UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Comparative Biomedicine and Food Science

Second Cycle Degree (MSc)
in Biotechnologies for Food Science

Salting-Out Assisted Liquid-Liquid Extraction for Simultaneous Determination of Steroid Hormones and Thyroxine by Using LC-MS/MS: Application on Fish Plasma

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Abbreviations and acronyms

ACN	Acetonitrile
ACTH	Adrenocorticotropic hormone
C18	Octadecyl silica
CMIA	Chemiluminescent magnetic microparticle immunoassay
Conc.	Concentration
CV (%)	Correlation coefficient
ELISA	Enzyme-linked immunosorbent assay
EIA	Enzyme immune assay
ELLA	Enzyme-linked lectin assay
ESI	Electrospray ionization
ESI+	Positive electrospray ionization
HPLC	High performance liquid chromatography
H PI-axis	Hypothalamus pituitary interrenal-axis
HPT-axis	Hypothalamus pituitary thyroid-axis
HSP	Hansen solubility parameters
i.e.,	In essence
ISTD	Internal standard
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MEOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry/Mass spectrometer
NIS	Sodium-iodide symporter

PP	Polypropylene
PPT	Protein precipitation
QC	Quality control
RIA-kit	Radioimmunoassay
SALLE	Salting-out assisted liquid-liquid extraction
SD	Standard deviation
SPE	Solid phase extraction
Tg	Thyroglobulin
TIC	Total ion chromatogram
TH	Thyroid hormone
TSH	Thyroid stimulating hormone
tR	Retention time
TRH	Thyrotropin-releasing hormone
TRE	Thyroid hormone response element
TT4	Total tetraiodothyronine
T3	Triiodothyronine
T4	Tetraiodothyronine
qRT-PCR	Quantitative Realtime polymerase chain reaction
UHPLC	Ultra-high performance liquid chromatography

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Summary

Most biological fluids contain hormones, sometimes in small amounts, whose detection and quantification as biological markers can be crucial. Measuring the level of steroid and thyroxine hormones is a key step to underfunding the overall fish physiology. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) combines the high sensitivity of MS with the significant physical separation capacity of LC, becoming the method of choice for hormone analyses. However, a throughput, fast, reliable, simple, and environmentally friendly sample preparation method is still missing, and research is needed to carefully extract those hormones from the biological sample.

This study aimed to develop a novel salting-out assisted liquid-liquid extraction (SALLE), a method to simultaneously extract L-thyroxine, testosterone, cortisone, and cortisol from human and fish plasma samples. The analysis was performed by using reversed-phase Acquity ultra-high-performance liquid chromatography with the column BEH C182.1x 50mm ID with 1.7 µm particle size (Waters) connected to a triple quadrupole Quattro Premier XE mass spectrometer (Waters) with ESI+ mode and MRM channel for ion monitoring. To achieve high extraction recovery, the performance of four different organic solvents and NaCl and $(NH_4)_2SO_4$, as salting-out reagents, was evaluated. The obtained result shows that SALLE with acetonitrile, as an organic solvent, and ammonium sulfate, as a salting-out reagent, was effective both in terms of reducing matrix interference and increasing extraction recovery. Additionally, acetonitrile with 10% methanol and saturated ammonium sulfate was selected for the current SALLE experiment, as they yield high analyte recovery and less ion suppression.

Finally, the optimized SALLE method was validated according to the Eurachem guide. The method showed high linearity ($R^2 \geq 0.997$) for all hormones. The limit of detection (LOD) and limit of quantifications (LOQ) ranged between 0.01 to 0.18 ng/mL for all hormones. Furthermore, the recoveries of the method were 76% to 95% for total thyroxine (TT4), 55 % to 74% for cortisol, 81 % to 96 % for cortisone, and 90% to 102% for testosterone, respectively. Moreover, the accuracy of the method was calculated as the bias in (RSD%) and found to be < 8% for TT4 and testosterone and <10 % for cortisone. Similarly, both repeatability and reproducibility of the method were found to be (RSD%) <15% across all hormones. Finally, the developed and validated method was applied to the fish plasma samples and the obtained results were good for all hormones. This thesis finding provides a significant contribution for FishLab AS and other laboratories aiming to identify biomarkers of stress, smoltification, and maturity in fish plasma samples.

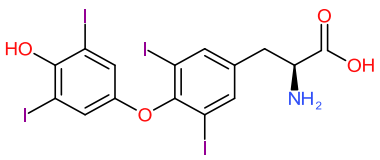
1. INTRODUCTION

1.1 Scope and aim of the study

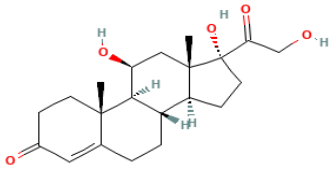
The small startup company FishLab AS is establishing biomarkers to identify stress, smoltification, and sexual maturity in fish through measuring cortisol, cortisone, thyroxine, and testosterone both in fish feces and blood by using liquid chromatography-tandem mass spectrometry (LC-MS/MS). This is to reduce long-lasting and repeated stress monitor the stage of maximum seawater tolerance, and fish sexual maturity for harvesting. Hence those hormones mainly play a crucial role in maintaining the immune system, smoltification process to optimize fish harvesting. Although LC-MS/MS is the ideal method for simultaneous quantification of hormones, simple and efficient sample preparation is needed. While SALLE was reported as a simple and efficient type of sample preparation, there is no study reported regarding steroid and thyroxine hormone extraction through this technique in both human and fish plasma. Based on this fact the aim of the current study was (i) to develop and validate sensitive and selective LC-MS/MS method for simultaneous quantify of steroid hormone and L- thyroxine in plasma sample (ii) to develop a quick, reliable, and reproducible SALLE method of sample extraction for the simultaneous extraction of steroid hormone and L- thyroxine in plasma sample (iii) to verify the application of the method in fish plasma samples.

The method was developed and validated on commercial quality control human serum samples due to the limited availability of fish plasma samples enough for all experiments. Additionally, the assigned concentration value of the quality control sample was used to assess the accuracy of the novel method. In this study we measured total concentration levels of steroid and thyroxine hormones.

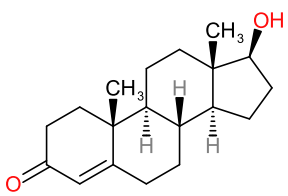
L-thyroxine



Cortisol



Testosterone



Cortisone

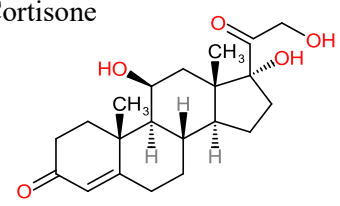


Figure 1: shows the molecular structure of targeted hormones, captured from https://pubchem.ncbi.nlm.nih.gov/compound/ L-thyroxine testosterone, cortisol, and cortisone

1.2 Fish welfare

Fish live in different ecosystems according to their habitat and diet. Humans interact with this process in different ways causing an effect on fish welfare to a certain extent (Berlinghieri et al., 2021). Feelings, health, and function are the main issues to be considered welfare (Ellis et al., 2012). Taking into consideration these three categories, animal welfare can be categorized from good to very poor. Though welfare is not an easy word to define and assess according to Stevens et al. (2017), good welfare can be defined as an absence of pain and fear, with five freedom. Thus, the five-animal freedom comprises quality of life such as freedom from hunger thirst, discomfort, pain, injury, and disease. Indeed, welfare can also be defined as the ability of the animal to cope and acclimate to its environment without being forced beyond its physical capacity. Accordingly, welfare can be measured and evaluated (Segner et al., 2012; Volpato, 2009). Behavior represents the reaction to the environment as fish perceive it and is, therefore, a key element in fish welfare. This includes good production and productivity according to Martins et al. (2012). Welfare in farmed fish, using both function and feeling-based approaches, indicates foraging behavior, ventilation activity, and aggregation. The consequences of good or poor welfare result in fish behavioral change, thus understanding this alternation can be useful to develop indicators of fish welfare, which requires adequate nutrition, good health, appropriate water environment, the expression of species/specific behavior, and safety (Kristiansen & Bracke, 2020; Størkersen et al., 2021).

Understanding fish feelings (e.g., stress) is important in monitoring their welfare and functioning. Stress farming conditions may weaken the welfare of the fish, which affects the health status. Consequently, it favors disease as well as lowers the performance of fish (Martins et al., 2012). Although farmers are conscious of the meanings of fish stress, they have often inadequate understanding related to both basic biological principles of animal stress and how it is associated with animal welfare. Likewise, in a commercial well designed practicable fish farming system, it is common that fishes may be subjected to some level of not avoidable stress, which includes environmental factors with combined diet and husbandry (Kalamarz-Kubiak, 2018; Luis O.B.Afonso, 2020).

1.3 Hypothalamus- pituitary-interrenal-axis in fish

Hypothalamus-pituitary-adrenal-axis (HPI-axis), which is the main connection between the endocrine and nervous system in mammals, is equivalent to the hypothalamus-pituitary-interrenal-axis in fish. The main component of the neuroendocrine system gives the response to stress and allows animals to maintain physiological homeostasis through adaptation (Mommsen et al., 1999; Sheng et al., 2021). In the brain, the hypothalamus controls the adrenal cortex, the liver, the thyroid, and the gonadal gland. The adenohypophysis mediates other endocrine glands

and non-endocrine targets through the secretion of hormones (Norris & Hobbs, 2006). In teleost, the HPI-axis regulates the internal cell of the head kidney, which is involved in the secretion and release of the major corticosteroid, called cortisol (Kalamarz-Kubiak, 2018). Thus, under stressful conditions fish launch neurochemical signaling such as releasing cortisol into the blood circulation via the activation of the HPI axis (Aerts et al., 2015).

1.3.1 Stress and Stress response in fish

The concept of stress in fish by Schreck & Tort (2016) clearly describes that the life of fish is filled with facing, coping with, overcoming, and recovering from threatening challenges resulting in a physiological cascade of processes that help the organism react to the stressors. In the same way, stress is a behavioral, physiological comeback to the regarded stressor, which can be chronic or adaptative and can damage health, leading to poor reproduction (Stevens et al., 2017).

Authors define the term stress in different ways. Stress is the state at which an environmental insultation requirement overcomes the natural regulation capacity of the organism (Laberge et al., 2019; Mommsen et al., 1999). Thus, it is an avoidable condition that demands a capacity for maintaining stressors. Schreck and Tort (2016) defines stress as "*a physical cascade that arises at the time the organism is trying to resist death or adaptive response to regenerate homeostasis norm in face of stressor*". Similarly, stress is sometimes described as a physiological response of fish and other vertebrates to threatening situations or stressors (Schreck & Tort, 2016; Wang et al., 2020). This threat can range anywhere from very short life (acute) to permanent (chronic). Indeed, fish can be exposed to stress naturally and/or to biotic stressors (e.g., exposure to predators) (Iwama et al., 1999). Moreover, they are vulnerable to the extensive human-made abiotic stressors at different stages of their life, ranging from aquaculture (e.g., during caught in the net) to removing them from water for laboratory purposes, overcrowding in which small fish are particularly susceptible due to the dense stock. In addition, markets, disturbing water quality (e.g., pH, temperature, salinity, and low dissolved oxygen), disease, and nutrition can be considered as abiotic stressors (Kalamarz-Kubiak, 2018; Schreck & Tort, 2016; Stevens et al., 2017).

Therefore, a physiological stress response to threatening conditions forces them to save energy to overcome and survive stressors, which results in an effect on the entire body. As a consequence, the negative response contributes to the development of disease and pathogens, potentially impairing fish reproductive ability and metabolism (Kalamarz-Kubiak, 2018; Sadoul & Geffroy, 2019). Consequently, this response is introduced immediately following the perception of the stressor, which then leads to eustress (adaptive response) or its consequences (distress) (Wang et al., 2020). The dynamics of response, which result in different severity of

stressors, vary within and among species. Indicators of variation can be found through genetics and nutrient availability or another environmental factor (Wagner et al., 2016).

The physiological stress response then includes the communication between tissues and cells that can occur via the central nervous system through the release of chemical messengers (namely, hormones) from the endocrine system (Barton, 2002). According to Laberge et al (2019) and Wu et al (2017), the changes in the endocrine system including measurable levels of corticosteroids are the primary physiological response to stress, followed by the production of glucose from metabolism via gluconeogenesis, glycogenolysis, and lipolysis. This process results in a change of behavior and may overwhelm survival (Barton, 2002; Wu et al., 2017). The stressor, which brings physiological response in the HPI-axis is measurable through physiological variables, such as the increased plasma levels of cortisol, as it comes from adrenocortical cells and of catecholamine (e.g. epinephrine and norepinephrine arising from chromaffin cell) (Norris & Hobbs, 2006). The overall understanding of fish stress physiology is of great importance to providing good welfare and for secure good productivity in aquaculture (Pankhurst, 2011).

1.3.1.1 Cortisol and stress in fish

Stress in fish can be detected through different physiological, psychological, and behavioral responses (Stevens et al., 2017). Apart from the complexity of the stress response process, the involvement of the HPI-axis through neurochemicals includes the secretion of cortisol (Tort et al., 2019). Thus, measuring cortisol is the most commonly used method to evaluate stress in fish (Carbajal et al., 2019). Additionally, the cortisol concentration is an additional natural information about the fish welfare condition. Thus, cortisol gives suggestions on natural reactions against stressors, which can be acute or chronic stress responses. The physiological stress response variation within fish species, reproductive status, and sex results in a diverse amount of cortisol, corticosterone, 11-deoxycortisol, and cortisone, which are core components of corticosteroids in fish plasma (Cao et al., 2017; Milla et al., 2009).

All physical, chemical, and further perceived stressors provoke a primary, secondary and whole animal response in fish, which are all achieved through the activation of the HPI-axis (Barton, 2002; Lupica & Turner, 2009). In most scenarios, this activation results in efficient responses towards the stressor via activation of the physiological stress response. Initially, neuroendocrine response to stressors has been readily measured via the concentration of cortisol, and this can alter fish behavior, marking physiological and brain functions (Sundh et al., 2010).

1.3.1.2 Cortisol as a biomarker of stress in different fish matrix

Although behavioral stress evaluation such as scratching body surface, change in color, clamping fin, social isolation, and grasping, the interpretation and the evaluation of the stage of stress in fish are subjective and difficult to interpret (Stevens et al., 2017). Numerous studies have shown that the fluctuation of cortisol in different matrix is directly related to stress and fish welfare (Table 1)**.** Moreover, the most used method to analyze cortisol, like a fish stress response, is measuring its concentration in the circulating blood.

Plasma: plasma sampling is a relatively invasive method and a primary indicator of acute stress. As the concentration of cortisol increases soon after facing stress, circulating cortisol can be primally used to evaluate stress (Uren Webster et al., 2020)). Thus, plasma cortisol concentration is influenced by acute stressors like handling before sampling, which constantly elevates the production of glucocorticoid (Poltronieri et al., 2009). Moreover, the cortisol concentration in the plasma of infected Atlantic salmons is directly associated with the severity of the acute outbreaking disease to which salmons are exposed (Olsen et al., 1995). However, the plasma cortisol concentration falls back during chronic stress (Mommsen et al., 1999). Specifically, in salmonoid unstressed fish, the level of cortisol is 0-5 ng/ml. Indeed acute stress elevates the concentration to 40-200 ng/ml and falls to nearly 10 ng/ml in chronic stress, indicating that plasma cortisol cannot be a good marker for chronic stress (Pickering & Pottinger, 1989). Furthermore, plasma cortisol does not show variation either for chronically stressed fish or for non-stressed fish in the absence of additional acute stress (Samaras et al., 2021).

Homogenate of whole body and egg: The internal tissue from unstressed fish is more sensitive to ACTH than that of the stressed one, showing that ACTH in the circulation plays a vital role in modulating stress by regulating HPI responses (Mommsen et al., 1999).

Mucus: Like other vertebrates, fish exposed to stressors do produce hormones and another peptide by mucosal tissues to mediate the stress situation. Hence, those products will activate the HPI axis and result in neuroendocrine response at a systemic level, until the disappearance of the stressor. Nevertheless, the specificity of the stressor and fish species makes a variation in time of the activation of HPI and also, by consequence, of cortisol response, which is the end product of HPI-axis (Alsop & Aluru, 2011; Stevens et al., 2017b). Recently, skin mucus measurements, a new alternative to blood ones, are gaining attention due to their suitability for farmed fish and their application to free-ranging animals, which would offer many advantages (Peter, 2011). However, cortisol found in the skin mucus is very low in comparison to cortisol in plasma and faeces samples (Uren Webster et al., 2020). Hence the examination of mucus

cortisol is an important indication of chronic cortisol and evaluation of fish welfare but has some technical challenges during sampling (Sadoul & Geffroy, 2019).

Faecal: Even though, the amount of cortisol in faeces is quite low, this measurement is less invasive and directly corelated to the plasma concentration if the sampling method includes the avoidance of cortisol degradation, contamination, and the effect of gut microbial (Sadoul & Geffroy, 2019). Also, cortisol, together with its metabolites, can be available and measurable in fish faeces (Figure, 2). Therefore, measuring cortisol in the faecal sample is important to monitoring stress in fish (Lupica & Turner, 2009).

Gut microbiome: Uren Webster et al. (2020) found that there is a strong correlation between gut microbiota and measured cortisol. They indicated that the elevation level of fecal cortisol resulted in a change in the diversity of the fecal microbiome, which reduced the composition of *Carnobacterium* sp. and increase Clostridia and Gammaproteobacteria in juvenile Atlantic salmon after two weeks of exposure to a mild confinement stressor.

Water: Poor water quality is the most important environmental stressor in fish life (Stevens et al., 2017). Cortisol can be detected by sampling water to measure excreted steroids (Blackwell & Ankley, 2021).

Scales: scale cortisol plays a crucial role as a non-invasive method in expressing chronic stress in fish. A study conducted by Laberge et al (2019) indicated that the exposure of fish to air for a short period did not influence scale cortisol. On the other hand, a high cortisol level in scale was determined with delay for about one week and differences in cortisol amount across the body of the studied fish. Thus, unlike plasma cortisol concentration, scales cortisol shows a significant difference for chronically stressed and non-stressed fish (Samaras et al., 2021).

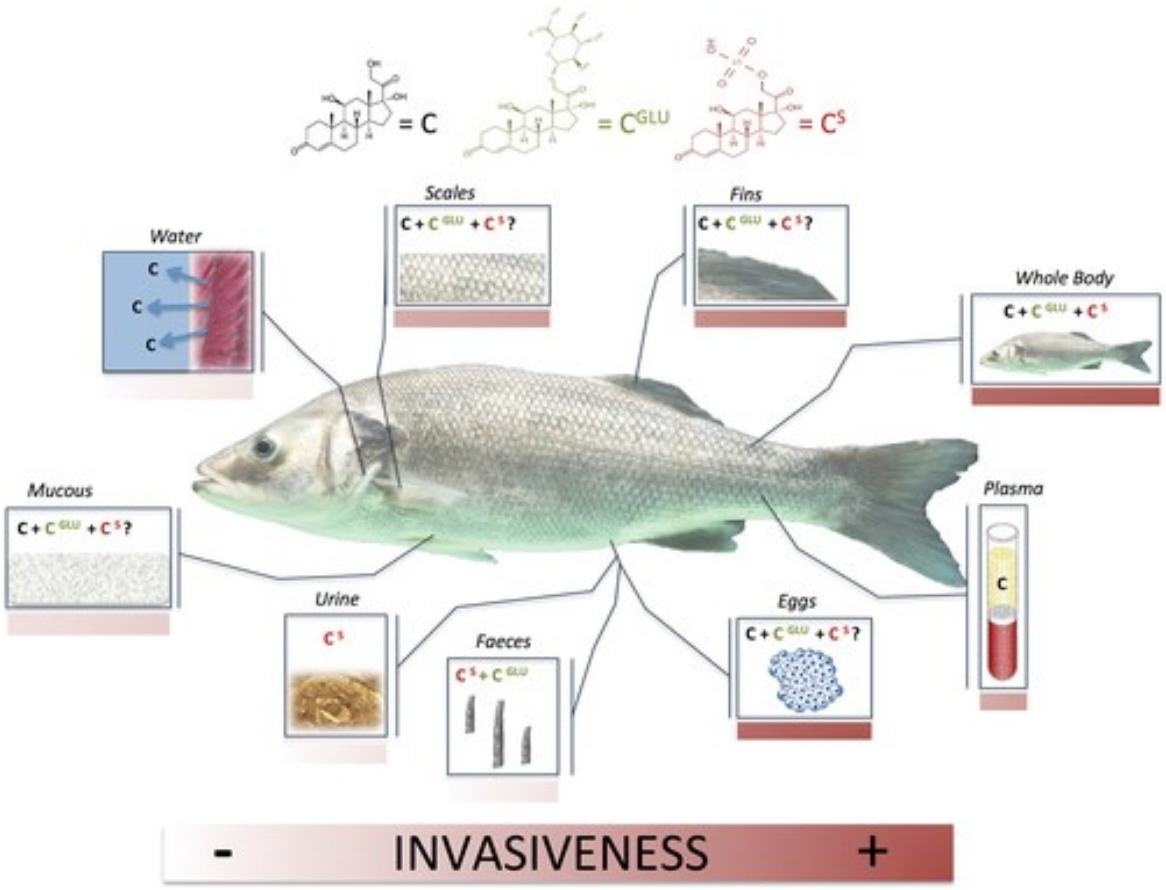


Figure 2: Shows cortisol in different parts of the fish body, the – and + sign indicates the level of invasiveness of the method. C=(cortisol), C^{GLU}=glucuronidase, C^{S}= cortisol sulphated (Sadoul & Geffroy, 2019).

1.3.2 Cortisol biosynthesis, secretion, and metabolism

Steroid hormones are synthesized from a cholesterol backbone and enzymatically transformed into various classes of steroids. Hence, steroids are the major group of signaling molecules of the endocrine system (Boggs et al., 2016; Sharma et al., 2018). In teleost, corticosteroids are synthesized inside the anterior part of the kidney of the internal tissue (Milla et al., 2009). Those cells can be found in layers, strands, and cords around the walls of posterior cardinal veins and their branches running through the head kidneys (Mommsen et al., 1999). Also, the steroidogenic cell (i.e., inter renal cells) is close to the chromaffin cells, forming small clusters at the head of kidneys along the posterior cardinal vein (Figure, 3). As in other mammals, cortisol biosynthesis in fish consists of many enzymatic microsomal pathways (Mommsen et al., 1999; Reid et al., 1998). Glucocorticoids (GC), the main stress hormone, mediated this process by being mimicking cortisol. Up-on stress, GC levels increase and reach the peak from basal concentration very rapidly to cope with acute stressors (Reid et al., 1998).

