OSLOMET

Master´s Thesis

Master´s degree in Biomedicine
November 2021

Development of a liquid chromatography-tandem mass spectrometry analysis for cortisol as a stress biomarker to monitor fish welfare

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5600

60 ECTS

Faculty of Health Sciences

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Acknowledgement

Abstract

Background: Norway is the world's biggest salmon producer and the salmon industry has grown to become Norway's second largest export industry in value, after oil and gas. Despite the impressive growth, the industry is still facing issues around animal welfare and fish health. Stress can be a triggering cause of compromised fish health and disease outbreaks. Cortisol is the most important glucocorticoid and plays a key role in the stress response of an animal. However, limited analytical tools are available for assessing cortisol and it is challenging to sample fish non-invasively. FishLab AS wants to investigate whether it is possible to quantify cortisol in fish feces with liquid chromatography-tandem mass spectrometry (LC-MS/MS) and use this method as a tool to monitor fish welfare.

Aim: The aim of the current project was to develop and validate a high-quality LC-MS/MS method to assess stress in fish by measuring cortisol in fish feces.

Methods: Sample preparation was improved by optimization of cortisol extraction, derivatization, separation and detection. Enzymatic, acidic and basic hydrolysis were evaluated for maximizing cortisol deconjugation. Liquid-liquid extraction (LLE) and salting-out assisted liquid-liquid extraction (SALLE) were evaluated as cortisol extraction methods using different organic solvents with the addition of sodium chloride (NaCl). Signal enhancement was explored through derivatization with 4-aminobenzoic hydrazide (4-ABH). Liquid chromatography (LC) separation of derivatized cortisol (cortisol-4-ABH) was investigated under gradient condition using water solution with either 0.2% ammonia hydroxide or 0.2% formic acid with 0.2% methanol water solution. Finally, cortisol-4-ABH was detected using tandem mass spectrometry (MS/MS) with optimum multiple reaction monitoring (MRM) transitions for cortisol mono-hydrazone products under electrospray ionization in positive mode (ESI+). The use of deuterium labeled internal standards determined cortisol concentrations. The method was then validated for repeatability, intermediate precision, recovery, linearity, limit range, limit of detection (LOD) and quantification (LOQ).

Results: Cortisol deconjugation was most efficient when using Helix Pomatia with 2 M ammonium acetate buffer of pH 6 at 1 h incubation. LLE with tert-butyl methyl ether (MTBE) and 100 μl NaCl was the most preferable method for cortisol extraction with less ion suppression and acceptable percentage of relative extraction recovery (REC%). Cortisol-4-ABH provided much higher ESI+ response than underivatized cortisol. A derivatization step was therefore included in the method. A 0.2% ammonium hydroxide

solution provided highest analytical sensitivity for cortisol mono-hydrazone detection. The repeatability coefficient of variation in percent (CV%) were 16.7 and 11% for individual and replicate samples. The method intermediate precision CV% was 10.5% and relative spike recovery in percent (R´%) were 114, 126 and 127%. A linear regression model obtained from calibration curve exhibited a linear range within 0.09 – 100 ng/mL with a regression coefficient (R^2) of 0.997 – 0.999. The LOD and LOQ for cortisol were 0.04 and 0.09 ng/mL.

Conclusion: Fish feces samples gathered from off- and onshore industrial fish farms showed cortisol levels highly above LOD and LOQ. The majority of samples contained 5 – 12 ng/g however, a subset of fish displayed clearly elevated levels about 3 – 4 times higher than the average observed. These differences are much higher than the intermediate precision of the method, and therefore suggest that the developed method could be a useful tool to assess the stress level in fish.

Sammendrag

Bakgrunn: Norge er verdens største lakseprodusent og laksenæringen har vokst til å bli Norges nest største eksportnæring i verdi etter olje og gass. Til tross for den imponerende veksten, står industrien fortsatt overfor problemer rundt dyrevelferd og fiskehelse. Stress kan være en utløsende årsak til nedsatt fiskehelse og sykdomsutbrudd. Kortisol er det viktigste glukokortikoidet og spiller en viktig rolle i stressresponsen hos dyr. Derimot er begrensede analytiske verktøy tilgjengelig for å vurdere kortisol samtidig er det utfordrende med å samle inn fiske prøve på en ikke-invasiv måte. FishLab AS ønsker å undersøke om det er mulig å kvantifisere kortisol i fiskeavføring med væskekromatografi-tandem massespektrometri (LC-MS/MS) og bruke denne metoden som et verktøy for å overvåke fiskevelferd.

Formål: Hovedformålet med det nåværende prosjektet var å utvikle og validere en høykvalitets LC-MS/MS metode for å vurdere stress hos fisk ved å måle kortisol i fiskeavføring.

Metoder: Prøveforberedelse ble forbedret ved optimalisering av kortisol ekstraksjon, derivatisering, separasjon og deteksjon. Enzymatisk, sur og basisk hydrolyse ble evaluert for å maksimere kortisol dekonjugering. Væske-væske-ekstraksjon (LLE) og salte-ut assistert væske-væske-ekstraksjon (SALLE) ble evaluert som kortisol ekstraksjons metoder ved bruk av forskjellige organiske løsningsmidler med tilsetning av natriumklorid (NaCl). Signalforbedring ble studert gjennom derivatisering med 4-aminobenzosyre hydrazid (4-ABH). Væskekromatografi (LC) separasjon av derivatisert kortisol (kortisol-4-ABH) ble undersøkt under et gradientforhold ved bruk av vannløsning med enten 0,2 % ammoniakkhydroksid eller 0,2 % maursyre med 0,2 % metanol vannløsning. Til slutt ble kortisol-4-ABH detektert ved bruk av tandem massespektrometri (MS/MS) med optimal multippelreaksjonsovervåking (MRM) for kortisol mono-hydrazon produkter under elektrosprayionisering i positiv modus (ESI+). Bruken av deuteriummerkede intern standarder bestemte kortisol konsentrasjoner. Metoden ble deretter validert for repeterbarhet, intermediær presisjon, gjenfinning, linearitet, grenseområde, grense for deteksjon (LOD) og kvantifisering (LOQ).

Resultater: Kortisol dekonjugering var mest effektiv med bruk av Helix Pomatia og 2 M ammonium acetat buffer ved pH 6 og 1 times inkubering. LLE med tert-butylmetyleter (MTBE) og 100 μl NaCl var den mest foretrukne metoden for kortisol ekstraksjon med minst ione suppresjon og akseptabel relativ ekstraksjon gjenfinning i prosent (REC%). Kortisol-4-ABH ga mye høyere ESI+ respons enn ikke-derivert kortisol. Et derivatiserings

trinn ble derfor inkludert i den endelige metoden. En 0,2 % ammoniumhydroksid løsning ga høyere analytisk sensitivitet for kortisol mono-hydrazon deteksjon. Repeterbarhet variasjonskoeffisient i prosent (CV%) var 16,7 og 11 % for individuelle og replikate prøver. Metodens intermediær presisjon i CV% var 10,5 % og relativ spiket gjenfinning i prosent (R´%) på 114, 126 og 127 %. En linear regresjons modell hentet fra kalibreringskurven viste et lineært område innenfor 0,09 – 100 ng/mL med regresjonskoeffisient (R^2) verdi 0,997 – 0,999. Grensen for deteksjon (LOD) og kvantifisering (LOQ) var 0,04 og 0,09 ng/mL.

Konklusjon: Fiskeavføringsprøver samlet fra off- og onshore industrielle oppdrettsanlegg viste kortisol nivåer høyt over LOD og LOQ. Flertallet av prøvene inneholdt 5 – 12 ng/g derimot viste en undergruppe av fisk klart forhøyet nivå som var omkring 3 – 4 ganger høyere enn gjennomsnittet som ble observert. Disse forskjellene er mye høyere enn den intermediære presisjonen til metoden og antyder derfor at den utviklede metoden kan være et nyttig verktøy for å vurdere stressnivået i fisk.

Abbreviations

Abbreviation	Meaning
4-ABH	4-aminobenzoic hydrazide
11β-HSD1	11 β-dehydrogenase type 1
11β-HSD2	11 β-dehydrogenase type 2
ACTH	adrenocorticotropic hormone
b	absolute bias
b	y-intercept
b%	relative bias in percent
BEH	bridged ethylene hybrid
BSC	octadecyl
C18	octadecyl
CaCl$_2$	calcium chloride
CID	collision-induced dissociation
Cortisol-4-ABH	derivatized cortisol
CRF	corticotropin-releasing factor
CV%	coefficient of variation in percent
d4-cortisol	cortisol internal standard
d4-cortisol-4-ABH	derivatized cortisol internal standard
DC	direct current
DNA	deoxyribonucleic acid
EC	enzyme commission
EC 3.1.6.1	arylsulfatase
EC 3.2.1.31	β-glucuronidase
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
ESI+	electrospray ionization positive mode
ESI-	electrospray ionization negative mode
ESI-MS	electrospray ionization-mass spectrometry
fM	femtomolar
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
H$_2$SO$_4$	sulfuric acid
HIF-1	hypoxia-inducible factor 1
HPI	hypothalamic-pituitary-interrenal

HPLC	high-performance liquid chromatography
HSPs	heat shock proteins
IS	internal standardization
ISTD	internal standard
IUBMB	International union of Biochemistry and Molecular Biology
k	multiplier used in calculating limit of detection
K$_2$CO$_3$	potassium carbonate
K$_a$	acid dissociation constant
KCl	potassium chloride
k$_Q$	multiplier used in calculating limit of quantification
LC	liquid chromatography
LC/ESI-MS/MS	liquid chromatography electrospray ionization-tandem mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LJ	Levey-Jennings
LLE	liquid-liquid extraction
LLOQ	lower limit of quantification
LOD	limit of detection
LogP	logarithm of partition coefficient
LOL	limit of linearity
LOQ	limit of quantification
m	slope
m/z	mass-to-charge
MALDI/MS/MS	matrix-assisted laser desorption/ ionization-tandem mass spectrometry
MeOH	methanol
MgSO$_4$	magnesium sulfate
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	tert-butyl methyl ether
Na$_2$SO$_4$	sodium sulfate

NaCl	sodium chloride
NH₃	ammonia
(NH₄)₂SO₄	ammonium sulfate
NH₄Cl	ammonium chloride
nM	nanomolar
NTNU	Norwegian University of Science and Technology
P	partition coefficient
P	partition ratio
pH	potential of hydrogen
pKₐ	negative logarithm of acid dissociation constant
PP	polypropylene
Q1	first quadrupole
Q2	second quadrupole
QC	quality control
R²	regression coefficient
R´%	relative spike recovery in percent
REC	extraction recovery
REC%	relative extraction recovery in percent
RF	radiofrequency
RNA	ribonucleic acid
ROS	reactive oxygen species
RPLC	reversed-phase liquid chromatography
RSD	relative standard deviation
s	standard deviation
S/N	signal-to-noice
SALLE	salting-out assisted liquid-liquid extraction
SD	standard deviation
SM1	first mass analyzer
SM2	second mass analyzer
SPE	solid phase extraction
SRM	selected reaction monitoring
TFA	trifluoracetic acid
TWIG	travelling wave ion guide
UHPLC	ultra-high performance liquid chromatography
ULOQ	upper limit of quantification

UPLC	ultra-performance liquid chromatography
USI	ultra-spray ionization
UV	ultraviolet
V-HV	vacuum high vapor
$\mathbf{V_{aqeous}}$	fixed volume of biological solvent
$\mathbf{V_{organic}}$	fixed volume of organic sample
$\overline{x}$	mean
$\overline{x}'$	mean value of spiked sample in recovery experiment
$\mathbf{X_{ref}}$	reference value
$\mathbf{X_{spike}}$	added concentration in recovery experiment

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1. Introduction

1.1 Bakground

The Norwegian salmon industry has had a fantastic growth since the beginning of 1970´s as is the world´s leading to the foremost producer of Atlantic salmon. The annual production reached more than 1.300.000 tons of salmon which in 2018 represented a value of 65 billion NOK [1]. This makes aquaculture to the second largest export industry in Norway after oil and gas [2]. For comparison, the Norwegian production of salmon [3] is 3-4 times higher than the total production volume of cattle, swine, sheep and chicken in Norway combined [4].

Despite the success, the industry is still facing issues around animal welfare and fish health which need to be resolved [1]. A clinical disease outbreak leading to high mortality is often triggered by a combination of stress and subclinical viral or bacterial infections [5]. It is well known that stress can be induced by e.g. physical handling or challenging environmental conditions that may involve undesirable quality of water, nutrition, mechanical and thermal treatments and transportation [6]. From the salmon is transferred to sea pens, until the fish reach a slaughter weight of 5 kg, average mortality rates are currently about 16-22% [7].

The high mortality is a concern in terms of economical loses and sustainability but even more in terms of animal welfare [1]. The Norwegian Animal Welfare act protects all vertebrates, including fish [8]. Fish are not only able to observe, learn, and memorize time and place [9] but recent research has revealed that fish also perceive pain [10]. Therefore, in order to prevent suffering and reduce fish mortality in fish farms it is important to develop good methods to continuously monitor fish welfare. However, it is difficult to improve survival rates because poor fish health typically has multi-factorial causes [6] as well as limited or unavailable tools for quantitatively assessment of stress [9].

A previous study performed by the Norwegian University of Science and Technology (NTNU) involved measuring cortisol from fish feces using enzyme-linked immunosorbent assay (ELISA) technology. This study concluded that the method was non-invasive as well as fast in detecting stress levels in farmed salmon. The results showed correlation between stress response and illness, and the ELISA method was considered as an applicable tool for monitoring fish welfare. However, the method will currently not be utilized as commercial analysis routine. [11]. However, immunoassays have been used to measure fecal cortisol metabolites [12] although, this method have certain limitations with regards to limited linear range, matrix effect, suboptimal specificity [13] and cross-reactivity [14, 15]. The use of cortisol as a stress biomarker for fish needs to be investigated further by controlled experiments, preferably by using a high-quality analytical method.

Several analytical methods have been proposed for analyzing cortisol in various matrices. Earlier studies have included gas chromatography-mass spectrometry (GC-MS) for urine cortisol [16, 17] and matrix-assisted laser desorption/ ionization-tandem mass spectrometry (MALDI/MS/MS) for serum cortisol [18]. However, GC-MS is a time consuming analysis that often requires a derivatization step. In comparison, due to its high sensitivity and specificity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) meets the requirements as high-quality analytical method for steroid analysis [14]. This approach has shown successful results for determination of cortisol in serum [19, 20] and urine [14, 21]. Due to the absence of commercial laboratories offering a routine analysis of cortisol in fish, FishLab AS wants to investigate whether it is possible to quantify cortisol in fish feces with LC-MS/MS and use this method as a tool to monitor fish welfare.

For adequate LC-MS/MS analysis, optimization of selectivity and sensitivity need to be achieved. This is ensured by adding a suitable sample preparation procedure prior to the analysis. Such procedures can remove unwanted matrix compounds and achieve analyte enrichment to create signal enhancement [22]. Different platforms used as sample preparation procedures for steroid hormone analysis are enzyme hydrolysis [16, 23], derivatization [24, 25], liquid-liquid extraction (LLE) [24] and salting-out assisted liquid-liquid extraction (SALLE) [26, 27].

1.2 Stress physiology in fishes

Stress is a physiological state present in fish [28], whereas prolonged or repeated stressor exposure is commonly associated with compromised health effects [28, 29]. To determine this physiological state, some considerations must be noted such as the variation between unrelated and related species. This divergence can be the result from intraspecific and interspecific factors. Although, similar responses to the same stressor can occur for unrelated species. Other differences that can vary due to these two factors are post stressor responses denoted as primary, secondary and tertiary response. These stress responses have indicators that may alter in terms of repeated exposure which can affect the ultimate response. Measuring stress indicators can provide critical information about fish health, welfare and performance [28]. This may include decrease in fitness and reproduction, tissue atrophy and immunosuppression [12]. Therefore, gathering such knowledge can improve fish's future survival and growth. Indication for stress physiologies can be done by measuring primary, secondary and tertiary indicators to acute or chronic stressor exposure [28].

1.1.1 Primary stress response

Stress is the adaptive response that re-establish the homeostasis when an individual gets exposed to a stressor [29] like feeding restrictions, competition, predator, hypoxia, raised water temperature and pollution [28]. The primary stress response in fish is the activation of the brain-sympathetic-chromaffin cell (BSC) and hypothalamic-pituitary-interrenal (HPI) axis as illustrated in Figure 1. The BSC axis then produces and releases catecholamines known as epinephrine and norepinephrine hormones from chromaffin cells in the head kidney. HPI axis activation produces and releases the corticotropin-releasing factor (CRF) from the hypothalamus that stimulates the corticotropic cells in the pituitary to release adrenocorticotropic hormone (ACTH). This hormone will trigger the secretion of the glucocorticoid hormone cortisol from the interrenal cells located in the head kidney. Cortisol is the predominant glucocorticoid hormone and acts to alter fish behavior, metabolic and physiological relations [30] and is commonly quantified to indicate stress [28].
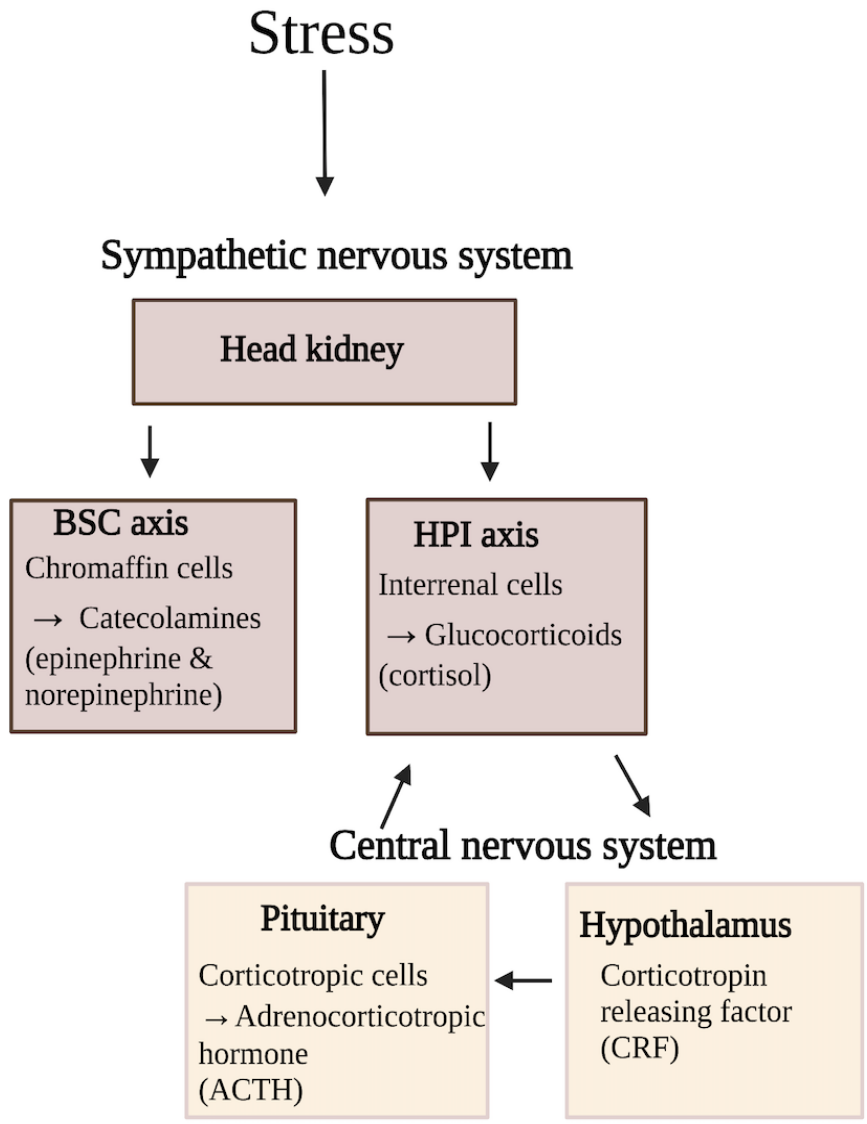
Figure 1: Primary stress response in fishes.

The primary stress response activates the brain-sympathetic-chromaffin cell (BSC) and hypothalamic-pituitary-interrenal (HPI) axis. The BSC axis produces and releases catecholamines epinephrine and norepinephrine from chromaffin cells in the head kidney. HPI axis activation produces and releases the corticotropin-releasing factor (CRF) from the hypothalamus that stimulates the corticotropic cells in the pituitary to release adrenocorticotropic hormone (ACTH). ACTH release triggers the secretion of the glucocorticoid hormone cortisol from the interrenal cells located in the head kidney. Illustration is created by the author using [30] for inspiration and Biorender.com.

1.1.2 Secondary stress response

Exposure to a stressor also includes a secondary response after glucocorticoid release. This helps with recovery and survival that involves cellular and molecular alterations. Range of stressors can lead to alterations in gene expression, apoptosis, deoxyribonucleic acid (DNA) repair, cell cycle arrest, removal of cellular and molecular debris. Environmental stressors can cause oxidative stress by producing reactive oxygen species (ROS) that can damage proteins, ribonucleic acid (RNA), DNA and lipids. If ROS production exceeds antioxidant levels it will cause oxidative stress that promote telomere shortening which triggers cellular and possibly organismal senescence. Another cellular stress response is the activation of hypoxia-inducible factor 1 (HIF-1). This transcription factor controls the expression of heat shock proteins (HSPs) that repair, fold and catabolize proteins. Other secondary responses include changes in glucose levels, immune system, acid-base balance, metabolism and ion balance [28].

1.1.3 Tertiary stress response

The tertiary response includes the whole-organism response such as changes in behavior, metabolism, cardiac activity, fitness, survival, swimming performance, disease resistance [28] and reflex impairment [31]. Fish can change their behavior due to predator avoidance, food acquisition and habitat selection. Other changes that can be caused by stress is their condition related to mass, length or both of the entire organism or organs. In fact, organosomatic indices are commonly used as stress indicators due to its simplicity, however it requires lethal sampling [28].

1.1.4 Stress indicator

Primary and secondary responses can provide information about future survival and performance thus, predict the outcome from the exposure to stress stimuli. This way, primary and secondary responses can provide information that can prevent future health consequences. There are many advantages using these indicators however, they require laboratory equipment for quantification purposes. Unlike tertiary response, quantification can be done without lab work and data can be easily collected to determine a physiological state and interpret it out in the field. However, interpretation of this data may require specialists like ethologists, physiologists and field ecologists. Another disadvantage with tertiary responses is that primary and secondary indicators do not necessarily correlate with tertiary indicators [28].

1.1.5 Stress biomarker for monitoring fish welfare

Steroid hormones have been proposed as a biomarker for monitoring fish welfare [30]. Steroid hormones are derived from cholesterol and categorized into five groups known as androgens, estrogens, progesterone, glucocorticoids and mineralocorticoids. The main glucocorticoid steroid hormone cortisol [32] is secreted through stressful conditions that can involve factors such as shock, toxins and heat as well as the conditions mentioned earlier in Section 1.1. Successful attempts to use cortisol as a stress indicator in fish have been reported. Blood matrices have commonly been used for steroid analysis, however less invasive cortisol measuring methods have now been more desirable in order to indicate stress physiologies, this includes analysis of mucus, fins, feces and water [33].

