look like through the lens of cortisol monitoring, it would likely manifest through changes and correlations in the stress and health profiles of the population due to public health interventions, stress-reduction strategies and improved social/economic conditions. The effort to be able to measure and understand these changes and correlations is certainly worthwhile.

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List of Abbreviations

Acronym	Definition	Page
ANOVA	Analysis of Variance	7
APCI	Atmospheric Pressure Chemical Ionization	22
BEH	Bridged Ethylene Hybrid	37
CALUX	Chemically Activated LUciferase gene eXpression	18
CAR	Cortisol Awakening Response	12
CAS	Chemical Abstracts Service	11
CID	Collision Induced Fragmentation	38
DNA	Deoxyribonucleic Acid	9
ECC	European Chemicals Council	16
ECHA	European Chemicals Agency	16
EDA	Endocrine-Disrupting Agent	17
ELISA	Enzyme-Linked Immunosorbent Assay	17
ELSI	Ethical, Legal, and Social Implications	55
ESI	Electrospray Ionization	16
GCMS	Gas Chromatography-Mass Spectrometry	17
HLB	Hydrophilic-Lipophilic Balance?	35
HPA	Hypothalamic-Pituitary-Adrenal	8
HPLC	High-Performance Liquid Chromatography	22
HRMS	High-Resolution Mass Spectrometry	18
HSD	Honestly Significant Difference	7
LCMS	Liquid Chromatography-Mass Spectrometry	5
LOD	Limit of Detection	3
LOQ	Limit of Quantification	3
MALDI	Matrix-Assisted Laser Desorption/Ionization	22
MPA	Mobile Phase A	37
MPB	Mobile Phase B	37
MSB	Mean Squares Between	50
MSW	Mean Squares Within	50
OASIS	Organic Adsorbent Silica Immobilized Support	35
OWASA	Orange Water and Sewer Authority	32
PLE	Pressurized Liquid Extraction	15
RPM	Revolutions Per Minute	35
SPE	Solid Phase Extraction	3
SRM	Selected Reaction Monitoring	37
SSB	Sum of Squares Between	50
SST	Sum of Squares for Treatment	49
SSW	Sum of Squares Within	50

Acronym	Definition	Page
STP	Sewage Treatment Plant	16
TGA	Total Glucocorticogenic Activity	18
TOF	Time of Flight	22
TSQ	Triple Stage Quadrupole	43
UAS	University of Applied Sciences	2
UHPLC	Ultra-High-Performance Liquid Chromatography	3
UPLC	Ultra-Performance Liquid Chromatography	20
WBE	Wastewater-Based Epidemiology	9
WWTP	Wastewater Treatment Plant	15

A: Calibration Curve Dilutions Procedure

Make the dilution in 2 steps:

1. First make a dilution of the analytical standard (AS) a step up starting from 2000 µg/L with pure solvent serial dilution by half (factor 2) (500 µL + 500 µL).
1. Then use these dilutions to make the final dilutions with internal standard (IS) spike solvent @ 200 µg/L. This way 2000 µg/L becomes 1000 µg/L and so on (500 µL + 500 µL) and IS becomes 100 µg/L.

Table 7: Calibration dilution in two steps

1: AS	Target C (µg/L)	2: AS + IS	Target C (µg/L)
1	2000	1	1000
2	1000	2	500,00
3	500,00	3	250,00
4	250,00	4	125,00
5	125,00	5	62,50
6	62,50	6	31,25
7	31,25	7	15,63
8	15,63	8	7,81
9	7,81	9	3,91
10	3,91	10	1,95
11	1,95	11	0,98
12	0,98	12	0,49
13	0,49	13	0,24

Needed for dilution:

1. Pure solvent (50% methanol) = min. 12 mL → 20 mL
2. IS spiked solvent = 10 mL

Stocks (for AS and IS) all start at = 2,5 mg/mL = 250000 µg/L

For IS spiked solvent:
Take 12 µL of crt-d4 + 12 µL of crn-d8 + 2976 µL pure solvent = 3000 µL of IS spiked solvent @ 1000 µg/L
From this, take 2000 µL + 8000 µL of pure solvent = 10 mL of IS spiked solvent @ 200 µg/L

For 2000 µg/L intermediate dilution of AS:
Take 24 µL of crt + 24 µL of crn + 2952 µL pure solvent = 3000 µL of AS @ 2000 µg/L
Arrange the vials in the 13 steps. Add 500 µL of pure solvent into each. Then take 500 µL of the AS @ 2000 µg/L and dilute down by half.