It is known that ACTH is the primary secretagogue for cortisol. However, numerous other hormones can indirectly modify cortisol secretion by modulating the ACTH-induced steroidogenesis. The stressor activates the brain's sympathetic chromaffin cell axis. Thus, it releases catecholamine, which is responsible for delivering the messages to the hypothalamus to release corticotropin, which, in turn, releases from the HPI axis (Figure 3). This makes the anterior pituitary secreting adrenocorticotropin hormone. This hormone leads to the secretion of cortisol in the blood circulation from the head of the kidney ((Kalamarz-Kubiak, 2018)). Accordingly, cortisol secretion is governed by HPI-axis (Mommsen et al., 1999). For example, both ACTH released from the pituitary cell and the internal cell of the head kidney is stimulating the release of corticosteroids and regulating the secretion of catecholamine in rainbowfish (Reid et al., 1998).

Because of its lipophilic nature, cortisol will be up taken into the cell via passive diffusion. Upon entering the cells, cortisol will bind to receptors, leading to its activation and metabolism. The metabolic clearance of cortisol from blood represents the net effect of tissue capacity for cortisol uptake and catabolism. Environmental factors such as stress, nutritional state, salinity can modify the clearance of cortisol (Stevens et al., 2017). Additionally, the cortisol effect on the target cell is mediated by genomic and nongenomic pathways (Kalamarz-Kubiak, 2018).

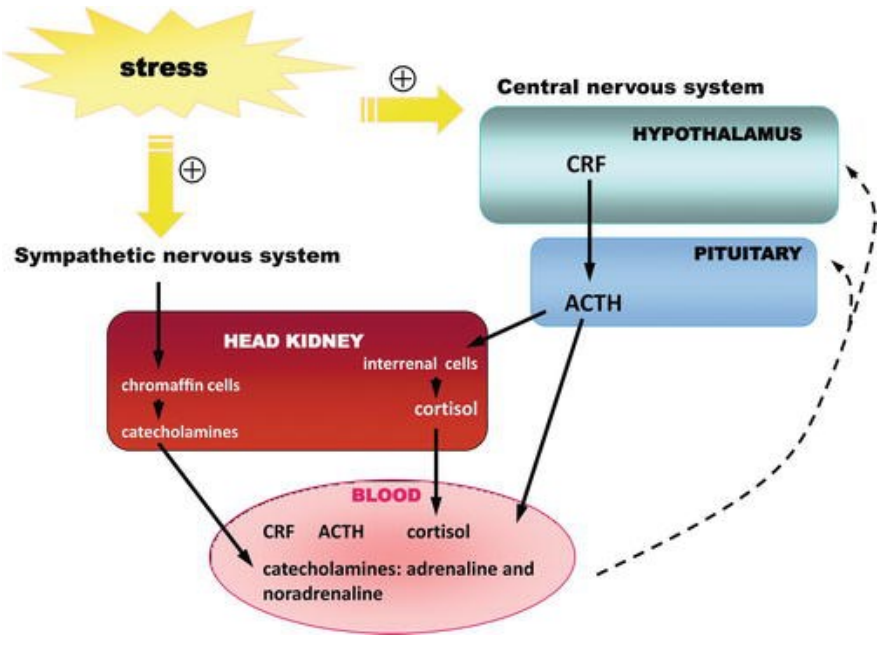


Figure 3:Activation of Hypothalamus pituitary internal-axis (HPI-axis) and releasing of cortisol in plasma circulation during stress in fish. CRF=(corticotropin-releasing factor) ACTH=(adrenocorticotropin hormone) (Kalamarz-Kubiak, 2018*)*.

1.3.3 Role of cortisol in fish

Cortisol, plays a vital role in osmoregulation, growth, reproduction, and metabolism and in the production of energy from glucose through the activation of the central nervous system to fight stress (Sharma et al., 2018; Wu et al., 2017). Krasnov et al (2012) and Thang et al (2017) indicate the role of cortisol in addressing all the functions of glucocorticoid, which include energy metabolism, gill epithelium cells proliferation, differentiation, control infection, and interaction between host and parasite through healing and repairing mechanism in Atlantic salmon skin.

Even though cortisol is brought by stress, its concentration is directly proportional to smolting as the fish expressions are not able to do osmoregulation and ion secretion. This happens because the passive movement of ions through the gill leads to ion losses and a decrease in plasma ion concentration for fish in saltwater (Stewart et al., 2016). According to Sundell et al (2003), finding the level of cortisol dramatically increased when fish migrated to salinity. Glucocorticoids such as cortisol, cortisone, and corticosterone in pacific salmon plasma rise at the time of smoltification (Stevens et al., 2017).

Table 1*:Cortisol as a biomarker of stress and detection method in different fish matrix*

Fish Matrix	**Method**	**Species**	**Exposure**	**Outcome**	**Reference**
Plasma	UPLC-MS/MS HPLC	*Tilapia spp*	Air exposure Exposure to lead and arsenic	Direct effect on bacterial cells by changing the phenotype of biofilm to heavy metal reduces cortisol level at treatment level and increases during recovery.	(Declercq et al., 2016) (Thang et al., 2017)
	CMIA ELISA	*Scophthalmus Maximus*	Dietary arginine and L-tryptophan supplements	Reduction of plasma cortisol and enhancement of innate immunity. Mediation of cortisol response and negative feedback.	(Costas et al., 2013) (Cabanillas-Gámez et al., 2018)
	RIA kit	Smallmouth bass		The endocrine stress response is affected by reproductive status when energy demand is higher.	(O'Connor et al., 2011)
	LC-/MS/MS	Female fat head minnows *Japanese medaka*	Water born fadrozole		(Blackwell & Ankley, 2021b)
	Transcriptome	European Sea bass	Handle net for 5minute	Hepatitis enzyme regulator due to various metabolic and	(Samaras et al., 2016)

				immunological processes.	
	EIA	Nile tilapia	Air, nitrate		(Wu et al., 2017)
Whole-body	ELLA	*Rhamdia Quelen*	Fluoxetine exposed		(Abreu et al., 2016)
	EIA	Small-sized fish	Handling by net		(Guest et al., 2016)
	ELISA	Zebrafish	Copper containing water	Stress.	(Pompermaier et al., 2021)
	UHLC MS/MS and RIA	Largemouth bass -fathead minnow -Zebrafish -*Juvenile silverside)*		The whole tissue homogenate is an alternative when the plasma sample is insufficient.	(Nouri et al., 2020)
	UPLC- MS/MS	Zebrafish		Impaired tissue growth and altered cortisol level.	
Scale	(RIA)	Goldfish		The rate of accumulation and clearance of cortisol (Crt) is much slower on the scale.	(Laberge et al., 2019)
Fecal	ELISA	Female		Non-invasive stress assessment.	(Lupica & Turner,

	HPLC	Parrotfish			2009)
Mucus	ELISA	Meager (*Argyrosomus regius*)	Hypoxia and netting with dietary Trp and Asp	Tryptophan mitigating acute stress. Asps elevate plasma and skin crt.	(Herrera et al., 2020)

1.4 Thyroid hormone and fish physiology

Like in other vertebrates, fish hypothalamic pituitary thyroid axis governs the releasing of thyroid hormone (TH). The thyroid gland is an enclave and diffused in other organs depending on the fish sp. (e.g. heart, cephalic kidney, and pharyngeal region) for many teleost's, with an exception for Lophiiformes and Tetraodontiformers in which the thyroid gland is encapsulated in a blood sinus (Blanton & Specker, 2007; Geven et al., 2007). The main function of the HPT axis is to regulate the thyroid gland which is responsible for the production of two essential hormones: triiodothyronine (T3) and tetraiodothyronine (T4) (Domenech-Coca et al., 2019; Rousset et al., 2000). The regulation of growth and secretion of the thyroid is mediated by thiotrophs at the cell level and adenohypophysis as a part of the pituitary. The availability of TH in the body will upregulate the secretion of thyroid-stimulating hormone (TSH) from the pituitary gland. In addition, the hypothalamus will block the production of TSH by regulating the production of thyrotropin-releasing hormone (TRH). In contrast, the anterior pituitary produces TSH to organize the function and morphology of the thyroid gland (Kim et al., 2018).

1.4.1 The role of thyroid hormone in fish

Although the thyroid gland acts differently depending on the animal, its main function is related to the maintenance of homeostasis (Deal & Volkoff, 2021a). As investigated by many researchers, the endocrine response system, and particularly the thyroid axis, regulates THs (T4) and T3, which have a similar function in different animal species. For instance, during their lifetime, THs play an important role in many biological processes like growth and differentiation, metamorphosis, reproductive events (Deal & Volkoff, 2021a; Tovo-Neto et al., 2018), regulation of metabolisms, and maintenance of the body homeostasis (Tanoue et al., 2018).

The thyroid axis senses and translates signals to maintain physiological conditions. In fish, this system has a significant part in the processes of metamorphosis and environmental adaptation (Taillebois et al., 2011). In addition, THs have a key role in the development of the nervous system, for continuous energy production and thermogenesis. Moreover, the THs balance body fluids, altering nutrient metabolism in the liver (Chen et al., 2018; Ortiga-Carvalho et al., 2016).

1.4.1.1 Role on fish metamorphosis

Practically in all fish species, THs are involved in the process that accompanies the transition from larvae to juvenile metamorphosis. During this process TH synthesis in larvae takes place in an endostyle that transforms thyroid into vertebrate thyroid tissue (Chen et al., 2018; Power et al., 2001).

In fish the aquatic life after embryonic development demands a complex life cycle. Thus, they need several different stages to colonize the diverse ecological niches to complete the final adult stage (Alibardi, 2019). Metamorphosis in fish is a process in which post-larvae undergo different anatomical, behavioural, and physiological changes for migration from freshwater to seawater, and vice versa. These processes support them to change feeding habitat and to take over the juvenile and adult freshwater environment. In post larva upon treatment with T4, the change in position of the mouth was significantly faster, suggesting that THs contributes to the true metamorphic event at the time of migration into the river (Taillebois et al., 2011). Moreover, from a study on the zebrafish exposed to triclosan (i.e. environmental toxicant that lowers TH levels), the rate of overall growth and metamorphosis was slightly declined the distance between pelvic and anal fin, which governs the speed of swimming and growth performance in female zebrafish (Stenzel et al., 2019). The same author stated that delay in metamorphosis's differentiation ratio weakened the reproductive ability upon the interruption of THs. In *Paralichthys olivaceous* larvae treatment with exogenous thyroid hormone has provoked premature metamorphosis (Sharma et al., 2018; Yu et al., 2019).

In flatfish, eye migration takes place during the process of metamorphosis by cell proliferation. This is under the expression of prolactin (PRL) protein that involves cell proliferation during eye migration and fin development at early stages. During metamorphosis this protein interacts with THs, inducing a signal pathway and the transcription of the prl gene, which is mainly expressed in the pituitary, brain, and intestine (Si et al., 2021). In similar species, THs exert their action on the change in body height during metamorphosis at the time of post-hatching development (Xu et al., 2016). The dietary tyrosine supplementation, which is the precursor of THs, significantly increases the metamorphosis process in *Senegalese sole*. (Pinto et al., 2010)

1.4.1.2 Role on shape and differentiation in fish

The well-studied zebrafish confirmed that the signal distribution of T3 and T4 has a notable role in bringing body shape in body height to length ratio. At the time of post-hatching, THs induce cell development in the body specifically at the dorsal edge and ventral edge (Xu et al., 2016). During amphidromous in *S. lagocephalus* fish feeding habits decreased due to changes in ecological habitat and the shift in the type of diet. This natural process forced them to modify the position of their mouth). Overall T4 accelerated the morphological changes of the mouth at the stage of metamorphosis to engulf the plankton plants. (Taillebois et al., 2011).

1.4.1.3 Role on fish reproduction

In many vertebrates, THs alone and cross-link with other endocrine hormones are well known in the regulation of fish reproduction through different mechanisms. THs regulates gene expression that takes part in gonadal sex differentiation and also controls Sertoli cell proliferation to diverge testicular development during different fish life stages (Tovo-Neto et al., 2018). Another study by Stenzel et al.(2019), confirmed that the disruption of THs by triclosan has a significant impact on fertility on all stages including egg production, fertility to development, and survival in zebrafish. In addition to this, a study conducted on rainbow trout clearly showed that T3 by itself and its combination with estradiol (E2) has a significant effect on growth and development in a dose-dependent manner in rainbowfish (Hegeman & Marlatt, 2021).

1.4.2 Thyroid hormone biosynthesis, secretion, and metabolism

Thyroid hormone production and storage depends on the thyroid follicles, which derive from the arrangement of thyroid epithelial cells (Fong, 2015). In many teleosts, THs cascades consist of three main steps. In the beginning, the central HPT-axis is initially responsible for biosynthesis, secretion, and maintenance for a given physiological state. In fish, the iodine from feed and water is up taken through gills, where it is oxidized to iodide and accumulated in follicular cells. Iodine constitutes 65 % of T4 and 58% of T3. Unlike other organisms, fish have a tremendously high vascularized gill surface and a subdivision of iodine pump that is capable of effectively absorbing iodine from water (Blanton & Specker, 2007). Thyroid follicles are responsible to pin down iodine through the protein called sodium iodide symporter (NIS) across the basolateral plasma membrane of the thyrocyte to produce thyroglobulin (Tg), the most abundant protein in the thyroid gland. Thyroglobulin has tyrosine residues for thyroid hormone synthesis. This step requires the presence of iodide peroxidase, which oxidizes iodide in the presence of H_2O_2 (Deng et al., 2021; Fong, 2015). The digestion of Tg in the lysosomes and endosomes by proteases results in the release of T4 and T3 into circulation. Every stage of synthesis and release of TH is stimulated by TSH, which is again regulated by TRH (Figure 4). Conversely, to maintain a normal physiological state, an excess amount of THs in circulation controls the secretion of TRH and TSH from the HPT axis via a negative feedback loop (Ortiga-Carvalho et al., 2016).

Secondly, the biological active hormone (T3) availability becomes peripherally controlled in the liver tissue, indicating the mono-deiodination of T4 on the outer ring, which makes T3 enter the peripheral tissue (Eales & Brown, 1993). This process is catalysed by 5` deiodinases (D1 and

D2) enzymes. On the other hand, T4 is also converted to an inactive form of T3 (rT3), which is excreted from the body by 5` deiodinases (D3) enzyme (Ortiga-Carvalho et al., 2016).

Finally, the synthesized T3 exerts its effect on a target cell, which regulates growth and development, metabolism, and reproductivity by being mediated via TR (Blanton & Specker, 2007). Thyroid hormone receptor can regulate gene expiration through the thyroid hormone response element (TRE), which is composed of repeated DNA sequences, (Terrien & Prunet, 2013). T3 binds to TR within a target cell at the site of the T3 target gene promoter. Thus, the binding of T3 to nuclear receptors regulates hormones through negative feedback, by amplifying their signal and activation in a tissue-specific manner (Figure, 4). Additionally, T3 can act extracellularly and non-genomically through non-nuclear TR and differ between fish species (Deal & Volkoff, 2021a). Furthermore, in fish plasma, several THs distributer proteins bind to TH and create its delivery or distribution. Zinc is a crucial factor in assisting the achievement of high TH binding to transthyretin especially in lower fish (Yamauchi, 2021).

Although some T4 is deiodinated to T3, TH is finally deiodinated by exerting its effect on metabolism in the cell and the iodide is recycled and excreted in the kidney (Eales & Brown, 1993). Thus, TH metabolism includes all biochemical reactions of iodothyronine and THs (Köhrle, 2019). Glucuronidation, sulfation, and deiodination are the main pathways of THs metabolism. Deiodination enzymes, namely iodothyronine D1, D2, and D3, play a vital role in catalyzing those pathways. The formation of T3 from T4, and further conversion of rT3 to T2, is under the effect of D1 and D2, whereas D3 breaks down T4 to rT3 and T3 to T2 (Peeters & Visser, 2000). Unlike other mammals, in fish only T3 conjugates TH that has undertaken the desulfation process. Thyroglobulin and rT3 are not undergoing this process (Finnson & Eales, 1999).

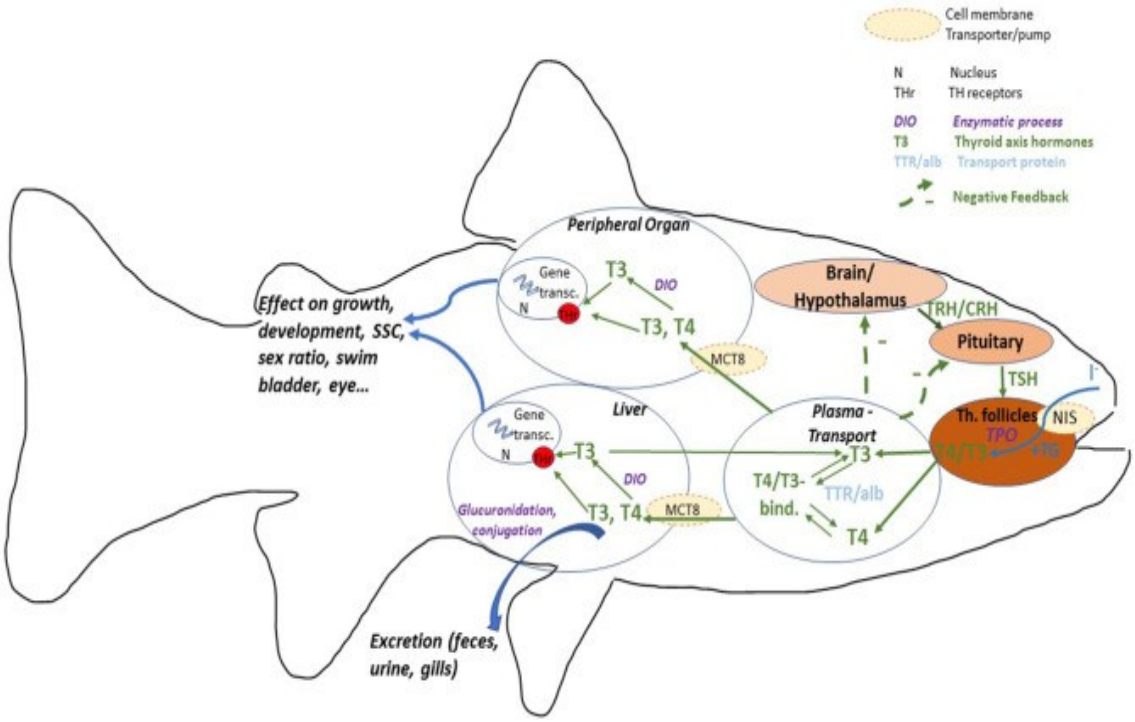


Figure 4: Thyroid hormone biosynthesis, secretion, and metabolism.

HPT (hypothalamus-pituitary-thyroid axis) produces TH (thyroid hormone), which controls the production, metabolism, and receptor-mediated effect of T3 (triiodothyronine) on the target cell. NIS (sodium-iodide symporter) is trapping the iodine up taken through the gill to produce TG (thyroglobulin) which is the precursor of T4 (thyroxine) and T3 under the control of TSH (thyroid-stimulating hormone) regulated by TRH (thyrotropin-releasing hormone) or CRH (corticotropin-releasing hormone. TTR (transthyretin) and Alb (albumin) are the major binding protein in fish. Deiodinases (DIOs) are responsible to activate and deactivate TH, which is then transported by MCT8 (monocarboxylate transporter8), which also assists THs to bind the TR (thyroid receptor to be activated (Dang et al., 2021).

1.4.3 Enzymatic regulation of thyroid hormone in fish

The THs homeostasis regulation turns out to be further complex due to the extrathyroidal deiodination pathway (outer ring deiodination) (Peter, 2011). Following secretion from follicles, the availability and disposal of the TH are regulated by different isoforms of deiodinated enzymes, which vary depending on the varying developmental stage and tissue (Deal & Volkoff, 2021a). The D1 is mediated by the production of T3, the most biological active THs in the liver, thyroid, and kidney. The D2 is described in the central nervous system, the muscles, and the pituitary, whereas D3, which only has the inner rind deiodination activity, is mainly expressed in the brain of animals (Peeters & Visser, 2000).

The outer ring deiodination of T4 occurs through the catalytical effect of D2, and D1 increases the level of T3 in the cell. At the same time, D1 can further mediate the degradation of TH.

Another enzyme, D3, catalyzes the inner ring deiodination and downregulates the availability of T3 through degradation of T3 to 3,3`-T2. For example, in the early development of zebrafish, knockdown of both D1+D2 and D3 results in a low level of T3 in different organs. This leads to declined development and weakened muscle function, swim bladder inflation, low amount of proteins, and decreased motility (Bagci et al., 2015).

1.4.4 Environmental pollution and thyroid hormones disruption in fish

Anthropogenic pollutants are the main cause of the endocrine disruption. Fish are mainly vulnerable to such pollution because of their closeness with water-soluble chemicals (agricultural pesticides, industrial by-products, animal manures, plastics, and heavy metals). Thus, the disruption of the endocrine axis has a great effect on fish physiological homeostasis and normal development (Table 2). In this regard, it can directly or indirectly affect the fish's immune system (Bagci et al., 2015; Kar et al., 2021; Tanoue et al., 2018). Considering this, the different chemicals present in the environment as a whole and as a trace act as THs disrupters, and this was evaluated by many researchers. Persistent environmental contaminants and polychlorinated biphenyl are well-known environmental pollutants and entail THs disrupting through altering THs metabolism in different mechanisms. Indeed, the chlorine substitutions at the 3' and 5' (meta-) positions on one or both phenyl rings make them competitively bind on both THs transporter and TRs. In addition to this, they lead to the reduction of THs synthesis through adverse effects on both thyroid gland structure and iodine transporter. Furthermore, they lead to increase excretion of T4 by altering further deiodination process (Blanton & Specker, 2007; Pocar et al., 2006; Preedy et al., 2009).