1.1.6 Cortisol metabolism

Cortisol metabolism is modulated through series of enzymatic reactions [13] as illustrated in Figure 2. The metabolism steps may require reduction at C-3 and/or C-20 and/or C-21, de(conjugation) and oxidation at C-11 [12]. The cortisol metabolite is irreversibly converted into its inactive cortisone metabolite by 11β-dehydrogenase type 2 (11β-HSD2) in the adipose tissue and liver. A reversible conversion of inactive cortisone to cortisol can be catabolized by 11β-dehydrogenase type 1 (11β-HSD1) in the colon and kidney [13]. Cortisol and cortisone metabolize into their tetra-metabolites known as tetrahydrocortisol, allo-tetrahydrocortisol and tetrahydrocortisone by 5a- and 5B-reductase [34]. Alterations of cortisol and its metabolite levels have shown an association to various disorders [35, 36], diseases and other pathological conditions [19, 21].
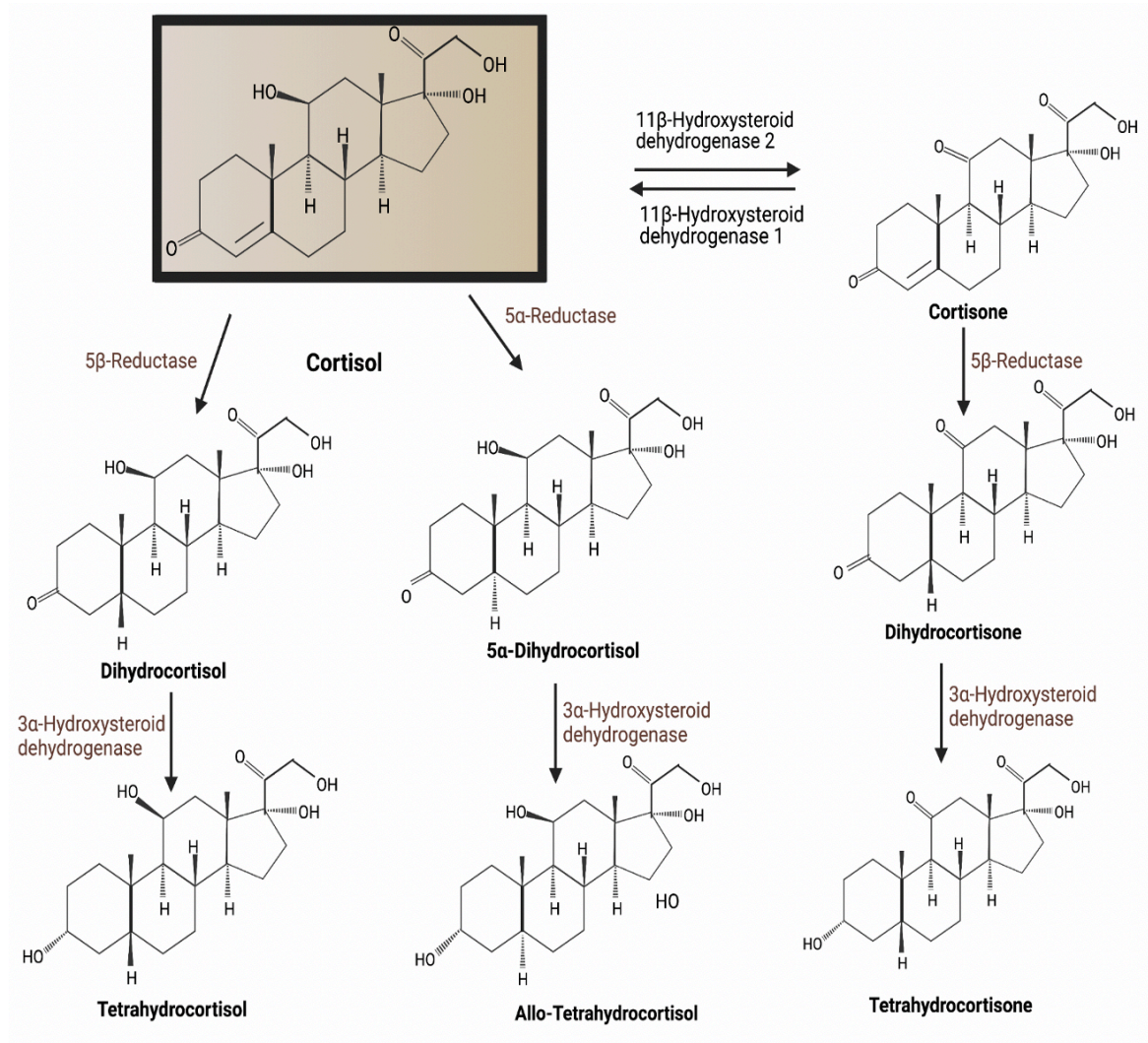
Figure 2: Cortisol metabolic pathway by various enzymatic reactions.

Illustration is created by the author using [37] for inspiration and Biorender.com.

1.2 Method development

1.2.1 Enzyme hydrolysis for signal enhancement

Cortisol and its metabolites can be secreted as free or hydrophilic conjugates of glucuronides and sulfides [34, 38]. Previous studies have stated that cortisol metabolites are predominantly excreted as glucuronide and sulfate conjugates [34]. This was also stated by another study where approximately 95% of the conjugated metabolites are sulfates and the rest is glucuronides [33]. A successful cleaving of glucuronide and sulfate conjugates by enzyme hydrolysis and optimization for hydrolysis conditions has been reported for cortisol and its metabolites [16]. Holoenzymes are active proteins with catalytic activity that increase the ultimate reaction rate to produce a product by acting on a selected substrate [39].

Selection of appropriate enzyme treatment prior to analysis can easily be done through the enzyme commission (EC) classification system. According to the International union of Biochemistry and Molecular Biology (IUBMB) enzymes are classified into six major classes [40]. These six main classes include 1. oxidoreductase, 2. transferases, 3. hydrolases, 4. lyases, 5. isomerases and 6. ligases. The EC classification system will provide a name and a four digital EC number to each enzyme. The first digit will be based on which main class the enzyme belongs to while the second and third are based on the reaction being catalyzed [39]. The fourth digit will indicate the number enzyme have in its sub-subclass [40]. Arylsulfatase (EC 3.1.6.1) enzymes are capable of catalyzing a sulfuric ester hydrolase reaction to release sulfate groups [41]. β-glucuronidase (EC 3.2.1.31) enzymes however, are capable of hydrolyzing a glycosidic bond releasing glucuronides [42].

LLE is a commonly used partitioning technique in sample preparation prior to steroid hormone analysis [32]. The purpose of this method is to selectively isolate targeted compounds by solvent extraction [43]. This involves two immiscible solvents to extract the analyte of interest from one aqueous phase (biological sample) into another immiscible phase (organic solvent) or the opposite way. To select an appropriate LLE technique, analyte(s) physicochemical properties have to be certified. This will include the analyte(s) partition coefficient (P) that will be calculated by the concentration ratio of the molecule´s neutral form in organic ($C_{Organic}$) and aqueous ($C_{Aqueous}$) solvent (Equation 1), whereas LogP is the logarithmic value of P. This logarithmic value can be used as an estimation for polarity of the analyte, therefore estimating the extraction efficiency in the chosen LLE method. Commonly used guidelines for logP values refer to very polar analytes as logP < 0, relatively polar as logP 0-1 and hydrophobic as logP > 1. This knowledge will tell if the extraction of the analyte can be easy or difficult by any organic solvent. The partitioning for an analyte is more efficient using a solvent with similar properties. Therefore, polar compounds will be present in aqueous phase while nonpolar compounds will diffuse over the liquid-liquid interface into the organic phase due to their higher solubility [44].

$$logP = \ log_{10} \ x \ \frac{C_{Organic}}{C_{Aqueous}} \qquad (1)$$

The pK$_a$ constant is the negative logarithmic value of the acid dissociation constant (K$_a$) (Equation 2) which is an indication of a compound´s basic or acidic strength. The potential of hydrogen (pH) constant determines the basicity or acidity of a solution and have a great influence on the pK$_a$ value thus, ionization capabilities. Acidic compounds are found in their unionizing state when pH is below pK$_a$ value and vice versa for basic compounds. The extraction recovery (REC) will be most sufficient in LLE and solid phase extraction (SPE) with reversed-phase extraction (RPE) when using pH where analytes are found in their uncharged form. The common rule is to use a pH value two units below pK$_a$ for acidic and two units above pK$_a$ for basic compounds [44].

$$pKa = \ -log_{10} \ K_a \qquad (2)$$

The REC is an estimation for LLE efficiency by the relation between partition ratio (P) and fixed volume of organic ($V_{organic}$) and biological ($V_{aqueous}$) sample (Equation 3). Improvement of REC can be done by choosing extraction solvent with higher P value than the targeted analyte and more extraction solvent. Selection of a fitted extraction solvent also includes other factors like density and volatility. Having a solvent with low boiling point makes evaporation less time consuming and having low density makes the transfer of organic solvent more easily as it becomes the top phase. General procedures for LLE include organic solvents, supplements and/or internal standard (ISTD) that will undergo centrifugation for phase separation. The phase containing the analyte of interest will then be collected for evaporation by vacuum centrifugation or nitrogen steam. Evaporation is necessary as the organic solvent is a strong eluent in liquid chromatography-mass spectrometry (LC-MS) and applying direct injection of the sample will lower the analyte retention. Also, in LLE the solvent is not miscible with water thus, direct injection in aqueous mobile phase used for reversed-phase liquid chromatography (RPLC) will not be feasible. Therefore, dryness of the organic solvent and reconstitution of the sample is needed before LC-MS analysis. Reconstitution can either be performed by adding the initial mobile phase solution or a solution with similar pH and polarity to the mobile phase. The LLE technique improves sample clean up, reduces interference like ion suppression [44] and has been applied in sample preparation prior to LC-MS/MS for multiplexed steroid hormone analysis [24].

$$REC = 100 \; x \; \frac{P \; x \; V_{Organic}}{P \; x \; V_{Organic} + V_{Aqueous}} \qquad (3)$$

Even though conventional LLE have several advantages, there are some limitations involving usage of toxic organic solvents, large quantities of sample and hydrophilic extraction difficulties. By this knowledge, SALLE is another efficient extraction approach that has been widely used in bioanalysis. This involves water-miscible organic solvent with the addition of salt to initiate phase separation [45]. The salting-out effect will lead to less solubility of nonelectrolyte substances in the aqueous phase, higher density [46] and viscosity [47] of the aqueous phase. Salting-out agents that have been commonly used in SALLE are ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate ($MgSO_4$) [45], calcium chloride ($CaCl_2$), potassium carbonate (K_2CO_3), sodium chloride (NaCl), potassium chloride (KCl) and sodium sulfate (Na_2SO_4) [46]. The most common water-miscible organic solvent used are acetonitrile, isopropyl alcohol and acetone. Since SALLE extracts are compatible with many analytical systems, evaporation can be avoided as opposed to LLE. SALLE extracts are suitable for analytical analysis by LC or gas chromatography (GC) with either mass spectrometry (MS) or ultraviolet (UV) detector. Although, LC-MS is the most frequent analytical system used for SALLE extracts [45].

1.2.3 Derivatization for signal enhancement

Sample matrices containing analytes with chemical instabilities, poor chromatographic retention and ionization can be prevented by adding derivatization step prior to the analysis. Derivatization is the process where analyte(s) chemical structure is modified by integrating new moieties that will act as functional groups. Such modification will create physiochemical alterations of the analyte as well as developing more desirable LC-MS performance. These moieties can either improve detection, separation and/or stability. Response-enhancing moieties such as charged or ionizable tags are commonly incorporated to improve detection sensitivity. This allows readily ionizable molecules to have better ionization capabilities. Derivatized compounds created have specific product ions and fragment ions that can be useful when applying multiple reaction monitoring (MRM) [22].

Steroids with low proton affinity have to undergo derivatization for sensitivity improvement. Derivatization reagents are chosen based on functional groups present in steroid hormones. These functional groups are mainly hydroxyl and ketones as shown in Figure 2 [25]. A previous study have listed 4-aminobenzoic hydrazide (4-ABH) reagent as an acceptable derivatization reagent for cortisol to produce mono-hydrazone products. The conversion of cortisol into hydrazone by 4-ABH is done through the attachment of an aromatic amine group as illustrated in Figure 3 [24].
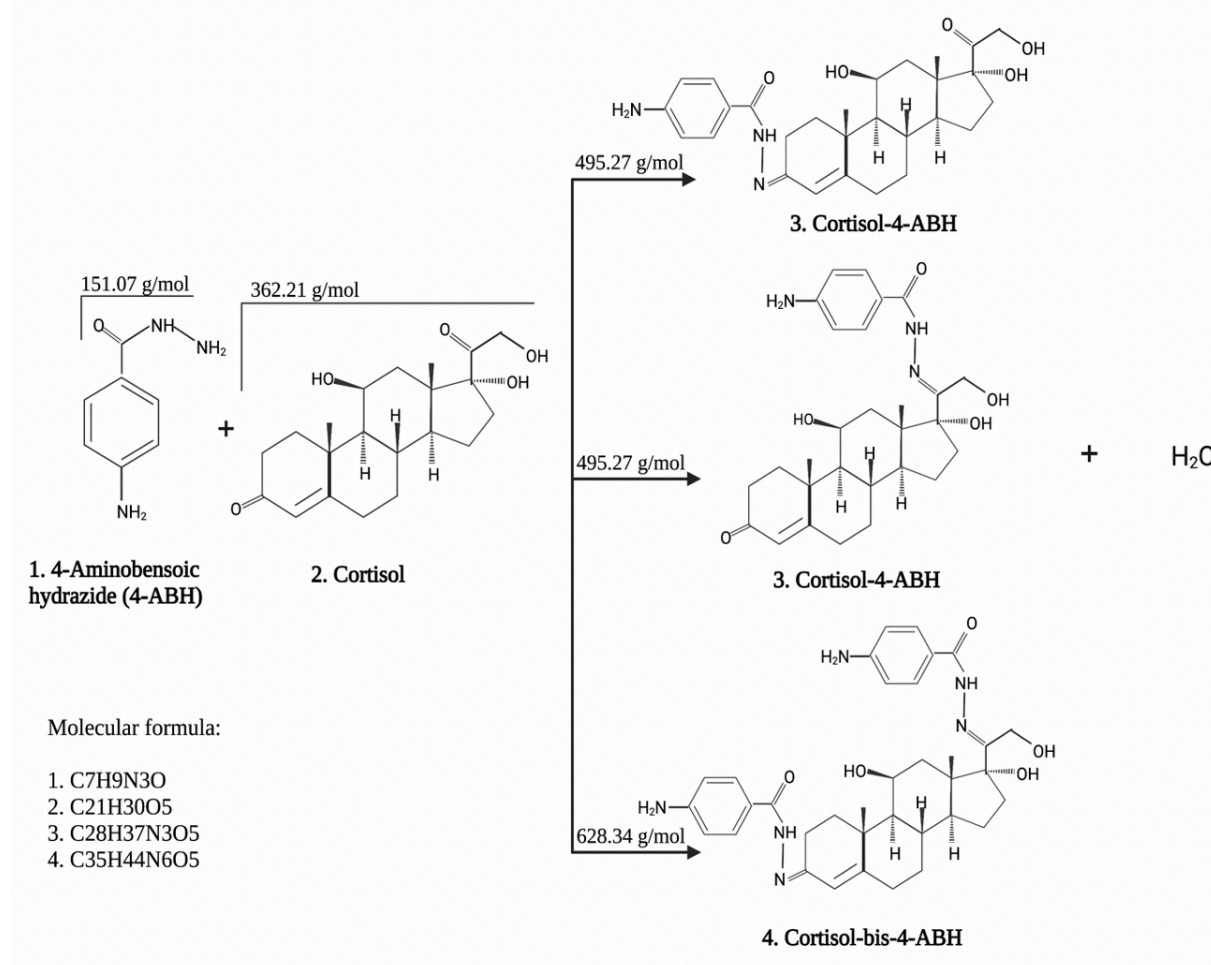
Figure 3: Cortisol derivatization.

Represents derivatization reaction between 4-aminobenzoic hydrazide (4-ABH) reagent and functional ketone groups in cortisol. Three possible chemically different hydrazone structures is shown in addition to molecular formula and isotopic molecular weight (g/mol). Illustration is created by the author using [21, 25] for inspiration and Biorender.com.

1.2.4 Liquid chromatography for analytical separation

The LC technique involves separating analyte(s) of interest from other compounds. Separation is accomplished by distribution via adsorption, partitioning or other interaction between a flowing liquid (mobile phase) and sorbent (stationary phase) packed inside a column. The mobile phase consists of a liquid mixture composed of A and B solvents that migrate down the column [48]. The most preferable mobile phase additives in LC includes formic acid, acetic acid, trifluoroacetic acid (TFA), ammonium formate, ammonium acetate and ammonium hydroxide [49]. High-performance liquid chromatography (HPLC) is a LC technique instrumented with small particle columns and high pressure systems. The most modularized form of HPLC is ultra-high performance liquid chromatography (UHPLC or UPLC) constructed with smaller particle columns and higher pressure systems [48]. This is an automated technique constructed with a solvent tray, column, column heater, sample manager, binary solvent manager, sample organizer coupled to a MS (Figure 4) [50]. The UHPLC analytical separation technique is able to provide faster separations [48].
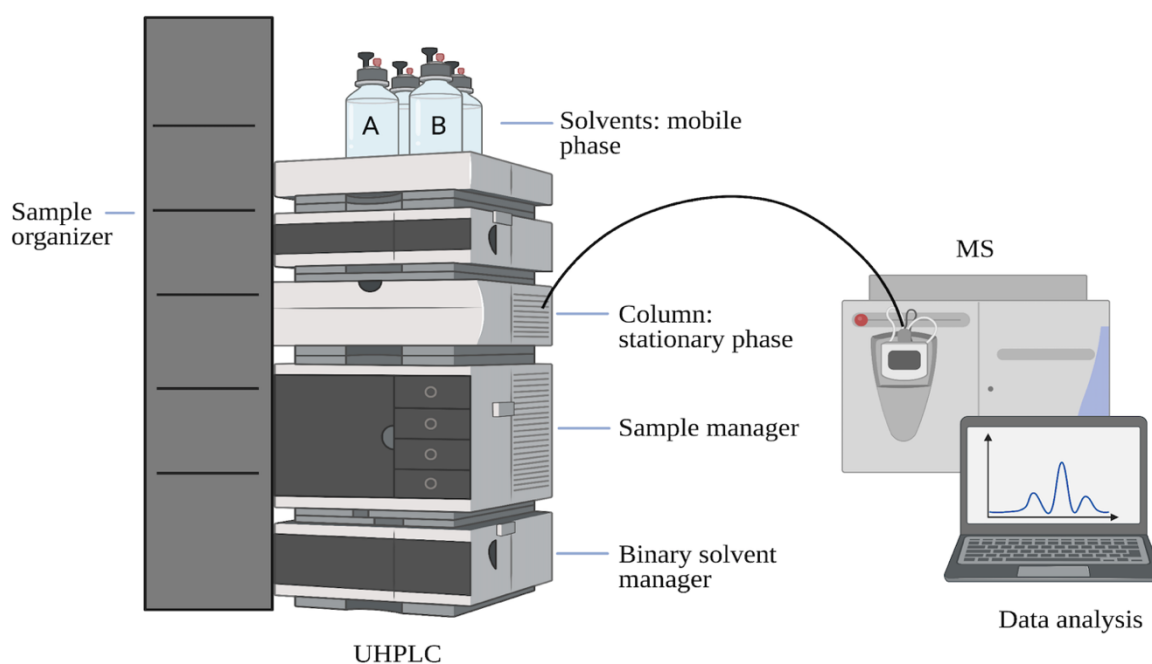
Figure 4: Ultra-high performance liquid chromatography (UHPLC) system coupled with mass spectrometer (MS).
UHPLC is constructed with a sample organizer, solvents (mobile phase), column (stationary phase), sample manager and binary solvent manager. Steroid hormone analysis is performed by analyte(s) separation in UHPLC and detection through MS for qualitative and quantitative analysis. Illustration is created by the author using [50] for inspiration and Biorender.com.

1.2.4.1 Reversed-phase liquid chromatography

To separate analyte(s) from other interfering compounds it must be retained and have sufficient interaction with the stationary phase [48]. To maintain such separation a RPLC separation technique is commonly applied for hydrophobic compounds [22]. The RPLC separation mode is based on the analyte(s) P value between a nonpolar stationary phase and polar mobile phase. A nonpolar stationary phase commonly used in RPLC is covalent bond hydrophobic groups known as octadecyl (C18) on a silica particle. The RPLC columns are mostly packed with pure silica or hybrid particles that have less surface silanol activity [48]. Unreacted silanol residues cause less efficient extraction and unwanted retention effects by interacting with the analyte(s) [22]. Another advantage with these particles is the improved pH range of 2-12 and peak tailing improvement. Waters Corporation improved the hybrid particle chemistry based on bridge ethylene groups also known as second-generation hybrid particles. The targeted nonpolar analyte(s) will interact with the C18 group bound to the bridged ethylene hybrid (BEH) particle stronger than polar analytes. Therefore, polar analyte(s) will elute first and nonpolar analyte(s) will elute last [48]. Elution is the process where hydrophobic interaction between the analyte and stationary phase is interrupted with an organic solvent or solvent mixture. The logP value can determine the elution solvent for LC-MS, whereas high logP requires a stronger organic solvent or solvent mixture. In order to mobilize analyte(s), a commonly used elution technique is by applying a concentration gradient. Normally, a LC gradient involves altering the concentration gradually of two solvents. By applying gradually increasing concentrations of an organic solvent, hydrophobic interactions will be interrupted thus, nonpolar analytes will be mobilized [22] as illustrated in Figure 5. The RPLC separation mode is commonly used in LC and is a suitable technique for analyzing nonpolar, medium polar and polar analytes [48].
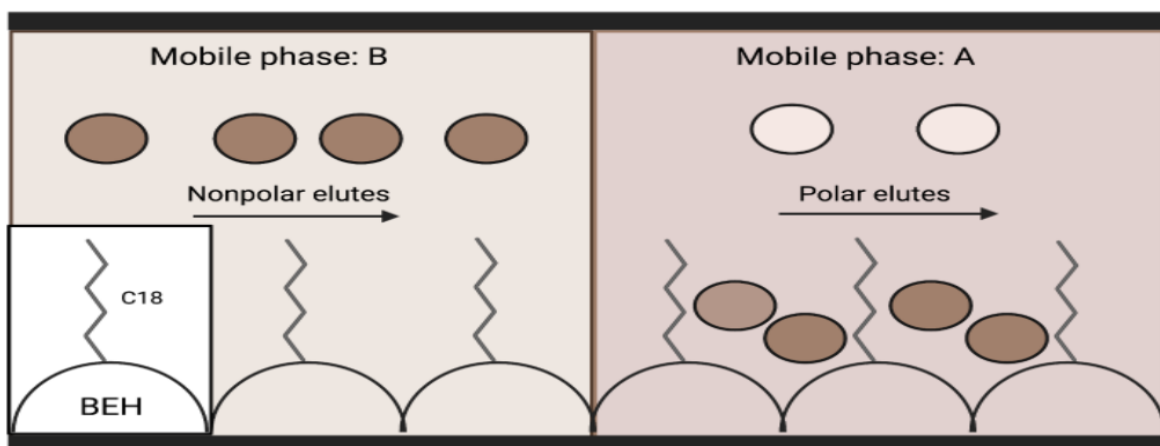
Figure 5: Reversed-phase liquid chromatography (RPLC).
Liquid chromatography (LC) column constructed with bridged ethylene hybrid (BEH) particles bounded with octadecyl (C18) in reversed-phase liquid chromatography (RPLC). Polar mobile phase A causes polar analyte(s) to elute first and nonpolar mobile phase B causes nonpolar analyte(s) to elute last. Illustration is created by the author using Biorender.com.