In juvenile minnows, chlorinated poly-fluoroalkyl ether sulfonates were found to be affect the THs and metabolic synthesis through downregulating the mRNA expression of genes such as those encoding for deiodinase, thyroid peroxidase, thyroglobulin, sodium iodide symporter, corticoid releasing hormone, thyroid hormone receptor (Guo et al., 2020). A recent study by Chu et al. (2021) evaluated 2-Ethylhexyl-4-methoxycinnamate activity on embryo-larval and adult zebrafish. Following the exposure, genes related to thyroid hormone were downregulated and the resulting T4 and T3 amount decreased. On the contrary, in adult zebrafish, the T3 concentration in blood circulation was reduced in a dose-dependent exposure.

Another study examined the chemical gut-brain axis by exposing zebrafish to oxytetracycline in water, resulting in a modification in gut microbiota and serotonin homeostasis. As expected, the thyroid receptor and both D2 and D3 enzyme-related gene expression were decreased in the brain. Results from intestinal rRNA showed an alteration of Proteobacteria and Fusobacteria diversity (J. Li et al., 2020). Tris-(1,3-dichloro-2-propyl)-phosphate caused sex-dependent

alteration in thyroid hormone and behavioural change in adult female zebrafish. Interestingly, the increase in the level of T3 and reduction in T4 detected in the F1 egg indicated that thyroid hormone disruption was inherited from mother fish (Si et al., 2021).

Table 2: shows thyroid hormone disrupter and method of detection in different fish matrix. DEHP (di- ethylplathalate), MEHP (mono-ethyl plathalate) and PCB (poly chlorinate bis phenyls).

Fish Matrix	Method	Exposure	Concentration	Mechanism	Species	Reference
Fish Plasma	ELISA	Season variation	Different temperatures and pH dissolve oxygen			(Sharma et al., 2018)
	LC-MS /MS and RIA				Fathead minnows	(Noyes et al., 2014a)
Whole-body TH	ELISA, qRT-PCR	BPS-Bisphenols	1,3,10,30, 30µg/	Change in the expression profile genes related to HPT- axis	Zebrafish embryo	(D. Zhang et al., 2017)
Larvae homogenate	ELISA, qRT-PCR	Phthalates, DEHP and MEHP	1.6 to 200µg/l	Changes in the transcription of genes involve in HPT- axis and exert toxicity	Zebra larvae	(Li et al, 2014)
Whole-body, T4 T3	HPLC-LCP-MS				Zebrafish	(Ti et al, 2001)
tadpole serum 11 TH	LC-MS/ MS			Tadpole development		(Hansen et al., 2016)

Embryo	UPLC-MS/MS	PCB	10μM		Zebrafish	(Chen et al., 2018)
Whole-body	LC-MS/MS			*Paralichthys olivaceus*		(Jesus et al., 1991)

1.4.5 Communications between thyroid axis and endocrine stress axis

The multi-step communication between the thyroid axis and endocrine stress axis is advantageous for the fish to cope with the demand that arises during a stressor challenge. This complex interaction at the hypothalamic-pituitary levels, as well as at the levels of hormonal actions, supports a role for thyroid hormones in stress response in fish (Peter, 2011). The thyroid works in tandem with the adrenal gland to handle small amounts of stress. During stress, the body releases cortisol, which enhances various bodily functions (Schreck & Tort, 2016). Recently Deal and Volkoff (2021b) reported that TH metabolism and function had been affected by energy balance. Additionally, THs played a crucial role in mediating osmoregulation and metabolism, which evidenced its role in stress response in fish.

Peripheral and neurohormones regulate both growth and reproduction. The study on the model goldfish that undergoes seasonal reproductive cycle with the effect of gonadotropin hormone-releasing hormone (GnRH), and T3 indicated that the examined hormones have a crucial part and mutual mechanism on regulating reproduction and growth in goldfish (Blanco, 2020). Somatotrophs (which produce growth hormone), thyrotropes (thyroxin), corticotropes (adrenocorticotropin), lactotrophs (prolactin), and gonadotropins (follicle-stimulating hormone and luteinizing hormone), release pituitary hormone on the target organ while the hypothalamus influences each of them with specific signals (Figure 5).

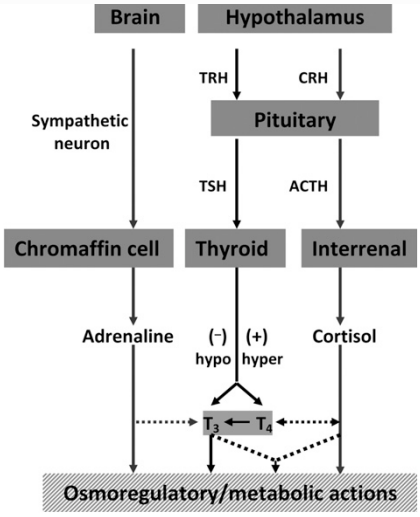


Figure 5. Intercommunication between Hypothalamus-pituitary-internal-axis (HPI-axis), Hypothalamus-pituitary-thyroid-axis (HPT-axis), and brain-sympathetic-chromaffin cell (BSC). HPI and BSC release cortisol and adrenaline upon activation by stress. Thyroxine (T4) and triiodothyronine (T3) concentrations stay normal in unstressed fish. Upon stress, fish with altered thyroid hormone (TH) can perceive stress and altered their level of cortisol, which alters metabolic regulation and osmoregulation. The dotted line indicates the site of those hormones' interaction to regulate normal metabolism and osmoregulation. (Peter, 2011).

1.5 Chromatography and molecule separation

Chromatography is an analytical tool, which plays a crucial role in the separation of a mixture of related components in a sample matrix into individual components for quantitative and qualitative analysis (Basharat et al., 2021). Within the field of analytical chemistry, chromatography is the single most applied laboratory technique. This is due to its wide range of applications, including for example bioanalysis, environmental monitoring, pesticide screening, forensics and toxicology, pharmaceutical, food quality and petrochemical analysis. Liquid chromatography, gas chromatography, paper chromatography, and thin-layer chromatography are the main types of chromatography (Coskun, 2016).

This set of techniques separates sample components depending on different partition coefficients of the two important phases, which are mobile phase, and stationary phase (Thammana, 2016). As a result, separation occurs based on interactions of the sample with the mobile phase and stationary phase. The physical state of these two-phases results in different separation characters of molecules (Coskun, 2016).

1.5.1 Liquid chromatography

Liquid chromatography (LC) is an ideal technique for many researchers due to its wider application of identification, purification, and quantification of metabolomics, proteomics, pharmaceutical, forensic, and other environmental monitoring. High-performance liquid chromatography (HPLC) is the improved form of column chromatography and is the separation technique that consists in high pressure of liquid or solvent mobile phase through the column of porous material with stationary phase on its surface. The solvent flows through the column filled with particles adsorbent material with the assistance of a high-pressure pump. This results in the separation of components of a sample based on their different affinities to stationary phase and solubility in the mobile phase. Thus, causing different partitioning between the mobile phase and stationary phase (Thammana, 2016). However, different variables such as pH, temperature, mobile phase composition, and choice of the column, need to be considered for efficient separation (Nikalje, 2013). Nowadays, HPLC is modified to ultra-high performance liquid chromatography (UHPLC) to increase chromatographic efficiency and reduce the time needed for the separation (Basharat et al., 2021).

UHPLC works on the same principle as HPLC, but with a smaller particle size which requires higher pressures (about 1000 bar). The increased chromatographic efficiency and increased mobile phase linear velocity can be achieved by using particle size less than 2.5µm, typically down to 1.6µm. Thus, the speed is improved due to high linear velocity, which can be applied without increasing the plate height, because particle size significantly reduces the C-term of the

Van Deemter equation. Therefore, ultra-high-performance separation is achieved both in terms of chromatograph efficiency and speed of analysis (Krull et al., 2009; Rathod et al., 2019).

1.5.1.1 Reversed-Phase High-Performance Liquid chromatography

The effectiveness of HPLC is regulated by appropriate column selection, which can be classified as reversed phase (RP) and normal phase (NP), based on the removal process of the desired solute from the mobile phase. Unlike NP-HPLC, in RP-HPLC the mobile phase is more polar, and the stationary phase is an immobilized hydrophobic functional group on the particle surface (Aguilar, 2004). Thus, the mobile phases get polar liquid pass through non-polar stationary phase. The separation of analytes occurs inside the pore space, and they get adsorbed onto a hydrophobic surface or hydrophobic functional groups as stationary phase bonded to a silica particle surface. Hence, sample compounds are separated based on their hydrophobic character, where too polar analytes have different selectivity and elute very quickly (Buszewski & Noga, 2012). Furthermore, the drawback with limited range of pH and tailing for the basic analyte is overcome by silica gel surface with incorporated carbon, such as C4, C8, and C18, making available versatile stationary phases that can withstand high pressure. C18 is the most commonly used stationary phase for separation of peptides and small molecules (Nikalje, 2013).

1.5.1.2 Main component of UHPLC

Solvent reservoir: The reservoir holds the solvent. In the RP-HPLC aqueous and organic solvents are conventionally installed on the UHPLC channels as A and B, in which A contains water or aqueous solvent and B contains organic solvent, such as acetonitrile and methanol. These solvents carry the analyte throughout the system (Guzzetta, 2001).

UHPLC pump is an important component of UHPLC, providing the flow of the needed amount of fluid or mobile phase from the solvent reservoir to the column assisting the system, that has to work correctly to measure the analyte concentration (Letter, 2020). The required pump pressure for delivery of volumetric flowrate, necessary for a certain mobile phase linear velocity of a column of a certain inner diameter, depends mainly on the particle size, but also mobile phase composition and temperature in the column since liquid viscosity decreases at elevated temperatures (Thammana, 2016).

Sample injector: It can be manual infusion or automated with the computer. In any way, the autosampler is used to introduce the sample to a phase stream that carries the sample into the high-pressure column. UHPLC sample injector uses small volumes of samples.

UHPLC column: Column is a tubular structure made from stainless cleaned steel, and it is the vital part where the real separation occurs. As it affects the whole separation process, the

component of the column can be specified as bonded phase (column dimension X particle size X pore size). Generally, shorter columns are cost-effective and generate less back pressure (Guzzetta, 2001). To achieve high speed and sensitivity, significant advancement on the HPLC column was made. Taking the advantage of this advancement, the ACQUITY system decreased the particle size to 1.6 -1.8 µm, which can deliver the mobile phase under the pressure up to 1,241 bar, providing a new level of chromatographic performance (third-generation hybrid particle technology) (www. waters.com/up columns, 2014).

Detector: The separated component pass to the detector following the analytical column before passing to the waste reservoir. Thus, the detector differentiates the separated bands of analytes as they elute from the column by a high-pressure pump. The signal from the detectors can be assembled on data collection devices or integrators. Recently, mass spectrometry is the common detector that can be combined with LC (Ho et al., 2003).

1.5.2 Mass spectrometry

Mass spectrometry (MS) is an analytical technique that studies the systems by the formation of gaseous ions, separating ionized particles such as atoms and molecules, which are characterized by using different ratios of their charges to their respective masses (i.e. mass/charge ratio, *m/z*) and relative abundance (Murayama et al., 2009). This can generate ions including protonated ions and fragmented ions from parental molecules. As a result, MS provides adequate evidence about given molecules such as molecular weight, molecular formula, and molecular structure after they are converted to ions. The speed, unmatched sensitivity, and broad area of application define it as an outstanding analytical method (Hoffmann & Stroobant, 2007). Additionally, the analytical application on the structural study, quantitative measurements of small metabolite in complex biological samples with high throughput, makes it a favorable instrument (Ho et al., 2003).

1.5.2.1 Ion source

The first step in the mass spectrometric analysis is the production of gas-phase ions through soft or hard ionization technique to negative or positive charge for the analyte of interest (Ho et al., 2003). With electron impact ionization (EI) which is a hard type of ionization, the produced molecular ion undergoes fragmentation to give radical cation (odd ion), even ion, and a molecule. The primary product derived from molecular ions can undergo further fragmentation (Hoffmann & Stroobant, 2007). Soft ionization methods such as electrospray ionization (ESI) and Matrix-assisted laser desorption ionization (MALDI) are widely used for the analysis of biomolecules.

1.5.2.1.1 Electrospray ionization (ESI)

ESI is the application of a soft ionization technique, in which the processes are supported by a gas flow that is coaxial to the capillary. This ionization method gained more attention for its fundamental role in the structural study and quantification of large molecules (Hoffmann & Stroobant, 2007). As per its principle, ESI transfers ionic species from the liquid phase into the gas phase, which is compatible with MS. Ion sources for ESI have different designs, depending on manufacturer of MS instrumentation. The ZSpray™ ion source is used in most MS instruments from Waters. The solution in the stainless-steel capillary tube initially forms a spray of droplets at atmospheric pressure (Figure 6A). Smaller and smaller droplets are formed by solvent evaporation Then, the charged droplet is produced. This is due to the opposite effect of electrostatic repulsion and surface tension. In a positive ion mode, each droplet is positively charged due to the presence of H^+, Na^+, or K^+. This is related to the number of basic sites on the molecule that can be protonated at low pH. As many analytes are acids, the protons are often the main contributors to the net droplet charge (Hoffmann & Stroobant, 2007). The spray is directed towards a small orifice leading towards the first vacuum stage of the MS instrument (Figure 6). Thus, the analyte ions, neutrals and solvent molecule moves toward the vacuum of the MS instrument under the stimulus of gas flow. In the interface between the outside atmosphere and the inside high vacuum, ions are separated from neutral molecules, such as mobile phase components. The beam of ions from analytes will be attracted 90 degrees towards the extraction cone (second interface) in the same direction to the transfer optics which is operated at somewhat lower pressure than the first interface (Figure 6B).

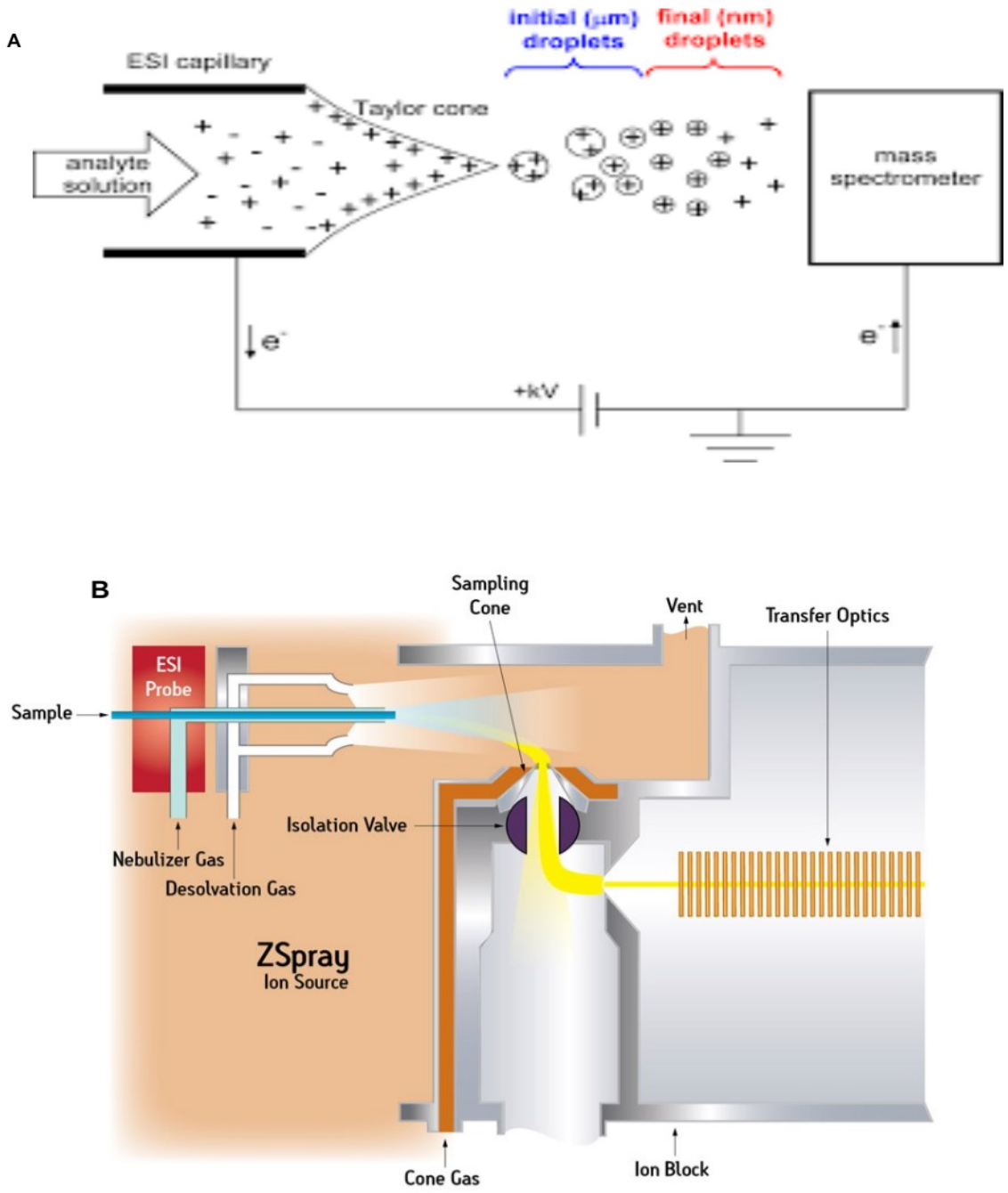


Figure 6: Shows (A) nanometer droplet by electrospray ionization. The chromatographically separated liquid analyte riches electrospray ionization where the liquid solution forms the Taylor cone. The gases charged droplets are then produced and drawn to mass spectrometry for further separation and determination (Konermann et al.,2012) and (B)API- ZSpray ion source. The transfer optics of the Quattro Premier is a T-wave type of ion guide

https://www.waters.com/webassets/cms/category/media/content_blocks/content_block_7.jpg

1.5.2.2 Mass analyzer and detector

Following the ionization, the ionized molecule travels through a magnetic or electrical field to promote the separation based on the mass-to-charge (*m/z*) ratio. Currently, the quadrupole mass analyzer is the most used due to its convenient filter, small size, economical, and interfaced with a wide variety of inlet systems (Ho et al., 2003). This mass analyzer contains 4 metal rods arranged in a unique oscillating pattern. Accordingly, applying a direct current (DC) and a superimposing radio frequency (RF) potential in equal and opposite phases, makes the ion separate and reach the detector. In MS/MS, the triple quadrupole is commonly used due to its capability to separate molecular ions and detect the fragment ions from a sample in a single instrument. Triple quadrupole instruments basically have two quadrupole mass filters and a collision cell between them. Original instrument design had a collision cell with a third quadrupole for ion beam focusing. Nowadays, the collision cell is typically with a hexapole or with a stack of electrostatic plates, such as travelling wave (T-wave) ion guide. Triple quadrupole instruments work under four scanning modes, which are parental ion scan, constant neutral ion loss scan, daughter ion scan, and multiple reaction monitoring (MRM) (Figure 7). Thus, the ionized molecular ion will be filtered and separated according to its mass to charge ratio in the first quadrupole. Furthermore, filtered ions undergo fragmentation in the collision cell by collision-induced dissociation (CID). Fragment ions are separated by the second quadrupole mass analyzer. This can be tuned to the desired mass for optimization of the fragment ion. Hence, the ions will be guided in the ion chamber providing focusing to achieve maximum selectivity of production, then separation based on their *m/z* ratio, and finally detection by an ion transducer detector (Yost & Enke, 1979). The most used detector in conjunction with the quadrupole mass analyzer is the high energy dynode. The electron multiplier enhances the signal originated by the ion hitting the detector with some slight variation (Hopfgartner et al., 2004).

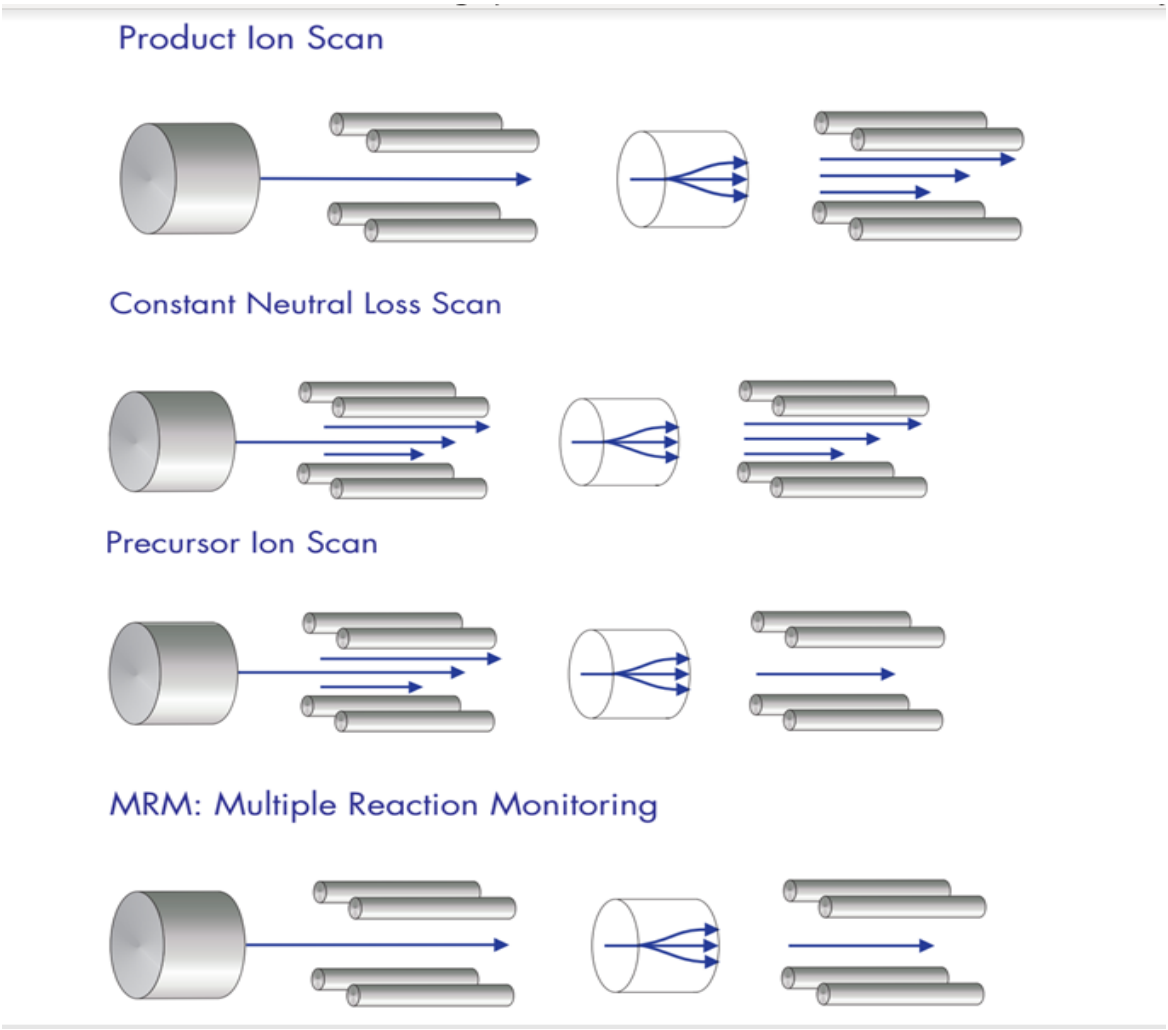


Figure 7: Shows tandem quadrupole mass analyzer working in several modes, such as scanning modes, which are parental ion scan, neutral ion loss scan, daughter ion scan, and multiple reaction monitoring to enable high selective quantification http://photos.labwrench.com/equipmentManuals/4131-1344.pdf.

1.5.3 Why LC-MS /MS for detection and quantification of hormones?

Biological fluids containing hormones and their small metabolites are small and valuable sample volumes for detection and quantification, as they mediate the function of the entire organ of the fish. They are key to assessing the hemostasis of fish and physiological processes, such as neural signaling and biological functions (Ke et al., 2014). The quantification of a level of steroid and thyroid hormones is an important step for understanding the overall fish physiology. This includes detection of stress in fish, growth, and development, osmoregulation, homeostasis controlling the volume of fluid and the level of glucose in circulation, and indicating the time for smoltification (Serrano et al., 2021).

The concentration of biological fluids, specifically hormones, in different parts of fish organs has been analyzed by different techniques. Among them, Enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), chemiluminescence microparticle immunoassay, liquid or gas chromatography coupled with mass spectrometry are the common methods used so far.

Immunoassay-based techniques are often used for the determination of steroid hormone concentration invertebrates. Considering this, Lupica and Turner (2009) and Guest et al. (2016) developed an ELISA method for efficient quantification of glucocorticoid levels in fish faeces and cortisol in the homogenized fish whole body, respectively. In contrast, a study by Dufour et al.(2021) indicates that the result from ELISA and RIA is higher than the result from LC-MS/MS for estrone detected in the serum of American bison. This was due to the cross-reactivity of antibodies with interfering compounds. Similarly, Abreu et al.(2016) reported duplicate concentrations of testosterone and estradiol measured by RIA and LC-MS/MS in a whole body of juvenile fish indicating viable binding of other interferences.

In addition to the situations in the development of high-affinity antibodies and screening for different species, there are various limitations with immunoassay-based methods. For instance, the cross-reactivity of antibodies leads to an overestimation of analytes, lack of specificity, limited multiplex ability, lack of reproducibility, low throughput, and inadequate level of selectivity at low concentration of hormones without derivatization (Hallmann et al., 2016; Noyes et al., 2014; Wang et al., 2020).

Other chromatography-based methods are GC-MS or GC-MS/MS, which provide high selectivity and represent significant tools for the study of metabolomes. They overcome the challenge of analyzing one analyte per one immunoassay kit since multiple hormones and their metabolites can be detected in a single injection. However, the analyte needs steps or two-step derivatization to be volatile and thermally steady (Wudy et al., 2018). In addition to this, GC-

MS /MS does not tolerate dirty samples and typically needs higher sample volume compared to LC-MS /MS (Ke et al., 2014).

Complying the advantage of the high sensitivity of MS, together with the significant physical separation capacity of LC, LC-MS/MS is the method of choice in different fields of study, including bioanalysis. The ideal separation and analysis of targeted and untargeted analytes make it the basic instrument for hormones and other small molecules. Moreover, the ability to detect several hormones at once, the applicability for low polarity and non-polar analytes, and the high specificity with accuracy make it a favorite technique for hormone determination in complex biological samples.

Accordingly, many studies have used this modern instrument for the simultaneous determination of hormones and their metabolites in different biological samples. Among them, Abreu et al. (2016) and Nouri et al. (2020) have developed an LC-MS/MS multiplex method for determination of 14 steroid hormones in low volume plasma samples with a limit of detection (LOD) of 0.012 ng/mL cortisol in 10 µL of serum. Furthermore, Ke et al (2014) and Serrano et al. (2021) quantified 13 steroids at the same time performing LC-MS/MS analyses. Currently, Blackwell and Ankley, (2021) quantified the concentration of 11 physiological important steroid hormones in 10µL of plasma sample with 0.05 to 1.0 ng/mL LOD. Noyes et al. (2014) and Hansen et al. (2016) detected total T4 and total T3 in 50 µL of teleost plasma and 11 thyroid hormones and their metabolites in picograms of plasma serum of amphibians (50 µL) by LC-MS/MS method, indicating that LC-MS/MS is a multiplex, very sensitive and robust analytical technique for biological fluids (i.e., hormones) and their metabolites.

1.6 LC-MS/MS sample preparation strategy

A sample is a representative and homogeneous portion of the whole matrix. Sample preparation is one of the key steps to obtain meaningful results from LC-MS/MS analysis. Thus, a good result starts from good sample preparation. On the other hand, this step contributes to the source of error in any analytical method. Furthermore, there is no definition of volume of sample in the bioanalysis, due to differences in matrix component. In bioanalysis, some ground principles are required such as choosing a suitable pre-treatment and sample preparation stages. Thus, the principles include low cost, suitability for the target analyte and analytical instrument, simplicity, allowing interference depletion without analyte losses, and containing the step to enrich or dilute sample, if needed. In this stage, the target analyte can be transformed to the chemical form that is suitable for its determination to increase the sensitivity and selectivity over the other interferences in the biological matrix (W. Li et al., 2019).

A bioanalytical method for small molecules in biofluids, such as hormones, needs pre-treatment to clean up the potential interferences including their metabolites. Considering the extraction of the small molecules from the whole blood, plasma, or serum, it is required to reduce the risk related to matrix effects, such as ion suppression, and other background interferences. This is due to serum samples containing a variety of interfering compounds, including proteins and phospholipids. The sample preparation before LC-MS/MS analysis needs to contain the steps of protein precipitation, as proteins cause clogging of the column and affect the chromatographic separation (Kvamsøe et al., 2020). On the other hand, adjusting the pH and organic solvent used for mobile phase, buffer, and aqueous composition results in a variation of the retention time and peak shape (Stuart Kushon & Erica Pike, 2012). Bearing in mind those factors, it is possible to achieve improvement related to the accuracy, precision, and robustness of the new and ready established sample preparation techniques, that were used for extraction of the target analyte from biofluid before LC-MS/MS analysis.

1.6.1 Protein precipitation

Protein precipitation (PPT) is a typical sample preparation technique making use of several water-soluble solvents, including acetone, acetonitrile, methanol, acid, and metallic ions (W. Li et al., 2019) . As the size of the sample gets higher, adapting fast and simpler methods is necessary. Protein precipitation is the method of choice in many areas of bioanalysis to adequately remove proteins from the other substance (Zhao & Juck, 2018). The aggregation of proteins in a sample depends on the organic solvent used and the volume of the solvent. In addition to this, pH and temperature are the factors needed to be considered to efficiently precipitate proteins from a given sample (Li et al, 2011).

This technique is still suffering from the effect of interference as the phospholipid is coextracted from the plasma sample. Stuart Kushon and Erica Pike (2012) examined the effect of phospholipid in ion suppression, in the entire gradient and degradation of the analytes when compared the amoxapine signal with sample extracted with PPT and phospholipids removal plate.

1.6.2 Solid-phase extraction

Solid-phase extraction (SPE) is the extraction technique based on the interaction between the sorbent in the stationary phase and the analytes, resulting in the absorbance of both the analyte and the interfering compounds. Thus, the choice of sorbent material allows the procedure to be modulated according to the analyte of interest as substance retained in the solid phase due to adsorption, the solute partition between immiscible phase, and Colombian interaction between molecules of opposite charge. SPE is one of the common sample preparation techniques widely

used in the extraction of small biomolecules like hormones. This technique is highly used in this area due to its capacity to selectively extract the analyte of interest and enrich compounds. Thus, the eluted will be collected and directly injected into LC-MS/MS or evaporated and resolubilized in a suitable solvent before analysis (W. Li et al., 2019).

Moreover, thyroid hormones were extracted from plasma after incubation with an acid solution to denature more proteins and avoid deiodination (Noyes et al., 2014). Results were clean extracts and high recovery of analytes. Similarly, Hansen et al. (2016) used SPE to extract and quantify 11 thyroid hormones and related metabolites in the plasma sample. On top of that, Hallmann et al. (2016) extracted a steroid hormone from fish of the southern Baltic Sea by using SPE cartilage, following enzymatic digestion to break proteins and homogenization of tissue samples. However, the issue related to a long time for the analysis, high cost, and non-environmentally friendly techniques, remaining under question when using this method.

1.6.3 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is a common sample extraction technique in which the analyte is extracted from the liquid phase and directed towards the water-immiscible liquid solvent phase (Figure 7). LLE results in simultaneous sample cleaning up and enrichment of the target. The principle of LLE is that the target analyte is needed to have less solubility in the water phase and high solubility in the water-immiscible solvent phase. As a result, solvents that have high solubility in water are not producing phase separation as the solvents dissolve in water (W. Li et al., 2019) . Thus, considerations such as the physio-chemical properties of the solvent used, and the density of the solvent needed must be considered. Hence, the solvent density needs to be lower than water as the aqueous phase floats on the top. The solvent with a low boiling point is selected as they result in faster evaporation during sample drying. The polarity of the analyte itself is also another factor to be considered. However, nowadays the polar analyte is also extracted by using water-miscible solvent from different matrix by using a high concentration of salt, known as salting out liquid-liquid extraction. Thang et al. (2017) extracted cortisol by LLE from a plasma sample to examine the effect of heavy metals on cortisol levels.

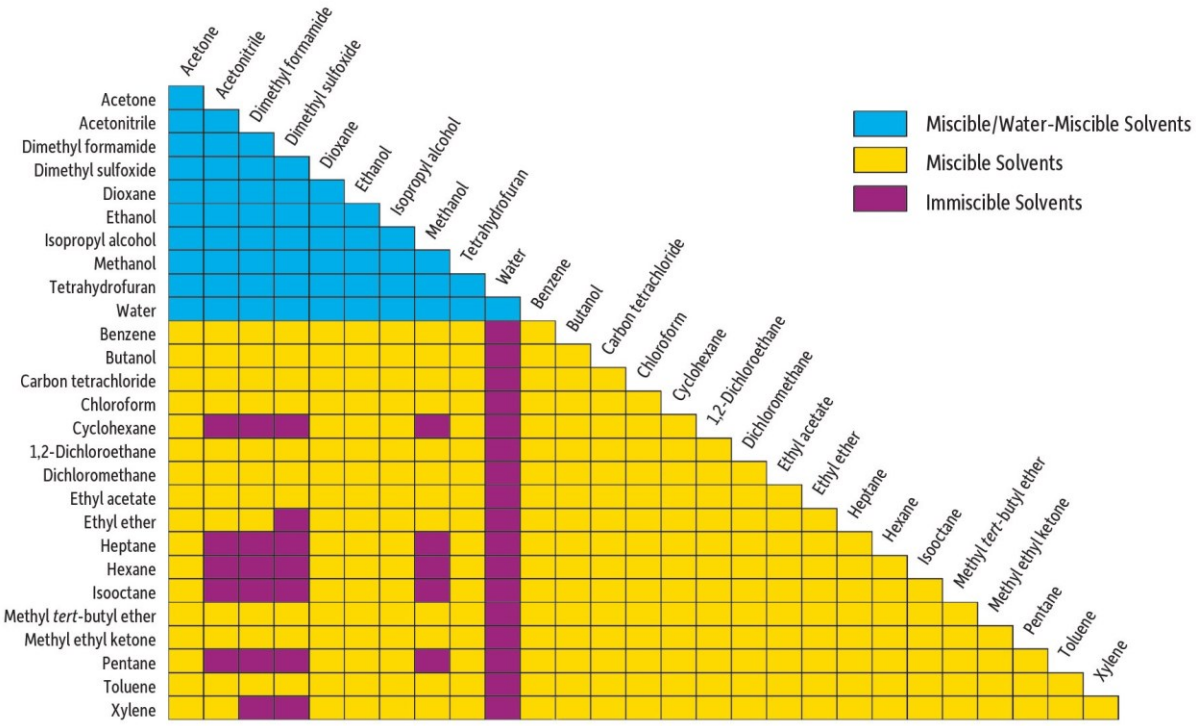


Figure 8: Shows solvent miscibility in water, the blue boxes are represented solvents suitable for salting-out liquid-liquid extraction. The yellow boxes with less miscible and purple boxes are immiscible solvents in water and each other, which are suitable for liquid-liquid extraction.

1.6.3.1 Salting-out assisted liquid-liquid extraction

Salting-out assisted liquid-liquid extraction (SALLE) is the homogeneous LLE technique by using water-miscible solvent (Figure 7) and a high concentration of salt to extract compounds with different polarities from water and different matrix such as biofluid (Kvamsøe et al., 2020). The SALLE method is the most reliable, fastest, and the most applicable for analytes with different polarities (Tang & Weng, 2013).

Compared to the conventional LLE and SPE, this technique is the most timesaving (fast enough for all needs), cost-effective, less laborious, and environmentally friendly for sample preparation method, which results in a very clear separation of analytes. In the meantime, it is comparable in providing clean extraction with both techniques. The other beauty of SALLE is that the solvent top phase is compatible with reverse phase LC column, and mobile phases, whichcan be directly injected or diluted (Sazali et al., 2019; Zhang et al., 2009). This favors SALLE over LLE where there is a need for evaporation of the non-polar solvent and change to a water miscible solvent before injection onto the LC column.

The solvent, salt and pH selection of the organic phase are the major factors to influence the efficiency of the SALLE technique. Acetonitrile, alone or combined with other simple alcohol,

is the most used solvent, while NaCl, $(NH_4)_2SO_4$, $MgSO_4$, $ZnSO_4$, and $NH_4CH_3CO_2$ are the most used salts. A study showed that ammonium salt is compatible with both LC and MS (Valente et al., 2013). On the other hand, the presence of ammonium in the supernatant is useful in terms of preventing the formation of sodium adduct, as Na is naturally found in methanol shipped and stored in soda-lime bottles (Kvamsøe et al., 2020). SALLE technique has a broad range of applications in different fields of study. Zhang et al. (2009) established SALLE for extracting lopinavir and ritonavir from plasma samples by adding magnesium sulfate. Similarly, Li et al. (2021) reported that SALLE is the effective sample preparation method resulting in wide range coverage of analytes in herbal medicines extracted from rat plasma, when compared to PPT, LLE, and sugaring-out liquid-liquid extraction (SULLE).

2. MATERIALS AND METHODS

In this section material and method used for this study are briefly presented.

2. 1 Chemical and materials

Analyte standards of L-thyroxine (Purity: 99%, Lot.BCBV2496), testosterone (Purity: 99.5%, Lot.BCBC786V), deuterated cortisol (Lot.EB0712), deuterated cortisone (Lot. MBBBB5535V) and 17β-hydroxyandrost-4-en-3-one-16,16,17-d7 (081M4053) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The two steroid hormones cortisol (Purity 99.4%, Lot.10216764) and cortisone (Purity:97.9%, Lot.M23G017) was obtained from Alfa Aesar (Ward Hill, MA, USA). 13C6-labeled thyroxine (Lot.3-EKP-26-3) was obtained from Toronto research chemicals. Methanol (MeOH) and acetonitrile (ACN) for HPLC, LC-MS grade-suitable for LC-MS UPC/UHPLC/ultra HPLC instrument and analytical grade formic acid (99-100%), methyl tert-butyl ether (MTBE), propanol, butanol were purchased from VWR (Rue Carnot Fontenay Sous Bois, Fr). Analytical 25% ammonium hydroxide and 100% acetic acid used for pH adjustment were purchased from Merck (Burlington, MA, USA). Sodium chloride (NaCl) 99.8% and ammonium sulfate 99.5% were purchased from Merck KGaA, Darmstadt Germany. The Autonorm™ Immunoassay serum Liq L-1 (LOT 1605243) was provided by Sero AS, Norway. The purified water to 18.2 MOhm was from Elga lab water (high Wycombe, United Kingdom). All sample preparation was conducted in a microcentrifuge tube, graduated, round bottom, 2 ml, PP. The samples were centrifuged in scan speed microcentrifuge Lab-tech, mini, 12,300 G (Labogene, LillerØd, Danmark) at 2300G and 4coRCF. 0.3 ml pp short thread micro-vial manufactured in Germany was used for sample injection to LC-MS/MS.

2.2 Preparation of the stock solution and calibration standards solution

The stock solution was primarily prepared separately for each hormone. L-thyroxine (38 ug/mL) was added into the volumetric flask containing 10 ml of water, 1 ml of 25% ammonium hydroxide, and 14 ml of methanol. The initial stock mix of cortisol (1 ml), cortisone (1 ml), and testosterone (1 ml) were mixed in 25 ml of methanol in a different volumetric flask. A final stock solution was then made in a single bottle by adding 0.2 ml of the initial stock mix from 960.9 ng/ml of cortisol, 921.4 ng/ml of cortisone, 867.3 ng/ml of testosterone, and 0.5 ml of 2000.0 ng/ml of L-thyroxine were prepared in methanol giving 10 ml of total volume.

The calibration solution was made by serial dilutions from the final stock mix. From zero to seven calibration solutions were made with a total volume of 10 ml of water containing 20% methanol. The solution consisted in different concentration ranges from 0 to 96.092 ng/ml for cortisol, 0-92.136 ng/ml for cortisone, 0-86.732 ng/ml for testosterone, 0-200 ng/ml for L-

thyroxine and 0-45 ng/ml. The detailed concentration of calibration solutions is indicated in the table (Table 3).

Table 3. calibration standard solution prepared in water containing methanol

Calibration STD	Methanol added first (mL)	Vol (mL) stock mix	Cortisol (ng/mL) 960.9	Cortisol (ng/mL) 921.4	Testosterone (ng/mL) 867.3	T4 (ng/mL) 2000	Total volume in water (ml)
STD #0	2	0	0.000	0.000	0.000	0.000	10
STD #1	2	0.01	0.961	0.921	0.867	2.000	10
STD #2	2	0.03	2.883	2.764	2.602	6.000	10
STD #3	2	0.06	5.766	5.528	5.204	12.000	10
STD #4	2	0.125	12.011	11.517	10.842	25.000	10
STD #5	2	0.25	24.023	23.034	21.683	50.000	10
STD #6	1.5	0.5	48.046	46.068	43.366	100.000	10
STD #7	1	1	96.092	92.136	86.732	200.000	10

A single solution of Internal standard (i.e., working solution) was prepared by spiking 0.5 ml of each ISTD of all analytes with the total volume of 250 ml methanol. Thus, the ISTD solution constituting the concentration of 67.2 ng/ml of D4 cortisol, 87.2 ng/ml D8 cortisone, 72 ng/ml D3 testosterone and 48 ng/ml thyroxine-13C6. All stock mix solutions, calibration solutions, and internal standard solutions were stored at 4 ºC and maintained at room temperature (21 °C) while they were used.

2. 3 Blood sample collection

Plasma samples from Atlantic Salmon were kindly provided by FishLab AS. The samples were derived from a commercial fish farm in southern Norway and had been collected as part of a previous research project. The average fish weight was ~4kg and they fed a commercial feed containing 38% fat. In brief, fish were anesthetized by an overdose of Finquel (MS-222) before sampling by Akvakulturdriftskriften (The aquaculture operations regulations §34). Blood samples were taken from the Vena caudalis by Vacuette containers containing lithium-heparin. After centrifugation, fish plasma was transferred to Eppendorf tubes and kept frozen (-18°C) before analysis.

2.4 Sample preparation and analyte extraction

In this thesis, new SALLE sample preparation methods were developed and validated on quality control human serum samples (Autonorm immunoassay). The validated method was applied to

extract both steroid and thyroxine hormones from fish plasma samples. Compared to traditional LLE and other methods, the newly established SALLE method is the simplest and most economical in terms of time and reagent used.

2.4.1 Salting-out liquid-liquid extraction

Salting-out liquid-liquid extraction is the most time-saving (fast enough for all needs), cost-effective, less laborious, and environmentally friendly sample preparation method that results in a very clear phase separation upon the addition of concentrated salt (Li et al., 2021). In this thesis, to develop promising SALLE method, different water-soluble organic solvents, such as acetonitrile, acetone, 1-propanol, 2-propanol, and methanol were tested, considering the Hansen solubility parameter of each solvent to obtain optimum solubility of analytes (Table 4). The hypothesis is that with a good match of HSPs between analyte and solvent, we expect the solvent to be a better solvent for the analyte, can dissolve more of the analyte and gives the higher extraction recovery. For protein precipitation and phase separation, saturated salts including NaCl and ammonium sulfate, have been tested. Furthermore, to stabilize and solubilize T4 in the organic phase different buffers and acids have been examined (i.e., ammonium hydroxide, acetic acid, formic acid) and trifluoroacetic acid (TFA) as an ion-pairing reagent.

Table 4 Hansen solubility parameter for hormones (thyroxine, cortisol) and solvents (acetonitrile, methanol, acetone, 1-propanol, and 2-propanol).