1.2.5 Mass spectrometry for analytical detection

MS is a analytical technique used in various fields of science that includes medicine, pharmacy, biochemistry and chemistry. The MS instrument consists of an automated inlet sample method coupled to an ion source, mass analyzer, detector and data system [51]. This methodology gathers qualitative (structure) and quantitative (concentration or molecular mass) information from biological samples [52] in picograms [53] to nanograms, therefore an effective method for detecting low amounts of samples. The purpose of this method is to identify compound(s) based on their molecular weight or atomic mass. The principle is firstly creating ions selectively by a chosen ion source from the eluate introduced by the inlet sample method. Further, ions created are mass analyzed and detected separately according to their mass-to-charge (m/z) ratio. The data system generates signals from the ionic m/z ratio detected and displays them in a mass spectrum for further data analysis [51]. By coupling MS in series commonly phrased as tandem mass spectrometry (MS/MS) additional sample separation, purification and derivative formation can be accomplished. Liquid chromatography electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) is a rapid analytical methodology for analyzing small and large molecules with various polarities and provides high sample throughput [52].

Electrospray ionization-mass spectrometry (ESI-MS) is commonly used in biochemical and chemical analysis for liquid samples and is therefore compatible with chromatographic separation techniques [54]. The electrospray ionization (ESI) process as illustrated in Figure 6, involves ionization of analyte(s) from an LC eluate to acquire a positive or negative charge. Compounds that have neutral characteristics can gain a charge by either cationization or protonation in either liquid or gaseous phase. Transfer of ionizable liquid into gas phase firstly involves a dispersion of the liquid. The beam from the tip of the electrospray will contain charged droplets generated by a capillary voltage. These charged droplets will then gain a polarity equal to the capillary voltage chosen. By the addition of a neutralizing gas such as nitrogen, the flow rate will be increased for the eluate. Charged droplets will then pass down their potential and pressure gradient from the electrospray tip towards the mass analyzer. By additional nitrogen gas and/or temperature, the liquid stream will be broken up and neutralized more efficiently [52]. When electrical charge on droplet surface has reached a critical limit known as rayleigh stability limit, smaller droplets will be created. Due to electrical repulson this will lead to ion emission that will be ejected onto the mass analyzer [49].
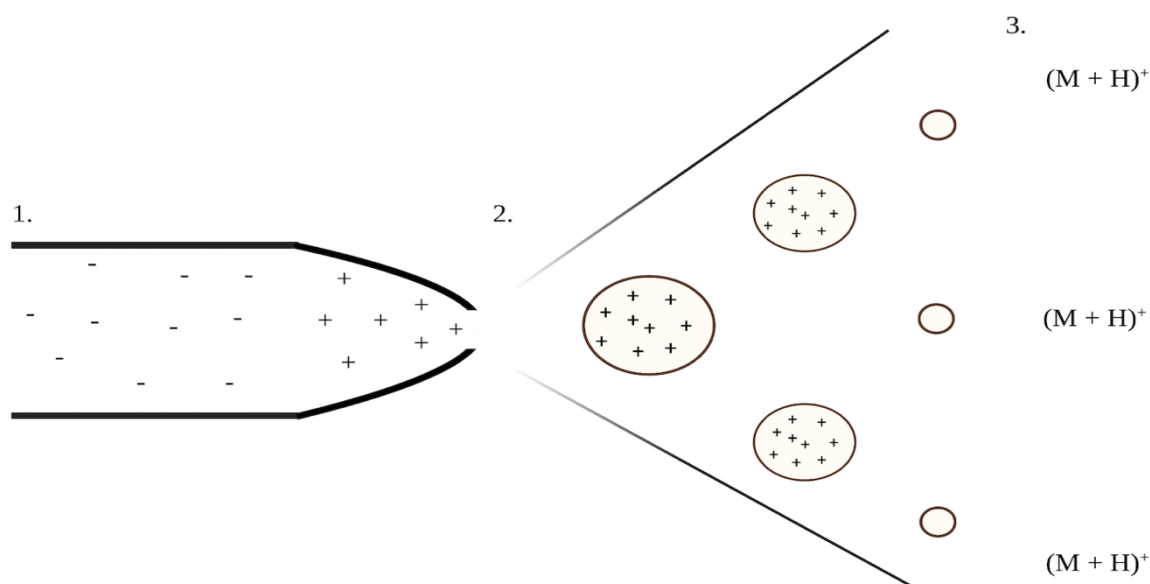
Figure 6: Electrospray ionization (ESI) process.

Charged ions are generated by a capillary voltage to a sample eluate (1). The beam from the electrospray tip will contain ESI selected (ESI+ or ESI-) charged droplets that will eventually be broken up to smaller droplets by adding gas and/or temperature (2). Electrical repulsion cause ion emission where remaining protonated ions ($[M + H]^+$) will be ejected onto the mass analyzer for further analysis (3). Illustration is created by the author using [52] for inspiration and Biorender.com.

ESI takes place at atmospheric pressure, therefore it is required to have an interfase to the high vacuum containing the mass analyzer. The interface can either contain series of cones or long capillary. In both cases, ions are transmitted to the high vacuum without breaking the vacuum [55]. One specific known ESI source design is the Z-spray with double orthogonal geometry design of the spray. The spray position enhance the sensitivity by separating the neutral compounds from highly charged droplets [56]. In Z-spray ESI, a beam of ions with Z-shape trajectories is created and directed towards the first mass analyzer. Ions will firstly pass through series of cones then onto travelling-wave ion guide (TWIG) operating under radiofrequency (RF) conditions, before passing onto the first mass analyzer [55] as illustrated in figure 7.
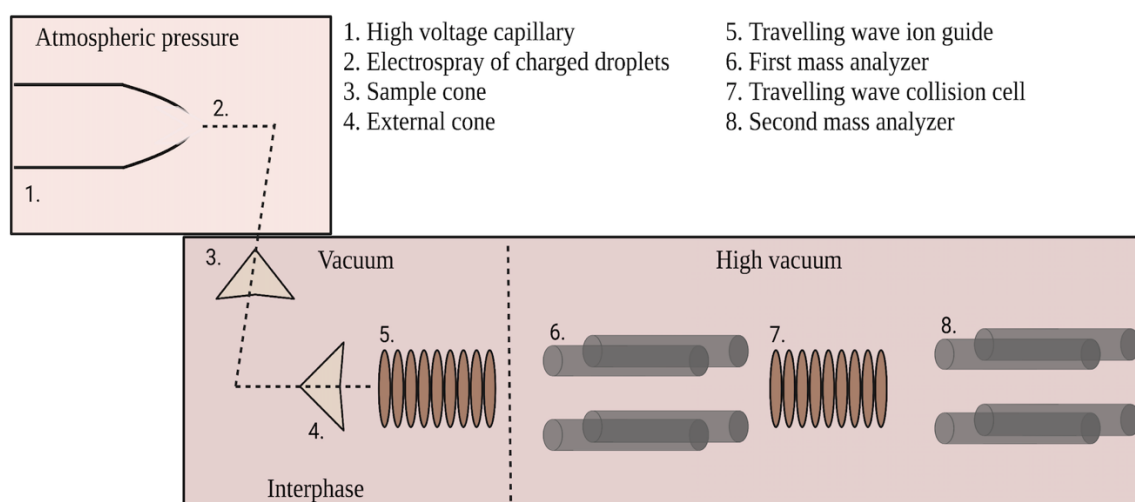
Figure 7: Z-spray with electrospray ionization (ESI) probe.

Z-shaped trajectories of charged droplets moving from atmospheric pressure into interphase before entering high vacuum towards the first mass analyzer. Simplified illustration created by the author used [55, 56] for inspiration and Biorender.com.

1.2.5.2 Quadrupole mass-analyzer for ionic filtration

Quadrupole mass analyzer act as a mass filter by allowing ions with specific m/z ratio to pass through. This device consists of four parallel rods aligned around a central axis as illustrated in Figure 8 [57]. Ion filtration can be accomplished by combining the direct current (DC) and RF voltages [58]. Creating a positive quadrupole field makes the beam consisting of positively charged ions accelerate towards negative rods and opposite for negatively charged ions [57]. However, if the polarity of the quadrupole field changes quickly the ions will be brought back to the center avoiding ion scattering. Consequently, ion trajectories are dependent on the m/z ratio determined by the combination of DC and RF voltages. Thus, combination of DC and RF voltages can selectively analyze ions on the basis of their m/z ratio [58].

1.2.5.3 Tandem mass spectrometry

MS/MS is a selective detection method for targeted compounds from complex matrices [53]. This analytical method contain mass analyzers and a collision cell. The mass analyzers can be coupled in series either in time (trapping instruments) or space (triple quadrupole, hybrid and sector instruments) [59]. Series of mass-analyzers allows for the selection of a particular ion to be further fragmented for specificity improvements. Mass analyzers does commonly work under both RF and DC voltages that is crucial for the mass selection while collision cell only works under RF conditions. Fragmentation is performed by adding a collision gas [58] such as nitrogen or argon [48]. The principle for using MS/MS (Figure 8) is firstly selecting ions based on their m/z ratio by the first mass analyzer (MS1) as well as filtering out ions with undesirable m/z ratios. The selected precursor ions are then colliding with a collision gas for further fragmentation in the collision cell to create secondary ions (product ions), a process known as collision-induced dissociation (CID). The product ions are then transmitted to the second mass analyzer (MS2) whereas ions passing through will be detected [58]. The commonly used data acquisition for MS/MS is selected reaction monitoring (SRM) or MRM [51]. The MRM technique enables to detect specific ions in MS/MS from complex matrices. The mass filtration from MS1 and MS2 clear away interfering compounds thus, creates high sensitivity and specificity for the detection. With MRM it is possible to detect numerous transition channels of various ions simultaneously [60]. For qualitative and quantitative analysis full scan mode is preferable for measuring picomolar (pM) to nanomolar (nM) samples while MRM measures femtomolar (fM) to nM. MRM transition channels and retention time gathered from one specific ion creates peaks that can be used for compound identification [61]. Each peak will then be presented in a MRM chromatogram that displays counts from a selected m/z value versus retention time [62]. Ideally, a chromatographic display will show no insufficient resolution causing peak overlap, diffusion causing peak broadening, column bleeding causing baseline drift [51] and peak tailing (Figure 9). Peak tailing can be caused by several factors, such as undesirable binding effects between analyte and stationary phase [63]. In RPLC, retention effect causing peak tailing can be due to hydrogen-bonding or van der Waals interactions between analyte and unreacted residual silanol groups [64].
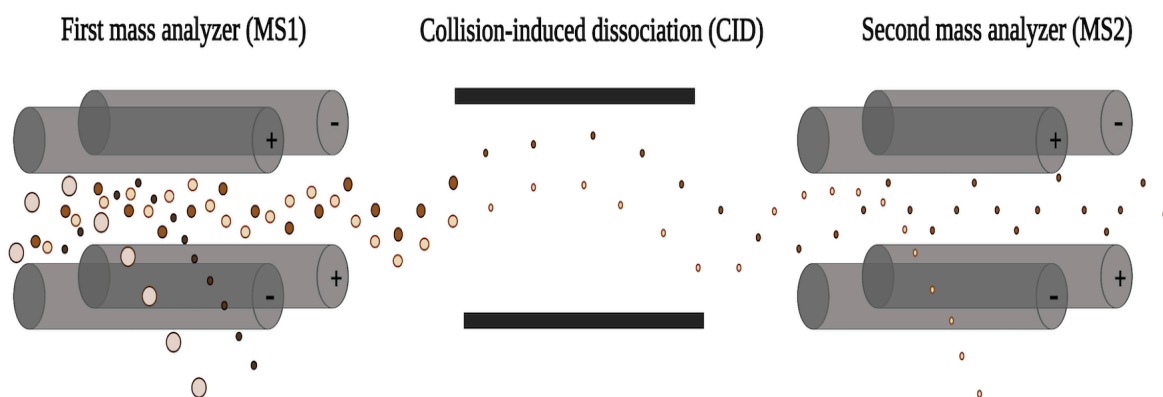
Figure 8: Tandem mass spectrometry (MS/MS).

Precursor ions will be selected based on their mass-to-charge (m/z) ratio in the first mass analyzer (MS1) and fragmented to product ions by collision-induced dissociation (CID). Selected product ions will then pass through the second mass analyzer (MS2) and onto the detector. Illustration is created by the author using Biorender.com.
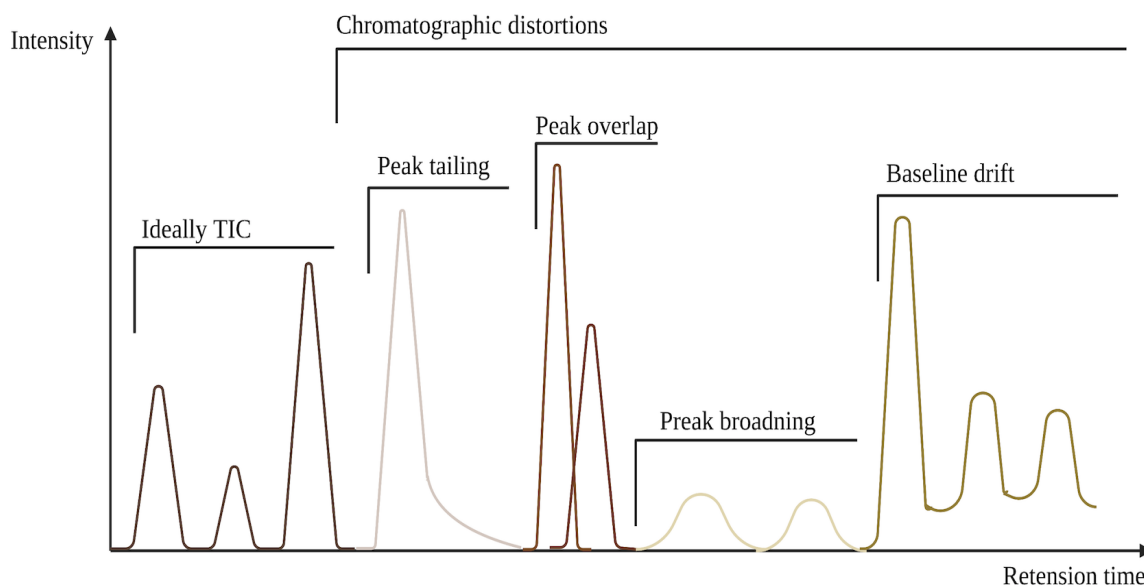
Figure 9: Ideal chromatogram and common chromatographic distortions.

Ideally a chromatographic display will show no insufficient peak overlap, peak broadening, peak tailing and baseline drift. Illustration is created by the author using [51] for inspiration and Biorender.com.

1.2.6 Quantitation and quantification by isotope dilution

To attain a correct quantitative analysis, instrumental calibration response versus concentration of analyte is needed. This can be achieved by using an internal standardization (IS) approach [51] as a calibration technique. This technique is able to compensate for the variations in loss that takes place in sample preparation, retention time, recovery, ionization efficiency and matrix effect [48]. The ISTD is a compound added at a known concentration level with similar retention time, ionization efficiency [51] and structure [48] of the targeted analyte. In MS, isotopic labeled analogs serve as ISTD, preferably ^{2}H (deuterium) ^{18}O and ^{13}C isotopes that will act as analogs of the targeted analyte. By adding the known concentration level of either of these isotope-labelled standards with different mass compared to the non-labelled compounds, IS termed isotope dilution can be achieved. The mass difference also creates avoidance of any chromatographic overlap between ISTD and analyte. The area ratio corresponding to intensity peaks of target analyte and internal standard (analyte:ISTD) delivered by MRM is the relative concentration of internal standard and analyte [51]. This will be determined by using the linear calibration curve whereas the peak area response of the analyte:ISTD on the y-axis corresponds to the concentration on the x-axis. The IS approach is a preferable method due to its compensating variations effects both during sample preparation and during the analysis. This way concentration in the original sample can be calculated based on the results from the prepared analysis sample [48]. The method is also preferable in detecting low levels of analyte in samples and is added during the sample preparation before the sample clean-up processing [51].

1.3 Method validation

1.3.1 Precision

Precision is evaluated through repeatability, intermediate precision and reproducibility of a method [65]. Precision represents the closeness of results to each other [66] and is presented as standard deviation (s), percentage coefficient of variation (CV%) or relative standard deviation (RSD) by using Equation 4,5 and 6 [67]. Normally 6 – 15 replicates should be used that should be representative in terms of analyte concentration, matrix, stability and independency [65].

$$s = \sqrt{\frac{\sum_{i=1}^{N} (x_i - \bar{x})^2}{N - 1}} \tag{4}$$

$$RSD = \frac{s}{\bar{x}} \tag{5}$$

$$CV\% = \frac{s}{\bar{x}} \; x \; 100 \tag{6}$$

1.3.2 Accuracy

Accuracy measures the closeness of a result to a reference value. Accuracy is typically determined by calculating the bias referred to in absolute terms such as absolute bias (b) or percentage of relatives such as relative bias (b%) and relative spike recovery (R´%). The b value is determined by the mean ($\bar{x}$) from series of replicates from a candidate method compared to a true value (x_{ref}), whereas b% is determined by the ratio between b and x_{ref} as shown in Equation 7 and 8. Calculating the R´% value is done through a recovery experiment using the mean value from non-spiked ($\bar{x}$) with several spiked replicates ($\bar{x}´$) and their true value (x_{spike}) as shown in Equation 9 [65].

$$b = \bar{x} - x_{ref} \tag{7}$$

$$b\% = \frac{\bar{x} - x_{ref}}{x_{ref}} \; x \; 100 \tag{8}$$

22

$$R'\% = \frac{\overline{x'} - \overline{x}}{x_{spike}} \; x \; 100 \tag{9}$$

1.3.3 Selectivity and sensitivity

Selectivity is a measurement used to determine how accurate the method is in distinguishing analyte(s) of interest from other interfering compounds within the matrix [68]. In other words, selectivity tells whether the method is free from interfering compounds within the sample matrix [67]. Interfering compounds may affect the calibration function in terms of proportional effects and/or translational effects. Proportional effect will change the slope of the calibration curve and not the intercept while translational effect will change the intercept and not the slope. However, standard additives will be able to adjust the proportional effects [65].

Sensitivity refers to a method´s capability to distinguish between small differences in analyte concentration. The slope in the calibration curve and precision of the measuring device will affect the sensitivity. When precision for each method is equal the sharpest calibration curve achieved will have the highest sensitivity. However, when slope for each calibration curve is the same, the method containing best precision will gain the highest sensitivity [67].

1.3.4 Limit of detection and limit of quantification

The lowest amount of analyte that can be detected by a given measurement procedure and at a known confidence interval is referred to as LOD [67]. The LOD value is gathered from samples containing no analytes such as blank samples. The results will be declared as positive if the value exceeds this critical value. LOD will be calculated by the sum of $\bar{x}$ and s values multiplied by a determined factor (k = 3) from blank measurements (Equation 10) [65]. The confidence level for k = 3 will then be 95% for the detection [67].

$$LOD = \bar{x} + (3\,x\,s)\qquad\qquad(10)$$

The lowest concentration that can be quantified is referred to as LOQ [68]. The LOQ estimation will also be determined by multiplying a selected factor (k_Q = 10) with s and $\bar{x}$ values gathered from blank sample replicates as shown in Equation 11 [65].

$$LOQ = \bar{x} + (10\,x\,s)\qquad\qquad(11)$$

1.3.5 Linear range and working range

Linear range is defined by the LOQ to the concentration value where calibration curve departs from its limit of linearity (LOL) [67]. Working range includes results that have an adequate uncertainty that extends from LOQ to concentration where significant deviation in sensitivity is noticeable as illustrated in Figure 10. There are two types of working ranges known as instrument working range and method working range. The instrument working range is determined by plotting concentration of calibration standards against instrumental signal response. The method working range can be presented as known sample concentration plotted against measured concentration. Between the lower and upper limit of the working range there will be a relationship present such as linear or curvilinear etc. This relation is important to decide whether or not the calibration process is satisfactory and that concentrations chosen for standard solutions expand over a certain range [65].
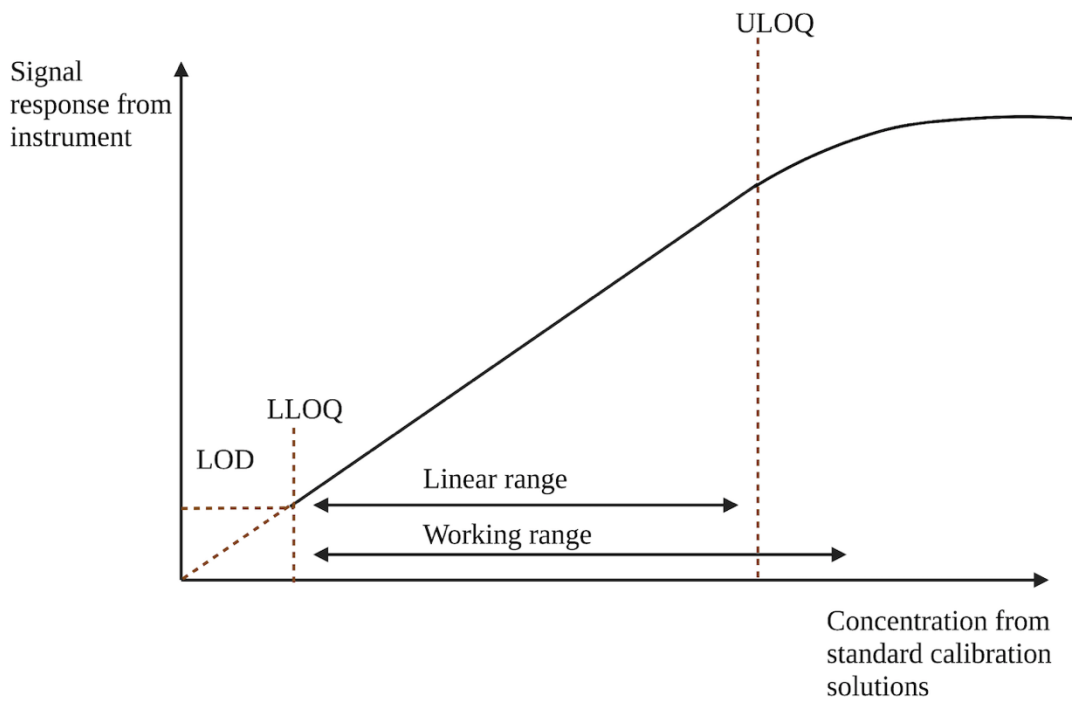
Figure 10: Desirable working- and linear range from instrumental procedure.

The curve is obtained by using expanding concentration values of calibrations standards plotted against the instrumental signal response. The working range will be decided from lower limit of quantification (LLOQ) to where calibration curve departs from its limit of linearity (LOL). Linear range will be at the range between LLOQ and upper limit of quantification (ULOQ). Illustration created by the author using [66] for inspiration and Biorender.com.

1.3.6 Matrix effect

LC-MS/MS is a highly sensitive and selective analysis approach however, it can be susceptible to a matrix effect known as ion suppression [69] or enhancement. However, ion suppression is more common than ion enhancement in LC coupled to MS [70]. Ion suppression affects the analysis precision, accuracy and detection capabilities regardless of the high sensitivity and selectivity [69]. This leads to lower signal-to-noise ratio (S/N), higher LOD, smaller linear range, linearity and false negative and false positive results. Ion suppression occurs during an early stage of ionization process in the chosen ion source. Suppression of the response might be due to competing co-eluents for available charge or surface area of the droplet in the interface of the MS detector. Other factors may involve mobile phase additives, equipment design, co-precipitation between analyte and matrix and neutralization of gas phase ions through deprotonation by acid- and base reactions [49].

Compounds that have ion suppression capabilities can either be endogenous organic or inorganic compounds in the matrices or exogenous compounds from contaminations during sample preparation. Endogenous compounds includes lipids, carbohydrates, peptides, amines, urea, ionic variants, highly polar compounds and metabolites similar to analytes of interest. Exogenous compounds includes polymer and plastic debris, calibration products, proton exchange agents, buffers, ion pairing reagent and detergent degradation [49]. This can also be due to compounds that have the same retention time as the analyte of interest or factors such as mass, basicity and high concentrations [69].