Hormones and solvents	Dispersion (δD)	Polarity (δP)	Hydrogen bonding (δH)
TT4	23.4	5.2	13.5
Cortisol	19.17	10.13	9.2
ACN	15.3	18	6.1
MeOH	14.7	12.3	22.3
Acetone	15.5	10.4	7
1-propanol	16	6.8	17.4
2-propanol	15.8	6.1	16.4

Eventually, the best combination of salt and solvent resulted in clear phase separation, and it was tested for further efficiency regarding matrix effect and yielding a high analyte extraction. For all tested experiments, the relative response and relative internal standard area were calculated according to Equation 1.2 and 3, respectively to determine extraction efficiency and ion suppression. Thus, the final method was chosen by compromising both values. All test experiments had three replicates. Sample matrix, calibration standard, and spiked sample were thrown to room temperature before analysis. Blank samples were water.

Relative response (%) was calculated from the response of replicates as:

Response = $\frac{\text{Area of analyte}}{\text{area of internal standard}}$ Equation 1

Mean $\bar{x} = \Sigma \frac{x_i}{n}$ Equation 2

Relative response (%) $\left(\frac{\text{mean responce of replicate}}{\text{highst mean responce of the series}}\right) * 100$ Equation 1.2

Relative internal standard area (%) = $\left(\frac{\text{mean internal standard of replicate}}{\text{highst meanintenal standard of the series}}\right) * 100$ Equation 3

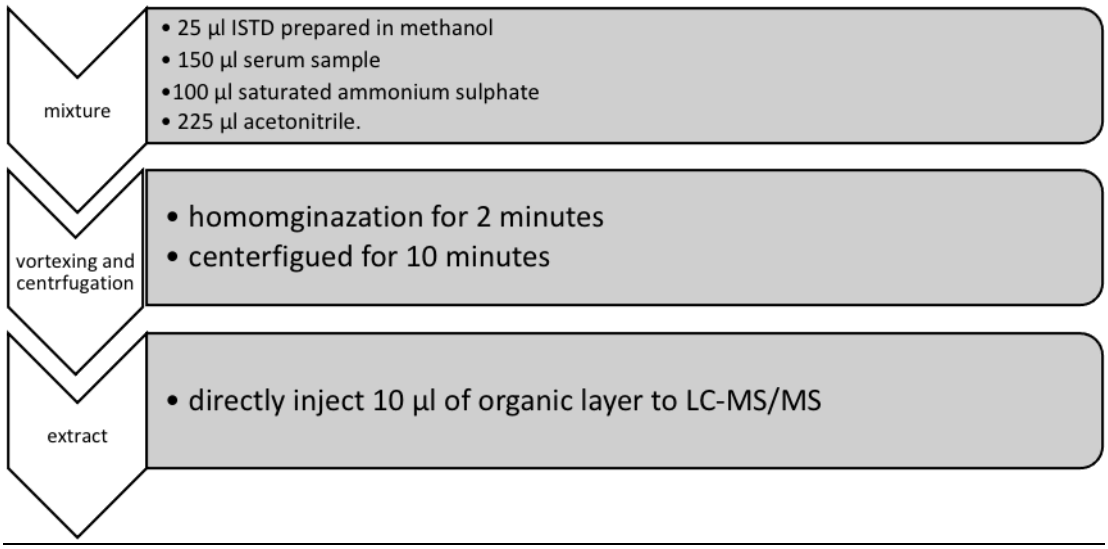
Accordingly, for the final SALLE experiment, acetonitrile, and ammonium sulfate were found to be promising, which resulted in fewer matrix effects (ion suppression) and sufficient relative response, which is a direct proportion to analyte recovery. Hence, the sample preparation procedure was started by adding 150 µL of serum sample into a vial containing 25 µL of internal standard (i.e., Isotopic label and deuterium-labelled). The sample was then supplemented with 100 µL of ammonium sulfate before organic solvent (225 µL acetonitrile) was added. Then, the sample was well homogenized for 1minutes before being subjected to centrifuge for 10 min in scan speed microcentrifuge Lab-tech, mini, 12,300 G (Labogene, LillerØd, Danmark) at 2,300G and 4coRCF centrifugation. Finally, the top phase was harvested and directly injected into the LC-MS/MS with 10 µL of injection volume. All the experiments conducted in this thesis contain:

1. Blank water: containing only water and 25 µL of internal standard
2. Blank reagent: containing reagents used for sample extraction such as acetonitrile, isopropanol, 1-propanol, and methanol spiked with 25 µL of the internal standard and 100 µL of ammonium sulfate. Those samples were used to generate information regarding ion suppression and solubility of analyte in the organic phase. Specifically, to monitor and verify if the endogenous matrix were coextracted with supernatant and coeluted at the same retention time, to avoid possible interfering with ESI and low sensitivity of the instrument in the determination of target analytes.
3. Serum sample (QCs), fish plasma and calibrator containing analytes were spiked with 25 µL of internal standard ammonium sulfate and organic solvent.
4. The spiked samples used for determination of method recovery and accuracy was prepared in quality control human serum sample by spiking with a stock solution containing different concentrations of individual analytes as, low (i.e. 19.22 ng/ml of cortisol, 18.43 ng/ml of cortisone, 17.35 ng/ml of testosterone, and 40 ng/ml L-thyroxine), medium (i.e. 28.83 ng/ml of cortisol, 27.64 ng/ml of cortisone, 26.02 ng/ml of testosterone and 60 ng/ml L-thyroxine) and high (i.e. 48.05 ng/ml of cortisol,

46.07 ng/ml of cortisone, 43.37 ng/ml of testosterone, 100 ng/ml L-thyroxine) concentrations.

5. All blank samples, a serum sample, fish plasma calibrator, and spiked samples were treated in the same way.

2.4.2 Optimization of SALLE Methods



2.5 Instrumental method development

2. 5.1. MS/MS method development

2.5.1.1 Instrumental calibration

Acquity UHPLC column was coupled with A quadrupole (hexapole collision cell) Quattro Premier XE mass spectrometer (Waters, Milford. MA, USA) with ZSpray™ ESI ion source. The instrumental setup was started by performing instrumental mass calibration, using salt clusters formed by introducing a solution of sodium formate in methanol. The solution was directly injected into the probe from a 250 µL syringe via a fused silica capillary with 75 µm internal diameter and with a 10 µL/min flow rate. Sodium-formate salt clusters generated ions in the ESI ion source. Therefore, the instrument was calibrated for the mass-to-charge ratio ranging from *m/z* 20 to 1999.

2.5.1.2 Multiple reaction monitoring (MRM) tuning

To determine, optimize the mass spectra for parental and daughter ion of all analytes, the MS/MS was tuned with the solution containing known concentrations of each compound separately. Different solutions were prepared for each analyte in methanol containing 100 µg/mL of cortisol, 100 µg/mL of D4-cortisol, 46 µg/mL of cortisone and D8-cortosone, 400 ng/mL of testosterone and D3-testostrone, and 3.8 µg/mL L-thyroxine, 4 µg/mL of 13C6 labelled thyroxine. The tuning solution was mixed with the mobile phase (30% A1 and 70% B1)

in a T-union via loop injection. The solution was infused into the ion source and the flow rate was set at 0.200 mL/min with an infusion volume of 10 µL/min L-thyroxine with its internal standards as they are non-polar compounds. While the composition of the mobile phase was 50 % A and 50 % B with 5 µL/min infusion volume for other steroid hormones and their internal standard.

Furthermore, for all analytes the protonated mass to charge ratios $[M + H]^+$ were calculated before mass tuning in ESI+ mode. To identify parental ions MRM channels of the first MS were set with the same parameter for each compound with the capillary cone voltage at +3 kV, the source temperature of 100 ºC, desolvation temperature of 350 ºC, desolvation gas flow 800 L/hr, cone gas flow 50 L/hr. However, in MS/MS the collision energy was slowly increased and established for individual compounds to obtain the daughter ion as indicated in Table 5. The collision energy was optimized, and Argon gas was used in the collision cells for activation (collision-induced dissociation) to produce more intense fragmentation of the molecular ion. For L-thyroxine, different MRM channels were established, and the most abundant fragments were chosen for quantification, and the others, assisted for confirmation of analytes. While for cortisol, cortisone, and testosterone only one MRM was monitored about their internal standard. For each MRM transition, the dwell time was set to 0.015 seconds.

2.5.2 LC method development

Development of chromatographic separation was achieved in gradient mode and was performed using reversed-phase Acquity UHPLC with the column BEH C182.1 x 50 mm ID with 1.7 µm particle size (waters). The column temperature was 50 ºC. Samples were stored in the autosampler at 10 ºC. High and low pH of mobile phase were tested, and the selection was based on the combination of signal strength (S/N) and separation efficiency. Formic acid (0.2 %) and ammonium hydroxide (0.2 %) have been tested as an aqueous mobile phase. For the entire experiment, 0.2 % of formic acid mixed in ultra-pure water was used as A1, methanol as B1. The linear gradient composition and the flow rate were tested and adjusted to the best result by injecting the known concentration of each analyte before being programmed to the LC inlet method. Thus, samples were injected with the infusion flow rate of 10 µL/min and mixed with the flow from the LC-pump at 200 µL/min.

Finally, the best chromatographic separation was achieved with initial linear gradient elution at 99% of A1 and 1% of B1 for 0.1 min, 50% B1 held for 1.6 minutes. Then, the level of B1 reached (70 and 80 %) until 3.10 min, and between 3.10 min to 5 min the level of B1 percentage increased to 99 % and remained the same for 1.9 minutes, returning to the baseline condition of 1 % B1 with 5.10 to 7.00 min. From 5.10 to 6.9 minutes the total flow rate was increased to 0.4 ml/min to regenerate the column and then, back to 0.2 ml/min (Table 5). Since the

hydrophobicity of our analyte was reduced by the pH of the mobile phase the retention time for all analytes was performed before 3.63 minutes, the rest of the time was for equilibration of the column between each sample.

Table 5: mobile phase gradient and flow rate

Time (min)	Flow (ml/min)	%A	%B	Curve
initial	0.200	99.0	1.0	initial
0.10	0.200	50.0	50.0	6
1.70	0.200	30.0	70.0	6
2.10	0.200	20.0	80.0	6
3.10	0.250	1.0	99.0	6
5.00	0.250	1.0	99.0	6
5.10	0.400	99.0	1.0	6
6.99	0.400	99.0	1.0	6
7.00	0.200	99.0	1.0	6

2.6 Method validation

Method validation is the step to evaluate the reliability, consistency, trueness, and robustness of the new established before its intended use (Kruve et al., 2015). In this thesis, a newly developed SALLE method was evaluated and validated according to the Eurachem guideline (*The Fitness for Purpose of Analytical Methods*, 2014).

2.6.1 Linearity

Linearity for all validation parameters, the calibration curve from the calibration standard solution was prepared in water containing 20% methanol and tested throughout the concentration of each analyte. The linearity $R^2 \geq 0.99$ was first established from the ratio of peak area to correspondent internal standard for each analyte by the linear regression equation.

2.6.2 Precision

Precision explains the variation caused by the random error of analysis including sample preparation process, chromatographic separation, and effectiveness of ionization source and selectivity of the MS (Eurachem, 2014). Hence, the intra-day precision was investigated by looking at the variation between samples run within the same day by the same analyst, while the inter-day precision was calculated from the result of consecutive five working days with identical concentration and sample preparation procedures. In this experiment, both intra series precision (repeatability) and intermediate precision (reproducibility) were calculated from 10

replicates of quality control serum samples. Thus, the samples were analysed every five days with calibration standards solution. The relative standard deviation (RSD %) was then calculated from both values by using equation 5. The same equation was used to calculate the intermediate precision.

$$Standard\ deviation, s \quad \sqrt{(\sum_{i=1}^{N}(x_i - \bar{x})^2)(N1)} \qquad \text{Equation 4}$$

Relative standard deviation, $RSD\% = \bar{x}/(s)$ Equation 5

2.6.3. Limit of detection (LOD) and Limit of quantification (LOQ)

Different factors need to be considered to detect the analyte at low concentrations. LOD is the most important parameter to know the lowest detectable concentration of analyte that is significantly different from zero with specific certainty. At the same time, it offers the information of the lowest concentration that can be detected using the methods. The lowest level at which the capability of the method can be accepted for a given application is the limit of quantification (Eurachem, 2014). To calculate both LOD and LOQ the mean average and standard deviation of the signal to noise of 10 replicates of the blank sample was calculated according to Equation 2 and 4, respectively. Then the slope of the calibration curve a, y-intercept b, and correlation coefficient (R^2) were used in the linear to calculate both parameters as follow:

LOD was calculated as follow

First, the average mean and the standard deviation of S/N of the blank samples were calculated. using Equation 2

$$y = bx + a \qquad \text{Equation 6}$$

a: is the y-intercept and it was 0 as we forced they-intercept to zero. and b: is the slope of the calibration curve.

Fitted into equation 6:

LOD: x=y/b Equation 7

If y =$\bar{x}\ of\ blank + 3(SD) of\ blank$,

LOQ: x= y/b Equation 8

Y= $\bar{x}\ of\ blank + 10(SD) of\ blank$

2.6.4 Method recovery and accuracy

Recovery represents the extraction efficiency of the analyte and indicates the loss of each analyte throughout the whole sample preparation at the same time (Eurachem, 2014). Whereas accuracy of the method indicates the bias due to systematic errors and estimates the variation between the true and the result from analyses by the given analytical procedure. Thus, the

variation can be from sample preparation, matrix effect and it determines the relative errors (Kruve et al., 2015). In this thesis, to calculate the recovery and accuracy of the established analytical procedure, three quality control samples were prepared by spiking known concentrations of each analyte (Section 2.4.1). Thus, the recovery was first investigated by comparing the ratio of the concentration of the spiked sample with the ratio non-spiked sample (Equation 10). While the accuracy of the method was estimated as the relative bias of the spiked sample (Equation 11).

Recovery%: $\left(\frac{measured\ difference}{true\ difference}\right) 100$ Equation 9

Then, accuracy was calculated from the relative bias of the spiked sample as follows

Absolute bias = $\left(\bar{x}\ sample\ concentration / assigned\ true\ value\right)$ Equation 10

Relative bias (%) = $\left(Absolute\ bias / assigned\ true\ value\right) * 100$ Equation 11

2.6.5 Linear range (working range)

The working range of the method is represented by the range between the lower quantifiable concentration of analyte and the upper concentration at which the level of the result has significantly deviated from analyte sensitivity (Eurachem, 2014). In this study, the response of calibration standard solution was plotted on the y-axis, while the concentration of the analyte was constructed on the x-axis. The plot was visually inspected for the deviation from the linearity.

2.7 Data analysis

The results from the LC-MS/MS were processed using the MassLynx and TargetLynx software. For further analysis, the data for all method validation parameters were performed by Microsoft Excel (ver.). Whereas the data representation and analysis were performed and presented by using OriginPro 2021b software (https://www.originlab.com/2021bAnnouncement). Statistical analyses made for statistical difference were performed by pairwise comparisons and Tukey test, to compare mean difference from the fish sample at the statistical significance of $P < 0.05$.

3. RESULTS

In this section, the results of instrumental method optimization, optimization of sample preparation, method validation, and application of the method on real fish plasma samples are presented.

3. 1 MRM transition for Thyroxine (T4), Cortisol, Cortisone, and Testosterone

To guarantee tuning at the correct *m/z* of analytes and internal standards, instrumental mass calibration was done before mass tuning. The MRM tuning was established in positive ion mode (ESI+) for all analytes. The mass tuning for cortisone, cortisol, and testosterone with their respective internal standard were previously accomplished for other studies. The tuning for L-thyroxine was done for this thesis to simultaneously quantify total T4 and steroid hormones. For analytes, the mass tuning was performed individually with the same mass of their respective internal standard. The cone voltage for the first vacuum entry was slowly adjusted to control the ion loss due to high voltage and to obtain clear spectra. The capillary voltage was tested from 1 kV to 3 kV and 3 kV was chosen for all analytes, whereas the collision energy was determined for individual hormones as indicated in Table 6. During mass tuning the protonated ion $[M+H]^+$ was considered as the mass to charge ratio of each analyte. Three MRM transition channels were monitored for thyroxine, while the steroid hormones were detected by one MRM transition for each. One MRM transition was applied for the internal standard of each analyte. Thus, the first abundant peak was used for quantification and the second peak was used for confirmation. In addition to this, the quantification of each hormone was performed with the ratio of analyte peak to the internal standard peak. The brief representative of MRM transition ion parameters was presented in Table 6.

Table 6: Optimized MRM transition, parental ion (m/z), daughter ion, dwell(s), cone voltage (cone), collision energy (Cen), and retention time (tR) for detection and quantification of analytes and internal standards are shown.

Compound name	Molecular (isotope mass)	Parental ion (*m/z*)	Daughter (*m/z*)	Dwell/ s	Cone (V)	Cen (V)	tR
Cortisol	362.21	363.2	121.1	0.015	33	23	2.87
D4 Cortisol		367.2	121.1	0.015	33	23	2.87
Cortisone	360.19	361.2	163.1	0.015	38	25	2.7
D8 Cortisone		369.2	169	0.015	38	25	2.7
Testosterone	288.21	289.3	97.1	0.015	33	20	3.63
D3Testosterone		292.2	97.1	0.015	33	20	3.63
Thyroxine	776.87	777.69	731.7	0.015	45	25	3.2
		777.69	604.9	0.015	45	40	3.2
		777.69	351	0.015	45	45	3.2
13C6 thyroxine		783.69	737.7	0.015	45	25	3.2

3.2 LC Separation of Thyroxine (T4), Cortisol, Cortisone, and Testosterone

LC method development started from adjusting the composition of the mobile phase, optimizing the linear gradient elution, and correcting the flow rate. In this thesis, column BEH C 18 2.1 mm ID and 50 mm length with 1.7 µm particle size was used throughout all experiments. We took the advantage of the broad range pH tolerance of this column and tested both high and low pH mobile phases to achieve high chromatographic separation. Thus, the aqueous part of the mobile phase was either 0.2% formic acid (A1) or 0.2% ammonium hydroxide in water. Gradient elution was with methanol as B1. It was observed that using ammonium hydroxide resulted in low signal for T4. However, the mobile phase with lower pH, 0.2% of formic acid resulted in narrow peaks and sufficient separation for all four compounds at different retention times tR (Table 5). We also tested different flow rates with a combination of the different gradients of the mobile phase B1. Thus, the total 7-minute run was with a high flow rate of 0.200 ml/min for the

first 2 min with 0.10 % of B1 to slowly reach 80.0 % of B1. For the first 2 minutes, the flow from the column was directed to the west, to reduce the contamination of the MS instrument. The gradient increased to 0.250 ml/min and reached 0.400 ml/min at 5-7 min and back to 0.200 ml/min. All analytes were eluted at the same time with their internal standard and relative retention time was considered both for detection and quantification. This was to control the degree of ion suppression causing a signal variation for the peak of the expected analyte. Based on the linear gradient of B1 percentage, cortisone and cortisol were eluted at 2.7 and 2.87 min, respectively. Additionally, T4 and testosterone eluted at 3.2 and 3.63 retention times.

3.3. Optimization of Sample Preparation and Pre-treatment

In this thesis, the main objective was to develop a fast, simple, and reproducible SALLE method of sample preparation for extraction of thyroxine and steroid hormones through evaluating and choosing sample extraction solvent and salting out reagent. This is to avoid analyte loss, removing interfering substances. Clear phase separation and concentrating the analyte to obtain the maximum extraction recovery. In addition, SALLE is not only time-efficient but also economical when compared to the traditional LLE method, which needs an additional step to evaporate the non-compatible solvent and resolubilize the dry extract with polar solvents before being introduced into LC-MS/MS.

In the optimization experiment, different water-miscible solvents in combination with salting-out reagents were examined. To choose more compatible solvents, the mean and RSD% of the result from each experiment with three (n=3) replicates were calculated by considering the peak area of internal standards as an indicator of ion suppression (i.e., the higher the S/N the higher detectability of the analyte). In all optimization experiments, the internal standard (ISTD) was added post-extraction. Any reduction of ISTD peak area is then caused by ion suppression from the co-extracted sample matrix components. The second criteria were the comparison of response factor, which is the ratio analyte peak area to the internal standard peak area. This response factor is directly correlated to the extraction recovery because the ISTD was added post-extraction. The final criteria were the observations of phase separation. However, it was challenging to have both values higher at the same times for different experiments. Hence, the decision made to scarify the relative response and the selection was based on the value of (S/N) ISTD. All SALLE experiments were performed using the same lot of quality control pooled human serum sample and ISTD as a model sample.

3.3.1 Observation of Phase Separation and Analyte clean up

3.3.1.1 NaCl as Salting out reagent with different Solvent

The first SALLE method was done by using saturated NaCl to assist the phase separation between two miscible liquid solvents. In this experiment, the efficiency of saturated NaCl (~5M) was evaluated by adding 300 µL to the vial containing 150 µL of serum sample before the addition of 300 µL of different organic solvent. After shaking the mixture for 1 min, the solution was subjected to centrifuge for 10 min and the phase was observed as indicated in (Figure 9).

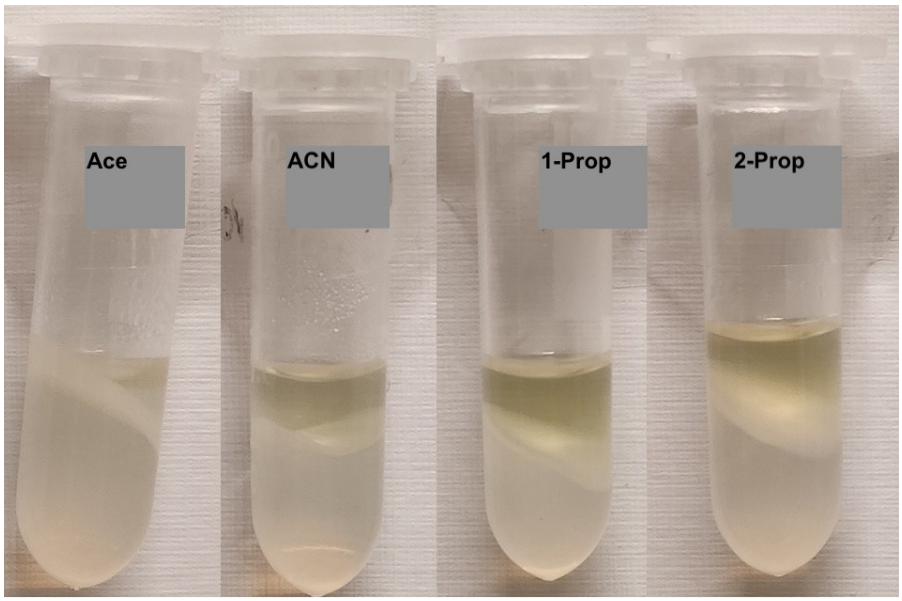


Figure 9: Demonstrating phase separation with different organic solvents and NaCl as a salting-out reagent. All experiments (n=3) consisted of 150 µL of the sample matrix, 400 µL of saturated NaCl (5M), and 400 extraction solvent. The picture shows from the left to right Ace (Acetone), ACN (Acetonitrile), 1-propanol, and 2-propanol.