Several chromatographic, sample preparations and calibration techniques prior to analysis have been suggested to reduce and compensate for ion suppression occurrence. Improvement of the chromatographic conditions need to create elution of analyte(s) where ion suppression is less apparent, meaning between solvent front and end of the gradient elution [69]. Improving separation and retainment of analyte(s) can be done through changing the column, particle size, temperature, flow rate, mobile phases, length and diameter of the column [49]. The most common sample preparation techniques utilized in LC are LLE and SPE for sample purification and better extraction efficiency obtained. Calibration methods involving external calibration, standard additives or ISTD have been introduced to compensate for the matrix effect, when matrix effect cannot be eliminated. However, the most frequently used technique involves ISTD approach [69].

1.4 Aim of study

The aim of this MSc assignment was to develop and validate a high-quality LC-MS/MS method to quantify cortisol in fish feces and use this as a tool to monitor fish welfare.

The purpose of the first part of the assignment was to develop a procedure to extract, modify, separate, detect and quantify cortisol in feces samples. This part included to find suitable conditions for enzyme hydrolysis, LLE, derivatization, liquid chromatography (LC) and mass spectrometry (MS).

The objective for the last part of the assignment was to validate the method and assess if the method was sensitive enough to detect differences of cortisol in feces samples from off- and onshore industrial fish farms. This part of the assignment included validation for repeatability, intermediate precision, recovery, linearity, limit range, limit of detection (LOD) and quantification (LOQ) according to the Eurachem guidelines [65].

2. Methods and materials

2.1 Materials

Analytes of cortisol and cortisone were purchased from Alfa Aesar (Ward Hill, MA, USA), testosterone from Sigma-Aldrich (St. Louis, MO, USA) while tetrahydrocortisol and tetrahydrocortisone were purchased from TRC (Toronto, CA). All organic solvents used for LC-MS/MS analysis were either analytical or LC-MS grade. Methanol (MeOH) used for chromatography and preparation of derivatization reagent-, stock- and stockmix solutions were LC-MS grade and purchased from VWR (Rue Carnot Fontenay Sous Bois, Fr). All solvents used in LLE were analytical grade involving tert-butyl methyl ether (MTBE), n-butanol and propanol except for LC-MS grade ethyl acetate and acetonitrile. All solvents used in LLE were purchased from VWR (Rue Carnot Fontenay Sous Bois, Fr). Mobile phase modifiers included analytical grade 99-100% formic acid purchased from VWR (Rue Carnot Fontenay Sous Bois, Fr) and analytical grade 25% ammonium hydroxide from Merck (Burlington, MA, USA). Preparation of acidic solutions for hydrolysis purposes was done by using 100% acetic acid that was purchased from Merck (Burlington, MA, USA). Isotopically labelled deuterium analogs of d4-cortisol, d7-cortisone and d3-testosterone were purchased from Sigma-Aldrich (St. Louis, MO, USA). For enzymatic hydrolysis β-glucuronidase and sulfatase activity from Helix Pomatia and Patella Vulgata were purchased from Sigma-Aldrich (St. Louis, MO, USA) while β-glucuronidase and sulfatase from Abalonase®+ and β-glucuronidase from Abalonase® was purchased from Ango (San Ramon, CA, USA). Derivatization processing by 4-ABH reagent was purchased from TCI (Toshima, TYO). Preparation of ASTM type I purified water (18.2 MOhm) was done by using a Milli-Q purification system (Millipore, Brussels, Belgium) and used in all experiments. All samples were centrifuged in Eppendorf centrifuge (Eppendorf, Hamburg, Germany) or Scanspeed micro centrifuge (Labogene, Lillerød, Danmark) at 2300 G and evaporated in Eppendorf concentrator plus^{TM} (Eppendorf, Hamburg, Germany) with vacuum high vapor (V-HV) at 60 °C. Overview of materials and manufactures are listed in Table 9, 10 and 11.

For data interpretation it has to be mentioned that because of interpretation difficulties and the need of further optimization to investigate cortisone, testosterone, tetrahydrocortisone and tetrahydrocortisol these data were not presented in this study. Therefore, qualification and quantification were only investigated and presented for cortisol. Data processing were performed by TargetLynx[TM] V 4.1 and MassLynx[TM] V 4.1 software
(Waters, Milford, MA, USA).

2.1.1 Physiochemical properties for materials

Physiochemical properties gathered from manufactures for organic solvents are listed in Table 1. This shows the organic solvents used in preparation of stock-, derivatization reagent- and stockmix solution, LLE, mobile phases and LC-MS/MS analysis.

Table 1: Physiochemical properties for organic solvents gathered from manufactures.

Solvent name	Boiling point (C°)	Density (g/cm³)	Solubility in water
Tert-butyl methyl ether	55.2	0.7405 at 20 C°	*Immiscible
Acetonitrile	81.60	0.782 at 20 C°	Miscible
Ethyl Acetate	77.1	0.902 at 20 C°	*Immiscible
n-Butanol	117-118	0.8098 at 20 C°	*Immiscible
Propanol	97	0.8053 at 20 C°	Miscible
Methanol	64.5	0.792	Miscible
25% Ammonium hydroxide	37.7	0.903	Miscible
99-100% Formic acid	100-101	1.22 at 25 C°	Miscible
100% Acetic acid	117.9	2.07	Miscible

* Generally regarded as immiscible with water

2.2 Preparations of stock solutions and derivatization reagent

A 25 µl stock solution was prepared for cortisol, cortisone, testosterone, tetrahydrocortisol and tetrahydrocortisone. Each solution contained 30.21 mg cortisol, 29.41 mg cortisone, 27.24 mg testosterone, 1.92 mg tetrahydrocortisol and 1.62 mg tetrahydrocortisone with MeOH dilution. Stock solutions were stored at 4 °C with concentration set to 48046 ng/mL cortisol, 46068 ng/mL cortisone, 43366 ng/mL testosterone, 76800 ng/mL tetrahydrocortisol and 67600 ng/mL tetrahydrocortisone.

A 25 µl stock solution was also prepared for internal standard of d4-cortisol, d7-cortisone and d3-testosterone. Each solution contained 0.84 mg d4-cortisol, 1.09 mg d7-cortisone and 0.9 d3-testosterone with MeOH dilution. Stock solutions were stored at 4 °C with concentration set to 33600 ng/mL d4-cortisol-d3, 43600 ng/mL d7-cortisone and 36000 ng/mL d3-testosterone.

A 250 mL derivatization reagent solution were made by adding 2578.2 mg 4-ABH with MeOH dilution and 1 mL HCl. The final concentration of the solution was set to 10.3128 mg/mL with 2 M HCl.

2.3 Preparation of quality control, stockmix and working standard solutions

Several quality control (QC) samples containing 500 µl aliquot from a homogenized sample processed as mentioned in section 2.5.1 was stored at -20 °C until use. Preparation of analyte stockmix was done by diluting 1 mL stock solution of cortisol, cortisone, testosterone and 2 mL stock solution of tetrahydrocortisol and tetrahydrocortisone in methanol to total volume of 25 mL and stored at 4 °C. Stockmix containing 480.5 ng/mL cortisol, 460.7 ng/mL cortisone, 433.7 ng/mL testosterone, 1536 ng/mL tetrahydrocortisol and 1352 ng/mL tetrahydrocortisone were then diluted to make series of working standard solutions as follows. Five replicates of eight working standard solutions were prepared by diluting 0, 0.02, 0.05, 0.1, 0.15, 0.2, 1 and 2 µl of stockmix with water to total volume of 25 mL. The diluted series of working standard solutions were further used to create calibration standard solutions for quantification purposes. Final concentration of each compound for eight calibration standard solutions are listed in Table 2. On the day of analysis one QC sample, two blank (water) samples and eight calibration standard solutions were prepared.

Table 2: Calibration standard solutions.

Concentration (ng/mL) values for eight calibration standard solutions containing cortisol, cortisone, testosterone, tetrahydrocortisol and tetrahydrocortisone.

Calibration standard	Cortisol	Cortisone	Testosterone	Tetrahydro-cortisol	Tetrahydro-cortisone
1	0.000	0.000	0.000	0.000	0.000
2	0.961	0.921	0.867	3.072	2.704
3	2.402	2.303	2.168	7.680	6.760
4	7.207	6.910	6.505	23.040	20.280
5	9.609	9.214	8.673	30.720	27.040
6	24.023	23.034	21.683	76.800	67.600
7	48.046	46.068	43.366	153.600	135.200
8	96.092	92.136	86.732	307.200	270.400

A 250 mL stockmix for internal standards were made by adding 0.5 mL from each stock solution of d4-cortisol, d7-cortisone and d3-testosterone with MeOH dilution. Stockmix concentration contained 67.2 ng/mL d4-cortisol, 87.2 ng/mL d7-cortisone and 72 ng/mL d3-testosterone and stored at 4 °C.

2.4 Preparation of acidic, basic and buffer solutions

Preparation of 25% ammonia (NH_3) and sulfuric acid (H_2SO_4) solutions at 2, 4, 6, 8 and 10% were made for hydrolysis purposes. Starting with water then transferring H_2SO_4 and NH_3 at different volumes from 0.9, 1.8, 2.7, 3.6 and 4.5 mL with a total volume of 44 mL. Additionally, acetic buffer solutions at pH 5 and 6 containing 2 M ammonium acetate were created to optimize pH condition for enzymatic activity. A 2 M ammonium acetate buffer to pH 6 were made by firstly adding 30 mL water before transferring 5 mL of 100% acetic acid and 25% ammonia. Additionally, 2 M ammonia acetate buffer to pH 5 were made using 5 mL 100% acetic acid and 3 mL 25% ammonia filled up with water.

2.5 Method development

2.5.1 Collection of fish feces samples and ethics

In this study the methodology startup is based on previous discoveries of cortisol and its metabolites in saliva reported by Nadarajah[24] et al. The fish feces samples from presumed stressed and non-stressed subjects were collected into individual 2 mL eppendorf vials (Thermofisher, MA, USA) and pooled samples from anonymous commercial marine off- and onshore industrial fish farms. This procedure was in accordance with the Animal Welfare Act [8]. All individual samples were stored at -20 °C while pooled samples were thawed and prepared as follows. Firstly, 2.5 g fish feces was measured into two 50 mL polypropylene (PP) tubes (Thomas Scientific, NJ, USA) mixed with 7 µl water and placed in 5 minutes centrifugation. Top layers created were transferred into one glass cup for homogenization where 500 µl were collected in 2 mL eppendorf vials. These samples were further utilized in method development processing for enzymatic hydrolysis, LLE and derivatization optimization and method validation.

2.5.2 Finding the optimum ionization conditions for electrospray ionisation

Derivatized and underivatized samples were made in order to investigate whether or not the signal response could be maximized by derivatization. Firstly, two glass vials were added 100 μl internal standard and 100 μl stockmix. Thereafter, added 200 μl methanol in one glass vial while the other contained 200 μl 4-ABH solution. After 1 h reaction, 700 μl water was transferred to both vials before direct injection into LC-MS/MS following the instructions from section 2.5.8 and 2.5.9.

2.5.3 Finding the most efficient hydrolysis treatment for cortisol deconjugation

Homogenization samples were treated with different enzymes for deconjugation of bound cortisol. Firstly, triplets of 20 µl β-glucuronidase/sulfatase (≥ 100000, ≤ 7500 units/mL from Helix Pomatia), 20 µl β-glucuronidase (> 50000 units/mL from Abalonase®), 20 µl β-glucuronidase/sulfatase (> 50000, > 400 units/mL from Abalonase® +) and 20 µl β-glucuronidase ($85000 \geq, 7500$ units/mL from Patella Vulgata) were prepared. All samples were added 50 µl 2 M ammonium acetate buffer of pH 6 before 1 h incubation at 60 °C. In addition to enzymatic treated samples, acidic and basic hydrolysis was also carried out in this experiment. Triplets of 20 µl acidic solution (H_2SO_4) and basic solutions (NH_4) at 2, 4, 6, 8 and 10% were made. Additionally, 500 µl of one QC, two blank samples and eight calibration standard solutions were used. All samples were then treated with 50 µl stockmix internal standard solution and LLE that included mixture of 700 µl MTBE and 100 µl 5 M NaCl. After 5 minutes centrifugation, 400 µl of supernatant was collected from each vial and transferred into new vials for 20 minutes evaporation in vacuum centrifuge. Fully dried samples were derivatized with 20 μl 4-ABH reagent for 30 minutes before adding 40 μl water. Finally, the total amount of 60 μl was transferred into new 0.3 mL micro vial (VWR, Rue Carnot Fontenay Sous Bois, Fr) adequate for LC-MS/MS analysis (section 2.5.8 and 2.5.9).

2.5.4 Finding the most suitable pH buffer solution for optimum enzyme activity conditions

Homogenized samples were added hydrolysis treatment with 20 µl Helix Pomatia and Patella Vulgata. Both enzymatic treated samples contained triplets with either 50 µl 2 M acetic ammonium buffer solution at pH 5 or 6 to compare optimal pH condition for enzymatic activity. All samples were then processed further as explained for enzymatic solutions with the addition of eight calibration standard solution, one QC and two blank (water) samples that were prepared as mentioned in section 2.5.3.

2.5.5 Finding the most optimum incubation time for enzymatic hydrolysis

Homogenized samples were treated with 20 µl Helix Pomatia and 50 µl 2 M ammonium acetate buffer solution of pH 6. Triplets of enzymatic samples were then made for each incubation time set to 1, 2, 3 and 24 h at 60 °C. All samples were further processed as explained for enzymatic solutions with the addition of eight calibration standard solution, one QC and two blank (water) samples as mentioned in section 2.5.3.

2.5.6 Finding the most sufficient amount of enzyme for enzymatic hydrolysis

Homogenized samples containing triplets of 10, 20, 30, 40 and 50 µl Helix Pomatia were made with 50 µl 2 M ammonium acetate buffer solution of pH 6 before 1 h incubation at 60 °C. All samples were further processed as explained for enzymatic solutions with the addition of eight calibration standard solution, one QC and two blank (water) samples as mentioned in section 2.5.3.

2.5.7 Finding the most efficient extraction method

Homogenized samples were treated with 20 µl Helix Pomatia and 50 µl 2 M ammonium acetate buffer solution of pH 6 before 1 h incubation at 60 °C. After incubation, all samples were treated with LLE that included triplets containing 700 µl water-immiscible organic solvents (MTBE, etylacetat and butanol) with 100 and 600 µl 5 M NaCl. Further, SALLE experiments were also included to compare the extraction efficiency for both methods. For SALLE experiments triplets containing 700 µl water-miscible organic solvents (acetonitrile and propanol) were used with the addition of 600 µl 5 M NaCl. All samples were then processed with 5 minutes centrifugation before transferring 400 µl top layer into new vials with 50 µl internal standard. All samples were then treated with evaporation and derivatization before LC-MS/MS analysis as mentioned in section 2.5.3.

2.5.8 Finding appropriate liquid chromatography conditions

The LC analysis was carried out for steroid hormone separation by using Acquity UPLC (Waters, Milford, MA, USA) with RPLC conditions. The UPLC analysis were equipped with a weak (water) and strong (methanol) wash station. The needle collecting the sample was washed before each run once with methanol (strong wash) and three times with water (weak wash) for re-equilibration. The temperature of the autosampler tray were set to 10 °C and column to 50 °C, respectively. The injection volume acquired for total runtime of 6 minutes was set at 20 μl with 0.25 mL/minutes flow rate. The internal standard d4-cortisol, d7-cortisone, d3-testosterone and analytes of interest were analyzed during each run. The RPLC separation mode was performed under gradient conditions using a BEH C18 column (100 mm x 2.1 mm, 1.7 µm) (Waters, Milford, MA, USA). The elution conditions contained two solvents (A, B) as mobile phase consisting of water with 0.2% ammonium hydroxide (A) and water with 0.2% methanol (B). Cortisol was eluted under linear gradient conditions using the inlet sample method for total run of 6 minutes that required 5 steps (Table 3).

Table 3: The liquid chromatography (LC) inlet sample method.

Method was used to analyze derivatized and underivatized cortisol and internal standards chosen for qualification and quantification purposes. Elution was performed in 5 steps using gradient conditions with 0.2% ammonium hydroxide (A) and water with 0.2% methanol (B). The injection volume acquired for total runtime of 6 minutes was set to 20 µl with 0.25 mL/min flow rate.

Step	Time (min)	Flow (mL/min)	A%	B%
1	0-3.5	0.25	99	1
2	3.5-4.0	0.25	30	70
3	4.0-4.5	0.25	5	95
4	4.5-4.6	0.25	5	95
5	4.6-6.0	0.25	99	1

2.5.9 Finding appropriate mass spectrometry conditions

The initial step for quantitation by MS was tuning processing for cortisol, d4-cortisol and derivatized cortisol (cortisol-4-ABH) and d4-cortsiol (d4-cortisol-4-ABH). Starting with making tuning solutions for each compound. The cortisol tuning solution contained 10 mL cortisol stock solution with 990 mL MeOH that contained final concentration of 12010 ng/mL. For d4-cortisol tuning solution added 10 mL d4-cortisol stock solution and 990 mL MeOH that contained final concentration of 12010 ng/mL. Cortisol-4-ABH tuning solution contained a mixture of 50 mL cortisol stock solution, 100 mL 4-ABH solution and 850 mL MeOH with final concentration of 16050 ng/mL. Tuning solution of d4-cortisol-4-ABH contained 100 mL d4-cortisol stock solution, 500 mL 4-ABH solution and 400 mL MeOH with final concentration of 3360 ng/mL. All tuning solutions were injected directly into the LC-MS/MS machine. The Quattro premier XE mass spectrometer (Waters, Milford, MA, USA) was coupled to the Acquity UPLC. All samples were injected 2 μl/min and diluted by the combination of 200 μl/min mobile phase. For underivatized compounds used 0.2% formic acid in water and methanol (50:50) while derivatized compounds used 0.2% ammonium hydroxide in water and methanol (50:50). The MS/MS instrument monitored each compound using electrospray ionization operating in positive mode (ESI+) using MRM. The MRM transition method used argon as the collision gas. The ion spray voltage and entrance potential were set to 1 unit with 50 V at 350 °C using 15 units resolution for first quadrupole (Q1) and second quadrupole (Q2). Nitrogen gas were used as desolvation and nebulization gas. Derivatization products and mass transitions for cortisol, d4-cortisol, cortisol-4-ABH and d4-cortisol-4-ABH were measured in each chromatographic run. The parameters dwell, cone and collision energies for MS detection were optimized for each analyte to obtain highest sensitivity (Table 4). A dwell time of 0.015 was used for all mass transitions monitored.

Table 4: Optimized tandem mass spectrometry (MS/MS) parameters and multiple reaction monitoring (MRM) transition channels for cortisol, d4-cortisol, cortisol-4-ABH and d4-cortisol-4-ABH detection.

Compounds	Cortisol*	d4-cortisol*	Cortisol-4-ABH*	d4-cortisol-4-ABH*
Molecular formula	C21H30O5	C21D4H26O5	C28H37N3O5	C28H33D4N3O5
Isotopic molecular weight	362.21	366.23	495.27	499.30
Derivatization products	-	-	Mono-hydrazone	Mono-hydrazone
Precursor ion (m/z)	363.2	367.2	496.25	500.25
Product ion (m/z)	121.1	121.1	119.8	119.8
Dwell (s)	0.015	0.015	0.015	0.015
Cone (V)	33	33	50	50
Collision (V)	23	23	35	35
Retention time	4.53	4.53	4.24	4.24

Deuterium analogues: d3, d4, d8 and derivatisation reagent: 4-aminobenzoic hydrazide (4-ABH)

- not specified

* Multiple reaction monitoring (MRM) transitions used in terms of qualification

2.5.10 The developed method conditions used on field samples

The procedure for the clinical investigation were based on the approved method developed in this project (Figure 11). Determination of stress physiologies was done by measuring total cortisol concentration levels in 96 individual feces samples. Firstly, samples were thawed and measured (Table 12) with 1 mL water in 2 mL eppendorf vials. Each sample were vortex-mixed for 10 – 12 seconds and centrifuged for 5 minutes. After obtaining phase separation, 500 μl of supernatant from each sample were transferred into new 2 mL eppendorf vials. Each vial was added a mixture of 20 µl Abalonase® and 50 μl 2 M ammonium acetate buffer of pH 5 and incubated for 1 h. Meanwhile, eight calibration standard solution, one QC and two blank (water) samples were prepared. All samples were then treated with internal standard, LLE, centrifugation, 20 minutes evaporation and derivatization as mentioned in section 2.5.3. Quantification was determined by calibration curve obtained from calibration standard solutions using equation y = ax + b gathered from linear regression analysis.
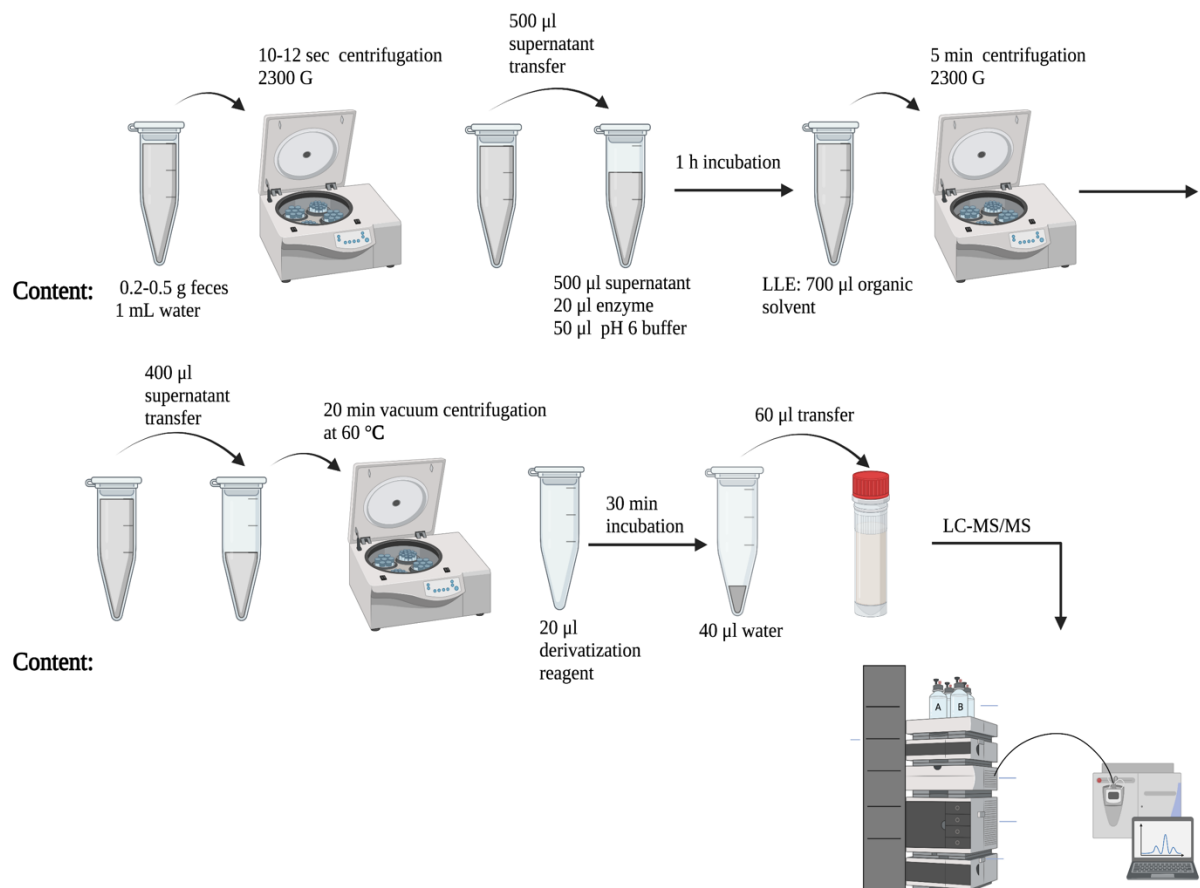
Figure 11: The developed method for cortisol quantification.