As indicated in Figure 9, the expected amount of phase separation did not occur when we used acetone as an extraction solvent. Furthermore, the cloudy color of the supernatant was observed.

Thus, the decision made was to increase the amount of saturated NaCl and the volume of organic solvent by 2 resulting in 300 µL of serum sample and 600 µL NaCl with 700 µL of all different extraction solvents. Likewise, both the first and the second experiment resulted in the vast variety of the top phase with the different solvents used. Additionally, the volume of the top phase was slightly higher than the amount of volume initially added for both, 1- propanol and 2- propanol. Hence, this experiment resulted in sufficient phase separation, and it was possible to collect the supernatant and directly injected it into the LC-MS/MS. The result of the second test was presented in Figure 9 after calculating the relative % ISTD area by equation 3 and relative response by Equation 1.2.

This comparison of relative internal standard and relative response with the same experimental setup guided us to choose the best solvent, resulting in a higher value of both parameters with low variation between the same replicate. Sticking to the result observed in all samples containing acetonitrile as an organic solvent, this approach resulted in a better internal standard area related to that obtained with other solvents. However, the relative response for TT4 was lower for this solvent when compared to 1-propanol and 2 propanol. Though, 2- propanol resulted in a high relative response. Contrary, it showed a lower internal standard area. Compared to acetonitrile there were more ion suppression observed when extracting with the other solvents (Figure 10A). Acetonitrile was found to be the most suitable solvent for SALLE, as it reduces matrix effects combined with an overall high extraction recovery for most of analytes. However, 2-propanol resulted in better extraction recovery for T4 (Figure 10B). Similarly, we took into consideration the result from phase separation and the variation in the same experiments (RSD%) and ion suppression. However, the clarity of phase separation, the internal area for blank sample, containing only acetonitrile as a reagent, and the low response of TT4 lead us to look for other salting-out reagents that will be shown in the following section (3.3.1.2).

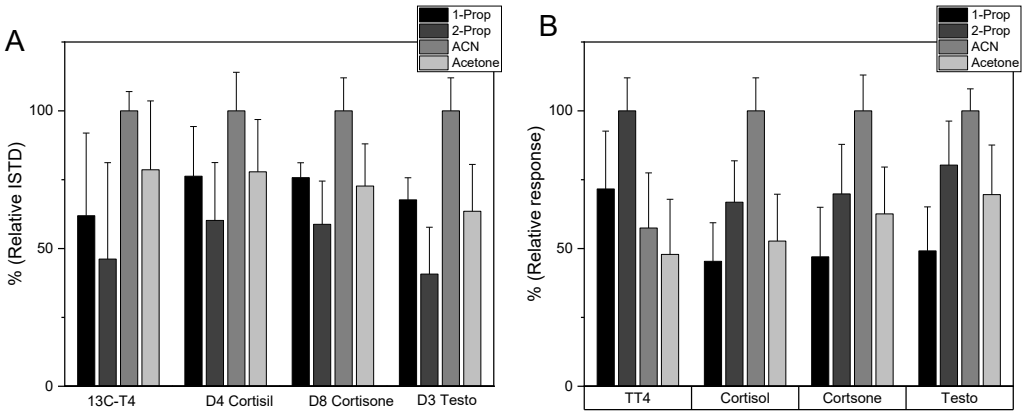


Figure 10: Bar plot representing (A)the relative internal standard peak area % and (B) relative analyte response for TT4, cortisol, cortisone, and testosterone with different organic solvents by combining the salting-out effect of NaCl.

3.3.1.2 Acetonitrile with Ammonium sulfate

Though acetonitrile was suitable for protein precipitation since NaCl resulted in cloudy top phase, which was due to incomplete phase separation and variation between the replicate of the same sample, we decided to examine other salting-out reagents. Thus, the saturated $(NH_4)_2SO_4$ (~5.6M) was prepared in pure water at the temperature of 25 °C. Thus, 50 µL of $(NH_4)_2SO_4$ was added to the vial containing 150 µL of a serum sample before the addition of 300 µL of

acetonitrile. After shaking the sample for 1 min and centrifuging it for 10 min we observed clear and high-volume phase separation.

As shown in Figure 11A, a sample prepared with acetonitrile and $(NH_4)_2SO_4$ resulted in a high relative internal standard area and low variation between the same replicate. The relative analyte response also showed higher extraction efficiency for TT4 by using $(NH_4)_2SO_4$ when compared to NaCl (Figure 11B). However, the extraction efficiency for cortisol was found to be low with $(NH_4)_2SO_4$ when compared to the result from NaCl. Based on these results, the conclusion was made to use $(NH_4)_2SO_4$ and acetonitrile for the SALLE method sample extraction.

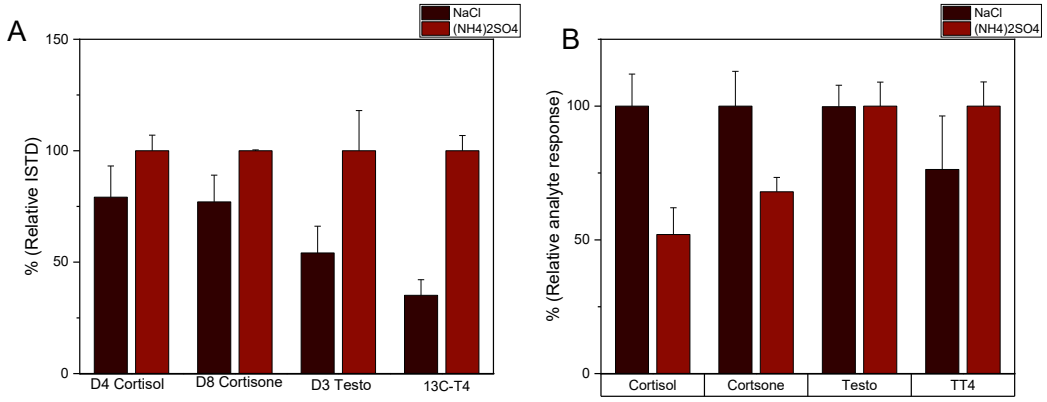


Figure 11: Bar plot illustrating (A) the relative internal standard peak area (%) and relative analyte response (%) for TT4, cortisol, cortisone, and testosterone extracted by acetonitrile with NaCl and $(NH_4)_2SO_4$ as a salting-out reagent.

3.4. Optimization of SALLE Experiment

3.4.1 Organic phase acid and base concentration and ion-pairing reagent

To optimize the concentration of TT4 in the organic phase and reduce the variation within the same sample, a systematic study was conducted. Thus, 0.5% of acetic acid (AA) was added to the top phase to reduce the pH (~5). The acidified samples contained 0.5% acetic acid combined with 300 µL, 50 µL, and 700 µL (sample matrix, $(NH_4)_2SO_4$ and acetonitrile), respectively. Thus, the acidification experiment aimed to neutralize the phenolic hydroxyl group of TT4 pKa value, which is 7.43, that would otherwise be partially ionized at the neutral pH of ~7. Based on the result from acidification, we planned to increase the pH (~10) by adding ammonium hydroxide (NH_4OH) to the same experiment set up. Hence, the second experiment was fortified with 0.05M of (NH_4OH) to increase the pH (~10). Nevertheless, the experiment with each buffer did not yield satisfactory results, either in ion suppression or extraction efficiency,

compared with acetonitrile alone. Relatively high pH was much more favoring protein solubility in the top phase.

Considering this, we decided to perform the experiments with ion-pairing reagents, such as trifluoroacetic acid (TFA) and formic acid (FA). In this case, we examined the optimum concentration of TFA, as it increases the hydrophobicity of the solution and causes ion suppression when added in high volume. As the concentration of TFA increased, the peak area of ISTD decreased. Thus, the optimum signal-to-noise ratio for both ISTD and analytes was achieved when using 0.01% of TFA. However, we decided to try formic acid as TFA also increase the solubility of small peptide and hydrophobicity of the top phase. Compared to all experiments with buffer and ion-pairing, 10% of formic acid resulted in slightly better extraction efficiency of TT4 as shown in figure 12B. Although, formic acid did not show a significant difference from the control experiment (containing only acetonitrile). As shown in Figure 12A, adding either acid or base resulted in increasing ion suppression for TT4. Based on this, we decided to optimize another parameter, such as salt concentration, the volume of solvent, and the addition of methanol to the top phase.

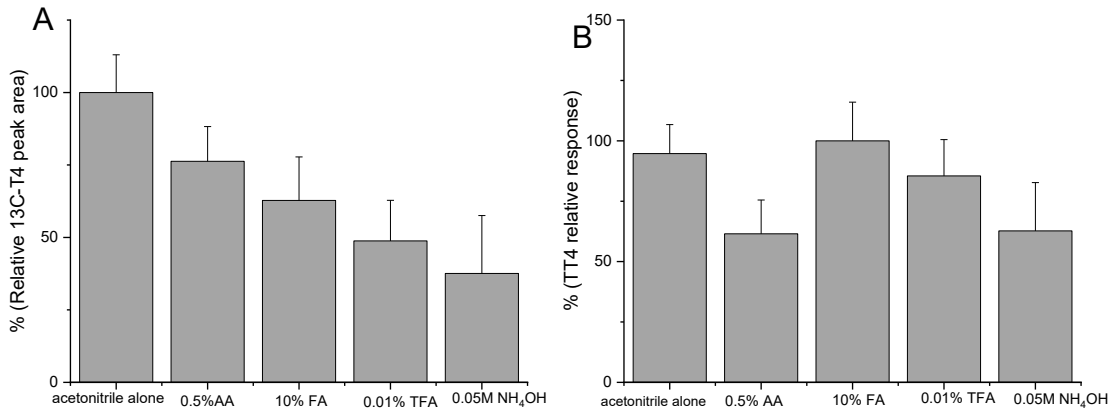


Figure 12: Bar plot representing the relative peak area of 13C-T4% (A) extracted with different and relative analyte responses for TT4 (B). All experiments contained acetonitrile as a solvent and $(NH_4)_2SO_4$ as salting-out reagent alone, with 0.5% of AA, 10% of FA, 0.01% of TFA, and 0.05M of NH_4OH to adjust the pH of the organic phase.

3.4.2 Effect of salt concentration on extraction efficiency

To optimize the extraction recovery and protein precipitation we evaluated the effect of salt concentration. This was done by taking into consideration the volume of organic solvent added to the sample. Thus, different ratios (i.e., 17%, 33%, 40%, and 50%) of salt to solvent were investigated both for clarity of the top phase and the extraction efficiency. Increasing the

amount of ammonium sulfate added to the solution resulted in the shrinking of the top phase. Contrary to this, a low concentration of ammonium sulfate resulted in a high volume of the top phase. In our previous experiment, we observed that adding high concertation of NaCl increased the volume of the top phase.

Though the high concentration resulted in concentrating supernatant, it also caused the hazy color supernatant and the protein molecules to start disaggregating and sticking to the wall of vials. Having this fact, slightly increasing the salt concentration from 38% to 42% resulted in the concentrated organic phase and clear phase separation. However, at the ratio of less than 38% the organic phase containing water and the volume were higher. The result from this experiment also showed that the analyte peak area was constant and got lower as the concentration of salt decreased (Figure 13A).

3.4.3 Effect of volume of acetonitrile on analyte enrichment

The next goal was to achieve the optimum extraction recovery of all analytes by concentrating the top phase (namely, analyte enrichment). Accordingly, the new experiment was performed using different volumes of acetonitrile (i.e., 250, 300, 400, 500, and 700 µL), which was added to the vial containing 150 µL of serum sample and before the addition of saturated ammonium sulfate (40%). As expected, analyte enrichment increased when using lower volumes of acetonitrile, which was observed as a higher peak area for the analytes (Figure 13B). Thus, the high peak area was directly proportional or dependent on the concentration of analytes. Optimum analyte detectability was achieved when 250 µL of acetonitrile was used. Previous experiments found acetonitrile with ammonium sulfate to produce high extraction efficiency and reduced matrix effects. This experiment found that it was possible to reduce the volume of acetonitrile solvent to increase analyte concentration and peak area, without losing signal caused by matrix effects. However, a further reduction in acetonitrile solvent volume was not practical.

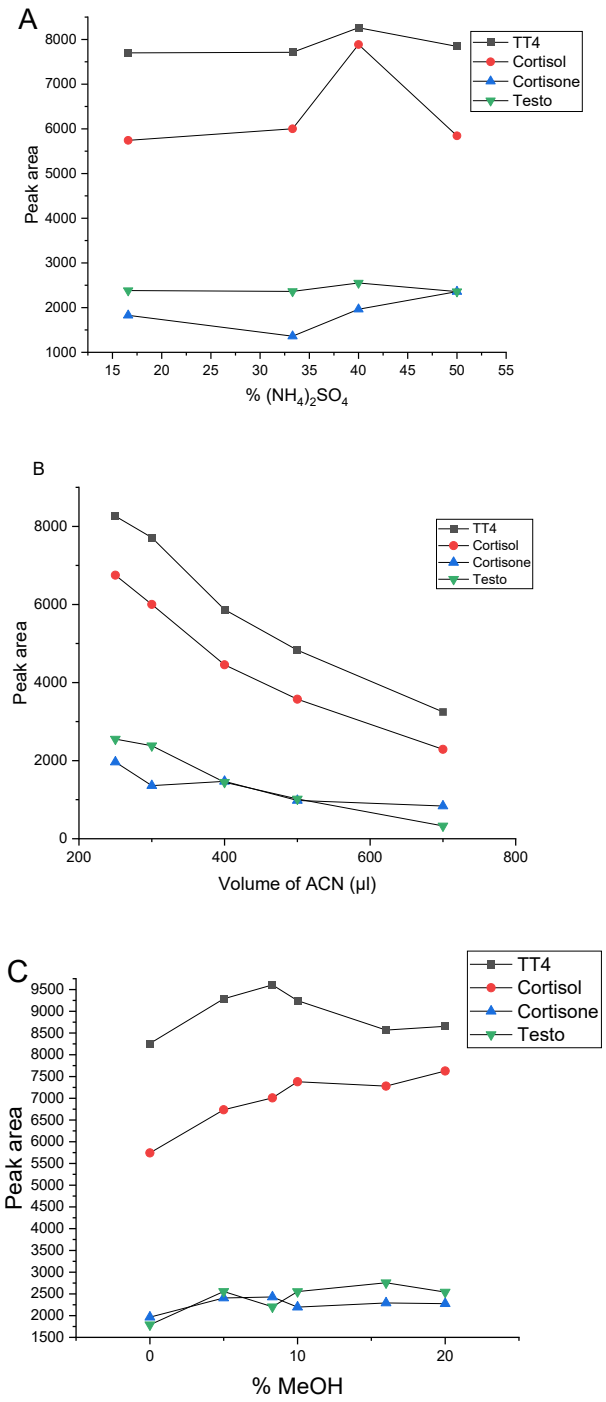


Figure 13: Graphs showing the effect of different optimization parameters (A). This figure shows the effect of salt on extraction efficiency when added in different concentrations (n=3). (B). The effect of ACN volume on analyte concentration (n=3) is shown. (C) shows the effect of methanol on the solubility of TT4, cortisol, cortisone, and testosterone when added in different methanol volumes at the same salt concentration (n=3).

3.4.4. Combination of acetonitrile with methanol

To increase the solubility of analytes and polarity of the organic phase, different volumes of methanol were added to acetonitrile (i.e., 5%, 8%, 10%, 16%, and 20%) (Figure 13C). As

expected, adding a small amount of methanol increased the solubility of analytes and kept them in the organic phase. Additionally, methanol increases the polarity of the analyte. However, it was noted that increasing the amount of methanol was favoring the water to come up to the top phase. As we increase the volume of methanol the supernatant got a cloudy appearance, and no phase separation was observed at 30% and higher. Thus, we decided to add 10% of the methanol to the final method, which was added to the serum sample as 25 µL of internal standard working solution, comprising a mixture of internal standards dissolved in methanol. The optimized SALLE method is shown in (Table 7).

Table 7: The optimized value of parameters for the final SALLE method

Parameter	ISTD in MeOH (µL)	Sample volume (µL)	Saturated $(NH_4)_2SO_4$ (µL)	ACN (µL)	Shaking(min)	Centrifugation (min)
Optimum cons.	25	150	100	225	1	10

3.5. Method validation

3.5.1. Linearity and linear range of the method

Both linearity and linear range of the method were determined by using the calibration standard solution prepared in water containing 20% of methanol from the stock mixture indicated in (Table 3). Thus, the calibration standard solution was treated as a real sample and run with all method validation parameters. The linearity and linear range were obtained by plotting the concentration of each hormone on the x-axis and the analyte responses on the y-axis, which fitted to the least linear regression equation (Equation 6). The method gave nice linearity, as observed by the coefficient of determination (R^2) ranging from 0.997 to 0.999 for all analytes as indicated in (Table 8). In addition to this, the method was showing the linearity overall calibration standard solution analyzed. Hence, all analytes showed the linearity on all working ranges, based on processed calibration solution. The calibration curve of all hormones was presented in (Annex 1).

3.5.2. Recover and accuracy

The recovery of the SALLE method was determined at three different concentration levels (specifically: low, medium, and high) of spiked samples in QCs human serum sample as indicated in (Section 2.4.1) for all analytes. As the stock mixture was prepared in methanol, the non-spiked samples were diluted with an equal small volume of methanol. This avoids the

variation due to sample dilution for spiked samples. Thus, the recovery of the optimized SALLE method for the low, medium, and high spiked samples were 76%, 92%, and 95% for TT4, 96%, 81%, and 87% for cortisone and 96%, 90%, and 102% for testosterone, respectively. Considering this, the accuracy of the method was calculated as a bias in (RSD%) of recovery at all three gradients of spiked samples according to (Equation 11). The result showed (Relative bias%) < 8% for TT4, <10 % for cortisone, and 8% for testosterone as indicated in (Table 8). Due to the interference substance with D4 cortisol, indicated in (Figure 15 D, and F) the extraction recovery of cortisol was relativity inconsistent.

3.5.3. Repeatability and Reproducibility

The intra and inter-day variation of the method was evaluated by analyzing ten (n=10) replicates of QCs samples processed on the same days and five consecutive working days. Thus, the relative standard deviation (RSD%) of the replicates was calculated according to Equation 5. As indicated in Table 8, both repeatability and reproducibility of the method were found to be RSD% <15 across all hormones. However, the calculated (RSD%) for cortisol was relatively high 17% for the intermediate precision.

3.5.4. Limit of detection and Limit of quantification

The limit of detection limit (LOD) and limit of quantification (LOQ) of the developed method were determined by processing calibration solution with ten blank samples. Subsequently, the response from the calibration curve was plotted on the y-axis whereas the concentration of all individual hormones was plotted on the x-axis. This was to achieve the slope and correlation coefficient $R^2 \geq 0.997$ of the curve. The mean and SD of the signal-to-noise ratio (S/N) of the blank sample were first determined as explained according to (Equation 2 and 4), respectively. Hence, the y-intercept was forced to the origin LOD, and LOQ were calculated by dividing 3X and 10X SD of S/N ratio blank sample to the slope of the calibration curve as indicated in (Equation 7 and 8). Thus, LOD and LOQ were obtained as 0.04 and 0.18 ng/mL for TT4, 0.2 and 0.15 ng/mL for cortisol, 0.07 and 12 ng/mL for cortisone and 0.01 and 0.08 ng/mL for the testosterone, respectively (Table 8).

Table 8: Precision, extraction recovery, LOD, LOQ linearity, and linear range

Parameters		TT4	Cortisol	Cortisone	Testosterone
Precision	Repeatability	13	14	14	13
(R.S.D) %	Intermediate	13	17	14	13
Recovery%	low	76(7.1%)	127	96(9.3%)	96(7.6%)
(Relative	medium	92(4.3%)	55.00	81 (4.6%)	90(4.3%)
bias%)	high	95 (1.7%)	75	87 (2.4%)	102 (2.6%)
LOD (ng/mL)		0.04	0.02	0.07	0.01
LOQ (ng/mL)		0.18	0.15	0.12	0.08
Linearity (R^2)		0.997-0.999	0.998-0.999	0.998-0.999	0.999
Liner range ng/mL		0.18-200	0.15-100	0.12-100	0.08- 90

3.6. Application of methods on Atlantic salmon plasma sample

The final aim of this thesis was to apply the newly developed and validated method to fish plasma samples. Thus, because of the difference in the plasma composition of the two matrixes, the phase separation has resulted in different colors and shapes (Figure 14). We observe the white flat layer between the aqueous and solvent phase during phase separation in human serum. However, it was only a big white precipitate layer in fish plasma. Additionally, an orange-yellow pigment in the human serum, which could be the bilirubin was not seen in the fish plasma sample. Moreover, the difference in the appearance of the water phase was also observed. In both matrixes, the shape of the supernatant was changed from circle to partial arc of the circle after methanol was added as indicated in (Figure, 14)

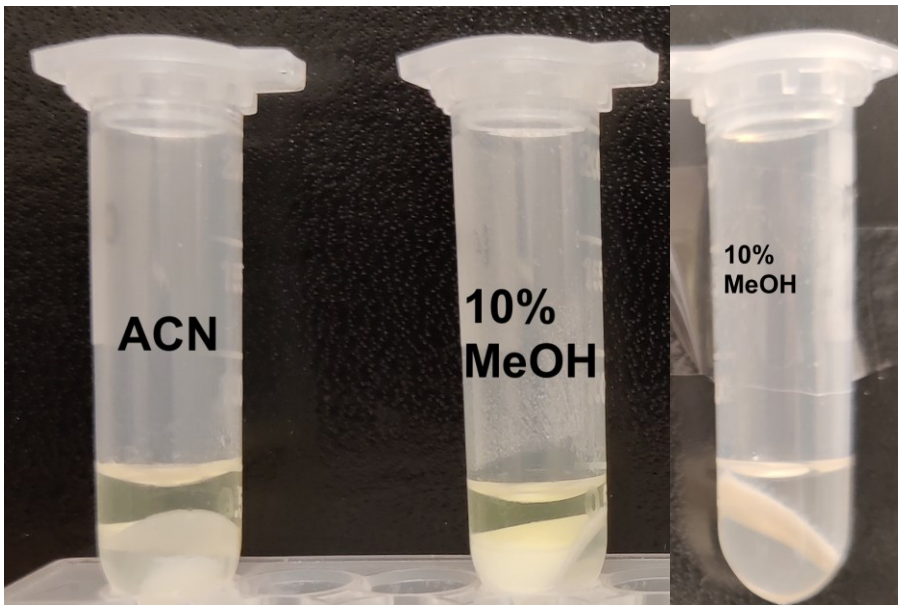


Figure 14: shows phase separation with acetonitrile alone and with 10% methanol as an organic solvent and ammonium sulfate as a salting-out reagent. The first from the left contains only acetonitrile and the second two contain 10% MeOH with acetonitrile with human serum and fish plasma sample, respectively.

Among all measured hormones the level of cortisol was quite elevated in fish plasma samples, particularly obtained from net 6 (Table 9). Thus, the measured value was significantly different in this net. However, the method suffered from interference affecting the accuracy for measurement of cortisol concentrations in both matrixes. This was due to the interference in the MRM channel for the deuterium-labeled internal standard of cortisol (i.e., D4 cortisol). Most interference was observed in the quality control human serum samples (Figure 15F), while there was no interfering substances present in the calibration standard solutions as shown in (Figure 15B). Thus, the measured level of cortisol resulted in high variation. Interestingly, the peak height of cortisol and its detectability in fish plasma were fairly comparable with QCs human serum and calibration standard solution (Figure 15A, C, and E). Hence, it should be fairly simple to overcome this interference in future work, for instance by using an internal standard with different types of labelling such as D5 cortisol.

Table 9: The mean value of total L-thyroxine (TT4), cortisol, cortisone, and testosterone (Testo) in the fish plasma sample

			Mean ± SE (ng/ml)			
	N	n	TT4	Cortisol	Cortisone	Testo
Net5	9	3	2.28 ± 0.38^{a}	14.13 ± 1.8^{b}	13.76 ± 1.10^{a}	0.32 ± 0.04^{a}
Net6	9	3	1.79 ± 0.25^{ab}	89.18 ± 7.89^{a}	32.93 ± 0.72^{b}	0.28 ± 0.04^{a}
Net7	9	3	1.23 ± 0.19^{b}	21.07 ± 2.23^{b}	18.52 ± 1.37^{c}	0.32 ± 0.04^{a}

Note: N= number of samples, n=number of replicates. Mean with different superscripts in the same column for the same compound are significantly different at P<0.05.

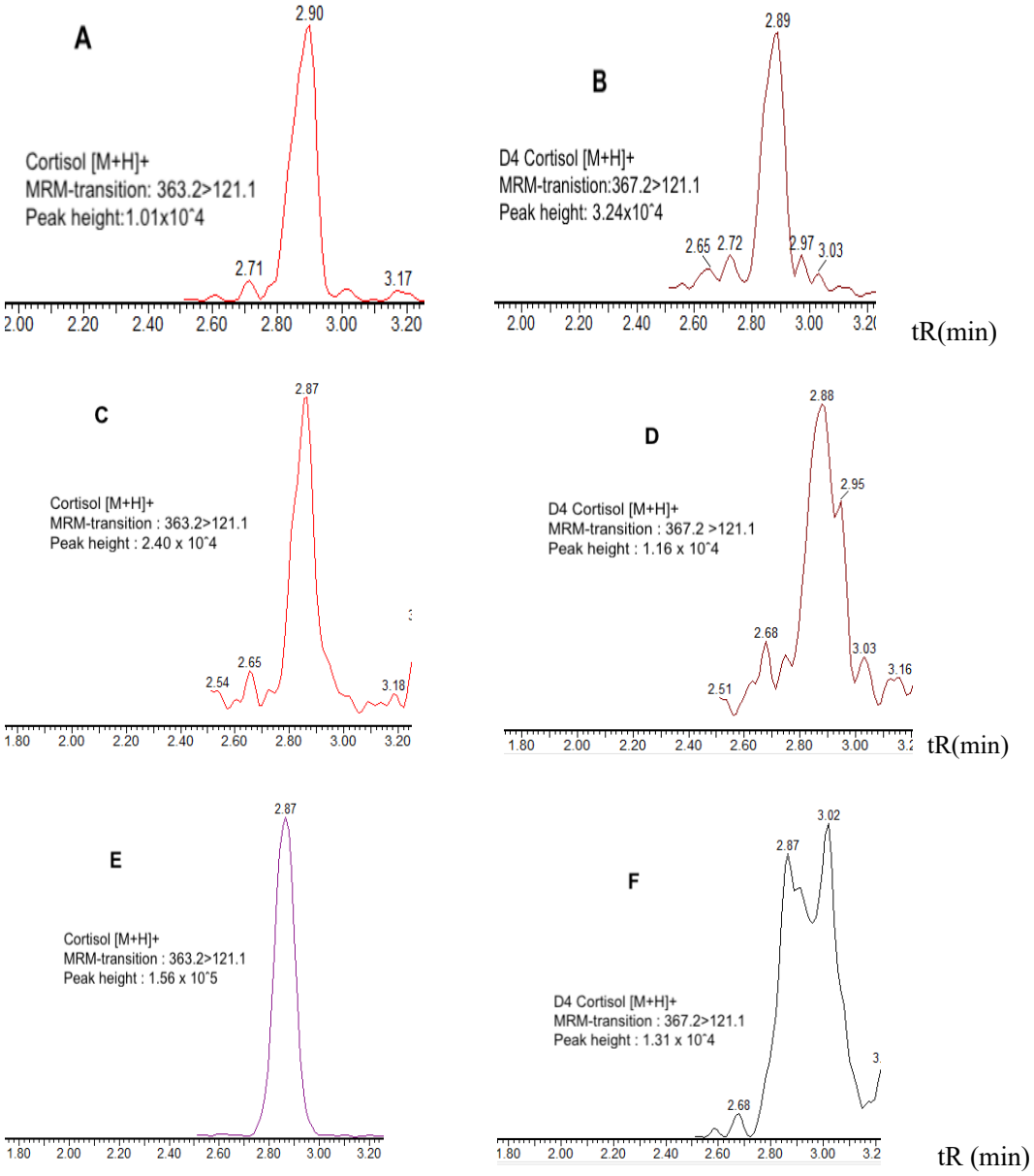


Figure 15: shows the MRM chromatograms of cortisol $[M+H]^+$ and D4 cortisol $[M+H]^+$ in calibration solution containing 2.88 ng/mL of cortisol (A and B) in fish plasma (C and D) and in quality control human serum samples (E and F) extracted by using acetonitrile containing 10% of methanol and ammonium sulfate.

Remarkably, the current method was sensitive and selective for cortisone in both matrix. Additionally, the concentration of cortisone was found to be correlated to that of cortisol in all net (Table 9). As indicated in figure 16A, B, C and D both cortisone and its deuterium labeled internal standard D8 Cortisone was eluted with no potential interfering substance. Thus, the peak height of internal standard in both matrix was fairly consistent in both matrix as they spiked with an equal concentrations of D8 cortisone.

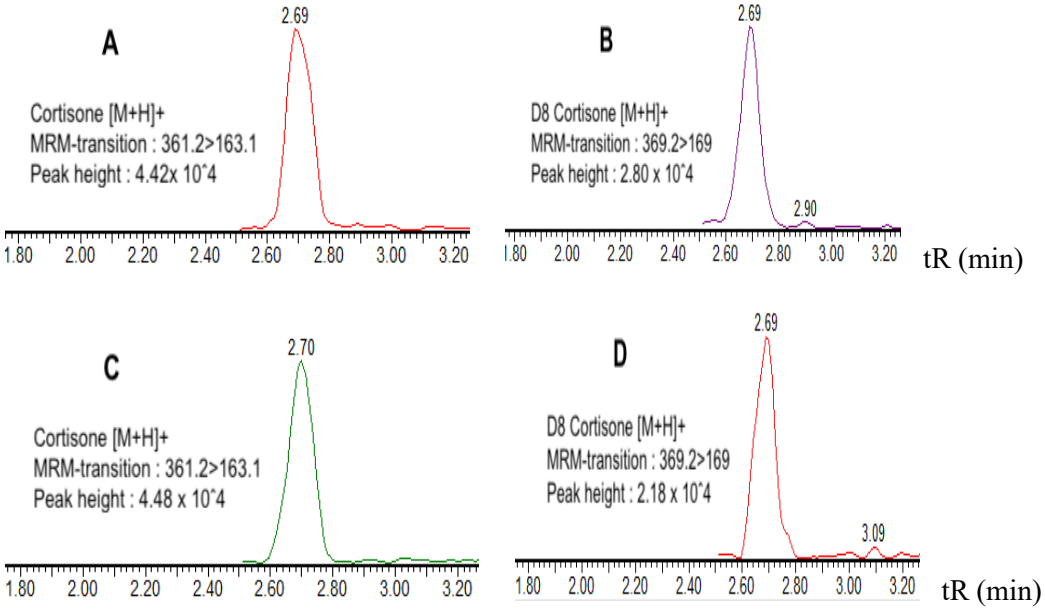


Figure 16: illustrates the comparable MRM chromatograms and peak intensity of cortisone $[M+H]^+$ and D8 Cortisone $[M+H]^+$ in fish plasma (A and B) and quality control human serum samples (C and D) extracted by using acetonitrile containing 10% of methanol and ammonium sulfate.

The concentration of testosterone in fish plasma was found to be very low and closest to the LOQ of the method in all fish (Table 9). Thus, we calculated the LOD and LOQ from the ratio of analyte peak to the ISTD peak, which was 0.28 ng/mL and 0.63 ng/mL, respectively. This indicates that the level of testosterone in fish was lower than the calculated LOD value. As indicated in the chromatogram (Figure 17 A) the peak of testosterone was not separated from the background noise. However, the peak height of D3 testosterone was shown a comparable signal-to-noise ratio when compared to a peak height of QCs (Figure 16 B and D).

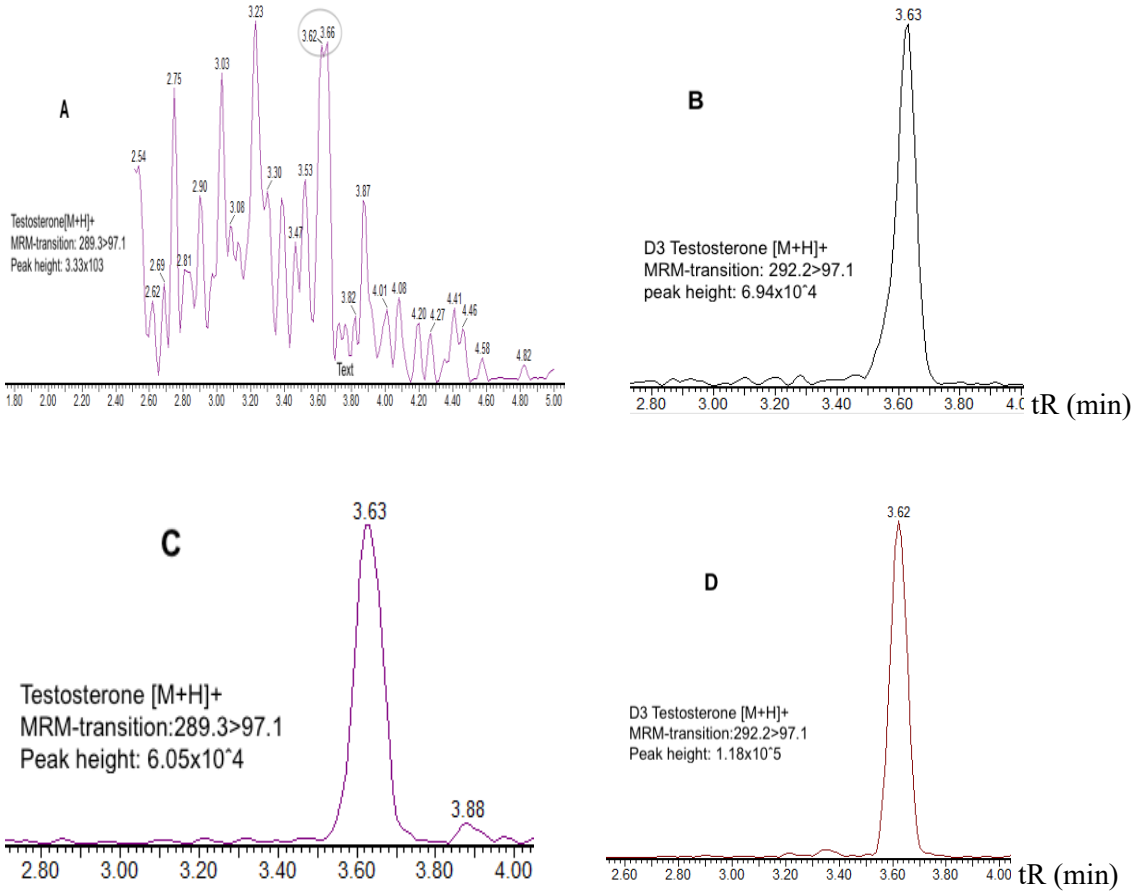


Figure 17: illustrates the comparable MRM chromatograms and peak intensity of testosterone $[M+H]^+$ and D3 testosterone $[M+H]^+$ in fish plasma (A and B) and quality control human serum samples (C and D) extracted by using acetonitrile containing 10% of methanol and ammonium sulfate.

The mean average of TT4 measured in fish plasma from the different net and individual samples were shown in (Table 9) and (Annex 2). However, a high variation between different samples was also observed. Similarly, it was indicating that the variation was from fish plasma matrix resulting in the low peak area of ISTD in the different samples, which was originally spiked with the equal concentration. This was caused by various degree of ion suppression in those samples. Thus, the matrix component in these samples resulted in high variation and reduced the detectability of TT4 in some of the fish plasma samples. Interestingly, the variations between the replicate of the same sample were comparable and consistent. Moreover, the level of TT4 in fish was also low in some samples. In most fish plasma samples, the measured peak height of 13 C6 was comparable between QCs (Figure 18 B and D).

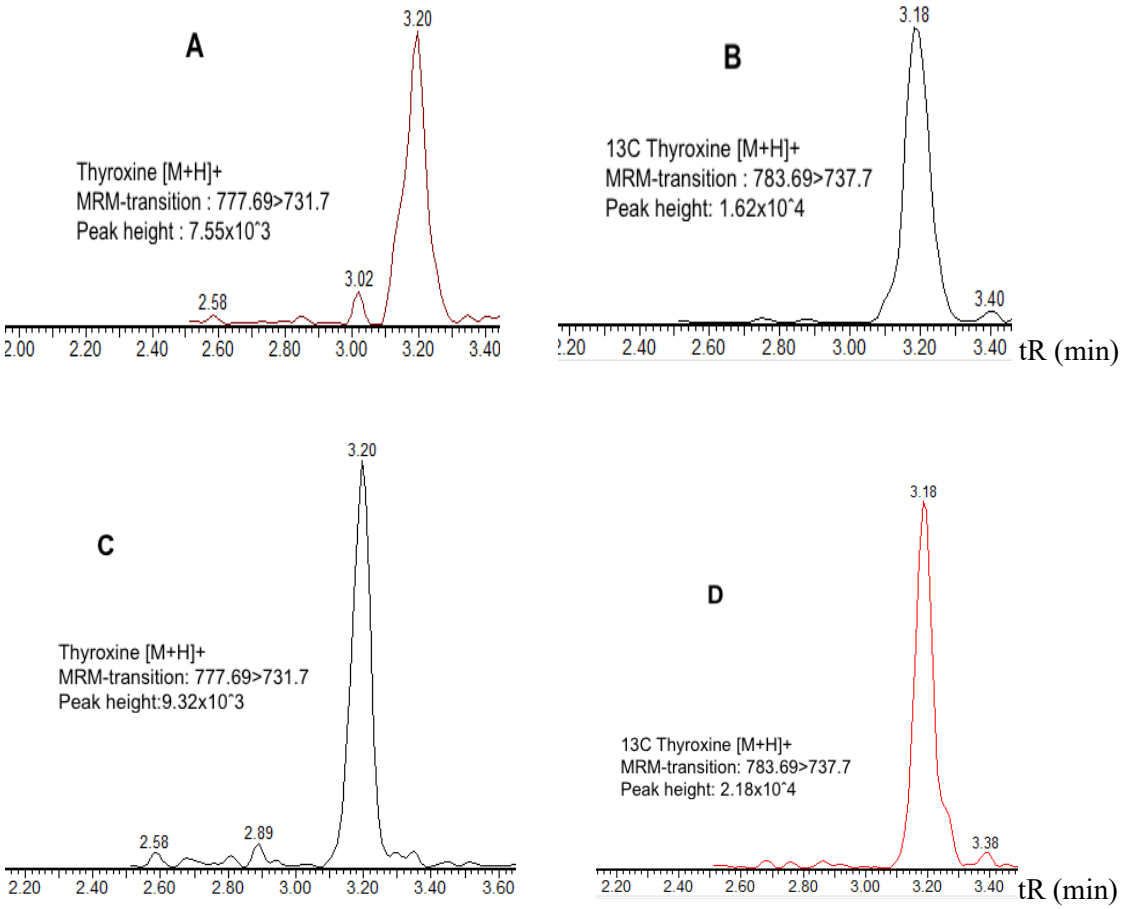


Figure 18: illustrates the comparable MRM chromatograms and peak intensity of cortisone $[M+H]^+$ and D8 Cortisone $[M+H]^+$ in fish plasma (A and B) and quality control human serum samples (C and D) extracted by using acetonitrile containing 10% of methanol and ammonium sulfate.

4. DISCUSSION

In this section the overall results from experimental optimization, such as analyte detection (ESI, and MRM) and separation (mobile phase and stationary phase), visual observation of SALLE vial, matrix effects and extraction efficiency with different solvent, method validation, and application of the novel method to fish plasma sample were briefly discussed and compared with the previous studies.

In this study, ESI+ mode was used as a source of ionization, according to previous laboratory examination (Nadarajah et al., 2017) and as it was reported by many authors who obtained a low limit of detection and high sensitivity for both thyroxine and steroid hormones (Blackwell & Ankley, 2021b; Boggs et al., 2016; Bråtveit, n.d.; Bussy et al., 2017; Hansen et al., 2016; Noyes et al., 2014b; Woźniak et al., 2017).

The capillary voltage for all analytes was set at 3 kV, which assist the droplet forming process of the electrospray ionization (ESI) to generate a high signal. Furthermore, the collision energy was set separately for each analyte and gradually increased to achieve the intense fragment ion by using argon gas in a collision cell. Interestingly, in our method, there was no sodium adduct $[M+Na]^+$ found. Hence, we only considered the $[M+H]^+$ during mass tuning. Overall, the MRM transitions in the established multiplex MS/MS method was selective enough for the detection and quantification of thyroxine, testosterone, cortisone, and cortisol.

Consequently, we chose methanol as a mobile phase B1 as it had good solubility for our analytes to assist elution from the stationary phase. As our results indicate, the pH of the aqueous part of the mobile phase was also important to be taken into the consideration. We tested 0.2% of ammonium hydroxide in water (A1) and that resulted in total loss of the T4 peak. This was likely due to the phenolic hydroxide group of this hormone and the fact that will be de-protonated and ionized at high pH of ~10 providing a negative charge. Additionally, the acidic group of this compound started to donate a proton and resulted in a negative charge which cannot be detected by (ESI+) mode. Furthermore, increased ionization of T4 made it more polar and would therefore reduce its retention time. In contrast, 0.2% formic acid in water (A1) resulted in good separation and high signal intensity for all analytes as it provides enough protons for the positive electrospray ionization (ESI+) process.

The LC separation was achieved with the total 7-minute run including column equilibration time. To ride off polar interferences that might have been coextracted during sample preparation, including salt, the flow from the column was directed to the waste during the first minutes of the chromatographic separation. In addition, it aims to reduce the contamination of the MS instrument. In the final optimized LC linear gradient elution method, all analytes were

eluted within 3.63 min, including non-polar compounds such as T4 and testosterone by increasing the gradient of B1. This was done to assist those hormones to pass through the nonpolar stationary phase as they otherwise would have retained for a long time. Therefore, the current method was fast enough to run comparably many samples in a short period when compared to other studies including Noyes et al.(2014) who used formic acid as aqueous phase buffer and achieved separation of T4 in 17 min. Likewise, (Ke et al., 2014b) used 0.2% of formic acid as aqueous mobile phase and quantified testosterone with 3.48 min retention time. The method developed by Domenech-Coca et al. (2019) achieved multiplex separation of cortisol, thyroxine, and testosterone at 6.09, 6.85, and 7.51 min, respectively. However, our method is comparable with (Yong et al., 2014) who used an isocratic mobile phase containing 0.1% formic acid in 70:30 methanol and water reported a 2.27% retention time for T4.

SALLE method is a relatively new, fast, simple, and cheap sample extraction technique that uses water-miscible solvent and the addition of high salt concentration to induce phase separation. It also effectively separates small biomolecules (Mariño-Repizo et al., 2018; J. Zhang et al., 2009). Moreover, it results in a higher recovery of analytes compared to simple protein precipitation as it concentrates the analytes of interest in the supernatant and it is suitable for automation (Kvamsøe et al., 2020). Thus, in this study, we developed the optimized first SALLE method to extract TT4, total cortisone, total cortisol and total testosterone from human serum samples and it was successfully applied to fish plasma samples.