The developed method for cortisol quantification was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The illustration is created by the author using Biorender.com.

The final method preparation was done automatically by a pipetting robot using 2 mL 96 PP deep well-plates (Thermo Fisher Scientific, Waltham, MA, USA) . This automation transferred 500 μl supernatant from feces sample, eight calibration standard solutions, one QC and two blank (water) samples. Thereafter, 20 μl Helix Pomatia with addition of 50 μl 2 M ammonium acetate buffer of pH 6 was added to feces samples. Plates were then capped with elastomeric cap to allow 1 h reaction before automatically transferring 50 μl internal standard, 700 μl MTBE and 100 μl 5M NaCl to all samples. Capped plates were centrifuged for 5 minutes before pipetting 400 μl top layer by the robot into new 2 mL 96 PP deep well-plates that was evaporated for 20 minutes by warm air stream. Fully dried extracts were added 20 μl 4-ABH and capped for 30 minutes to allow derivatization reaction occur. Finally, 40 μl water was added and the final volume of 60 μl was analyzed by the final LC-MS/MS method (section 2.5.8 and 2.5.9).

2.6 Method validation

2.6.1 Repeatability

Repeatability assessment was performed on 10 replicates from one pooled fish feces sample on the same day. These replicates were firstly made by homogenization before transferring 300 μl supernatant for each replicate. Additionally, 300 μl of eight calibration standard solution, one QC and two blank samples were prepared. All samples were treated with internal standard, LLE, centrifugation, evaporation and derivatization as mentioned in section 2.5.3.

Repeatability assessment was also determined for 96 individual measured fish feces samples on the same day. These samples contained a quantity of approximately 300 mg with 1 mL water. Samples were then vortex-mixed for 10-12 seconds before 5 minutes centrifugation. Thereafter, 500 µl supernatant was collected and added to new vials for hydrolysis treatment with 20 µl Abalonase® with the addition of 2 M ammonium acetate buffer of pH 5. Samples were then incubated for 1 h at 60 °C meanwhile, eight calibration standard solution, one QC and two blank (water) samples were prepared. All samples were treated with internal standard, LLE, centrifugation, 20 minutes evaporation and derivatization as mentioned in 2.5.3. Repeatability of the method was calculated by using Equation 6 and presented as CV% for both replicates and individual samples.

2.6.2 Intermediate precision

Intermediate precision assessment was performed by using 10 QC samples over 10 day period. All samples were treated with internal standard, LLE, centrifugation, 20 minutes evaporation and derivatization as mentioned in 2.5.3. Intermediate precision was then presented as CV% using Equation 6 and illustrated in a Levey-Jennings (LJ) chart (Figure 21).

2.6.3 Accuracy

Accuracy assessment was evaluated by measuring four replicates of non-spiked (A) and spiked samples at low (B), mid (C) and high (D) cortisol concentrations. These replicates was firstly made by homogenization as mentioned in section 2.5.1, where 1800 μl supernatant was transferred for each replicate with 50 µl of internal standard into 2 mL eppendorf vials. Stockmix solution was added at different volumes that included 0 μl for A, 50 μl for B, 100 μl for C and 200 μl for D. These replicates was also added water at different volumes using 200 μl for A, 150 μl for B, 100 μl for C and 0 µl for D. The known cortisol concentration for A, B, C and D was set to 0, 4.805, 9.61 and 19.22 ng/mL. Additionally, eight calibration standard solution, one QC and two blank (water) samples were prepared. All samples were treated with LLE, centrifugation, 20 minutes evaporation and derivatization as mentioned in 2.5.3. Accuracy of the method was then presented as R´% by using Equation 9.

2.6.4 Linearity and linear range

Linearity and linear range were assessed by the calibration curve obtained from calibration standard solutions. These standard solutions were then prepared as mentioned in section 2.5.3 with internal standard, LLE, centrifugation, 20 minutes evaporation and derivatization before LC-MS/MS analysis. Calibration curve was constructed by plotting concentration of analyte on x-axis against chromatographic peak area ratio of analyte to internal standard on y-axis. By using linear regression analysis the model perfect for calibration curve was determined as y = ax + b with y-intercept (b) and slope (m). The linearity was determined by regression coefficient (R^2) gathered from calibration curve data while linear range was determined where curve was linear.

2.6.5 Limit of detection and limit of quantification

In this study the LOD and LOQ was assessed by measuring 10 blank (water) samples containing no analyte of interest. Each blank sample was treated with internal standard, LLE, centrifugation, 20 minutes evaporation and derivatization as mentioned in 2.5.3. Evaluation of LOD and LOQ was done through calculations using Equation 10 and 11.

2.6.6 Matrix effect

Evaluation of matrix effect was done by comparing the internal standard area for LLE and SALLE to determine the extent of ion suppression. Sample preparation was performed as mentioned in section 2.5.7.

2.6.7 Statistical analysis

Statistical analysis was done in Microsoft Excel version 16.52. First, F-test was used to determine the significance level of variance. The outcome from F-test determined the appropriate statistical analysis for the data. Parametric T-test for equal or unequal data, analyzed the significance of difference between groups. Differences were considered as significant at $p < 0.05$ and not significant at $p > 0.05$.

3. Results

3.1 Method development

3.1.1 The most efficient hydrolysis treatment for cortisol deconjugation

Examination of cortisol deconjugation in fish feces samples were investigated for acidic, basic and enzymatic hydrolysis (Figure 12). The measurement of cortisol was determined by LC-MS/MS with MRM. The average amount of cortisol from triplet sample replicates after 1 h incubation at 60 °C created a range set from 0.6 – 26.5 ng/mL at maximum. The amount of total cortisol by sulfatase from Helix Pomatia showed much higher yield compared to β-glucuronidase activity from Patella Vulgata, Abalonase® and Abalonase®+. However, enzymatic treatments resulted in much higher amount for total cortisol compared to acidic and basic hydrolysis treatments. F-test two-sample variances were performed on mean concentrations values, the variance in these values was equal for some data sets and different for other data sets. The outcome from the F-test determined which statistical T-test to perform. The variance for treated samples (acidic, basic and enzymatic) were tested against variance for untreated (quality control) samples (Table 5). Based on datasets for 2, 6 and 10% H_2SO_4, 2 – 10% NH_3 and Abalonase®+, T-test for equal variances were used for statistically evaluation of differences in average amount of cortisol between untreated and treated samples. Whereas datasets for 4 and 8% H_2SO_4, Helix Pomatia, Patella Vulgata and Abalonase® used T-test for unequal variances for statistically evaluation of differences in average amount of cortisol between untreated and treated samples. One-tail and two-tail T-test was performed (Table 5) to determine the significance of difference. One-tail p-value decided if the values were significantly greater or less than a certain value while two-tail p-value decided if the significant difference were equal or unequal than a certain value. All acidic and basic experiments were significantly different ($p < 0.05$) with significantly lower value than quality control samples. In comparison, all enzymatic hydrolysis treatments excepts for Patella Vulgata were significantly different ($p < 0.05$) with a significantly higher value than quality control samples. Cortisol deconjugation was most efficient when using enzymatic treatment from Helix Pomatia.
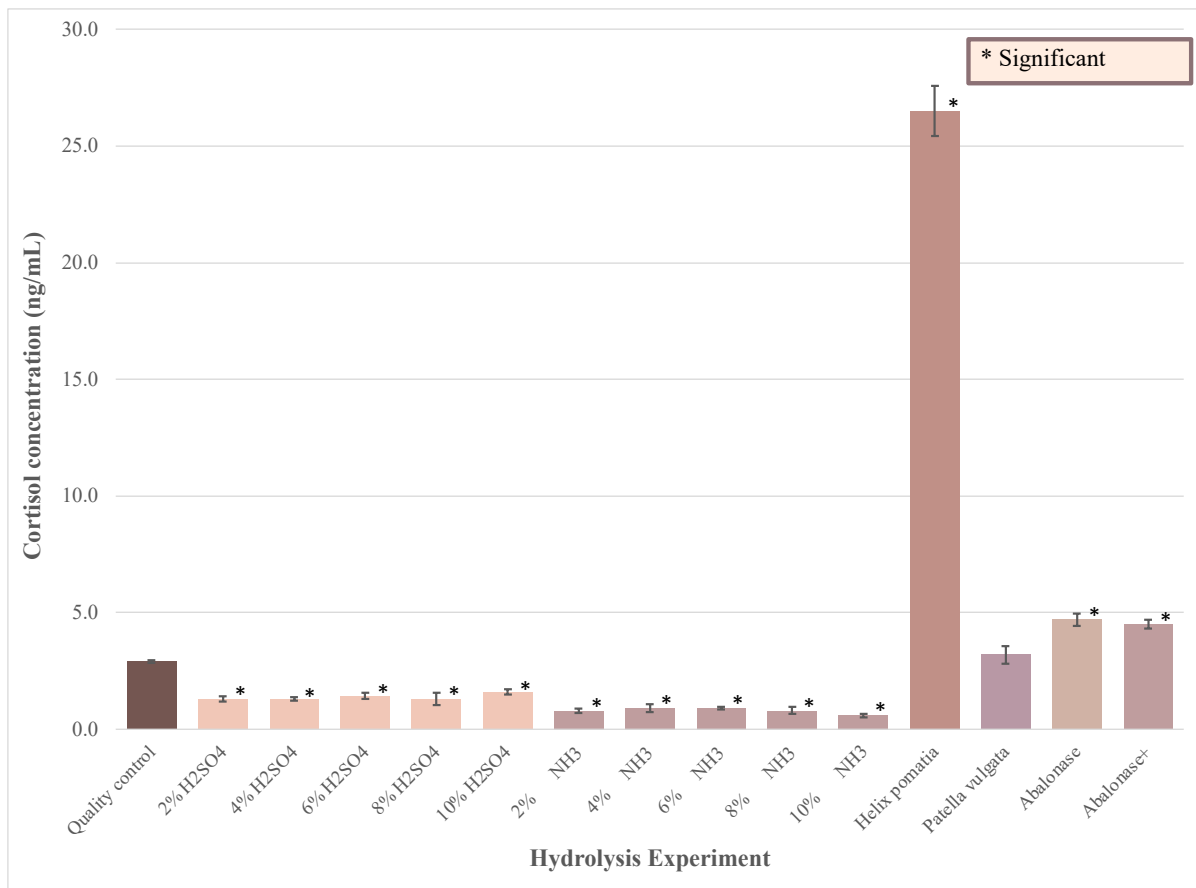
Figure 12: Hydrolysis experiments for cortisol deconjugation.

Average and standard deviation (±SD) values from triplet replicates of acidic (H_2SO_4), basic (NH_3) and enzymatic (Helix Pomatia, Patella Vulgata, Abalonase and Abalonase+) hydrolysis treatments and their impact on cortisol concentration (ng/mL). Statistical analysis performed on untreated (quality control) against treated (acidic, basic, enzymatic) show a significant difference ($p < 0.05$) except for enzymatic hydrolysis from Patella Vulgata ($p > 0.05$).

Table 5: Statistical evaluation of acidic, basic and enzymatic hydrolysis treatments.
Statistical analysis was gathered from F-test two-sample for variances and T-test for equal or unequal variances between untreated- (quality control) and treated (acidic, basic and enzymatic) samples.

Acidic hydrolysis	Quality control	2% H_2SO_4	4% H_2SO_4	6% H_2SO_4	8% H_2SO_4	10% H_2SO_4
Mean	2.93	1.35	1.31	1.44	1.31	1.62
Variance	0.003	0.015	0.005	0.019	0.065	0.012
P-value (F <= f) one-tail	-	0.179	0.405	0.152	0.049	0.214
P-value (T<= t) one-tail	*	1.78×10^{-5}	3.23×10^{-6}	3.19×10^{-5}	0.0043	2.63×10^{-5}
P-value (T<= t) two-tail	*	3.57×10^{-5}	6.46×10^{-6}	6.39×10^{-5}	0.0086	5.27×10^{-5}

Basic hydrolysis	Quality control	2% NH_3	4% NH_3	6% NH_3	8% NH_3	10% NH_3
Mean	2.93	0.81	0.87	0.92	0.86	0.62
Variance	0.003	0.010	0.024	0.004	0.022	0.004
P-value (F <= f) one-tail	-	0.242	0.120	0.472	0.132	0.441
P-value (T<= t) one-tail	*	3.07×10^{-6}	1.41×10^{-5}	1.02×10^{-6}	1.13×10^{-5}	6.61×10^{-7}
P-value (T<= t) two-tail	*	6.14×10^{-6}	2.81×10^{-5}	2.03×10^{-6}	2.27×10^{-5}	1.32×10^{-6}

Enzymatic hydrolysis	Quality control	Helix Pomatia	Patella Vulgata	Abalonase	Abalonase+	
Mean	2.93	26.5	3.19	4.65	4.52	
Variance	0.003	1.143	0.129	0.074	0.033	
P-value (F <= f) one-tail	-	0.003	0.025	0.043	0.093	
P-value (T<= t) one-tail	*	0.0003	0.168	0.004	6.5×10^{-5}	
P-value (T<= t) two-tail	*	0.0007	0.337	0.009	0.0001	

- All treated (acidic, basic, enzymatic) samples tested against variance for untreated (quality control) samples

* All treated (acidic, basic, enzymatic) samples statistically evaluated for amount cortisol (ng/mL) against untreated (quality control) samples.

3.1.2 The most suitable pH buffer solution for enzymatic activity conditions

Enzymatic activities were investigated based on their influence on cortisol concentrations (ng/mL). This was determined for Helix Pomatia and Patella Vulgata when using 2 M ammonium acetate buffer solutions of pH 5 and 6 (Figure 13). The measurement of cortisol was determined by LC-MS/MS with MRM. To compare which buffer solution resulted in highest yield of cortisol, three replicates containing one enzymatic treatment and one buffer solution was prepared. The average amount of cortisol from triplet replicates after 1 h incubation at 60 °C created a range set from 2.9 – 22.55 ng/mL at maximum. F-test two-sample variances were performed on mean concentrations values, the variance in these values were equal for some data sets and different for other data sets. The outcome from the F-test determined which statistical T-test to perform. The variance for treated samples of Patella Vulgata and buffer solution of pH 5 or 6 were tested against variance for untreated quality control samples (Table 6). Based on datasets from Patella Vulgate with buffer of pH 5, T-test for equal variances were used for statistically evaluation of amount cortisol between untreated and treated samples. Datasets from Patella Vulgata with buffer of pH 6 used T-test for unequal variances for statistical evaluation of amount cortisol between untreated and treated samples. One-tail and two-tail T-test were performed to determine the significance of difference (Table 6). One-tail p-value decided if the values were significantly greater or less than a certain value while two-tail p-value decided if the significant difference were equal or unequal than a certain value. Samples treated with Patella Vulgata were not significantly different ($p > 0.05$) from quality control samples using buffer of pH 6 however, showed a significant increase ($p < 0.05$) in cortisol concentration by using buffer of pH 5. Samples treated with Helix Pomatia showed a clear significant increase in cortisol levels compared to quality control. However, there was significantly lower cortisol levels when using buffer of pH 5 compared to buffer of pH 6. Therefore, suitable enzymatic activity was obtained when using 2 M ammonium acetate buffer of pH 6 resulting in highest amount of cortisol.
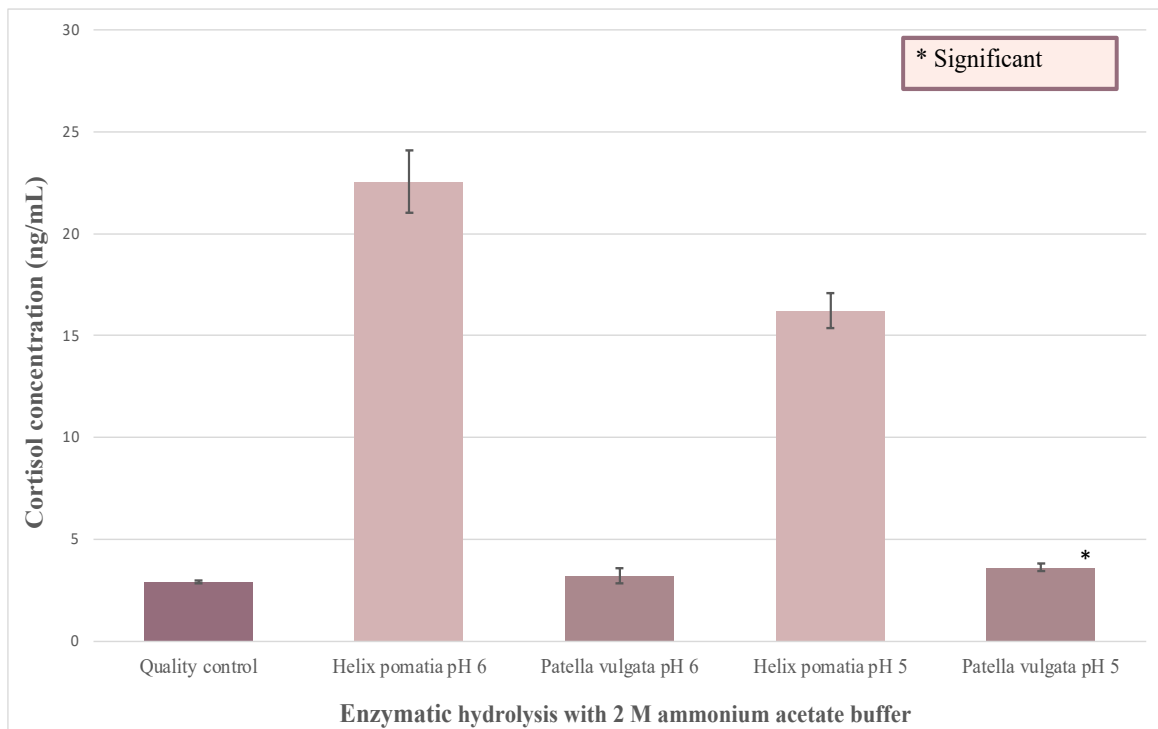
Figure 13: Enzymatic hydrolysis with buffer solution of different pH value.

Represents average and standard deviation (±SD) values of cortisol from triplet replicates of Patella Vulgata and Helix Pomatia with 2 M ammonium acetate buffer of pH 5 and 6. Each buffer solution show different effect on enzymatic activity and cortisol concentration (ng/mL). Statistical analysis of untreated quality control samples and enzymatic treated with Patella Vulgata was significantly different using buffer of pH 5 ($p < 0.05$) and not significantly different using buffer of pH 6 ($p > 0.05$).

Table 6: Statistical evaluation of Patella Vulgata hydrolysis with buffer of different pH value. Statistical analysis was gathered from F-test two-sample for variances and T-test for equal or unequal variances between untreated (quality control) and treated (Patella Vulgata with 2 M ammonium acetate buffer of pH 5 and 6) samples.

Samples	Quality control	Patella Vulgata with 2 M ammonium acetate buffer of pH 5	Patella Vulgata with 2 M ammonium acetate buffer of pH 6
Mean	2.93	3.62	3.20
Variance	0.003	0.037	0.129
P-value (F <= f) one-tail	-	0.082	0.025
P-value (T<= t) one-tail	*	0.002	0.168
P-value (T<= t) two-tail	*	0.004	0.337

- All samples treated with Patella Vulgata using 2 M ammonium acetate buffer of pH 5 and 6 were tested against variance for untreated quality control samples

* All samples treated with Patella Vulgata using 2 M ammonium acetate buffer of pH 5 and 6 were statistically evaluated for amount cortisol against untreated quality control samples.

3.1.3 The most optimum incubation time for enzymatic hydrolyzation

Incubation times of 1, 2, 3 and 24 h with Helix Pomatia was studied to find the most optimum incubation time for cortisol deconjugation (Figure 14). The amount of cortisol (ng/mL) was determined by LC-MS/MS with MRM. Incubation times of 1, 2 and 3 h had a great effect on average cortisol concentrations resulting in an increase from 2.93 ng/mL in cortisol samples to 24.05 ng/mL after 3 h. However, slight increase was observed between 1, 2 and 3 h incubation time as well as an decrease in amount from 3 to 24 h. Although, sustainable levels of cortisol is found to be above 20 ng/mL with Helix Pomatia hydrolysis, meaning most optimum incubation time for cortisol deconjugation can be achieved after only 1 h.
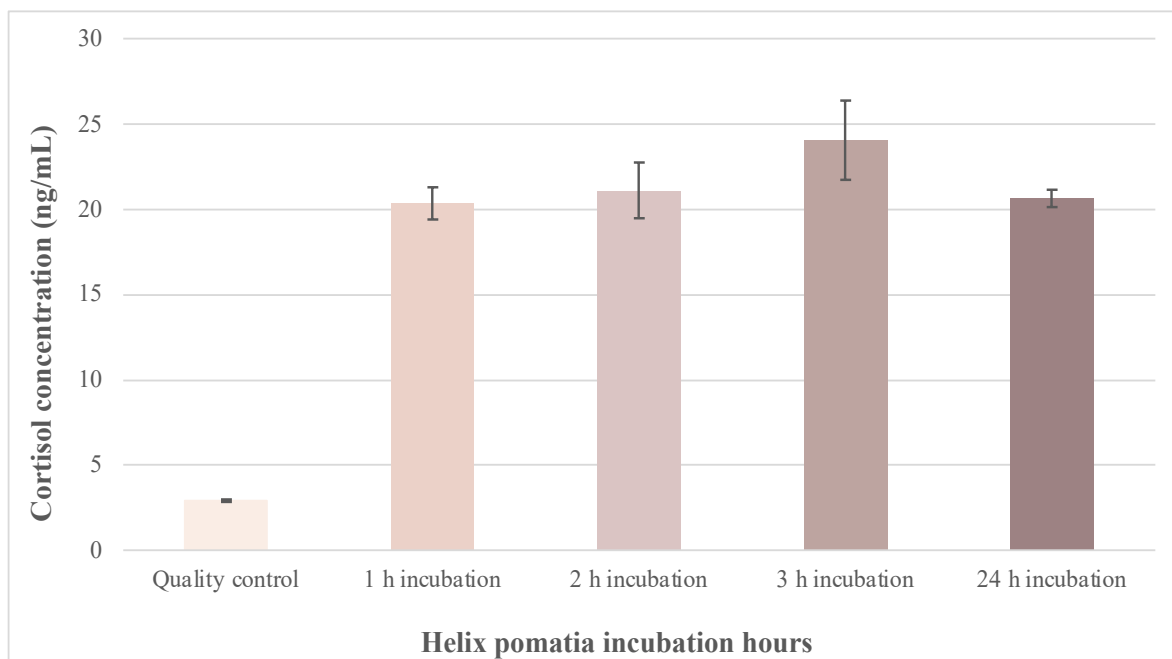
Figure 14: Hydrolysis treatment with Helix Pomatia at different incubation times.

Average and standard deviation (±SD) values of cortisol (ng/mL) from three replicates of untreated (quality control) and treated (2 M acetate buffer at 60 °C with Helix Pomatia) fish feces samples incubated at 1,2,3 and 24 hours.

3.1.4 The sufficient amount of enzyme solution for cortisol deconjugation

The influence of adding different amounts of Helix Pomatia solution on cortisol deconjugation in fish feces samples was studied (Figure 15). The amount of cortisol (ng/mL) was determined by LC-MS/MS with MRM. This was done for three replicates containing 10, 20, 30, 40 and 50 μl Helix Pomatia solution after 1 h incubation at 60 °C. The average value from each solution showed a clear trend that increasing the amount of enzyme solution will lead to higher yield in cortisol concentration.
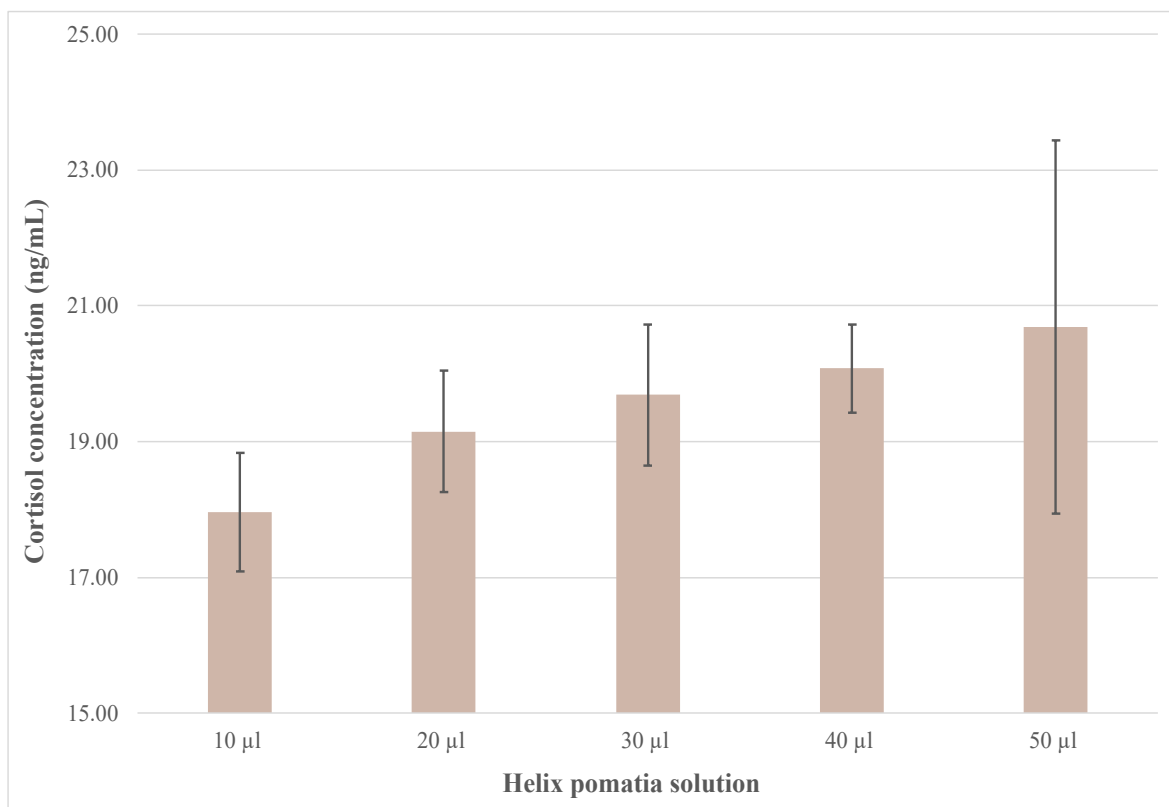
Figure 15: Hydrolysis treatments with different amounts of Helix Pomatia solution.

Average and standard deviation (±SD) values of cortisol (ng/mL) gathered from three replicates containing 10, 20, 30, 40 and 50 μl of Helix Pomatia solution.

3.1.5 The most efficient extraction method for cortisol

The cortisol extraction efficiency was evaluated for LLE and SALLE by examining the extent of ion suppression (Figure 16) and percentage of relative extraction recovery (REC%) (Figure 17) achieved from both methods. In LLE, triplets containing water-immiscible organic solvents (MTBE, etylacetat and butanol) were added either 100 or 600 µl 5 M NaCl. In comparison, SALLE experiments used triplets containing water-miscible organic solvents (acetonitrile and propanol) with 600 µl 5 M NaCl. Both LLE and SALLE methods were compared to each other and evaluated for degree of ion suppression by their impact on d4-cortsiol-4-ABH peak area. These experiments allowed also the study of ion suppression caused by co-extracted sample matrix components, since the internal standard was added after the extraction. The peak area for d4-cortisol-4-ABH obtained for each extraction method showed less impact by using LLE with MTBE and 100 µl 5 M NaCl. In comparison, higher amount of 5 M NaCl for MTBE resulted in much higher impact on d4-cortisol-4-ABH peak area. SALLE experiments using acetonitrile and propanol showed the highest impact on d4-cortisol-4-ABH peak area. However, SALLE experiments showed higher REC% compared to LLE experiments. Even though LLE with MTBE and 100 µl 5 M NaCl resulted in approximately 40% lower extraction recovery compared to SALLE with acetonitrile, the LLE method with MTBE also resulted in 400% less ion suppression. This resulted in a significant combined improvement in detectability, which is why LLE with MTBE and 100 µl 5 M NaCl is the most preferable method for cortisol extraction.
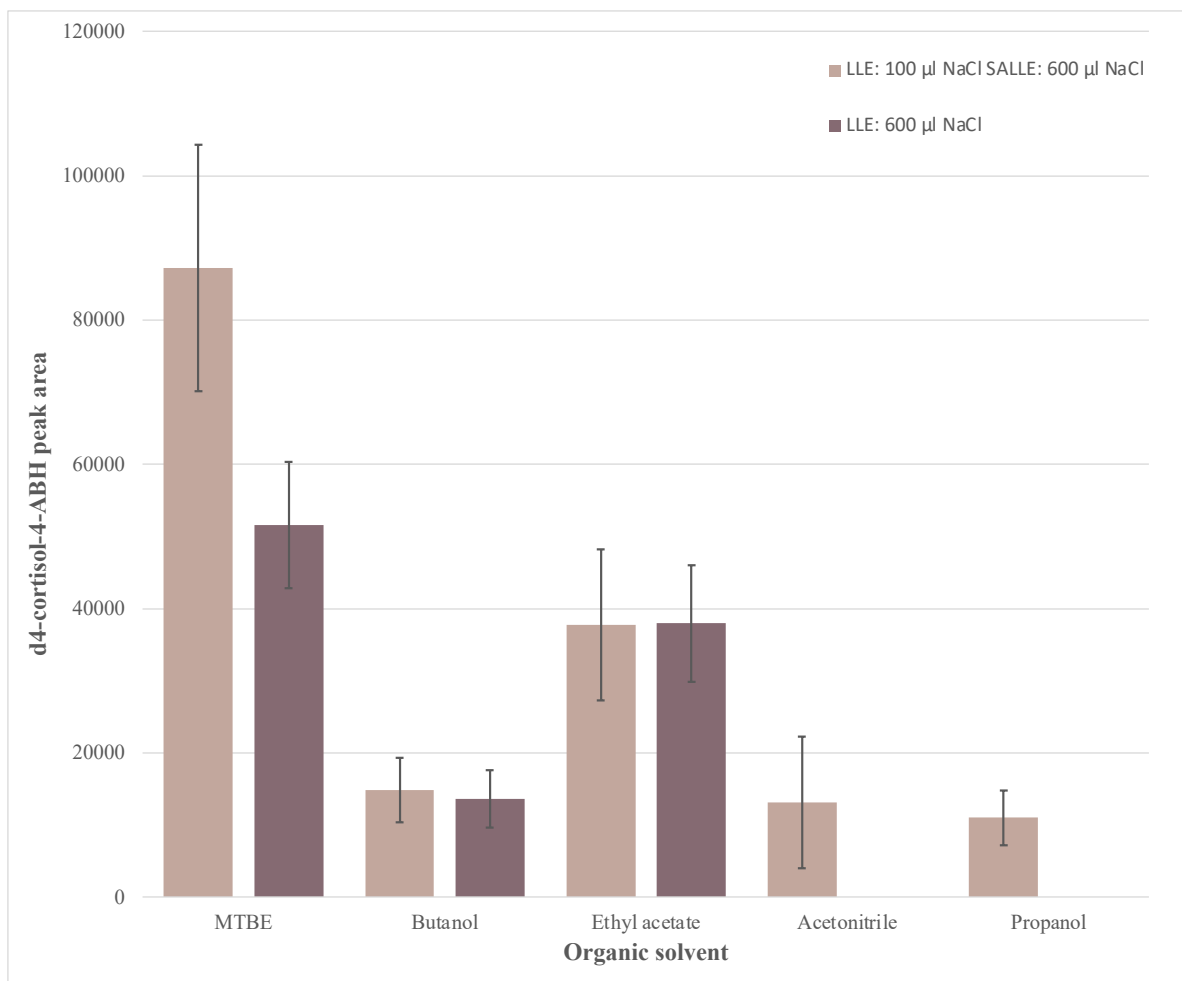
Figure 16: Different techniques for cortisol extraction.

Average and standard deviation (±SD) response values from three replicates of liquid-liquid extraction (LLE) and salting-out assisted liquid-liquid extraction (SALLE). The LLE and SALLE samples show different influence on the derivatized d4-cortisol (d4-cortisol-4-ABH) peak area. Each extraction method performed used different organic solvents that included water-immiscible (MTBE, butanol and ethyl acetate) for LLE and water-miscible (acetonitrile and propanol) for SALLE.
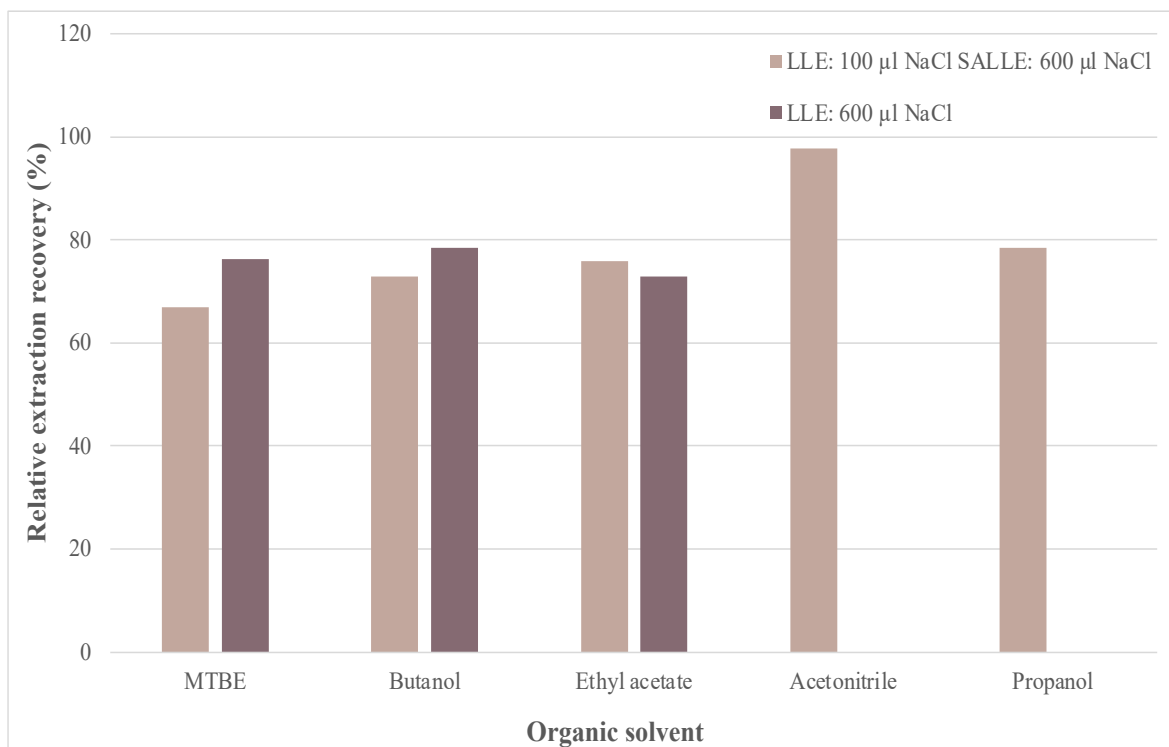
Figure 17: Percentage of relative extraction recovery (REC%) values from different cortisol extraction techniques.

Average REC% values gathered from three replicates of liquid-liquid extraction (LLE) and salting-out assisted liquid-liquid extraction (SALLE). Each extraction method performed used different organic solvents that included water-immiscible (MTBE, butanol and ethyl acetate) for LLE and water-miscible (acetonitrile and propanol) for SALLE.

3.1.6 The most efficient mobile phase for ion enhancement

The signal from cortisol-4-ABH mono-hydrazone products was investigated after ESI+ when using two different mobile phases (Figure 18). These mobile phases consisted of water with 0.2% formic acid- or 0.2% ammonium hydroxide and water with 0.2% methanol. This was evaluated based on the impact each mobile phase had on the peak area response for d4-cortisol-4-ABH (Figure 18A) and cortisol-4-ABH (Figure 18B). The signal response was determined by LC-MS/MS with MRM. Each mobile phase was used to analyze three replicates containing fish feces samples treated with Helix Pomatia deconjugated and incubation for 1 h at 60 °C. By evaluating these two peaks for both mobile phases, it was clearly less signal and more variation for 0.2% formic acid in water compared to 0.2% ammonium hydroxide in water. The analytical sensitivity for detecting cortisol mono-hydrazone products will therefore be highest and most efficient when using 0.2% ammonium hydroxide in water as mobile phase.

A
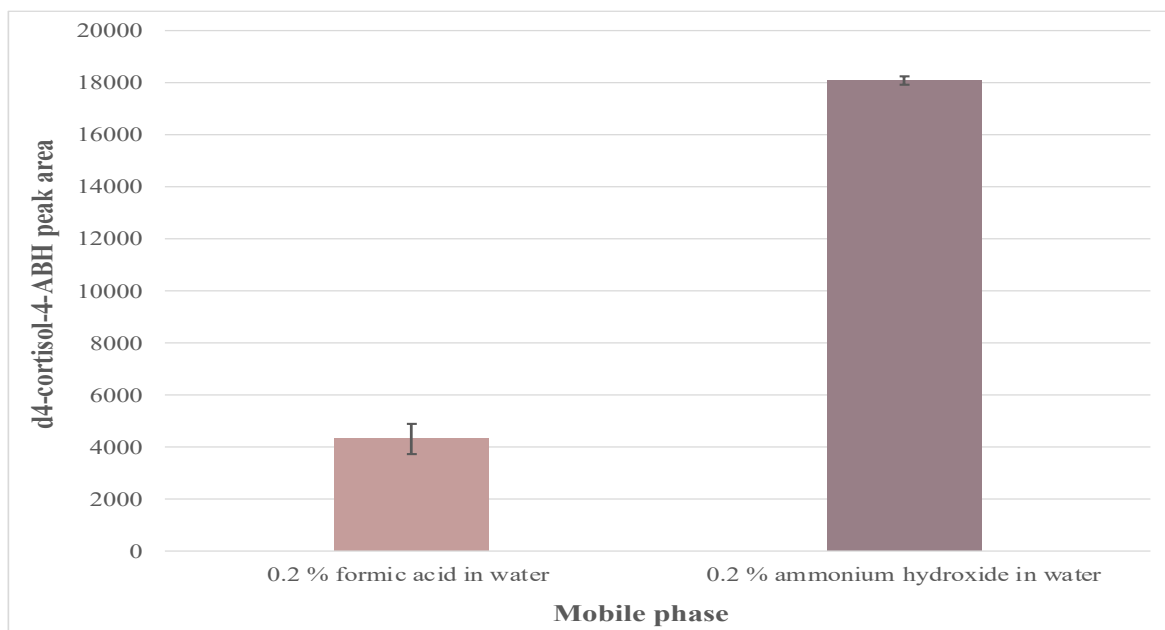
B
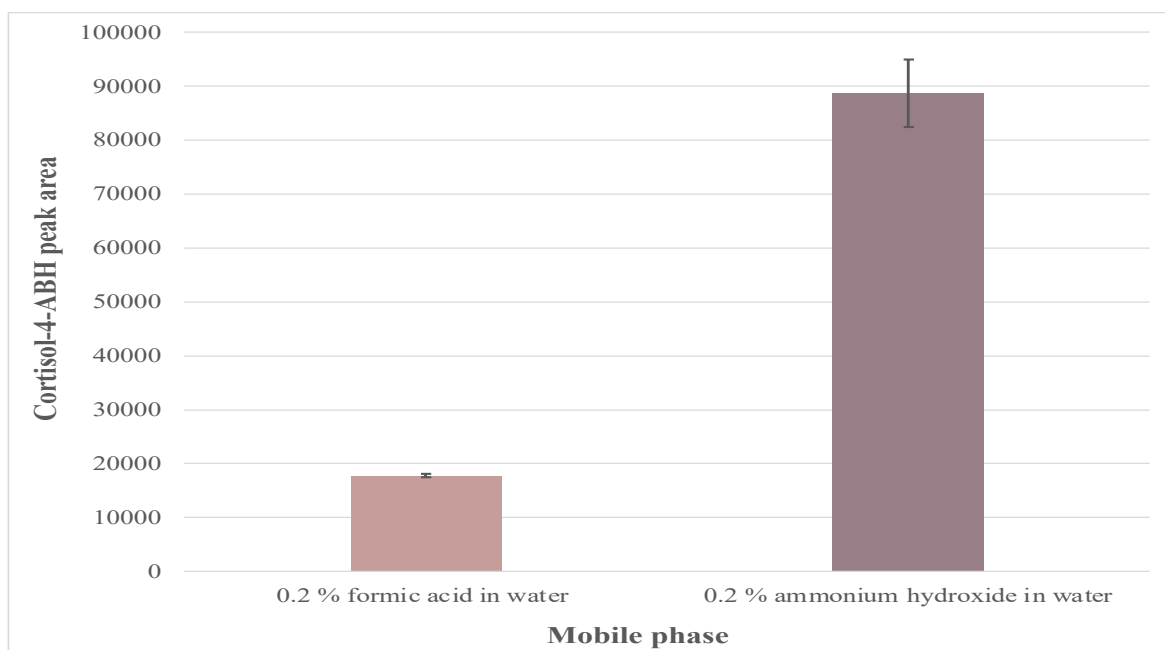
Figure 18: Signal response from different mobile phases.

Represents average and standard deviation (±SD) response values from three replicates. Replicate samples show different peak area response for derivatized d4-cortisol (d4-cortisol-4-ABH) (A) and cortisol (cortisol-4-ABH) when using 0.2% formic acid- or 0.2% ammonium hydroxide in water as mobile phase.

3.1.7 The appropriate liquid chromatography-tandem mass spectrometry conditions

The maximum signal response and transition channels were investigated for underivatized and derivatized cortisol (Figure 19). Samples were analyzed according to previously described LC and MS/MS conditions (section 2.5.8 and 2.5.9). A typical chromatogram from a LC-MS/MS analysis with MRM from 1100 μl of clear extract for cortisol, d4-cortisol, cortisol-4-ABH and d4-cortisol-4-ABH is shown in Figure 19. These chromatograms are obtained by monitoring precursor and product ions for cortisol and d4-cortiol with transition channels at 363.2 > 121.1 m/z and 376.2 > 121.1 m/z (Figure 19A-B). This was also done for cortisol-4-ABH and d4-cortisol-4ABH with transition channels at 496.25 > 119.8 m/z and 500.25 >119.8 m/z (Figure 19C-D). The total run was 6 minutes per sample with retention time set to 4.46 for cortisol and d4-cortisol. While cortisol-4-ABH and d4-cortisol-4-ABH showed a retention time at 4.32 and 4.33. The mobile phase contained water with 0.2% ammonium hydroxide and 0.2% methanol using gradient conditions. All interfering compounds were eluted before cortisol. This was done by using high gradient conditions with 0.2% methanol in water solution, thus removing most impurities before the analysis. The cortisol derivative provided much higher ESI+ response with 5.60 x 10^5 than underivatized with 5.84 x 10^4.

A
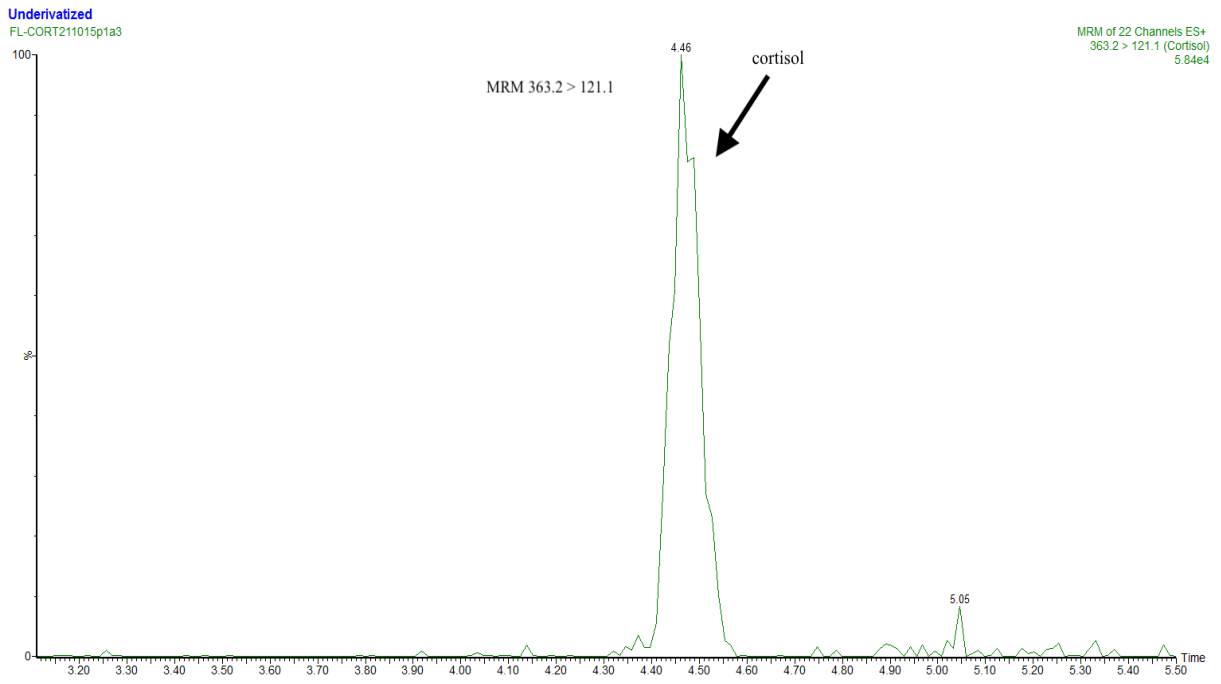
B
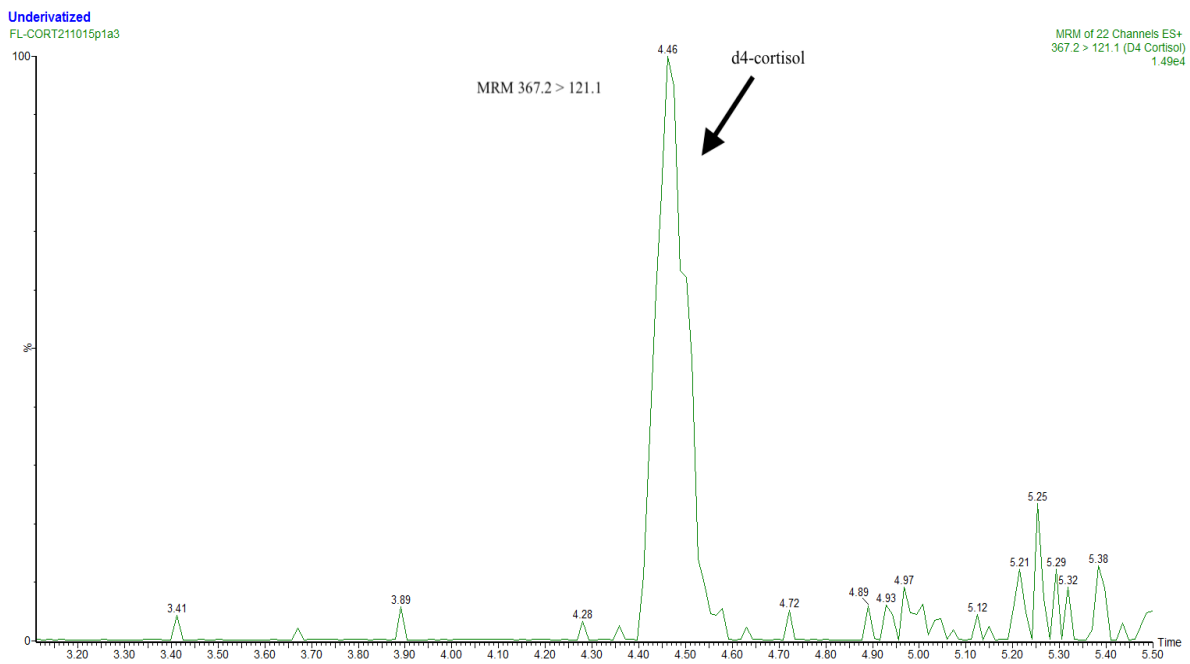
Figure 19 continuous on the next page