The first and most important parameter to consider when developing the SALLE method is the selection of extraction solvent and salting-out reagent. Thus, in this study, we performed the first SALLE experiment by using saturated NaCl as a salting-out reagent with combining the effect of different organic solvents such as acetonitrile, acetonitrile with methanol, 1-propanol, 2-propanol, and acetone. The selection of extraction solvent was based on the Hansen solubility parameter of analytes to reduce analyte loss due to low solubility. From the first tested experiment, all solvents resulted in different amounts of organic phase except for acetone, which showed the total shrinkage of the top phase and formed the lipid layers on the top of the aqueous phase when an equal amount of saturated NaCl was added. The extraction recovery for T4 was high when we used 2-propanol and high for cortisol with ACN. This is according to HSP theory, as T4 has low dP and high dH and 2-propanol has a much better match for dP and dH with T4. While ACN has high dP and low dH, which is the best match for cortisol. Moreover, increasing acetone volume with salt concentration resulted in better selectivity for TT4 and cortisol. However, by comparing this result to acetonitrile, acetone is not the solvent of choice as it causes the coextraction of lipids (Alzweiri et al., 2008; Schenck et al., 2002). In contrast to acetone, 1-propanol and 2-propanol resulted in a high volume of top phase due to the high hydrogen bonding between water and propanol. Then it is likely that some water entered

into the solvent phase. The other possible cause of ion suppression might be the phospholipids present in blood as it is known as the major interference and challenge for analysis of plasma samples by LC-MS (Woźniak et al., 2017)

Based on the SALLE result from NaCl and acetonitrile, it was emphasized that acetonitrile is the best organic solvent for SALLE due to its ability to produce a clear top phase, precipitate proteins, and providing low ion suppression for all hormones. In addition to this, the solvent has a wide range of applications for both polar and non-polar analytes, as it was suitable for the simultaneous extraction of our analytes. Thus, acetonitrile was the most commonly used solvent for SALLE-type samples for extracting multiplex molecules in one single sample (Du et al., 2014; Kvamsøe et al., 2020). Additionally, it's the low boiling point, low viscosity, low toxicity, and effective deproteinization (Jafari & Entezari, 2020; J. Zhang et al., 2009), make it applicable in all fields of LC-MS/MS studies. Similarly, in addition to the high power of protein precipitation of acetonitrile it is also a poor solvent for phospholipids (Alzweiri et al., 2008). The reduced lipid solubility in acetonitrile is most likely the best explanation to reduced ion suppression observed in SALLE with this solvent. Furthermore, it's a widely used solvent with RP-HPLC because of water miscibility, low viscosity when mixed with water, and for providing high chromatographic efficiency (Arakawa, 2018; Liu et al., 2019).

However, the selection of suitable salt for phase separation in SALLE is the second main issue. Thus, the ammonium sulfate was selected from the Hoffmeister series, which contains the series of salt-based on anion and cation. Based on our result, the efficiency of ammonium sulfate was high both in and resulted in an adequate top phase and protein precipitation. This is because ammonium sulfate has a high solubility in water due to its high ionic strength (Alshishani et al., 2017; Jafari & Entezari, 2020). On top of that, the ammonium salt is compatible with LC-MS/MS (M. Li, Wang, et al., 2021)

The optimization of the SALLE technique for optimum extraction recovery of TT4 was performed by conducting different experiments, such as adjusting the acid, base concentration of the organic phase and evaluating the effect of ion-pairing reagents. This experiment was planned for the fact that L-thyroxine has three pKa values which are pKa1 = 0,27 (carboxylic), pKa 2 = 7.43 (phenol) and pKa 3 = 9.43 (amine) group of the compound. This makes TT4 the most challenging analyte as it would probably ionize in almost all pH ranges (Yong et al., 2014). In our experiments, we had tested first by adjusting the pH of the organic phase (~5 and 10) aiming at neutralizing the phenolic group. However, at both pHs, there was no significant difference in the extraction efficiency of TT4. We observed that adding ammonium hydroxide to the sample solution resulted in increasing the solubility of small protein, and this was detected by the low signal of internal standard. Moreover, we examined the effect of ion-pairing

reagent on the extraction efficiency of TT4. This experiment was planned to neutralize the amine group as its ion pair with TFA. In addition to this, TFA can also reduce the pH of the solution. Thus, both the acidic group and phenolic group would be neutralized. To effectively study the effect of TFA the test experiment was evaluated with varying percentages of TFA. This is because TFA is particularly interacting with analytes, and it can cause ion suppression by reducing ion-pair formation in ESI droplets (Taylor, 2020). The result from this experiment concluded that 0.01% of TFA results in better analyte signal when compared to 0.005%, 0.015%, and 0.02% of TFA. However, this experiment was not included in the final method development, because it did not result in a higher signal when compared to the control experiment. Furthermore, we also demonstrated that the addition of formic acid resulted in good extraction efficiency of TT4; besides, it also resulted in a low internal standard area when compared to the experiment with only formic acid added. Therefore, both high and low pH could in theory increase the solubility of small proteins and peptides, which would result in ion suppression. However, (Domenech-Coca et al., 2019) used 10% of formic acid with ethyl acetate in LLE to extract L-thyroxine from a serum sample. Moreover, addition of ammonium sulphate by itself slightly reduced the pH (~6) of the solution, which might affect the partitioning of T4 into solvent phase. Though we don't have data for the control experiment with buffering to neutral pH.

The main parameters for the SALLE method optimization performed in this study were optimizing salt concentration, the volume of organic solvent, and the addition of methanol to increase the extractability of analytes. The ratio of organic solvent to protein precipitation and concentration of salts were optimized to achieve maximum extraction efficiency. In addition to this, the higher solvent volume in the SALLE experiment leads to a decrease in the concentration of analyte. This was results in low analyte enrichment, which was observed in the low peak area in our experiment. Thus, the measured peak area depends on the analyte concentration (i.e., analyte enrichment) of the injected sample on the LC-MS/MS instrument. However, the extraction recovery, if defined as the total amount transferred to the solvent phase, can still be the same. Considering this, decreasing ACN volume down to 250 µL resulted in a very concentrated top phase and still keeping a practical volume for manual removal of solvent or direct injection from the top phase.

Furthermore, different concentrations of ammonium sulfate resulted in different amounts of phase separation. The optimum extraction efficiency was achieved when 40% of saturated ammonium sulfate (i.e 100 µL of salt and 250 µL of ACN) was added. At a low salt concentration, the water started entering the top phase. However, as the concentration of salt increased, the top phase shrank down due to the interaction of the high dielectric constant of

water (Jafari & Entezari, 2020). However, there is an optimum value for the concentration of salt. Above those values the aqueous phase got saturated, and proteins started to precipitate and form a layer in the middle between the aqueous phase and solvent phase.

Furthermore, the effect of methanol on extraction efficiency was achieved through the addition of different volumes of methanol to the top phase. However, the addition of methanol to the ACN increased the volume of supernatant. This was due to the high interaction between methanol and water. However, adding a high salt concentration resulted in a reduced water amount in the top phase. Additionally, the shape of the supernatant and protein precipitation layer in the middle changed to a curve when adding methanol to acetonitrile. We also observed that when using 1- propanol and 2- propanol. Apart from this, it was observed that the addition of more methanol increased not only the volume of the top phase but also the matrix effect. This is due to the solubility of phospholipids being higher in methanol, which otherwise is very low in pure ACN (Alzweiri et al., 2008). Thus, we decided to add only 10% of methanol to the volume of acetonitrile as it resulted in a satisfactory combination of high extraction recovery and reduced ion suppression. Similarly, Zhao and Juck (2018) recommended 5 to 15% of methanol in ACN to increase the extraction capability of the analyte, if needed. In contrast to this, M. Li, Zhuang et al. (2021) reported that the addition of methanol in the (9:1) ratio resulted in ion suppression when compared to acetonitrile alone.

The optimized SALLE method was validated according to the Eurachem guideline (*The Fitness for Purpose of Analytical Methods*, 2014). The method showed high linearity ($R^2 \geq 0.997$) and a linear for all hormones. The initial detection limit and limit of quantification also showed that the method is sensitive enough to detect and quantify hormones in small concentrations.

Furthermore, the method showed acceptable precision and accuracy with high extraction recovery for TT4, testosterone, and cortisone. However, the estimated recovery of cortisol showed inconsistency due to matrix interference with the standard area of cortisol (D4 cortisol). In addition to this, the calculated value of LOD and LOQ also showed the sensitivity and selectivity of the methods. The LOD for testosterone and cortisol are comparable with the LOD reported by Noure et al. (2020), who used the LLE sample extraction method, and the recovery of our current method is higher than the recovery reported by the same author and lower than the LOD reported by Wozniak et al. (2017), which still reported the lower recovery than the current method 0.09 µL LOQ.

As per our final aim, we applied the newly developed method to the real fish plasma. Regardless of the difference in the composition of matrices in human serum and fish plasma, the method showed good detectability for each hormone in the fish plasma samples. However, the

variation between the solvent phase was observed during phase separation. The flat white layer, which could be protein that was not dissolved in the aqueous phase and precipitated when mixed with the solvent phase, forming a pancake-like structure in the middle after centrifugation was not observed in fish plasma sample. In addition to that, some proteins were precipitated in the bottom of the extraction vials when processing human serum samples. However, the large white precipitate was in between the solvent phase and the aqueous phase for fish plasma. This could be because the composition of both protein and lipid in those two sample types are different. In addition to this the serum contains less protein when compared to plasma, which is again lower than whole blood sample (Zhao & Juck, 2018).

Therefore, the lipid could also precipitate in the middle layer together with proteins as they have a lower density than the aqueous phase and a higher density than acetonitrile and has poor solubility in both phases. Additionally, the sample was originally collected from fish given a high fat feed (38%). This can make the plasma matrix more lipemic than the human QC sample applied in the present work. Though acetonitrile is known for its low solubility of phospholipids, the phospholipid effect on sample variation could still be an issue if there are large variation in lipid content of the fish plasma samples. Although, the method showed a significant difference between different net and even individual fish, the variation between those samples was very high when related to the QCs sample. From all measured hormones in fish plasma, the level of cortisol was quite elevated. However, the interference matrix with the D4 cortisol was still challenging in the fish plasma sample as it previoucly detected in QCs. Thus, the interference in both matrices was eluted with the closest retention time as the analytes. The interference came from sample matrices as it was not coeluted with the calibration standard. Remarkably, the peak area of the cortisol and detectability in fish plasma were fairly comparable with QCs human serum and show a high correlation with the level of cortisone in all nets. This verfied that the current method has a great potential for identification of stress biomarkers in fish plasma samples. Future work may investigate the possibility of using plasma cortisone as a better marker of stress than plasma corisol. A significant increase in cortisol is expected during the stressfull event of blood sampling, which may not be the case for plasma cortisone. It is likely that there is some delay in the metabolism of cortisol into cortisone, which is a metabolite of cortisol.

Although some variations from the fish plasma matrix component, which might be the challenge of phospholipid, we successfully measured TT4 in fish plasma samples. Thus, the matrices component of those samples resulted in high variation and reduced the detectability of TT4 in the fish plasma samples. Indeed, the current SALLE method is promising for the identification of smoltification combined with the level of testosterone. Increased levels of

testosterone indicate the sexual maturity of fish, although the level of testosterone was found very low in the few fish plasma samples analyzed in the present work. However, detectability of testosterone was good, probably due to efficient separation from coextracted matrix components, including phospholipids. Samples from fish is suspected to have a high phospholipid concentration. Hence, it would be interesting to apply the novel method for analyzing plasma samples from sexually mature fish, to investigate the possibility of detecting early maturation.

5 CONCLUSIONS

As far as we know, this is the first time that the SALLE sample extraction method has been accomplished to simultaneously determine the concentration of thyroxine, cortisol, cortisone, and testosterone by using LC-MS/MS from both human and fish plasma. Our result shows that the fast and efficient LC separation method, which was achieved by using 0.2% formic acid as aqueous phase (A1) and methanol as (B1) in a linear gradient elution method with a total 7-min run. We developed MS/MS method in ESI+ mode combined with triple quadrupole and MRM scanning mode. Among different organic solvents applied for extraction, acetonitrile resulted in less matrix interference and high precipitating of both protein and lipid. Combining with the high efficiency of ammonium sulfate as salting-out reagent for inducing phase separation, the method results in clear phase separation, protein precipitation, and better extraction recovery of analytes. Adding 10% of methanol to acetonitrile has even resulted in high recovery as it increases the extractability of hormones by fine-tuning the polarity of the solvent. The optimized SALLE method was validated according to the Eurachem guideline. Thus, the method showed high linearity (R^2 >0.997) for all hormones. The LODs and LOQs were also satisfactory for making the method sensitive enough to detect and quantify hormones at low concentration levels. Furthermore, the method shows acceptable precision and accuracy with high extraction recovery for TT4, testosterone, and cortisone.

However, the recovery estimated for cortisol was inconsistent and indicated unsatisfactory accuracy for this analyte. This was due to interference with D4 cortisol. Thus, we recommended to first identify the interfering substance and making use of different LC-column to fine tune this method for D4 cortisol or try using a different isotopic labelled cortisol such as D5 cortisol. Furthermore, the method fulfilled all the requirements accordingly, and, the method was applied on the real fish samples, for which it worked well for all hormones with the same variability for cortisol. It’s very important to analyze as many as possible fish plasma samples to verify the current method for further uses. This can be done in automation of the current SALLE method as it allows for direct injection from the top solvent phase and therefore is very suitable for automation on a 96 well microplate format.

In general, the newly developed method will solve the problem of analyte losses, cost, time, and long process during the sample preparation, as its suitable for direct injection to LC-MS/MS. Moreover, it is environmentally friendly and requires simple sample preparation steps for people who do not have an analytical chemistry background. Though, the current method is open for further identification of biomarkers of stress, smoltification, and maturity test in fish and human plasma samples. Further studies are needed to verify if the current method is applicable in other less invasive fish biological fluids and tissues.

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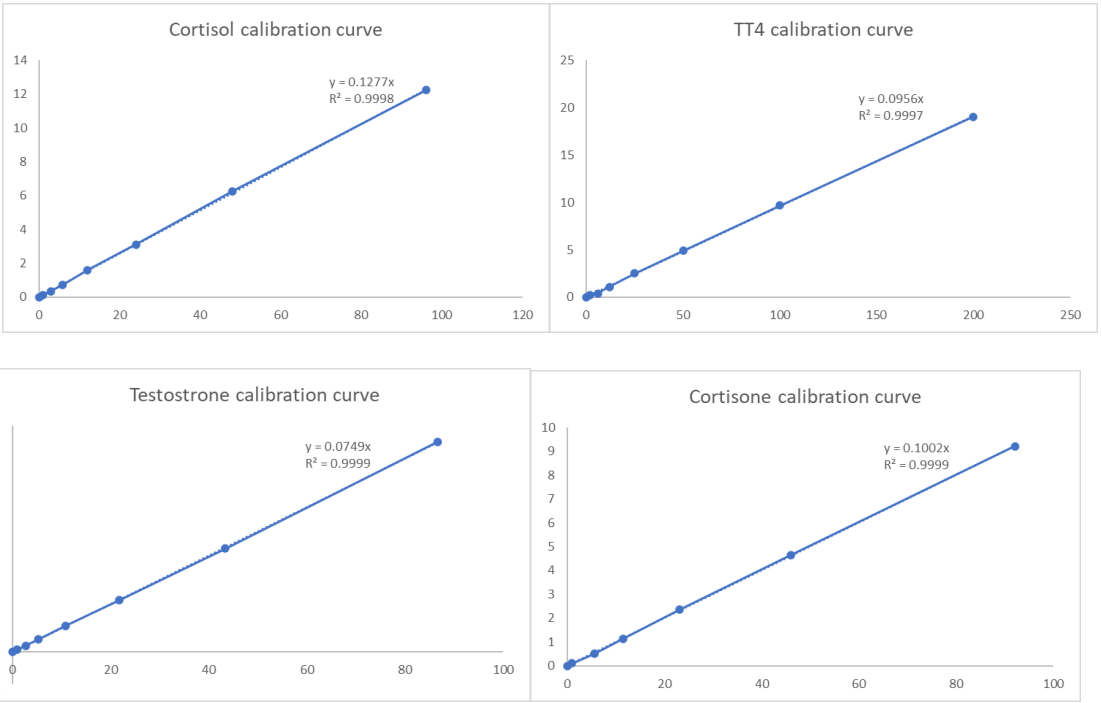
Annexes

Annex 1 – Calibration curve for cortisol, cortisone, total thyroxine, and testosterone.



Annex 2 – level of cortisol, cortisone, total thyroxine and testosterone measured in individual fish

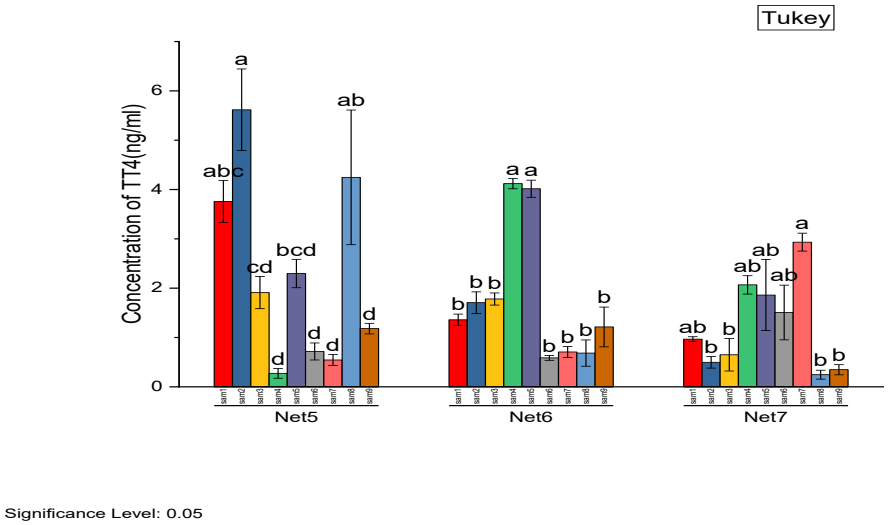


Figure 1. The mean variation of TT4 between individual fish in the same net. Mean with different superscripts in the same net are significantly different at P<0.05.

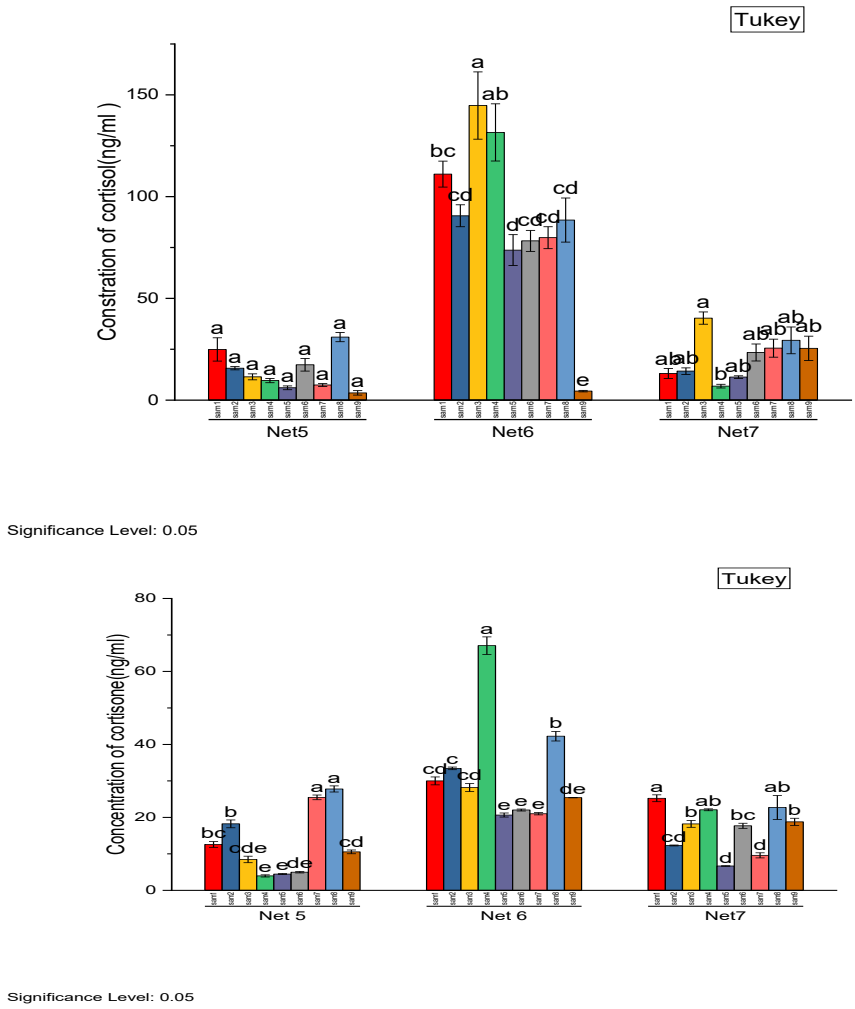


Figure 2. The mean variation of cortisol and cortisone between individual fish in the same net. Mean with different superscripts in the same net for the hormones are significantly different at P<0.05.

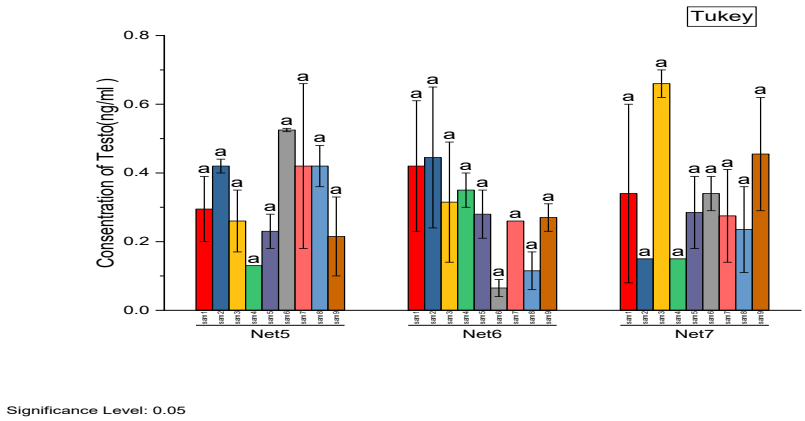


Figure 3. The mean variation of testostrone was measured between individual fish in the same net. Mean with different superscripts in the same net are significantly different at P<0.05.