C
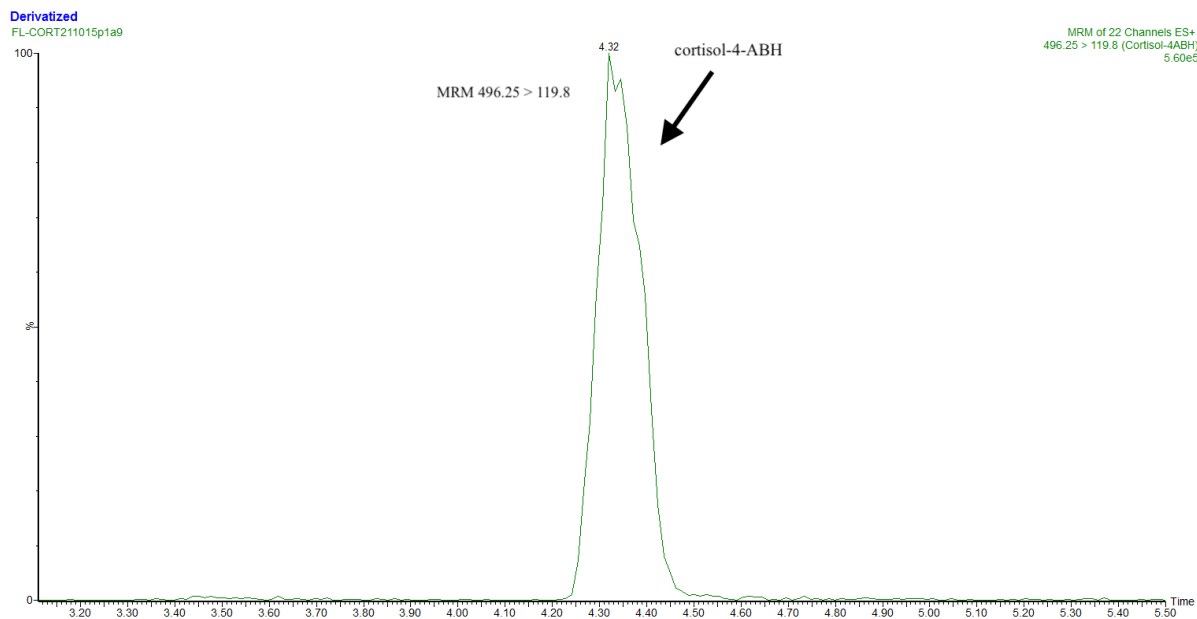
D
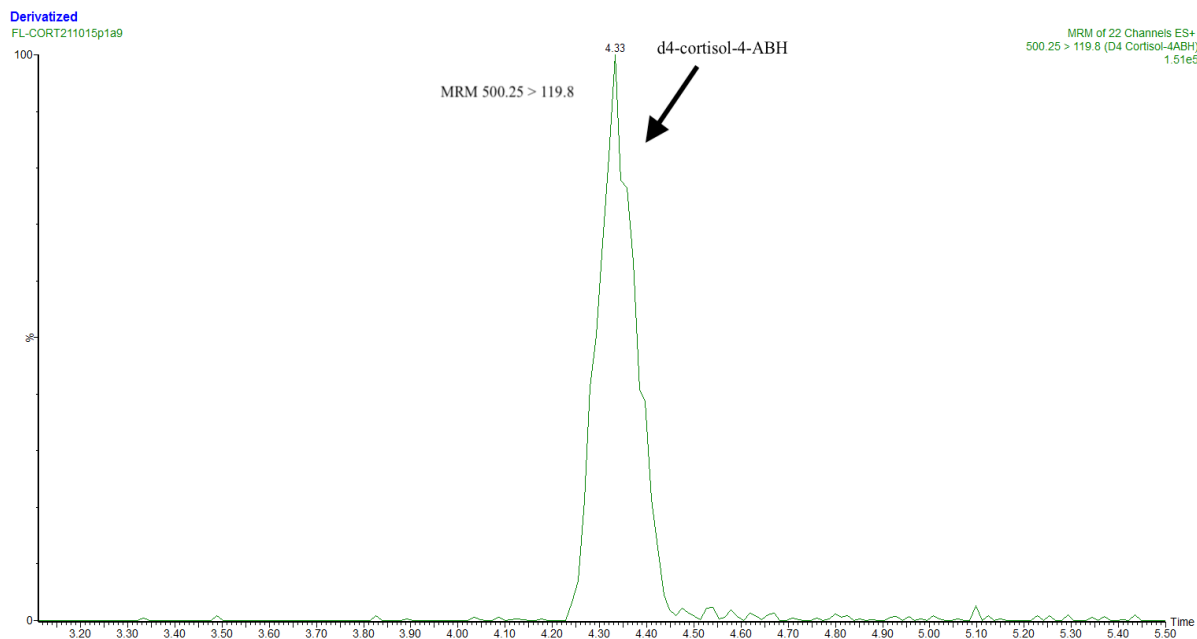
Figure 19: Multiple reaction monitoring (MRM) chromatograms.

Representing MRM chromatograms for underivatized cortisol (A) and internal standard (B) addition to derivatized cortisol (C) and internal standard (D) with 4-aminobenzoic hydrazide (4-ABH). Transition channels for cortisol was 363.2 > 121.1 m/z and 376 > 121.1 m/z for d4-cortisol with retention time set to 4.46. Transition channels for cortisol-4-ABH was 496.25 > 119.8 and 500.25 > 119.8 m/z for d4-cortisol-4-ABH with retention time set to 4.32 and 4.33 min. The response from electrospray ionization working in positive mode (ESI+) was 5.84×10^4 for cortisol and 5.60×10^5 for cortisol-4-ABH.

3.1.8 Cortisol concentration levels in field samples

The method´s ability to measure differences in cortisol levels in fish gathered from off- and onshore industrial fish farms was investigated. This was done by measuring total amount cortisol levels from 96 individual fish feces samples (Table 12). The total amount of cortisol from these samples are represented in Figure 20. The amount of cortisol was determined by LC-MS/MS with MRM. Each sample was treated with enzymatic deconjugation from Abalonase® with 1 h incubation at 60 °C. The majority of fish feces samples contained around 5 – 12 ng/g of cortisol while a few other samples showed levels under 5 ng/g. However, a subset of fish displayed clearly elevated levels about 3 – 4 times higher than the average observed. The developed method is therefore applicable to measuere differences in cortisol levels and could be a useful tool to assess the stress level in fish.
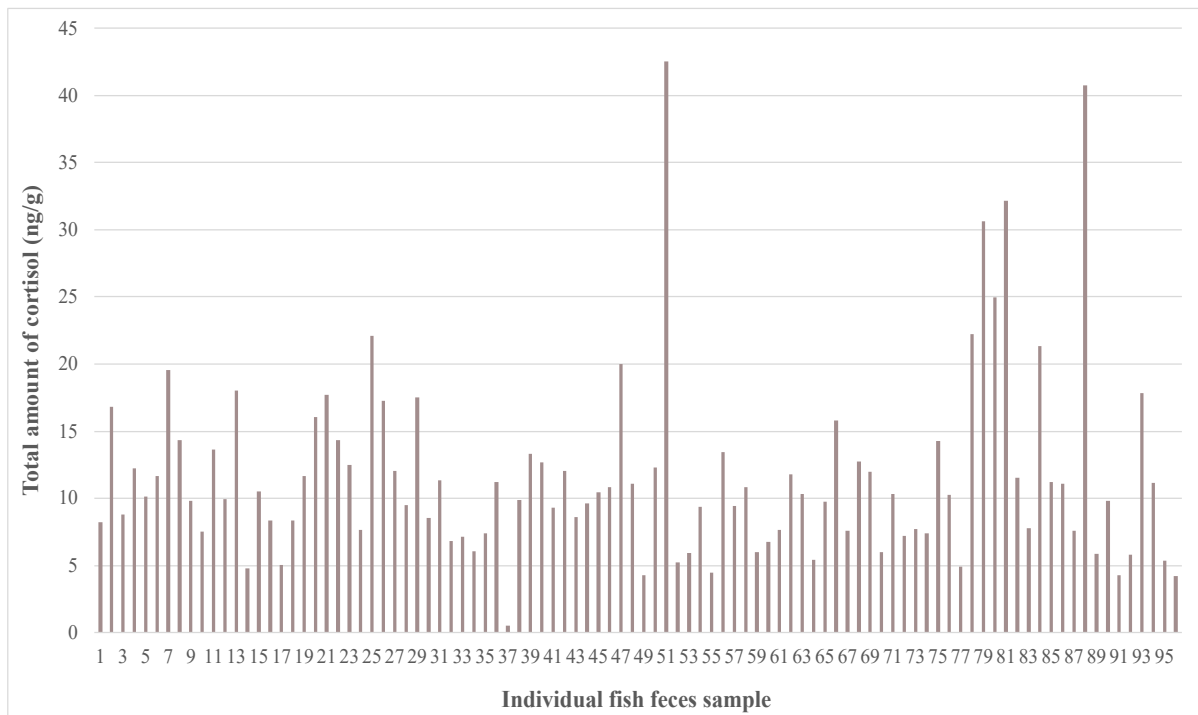
Figure 20: Total amount of cortisol levels from fish feces samples.

Represents the total amount of cortisol levels (ng/g) from 96 individual fish feces samples collected from off- and onshore industrial fish farms.

3.2 Method validation

3.2.1 Precision

To determine repeatability and intermediate precision of the method. Repeatability assessment was performed by using 10 replicates from one pooled fish feces and 96 individual measured fish feces samples on the same day. Intermediate precision assessment was performed by using 10 QC samples over a 10 day period. The concentration of cortisol (ng/mL) was determined by LC-MS/MS using MRM. Repeatability and intermediate precision was expressed as CV % that was calculated using Equation 6. Excellent precision were achieved for repeatability and intermediate precision with 16.7% for individual samples, 11% for replicates and 10.5% for QC samples.

The results for intermediate precision are also presented in a LJ chart (Figure 21). The LJ chart represents cortisol concentrations (ng/mL) measured from QC samples in a 10 day run. This graphical illustration includes calculated $\bar{x}$ value at 2.9 and $\pm 1 - 3$ standard deviation (SD) control limits at 3.2, 3.5, 3.8, -2.0, -2.3 and -2.6. This indicates that all data points were within the action limits that represents acceptable performance.
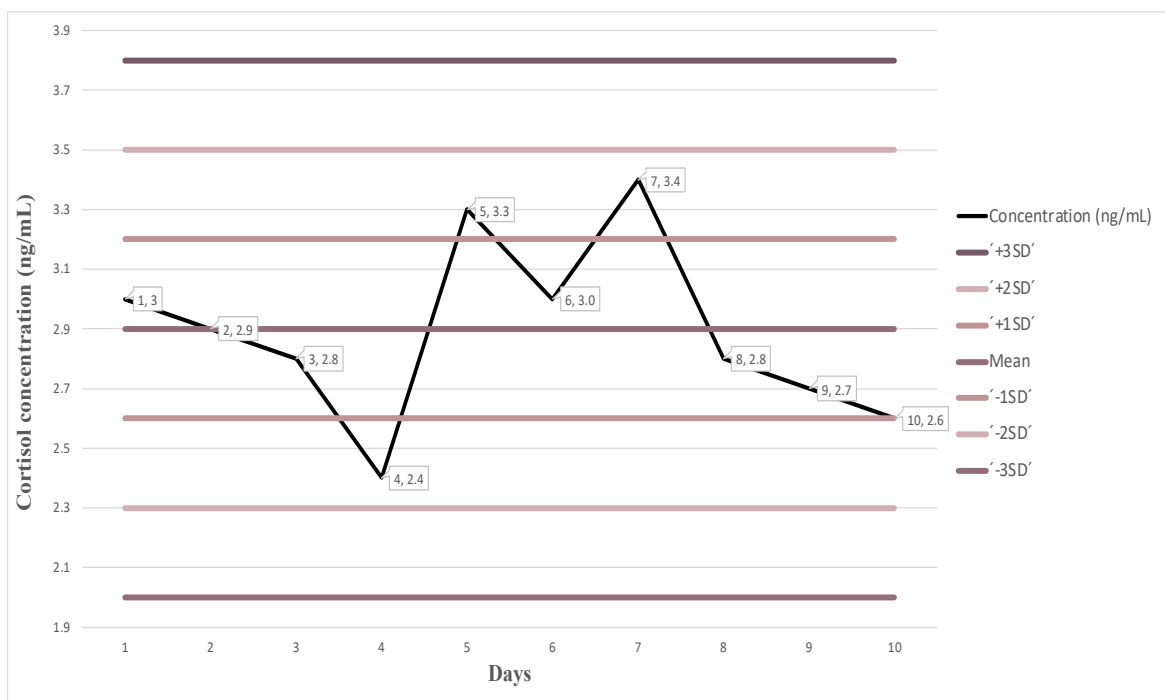
Figure 21: Levey-Jennings (LJ) chart.

The LJ chart show $\pm 1 - 3$ standard deviation (SD) control limits for intermediate precision measurements of the method.

3.2.2 Accuracy

Accuracy was determined by measuring four replicates of non-spiked (A) and spiked samples at low (B), mid (C) and high (D) cortisol concentrations. The concentration of cortisol for each sample was determined by LC-MS/MS with MRM. Accuracy was expressed as R´% by using the $\bar{x}$ value from non-spiked with several spiked replicates and their true value (Equation 9). The results from recovery experiment to assess the method´s trueness are represented in Table 7. Great accuracy was achieved for cortisol at low (B), mid (C) and high (D) concentrations (Table 7).

Table 7: Recovery experiment for accuracy assessment

Accuracy assessment of cortisol from recovery experiment was performed by using non-spiked (A) and spiked fish feces samples at low (B), mid (C) and high (D) concentrations.

Sample	A	B	C	D
Cortisol				
n	4	4	4	4
Mean [a]	1.49	6.96	13.6	25.9
$\bar{x}$ - $\bar{x}´$ [b]	-	5.48	12.1	24.4
X$_{Spike}$ [c]	-	4.81	9.61	19.2
R´% [d]	-	114	126	127

[a] Mean value of cortisol concentration (ng/mL)

[b] Mean of unspiked ($\bar{x}$) samples, mean of spiked ($\bar{x}´$) samples

[c] Known concentration added

[d] Percentage of relative spike recovery, $\frac{\bar{x}´-\bar{x}}{X_{Spike}}$ x 100

- Compared concentration of low (B), mid (C) and high (D) against non-spiked (A) samples

3.2.3 Linearity and linear range

Evaluation of the methods linearity and linear range was done by a calibration curve constructed from calibration standard solutions. All samples and calibration standards were added the same amount of internal standard and therefore, the calibration curve was made by directly plotting the concentration of cortisol on the x-axis against chromatographic peak area ratio of cortisol-4-ABH to d4-cortisol-4-ABH on y-axis. The calibration curve gathered from linear regression model exhibited good linearity range within 0.09 – 100 ng/mL and regression with acceptable R^2 value at 0.997 – 0.999. A typical internal standard calibration curve for cortisol was expressed by y = 0.233508x by forcing trend line through origin.

3.2.4 Limit of detection and limit of quantification

To evaluate the methods LOD and LOQ this was determined by the calibration curve and calculations using $\bar{x}$ of response values with the addition of 3 and 10 SD (Equation 10 and 11). The lowest cortisol concentration that could be detected (LOD) was 0.04 ng/mL. The lowest amount of cortisol quantification (LOQ) was set to 0.09 ng/mL.

3.2.5 Validation parameters summarization

This chapter provides a summary of the validation parameters gathered for the final method (Table 8). The parameters validated were repeatability, intermediate precision, accuracy, linearity, linear range, LOD and LOQ.

Table 8: Method validation parameters summarization.

Summarized validation parameters for cortisol quantification in fish feces samples collected from off- and onshore industrial fish farms.

Validation parameters	Cortisol
Repeatability (CV%)	Individual: 16.7% Replicates: 11%
Intermediate precision (CV%)	10.5%
Accuracy (R´%)	B: 114% C: 126% D: 127%
Limit of detection (LOD)	0.04 ng/mL
Limit of quantification (LOQ)	0.09 ng/mL
Linearity (R^2)	0.997 – 0.999
Linear range	0.09 – 100 ng/mL
Relative extraction recovery (%)	MTBE: ~ 67% Butanol: ~ 73% Ethyl acetate: ~ 76% Acetonitrile: ~ 98% Propanol: ~ 78%

4. Discussion

4.1 Method development

4.1.1 Hydrolysis treatment for cortisol deconjugation

Examination of cortisol deconjugation in fish feces samples were investigated for acidic, basic and enzymatic hydrolysis (Figure 12). Since cortisol and its metabolites can be secreted as free or hydrophilic conjugates of glucuronides and sulfides [34, 38] deconjugation is necessary and may be performed by either chemically or enzymatic methods. According to the hydrolysis experiments performed, β-glucuronidase and sulfatase activity from Helix Pomatia was the optimum treatment for cortisol deconjugation. It has been reported that deconjugation by Helix Pomatia treatment is beneficial [71]. Predominantly, cortisol metabolites are found bound to either glucuronide or sulfate while only a small portion is free [34]. This statement is also true for cortisol as in our findings show that enzymatic treated samples yielded higher amount of cortisol than untreated samples. This was only consistent with enzymatic and not chemical (acidic and basic) hydrolysis that resulted in cortisol decrease due to less sufficient deconjugation, which is not consistent with other studies [72, 73].

Only β-glucuronidase and sulfatase activity from Patella Vulgata were not significantly different ($p > 0.05$) from untreated samples. While other studies state that Patella Vulgata in comparison to Helix Pomatia was the best choice of hydrolysis [72], this was not in accordance with our findings. Most of the conjugated cortisol metabolites are bound to sulfate groups [33], this might also be accurate for cortisol when comparing enzymatic treatment from Helix Pomatia versus Abalonase® and Abalonase®+. The β-glucuronidase activity from Abalonase® resulted in higher yield of cortisol do to hydrolysis but significantly smaller amount compared to the additional sulfatase activity from Helix Pomatia. Further, Abalonase®+ with the same enzymatic content as Helix Pomatia, contained less sulfatase activity and amount of cortisol. Therefore, since Helix Pomatia had highest sulfatase activity and amount of cortisol one can assume that cortisol is more bound to sulfate than glucuronide groups. In fact, arylsulfatase activity have also been most commonly extracted from Helix Pomatia [74].

While enzymatic hydrolysis from Helix Pomatia seems to be the most suitable treatment for cortisol deconjugation, Patella Vulgata, Abalonase® and Abalonase®+ were not. One consideration is that enzyme preparations from Patella Vulgata, Abalonase® and Abalonase®+ contain other activities that can destroy steroids as reported by Graef, Furuya and Nishikaze[75]. Another consideration might be other competing substances for the available enzyme as reported by Dodgson and Powell[76]. There has also been stated that enzymes react differently depending on parameters such as amount of enzyme, pH, incubation time and temperature to obtain optimum deconjugation [77]. The difference in enzyme deconjugation activity can also be due to whether or not the enzyme is stable. According to Dodgson and Powell[76] the enzyme instability resulted in destruction of the enzyme. Therefore, further development in regards to optimization of enzyme activity conditions can be beneficial for the optimum deconjugation.

4.1.2 Suitable pH buffer solution for enzymatic activity conditions

Enzymatic activities were investigated based on their influence on cortisol concentrations (ng/mL). This was determined for Helix Pomatia and Patella Vulgata when using 2 M ammonium acetate buffer solutions of pH 5 and 6 (Figure 13). The optimum pH of Helix Pomatia and Patella Vulgata for cortisol deconjugation was 6 and 5 respectively, which is almost identical to the manufacture´s recommendations. The shift in pH optimum for hydrolysis might be due to presence of more than one enzyme [76]. This might be the case as the recommendations for β-glucuronidase and sulfatase activity were pH 5 for Helix Pomatia and pH 3.8 and 5 for Patella Vulgata. Additionally, shift in pH might depend on the compound being deconjugated as reported by Combie et al.[78]. However, according to the literature the optimum pH is around 5 for steroid deconjugation with Helix Pomatia, 6.0-7.5 for sulfatase [77] and 3.8 and 5 for Patella Vulgata [78]. This also might explain why the amount of cortisol was low due to less than optimal pH condition for β-glucuronidase activity found in Patella Vulgata solution. Samples at pH 6 showed no significantly differences and slightly significant differences at pH 5. There has been reported that increase in buffer concentration results in increasing enzymatic activity [76]. Therefore, further optimization for enzyme activity can be accomplished by altering pH conditions and increasing the ammonium acetate concentrations which might yield higher cortisol levels for both enzymatic treatments.

4.1.3 Optimal incubation time for enzymatic hydrolyzation

Incubation times of 1, 2, 3 and 24 h with Helix Pomatia was studied to find the most optimum incubation time for cortisol deconjugation (Figure 14). The incubation time set for 1-3 h deconjugation by Helix Pomatia showed positively increase in cortisol concentration. There has been stated that maximum hydrolysis can be achieved by using less incubation time in combination with higher amount of enzyme [75] and higher temperature leading to higher reaction rate [78]. This was observed by Ferchaud et al.[77] that increasing the temperature resulted in less incubation time for more efficient deconjugation. However, other hydrolysis efficiencies are not affected by temperature but rather by the amount of enzyme [77]. There has also been stated that prolonged incubation time can result in decreasing enzymatic activity [76] which is consistent with our findings where amount of cortisol decreased after 24 h incubation. According to our findings, cortisol is presumably mostly bound to sulfate thus, cortisol decrease might be due to declining arylsulfatase activity as reported by Dodgson and Powell[76]. Additionally, prolonged incubation time have proven to be less sufficient when enzymes contain other activities that can potentially destroy steroids as reported by Graef, Furuya and Nishikaze[75]. Therefore, higher yield of cortisol levels can be achieved by altering these two parameters making hydrolysis reaction less time-consuming and thus more beneficial and productive.

4.1.4 Suitable amount of enzyme solution for cortisol deconjugation

The influence of adding different amounts of Helix Pomatia solution on cortisol deconjugation in fish feces samples was studied (Figure 15). The deconjugation of cortisol was highly affected by the increasing amount of enzyme added which is consistent with previous studies [77, 78, 79]. Additionally, another study reported a similar increase for the majority of steroid hormones. However, only small differences were observed when adding 20, 40 and 80 μl [73]. This is also consistent with our findings, by adding 10, 20, 30, 40 and 50 μl only small differences were observed. Furthermore, there was a considerable variation between samples containing 50 μl of Helix Pomatia solution. This might be due to the fact that other glucuronides or sulfates are competing for the available enzyme. One study reported that other compounds hydrolyzed to higher extent when using increasing amount of enzyme [73].

Enzyme concentration is one of the factors that will affect enzyme kinetics. It has been reported by Combie et al.[78] that using higher concentration of Patella Vulgata resulted in higher yield. Therefore, effective deconjugation might be accomplished with less amount of enzyme solution when the enzyme concentration is increased.

4.1.5 Extraction method for cortisol

The cortisol extraction efficiency was evaluated for LLE and SALLE by examining the extent of ion suppression (Figure 16) and percentage of relative extraction recovery (REC%) (Figure 17) achieved from both methods. By adding the ISTD after the extraction, the reduction of ISTD signal response provided information about ion suppression caused by co-extracted matrix components. The peak area of d4-cortisol-4-ABH decreased when we increased the amount of NaCl with MTBE. In comparison, this was slightly observed for butanol and opposite effect for ethyl acetate. Additionally, adding increasing NaCl concentration would be more beneficial for the extraction as the separation factors increase by decreasing the solubility of water into organic phase and nonelectrolyte substances into aqueous phase [80] to induce phase separation [45]. However, NaCl have capabilities to lower water cohesion [81] by binding to water molecules [47] which will interrupt the weak interactions between water to other types of compounds as reported by Wannachod et al.[80]. This might lead to more compounds being extracted into the organic phase (Figure 22) causing more ion suppression and thus reducing the signal response for d4-cortisol-4-ABH.
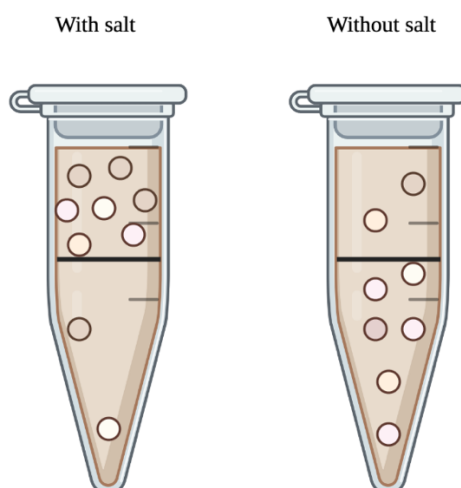
Figure 22: The effect of salt on the extraction method.

Salt affects the extraction by causing interruptions between the weak interactions of water to other types of compounds leading to more compounds being extracted into the organic phase. Illustration created by the author using Biorender.com.

Competing co-eluents for available charge or surface area of the droplet in the interface of the MS detector will cause ion suppression [49] affecting the analysis precision, accuracy and detection capabilities [69]. SALLE as opposed to LLE have the capability of extracting compounds that are more polar [45]. Thus, using propanol or acetonitrile will also influence d4-cortisol-4-ABH peak area by having competing substances present since highly polar compounds have great ion suppression capabilities [49]. Peak area for d4-cortisol-4-ABH was highly affected when using butanol, propanol and acetonitrile meaning that ion suppression from competing compounds have been present. As opposed to MTBE and ethyl acetate, d4-cortisol-4-ABH peak area was less effected meaning less ion suppression occurs especially for MTBE with 100 μl NaCl. Decrease in d4-cortisol-4-ABH peak area for SALLE treated samples might be due to triphasic occurrence. Partitioning is affected by several parameters such as phase composition, salt concentration [46], type of salt [82], temperature [80] and pH modifications [45].

In another study they used acetonitrile with ammonium sulfate resulting as the most sufficient extraction method [82]. Sulfates have the capability of brining a stronger salting-out effect. This is also accurate by the Hofmeister series [46] and explains why $(NH_4)_2SO_4$, $MgSO_4$ [45], $CaCl_2$, K_2CO_3, NaCl, KCl and Na_2SO_4 [46] are the most common salting-out agents. It has also been stated that salt can have a greater influence than the extraction solvent itself on the results [45]. Therefore, further examination would be to maintain a biphasic system by altering these parameters that might show an improvement of ion suppression. Even though SALLE resulted in highest extent of ion suppression this extraction method also proved to gain the highest yield of cortisol. Additionally, by increasing the addition of NaCl altered the REC% levels that showed a slight increase for LLE with MTBE and butanol and decrease for ethyl acetate. However, since the detection limit for the analysis will be affected by these two parameters combined the extraction method containing less ion suppression would be the most profitable.

4.1.6 Mobile phase for ion enhancement

The signal from cortisol-4-ABH mono-hydrazone products was investigated after ESI+ when using two different mobile phases (Figure 18). These mobile phases contained water with 0.2% formic acid- or 0.2% ammonium hydroxide and water with 0.2% methanol. The peak area for both cortisol-4-ABH and d4-cortisol-4-ABH show a proportional increase, which is expected as to their similar structures [48] being presumably equally affected. However, the graphic illustration unexpectedly showed higher signal response for cortisol mono-hydrazone products by using 0.2% ammonium hydroxide in water. The cortisol-4-ABH compound has a basic and hydrophobic (logP > 1) character in water with 0.2% formic acid and 0.2% ammonium hydroxide with pH ∼ 3 and ∼ 11. Since protonation occurs when pH is below pKa by two units [83] and two units above for deprotonation [84], our expectations were opposite of what the results showed. In literature, suppression of ionization can be accomplished when using a pH mobile phase that is 2 units above pKa value [83]. However, these findings are in good agreement with another study performed by Peng and Farkas[83].

According to Peng and Farkas[83] the increase in response with high pH mobile phase might be due to the poorly ion formation when using acidic additives, more sufficient proton generation mechanisms when using basic additives such as droplet desolvation or presence of applicable proton donors. The ammonium hydroxide solution in water will contain small amounts of ammonium ions (NH_4^+) and hydroxide ions (OH^-) and larger amounts of ammonia (NH_3) and water (H_2O) [85]. One assumption would be that NH_4^+ act as the proton donor as concluded by Peng and Farkas[83], creating protonated mono-hydrazone products. The protonation transfer from presumed NH_4^+ proton donor appears to happen in either liquid phase [86] or gas phase [87] leading to the following two equations (Equation 12 and 13) [83].

$$NH_4^+ \ (l) + M \ (l) \rightarrow [M + H]^+(l) + NH_3 \ \uparrow \tag{12}$$

$$NH_4^+ \ (g) + M \ (g) \rightarrow [M + H]^+(g) + NH_3 \ (g) \tag{13}$$
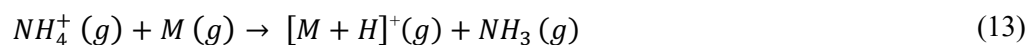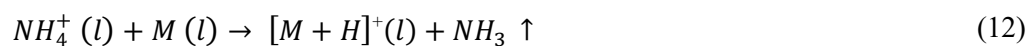
We suggest that proton transfer will appear in highly charged droplets (liquid phase). By applying a positive capillary voltage this will attract all negative ions and repulsion of all positive ions, making each droplet contain presumably hydrazone products, NH_3, NH_4^+, H_2O and methanol. With the addition of nitrogen desolvation gas smaller droplets will reach their rayleigh stability limit hence smaller droplets will be created. This will lead to repulsion and finally ion emission [49] of the protonated mono-hydrazone products onto the mass analyzer as illustrated in Figure 23.
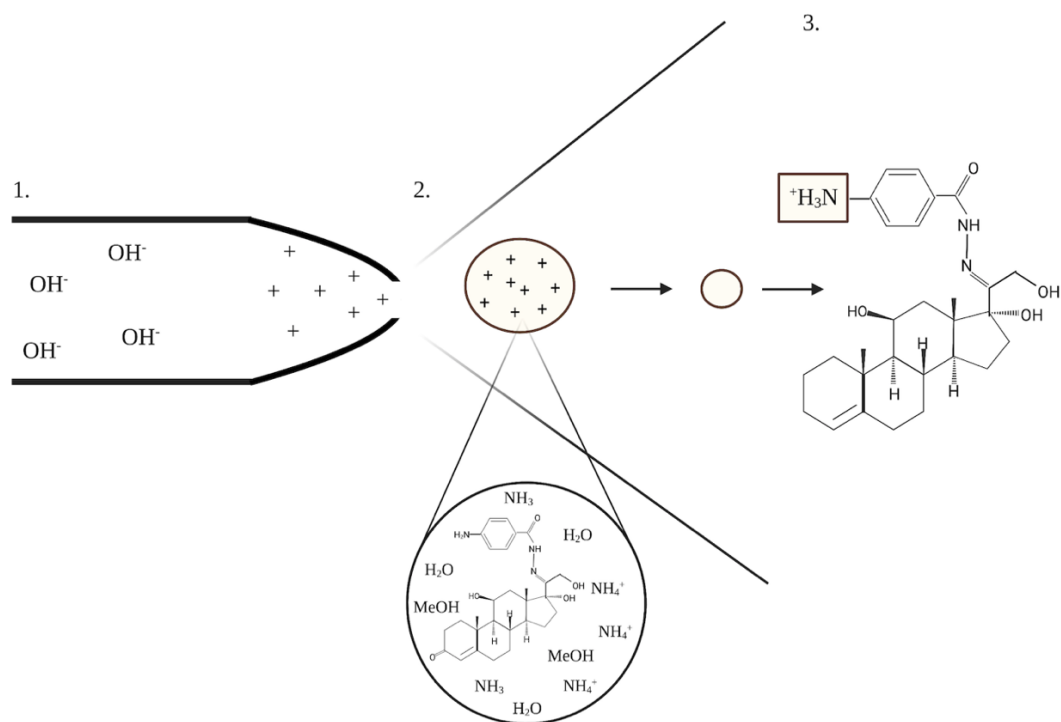
Figure 23: Electrospray ionization working in positive mode (ESI+) with high pH mobile phase.
This simplified ESI+ illustration show the suggested proton transfer mechanism to create protonated mono-hydrazone products when using water with 0.2% ammonium hydroxide solution and water with 0.2 % methanol solution as mobile phase mixture. Applying a positive capillary voltage will attract all negative hydroxide ions (OH⁻) and repulsion of all positive ammonium ions (NH_4^+) (1). This will make each droplet contain NH_4^+ together with ammonia (NH_3), water (H_2O) and methanol (MeOH) (2). With the addition of nitrogen desolvation gas this will lead to smaller droplets and finally ion emission of the protonated mono-hydrazone products onto the mass analyzer (3). Illustration created by the author using Biorender.com.

4.1.7 Liquid chromatography-tandem mass spectrometry conditions

The maximum signal response and transition channels were investigated for underivatized and derivatized cortisol (Figure 19). The 4-ABH hydrazide derivatization reagent was used to produce cortisol mono-hydrazone products by introducing an aromatic amine group. Sensitivity and signal enhancement with ESI+ was then achieved from 4-ABH derivatization procedure with high pH (~11) mobile phase consisting of water with 0.2% ammonia hydroxide. The ESI+ signal improvements when combining high pH mobile phase with 4-ABH reagent, were also reported in another study, where ammonia hydroxide compared to formic acid mobile phase provided signal enhancement [24]. However, several other studies have indicated that ESI in negative mode (ESI-) using gradient elution consisting of formic acid in combination with methanol [88, 89] or acetonitrile [23, 90] provide satisfactory results for analyzing cortisol and/or its metabolites. However, these studies did not include a derivatization step in addition to using different mobile phases, one can therefore assume that mobile phase and analyte structure in LC separations have great influence on ionization capabilities. In our study the most sufficient LC separation for cortisol mono-hydrazone products consisted of gradient mixture of water with 0.2% ammonium hydroxide and 0.2% methanol, that proved to be more beneficial than formic acid due to the derivatization step included as discussed in Section 4.1.6. Even though derivatization proved to have beneficial effect for signal improvements with ESI+ other ionization techniques have proven to be better than ESI such as unispray ionization (USI) in positive mode for cortisol using gradient conditions with 0.1% formic acid in water and methanol containing 0.1% formic acid [91]. Thus, signal enhancement might be improved by using other mobile phases and/or ionization techniques. Despite this assumption, chromatograms gathered from this study showed acceptable signals and peak heights. However, peak splitting was observed as well as peak interference for underivatized samples. The addition of internal standard has, however, created a more precise and accurate method by compensating for the matrix effects as observed in other studies [16].

As previously described ESI+ with high pH mobile phase was found to be most beneficial for the detection of mono-hydrazone products with [M + H]$^+$ as the chosen precursor ion. The product ions gained from CID provided a fragmented cation [M]$^+$ at 119.8 m/z (Figure 19C). This CID ion proved to be the most important product ion for cortisol-4-ABH which is consistent with another study [24]. We believe that most likely the proton donated from NH$_4^+$ will be attached onto the amine group located at the aromatic amine group. Another presumption it that the fragmented cation at 119.8 m/z corresponds to a C7H6NO group thus, bond breaking will occur between a ketone group and amine group. This group will gain a positive acylium ion due to electron delocalization. The MRM transition can be explained by Figure 24. Keeping water with 0.2% ammonium hydroxide and 0.2% methanol gradient mixture with ESI+ was preferred for the transition channels gathered from MRM analysis for cortisol-4-ABH detection.

1.

C28H38N3O5$^+$
Exact mass: 496.28

2.

3.

C7H6NO$^+$
Exact mass: 120.04
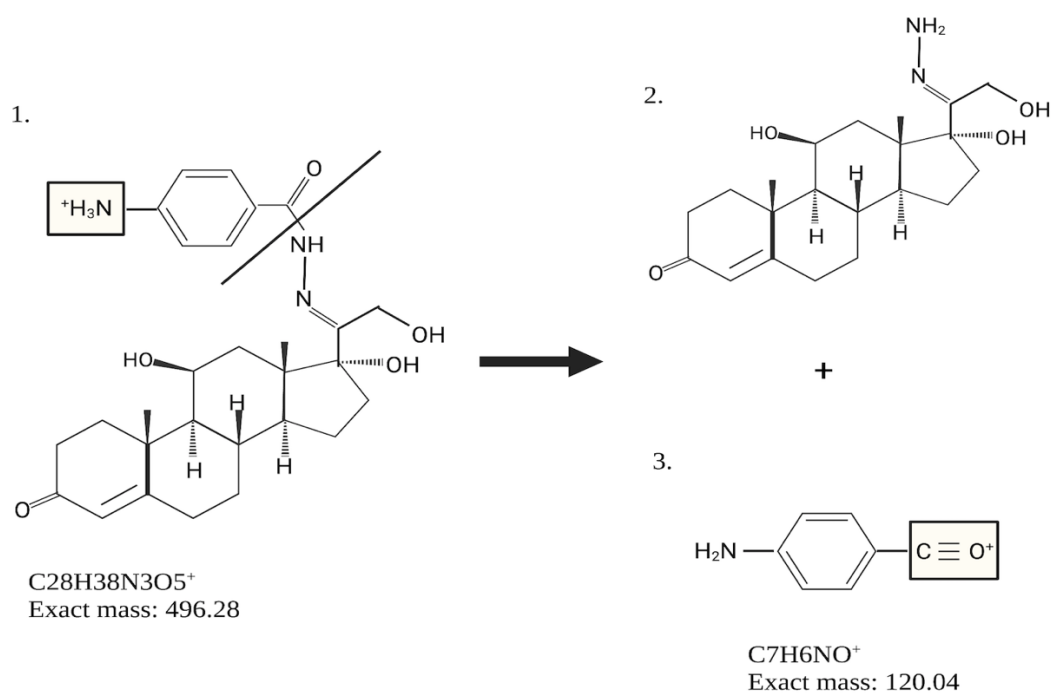
Figure 24: Multiple reaction monitoring (MRM) transitions for derivatized cortisol (cortisol-4-ABH). The protonation of the precursor ion [M + H]$^+$ will most likely occur at the aromatic amine functional group (1). Fragmentation of the precursor ion by collision-induced dissociation (CID) creates a positive acylium product ion [M]$^+$ (2). Additionally, part of the precursor ion will receive a proton by a re-arranging reaction in the collision cell where it is lost as neutral (3). Illustration is created by the author using Biorender.com.

4.2 Method validation

A validation procedure was performed according to Eurachem guidelines [65]. Summarization of optimized validation parameters for cortisol quantification are presented in Table 8. Most of the cortisol concentration levels measured from off- and onshore industrial fish farms were highly above LOD and LOQ values set at 0.04 ng/mL and 0.09 ng/mL respectively (Figure 20). Additionally, the ability to measure cortisol levels at 0.09 –100 ng/mL range makes the developed method well suited for cortisol detection and quantification. The linear regression analysis also provided excellent R^2 values at 0.997 – 0.999. Repeatability and intermediate precision measurements showed acceptable results with CV% above 10.5%. Great accuracy was also achieved for cortisol at low, mid and high concentrations with R´% range between 114 – 127%. The REC% value gathered from different organic solutions used in either LLE or SALLE (Figure 17) did also show excellent recovery values in the range ~67 to ~98%. All of the data points in LJ quality control plot (Figure 21) were within the action limits that represents acceptable performance.

5. Conclusion

The aim of this master thesis project was to develop a high-quality LC-MS/MS method for cortisol quantification in fish feces samples, that were optimized and validated according to Eurachem guidelines. The developed LC-MS/MS method proved to be precise and accurate with excellent LOD, LOQ, linearity and linear range. Investigation on hydrolysis conditions, extraction techniques and derivatization were examined. This showed that Helix Pomatia with 2M ammonium acetate buffer at pH 6 buffer solution for 1 h was most suitable conditions for conjugated cortisol hydrolysis. LLE with MTBE and 100 μl NaCl resulted in less REC% compared to SALLE. However, LLE resulted in much lower ion suppression and therefore the best choice for cortisol extraction. Including a 4-ABH derivatization step provided higher ESI+ response and for practical reasons no longer than 30 minutes was chosen. Signal enhancement was also achieved by using high pH mobile phase containing water with 0.2% ammonium hydroxide instead of water with 0.2% formic acid solution with 0.2% methanol water solution gradient mixture. It is therefore strongly recommended to use high pH mobile phase in RPLC for cortisol-4-ABH as the precursor ion $[M + H]^+$. Fish feces samples gathered from off- and onshore industrial fish farms showed cortisol levels highly above LOD and LOQ. The majority of samples contained 5 – 12 ng/g however, a subset of fish displayed clearly elevated levels about 3 – 4 times higher than the average observed. These differences are much higher than the intermediate precision of the method, and therefore suggest that the developed method could be a useful tool to assess the stress level in fish.

Further work should include optimization for cortisol deconjugation by altering parameters such as pH, incubation time, amount of enzyme, temperature, buffer and enzyme concentration. In addition to improvement of signal enhancement by using other alternatives for mobile phases and ionization sources. Further work should also include extraction improvements of LLE by altering parameters such as phase composition, salt concentration, type of salt, temperature and pH modifications. Further research questions are related to establishment of a normal range for cortisol levels in fishes, that will require more data to be analyzed by the final method.

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Appendix

A1: Materials

A1.1 Compounds

Table 9: Analytes, internal standards and reagent used in this master thesis.

Analyte	Producer
Cortisol	Alfa Aesar
Cortisone	Alfa Aesar
Testosterone	Sigma-Aldrich
Tetrahydrocortisol	TRC
Tetrahydrocortisone	TRC
Internal standard	
D4-cortisol	Sigma-Aldrich
D7-cortisone	Sigma-Aldrich
D8-testosterone	Sigma-Aldrich
Reagent	
4-aminobenzoic hydrazide (4-ABH)	TCI

A1.2 Solutions and solvents

Table 10: Enzyme solutions and organic solvents used in master thesis.

Organic solvent	Producer
99-100% formic acid	VWR
25% ammonium hydroxide	Merck
Acetonitrile	VWR
Ethyl acetate	VWR
Methanol	VWR
n-butanol	VWR
Propanol	VWR
Tert-butyl methyl ether	VWR
Enzyme solution	
Abalonase®	Ango
Abalonase®+	Ango
Helix Pomatia	Sigma-Aldrich
Patella Vulgata	Sigma-Aldrich

A1.3 Equipment's and instruments

Table 11: Lab equipment and instruments used in this master thesis.

Equipment	Producer
0.3 mL micro vial	VWR
50 mL polypropylene tubes	Thomas Scientific
96 polypropylene deep well plates	Thermo fisher
2 mL eppendorf vials	Thermo fisher
Instrument	
Acquity UPLC	Waters
BEH C18 column	Waters
Eppendorf centrifuge	Eppendorf
Eppendorf concentrator	Eppendorf
MassLynx™ V 4.1	Waters
Milli-Q purification system	Millipore
Scanspeed micro centrifuge	Labogene
TargetLynx™ V 4.1	Waters
Quattro premier XE mass spectrometer	Waters

B1: Raw data

B1.1 Sample overview

Table 12: Fish feces samples overview.

Represents the amount of 96 individual fish feces samples measured in gram and cortisol concentration (ng/mL) levels from supernatant.

Vial#	Gram	Cortisol (ng/mL)
A1	0.3501	2.13
A2	0.2880	3.76
A3	0.3451	2.26
A4	0.3420	3.12
A5	0.3994	2.89
A6	0.3534	3.04
A7	0.3077	4.6
A8	0.2628	2.98
A9	0.3476	2.53
A10	0.3116	1.78
A11	0.3182	3.29
A12	0.3610	2.64
A13	0.3395	4.57
A14	0.3042	1.11
A15	0.1910	1.69
A16	0.3359	2.1
A17	0.2420	0.98
A18	0.4345	2.53

A19	0.1513	1.53
A20	0.3324	4
A21	0.3154	4.25
A22	0.2733	3.07
A23	0.3851	3.48
A24	0.3044	1.78
A25	0.2582	4.53
A26	0.2621	3.58
A27	0.3252	2.96
A28	0.2960	2.17
A29	0.2948	3.99
A30	0.2641	1.78
A31	0.4091	3.29
A32	0.3248	1.67
A33	0.2215	1.29
A34	0.3053	1.42
A35	0.3143	1.77
A36	0.3254	2.76
A37	0.2238	0.09
A38	0.3342	2.47
A39	0.2621	2.76
A40	0.3132	3.02
A41	0.2440	1.82
A42	0.2242	2.21
A43	0.4114	2.51
A44	0.2261	1.77
A45	0.3136	2.49
A46	0.2708	2.31
A47	0.3875	5.59
A48	0.2247	2.04
B1	0.3647	1.14
B2	0.2971	2.81
B3	0.3370	10.72
B4	0.3485	1.35
B5	0.2971	1.36
B6	0.3616	2.49
B7	0.3110	1.06
B8	0.3216	3.27
B9	0.3678	2.54
B10	0.2571	2.22
B11	0.2911	1.35
B12	0.5329	2.34

B13	0.2783	1.67
B14	0.3253	2.89
B15	0.3085	2.43
B16	0.2737	1.16
B17	0.3471	2.51
B18	0.3363	3.98
B19	0.4368	2.3
B20	0.4448	3.93
B21	0.3893	3.35
B22	0.4429	1.84
B23	0.3034	2.41
B24	0.3644	1.93
B25	0.3121	1.83
B26	0.2510	1.49
B27	0.3584	3.77
B28	0.2837	2.27
B29	0.3569	1.29
B30	0.2714	4.74
B31	0.3042	7.14
B32	0.3315	6.22
B33	0.2836	7.11
B34	0.2417	2.24
B35	0.3599	2.06
B36	0.4208	6.31
B37	0.3487	2.9
B38	0.3129	2.65
B39	0.2905	1.71
B40	0.3113	9.67
B41	0.2583	1.2
B42	0.2407	1.91
B43	0.3667	1.14
B44	0.3513	1.51
B45	0.3103	4.22
B46	0.2338	2.11
B47	0.3385	1.35
B48	0.4982	1.4