Kynurenine to tryptophan ratio as a biomarker of acute stress in fish

By

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A thesis submitted to the Faculty of Graduate Studies of the
University of Manitoba
in partial fulfilment of the requirements of the degree of

Master of Science

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ABSTRACT

The aim of this study was to determine the kynurenine (KYN) to tryptophan (TRP) ratio in fish liver and brain tissue to assess its usefulness as a biomarker of acute stress exposure. The activity of the IDO enzyme in the kynurenine pathway is known to be activated during periods of stress, infection, inflammation, and immunomodulation. The enzyme activity was measured indirectly using the ratio of TRP and its primary metabolite KYN. Laboratory held rainbow trout (*Oncorhynchus mykiss*) were subjected to an acute physical stressor and kynurenine, TRP, serotonin (5-HT), and cortisol were measured in liver and brain tissues extracts sampled at 4- and 48-hrs post-stress exposure. The analytical method used to extract and detect our analytes was based on lyophilization; liquid-solid extraction followed by isotope dilution high-performance liquid chromatography positive ion electrospray tandem mass spectrometry. The [KYN]/[TRP] ratio (KTR) was greater in fish liver and brain in the 48-hrs post-stress exposure group ($n=8$) relative to controls ($n=8$, $p<0.05$); a similar increase was not observed in fish in the 4-hr post-stress exposure group. The levels of TRP in the liver and brain samples did not decrease substantially and the increase in the KTR can be attributed to the increase in KYN levels. Hepatic and brain cortisol levels were also elevated in fish from both stress-induced groups relative to their respective controls implying that cortisol responded more quickly to the stressful stimulus than KYN, TRP, and 5-HT. My results suggest that (i) cortisol responds more quickly to an induced stressor than KYN and TRP and (ii) KTR is a promising acute stress diagnostic biomarker in fish (iii) KTR increases is due to KYN increasing and TRP slightly decreasing. The broad scope of TRP metabolism in organisms suggest that the KTR may also be a useful biomarker for stress in different species. Efforts are ongoing to assess whether the KTR can be used as a biomarker for chronic stress in fish exposed to aquatic contaminants and other environmental stressors and if similar assessments can be made on tissues collected *via* non-lethal approaches.

DEDICATIONS

I would like to express my gratitude and appreciation for my family, friends and colleagues for their support and encouragement during my graduate studies. To my supervisor, Dr. Gregg Tomy, thank you for always being happy to answer my questions, provide guidance, wisdom, and at times some much-needed laughter, your support, dedication and trust made this thesis a success. In the Centre for Oil and Gas Research and Development (COGRAD) group I would especially like to thank Patrique Bulloch, Thor Halldorson and Lisa Oswald for all their assistance and patience. A great appreciation also goes to Dr. Jason Raine and Patrique Bulloch for performing the acute stress exposure study and sample collection at the Toxicology Centre at the University of Saskatchewan. The sound contributions, critics, edits, and advice of Dr. Alison Holloway and Laiba Jamshed were invaluable and crucial for the completion of my thesis. I would also like to thank my M.Sc. committee members, Dr. Mazdak Khajehpour and Dr. Christian Kuss for their advice, questions and supervision. Finally, a great thanks to the Department of Chemistry at the University of Manitoba and the COGRAD laboratory for providing a wonderful learning environment and well-equipped laboratory to perform sample extraction and analysis as well as the funding to complete my programme and research.

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Chapter 1. Introduction

Fish in the wild, in aquaculture, and in experimental settings can be exposed to many stressors which include changing water quality and salinity, temperature variations, changes to oxygen availability, environmental pollutants and contaminants, predators, parasites, and aquaculture activities, such as high-density stocking and rearing, hypoxia, net catching (Flik *et al.*, 2006; Wu *et al.*, 2015; Machado *et al.*, 2019). Stress is defined as a state in which a series of adaptive responses re-establish the organism's homeostasis following exposure to a stressor (Schreck and Tort, 2016). These adaptative responses to stress are crucial for the organism's survival and require a dynamic interaction with the ever-changing environments and ecosystems (Akhtar *et al.*, 2013a; Akhtar *et al.*, 2013b; Lushchak, 2011). The vast majority of stress responses are mediated by changes in stress hormones levels, such as catecholamines (adrenaline and noradrenaline and their precursor dopamine) and glucocorticoids (mainly cortisol) (Schreck and Tort, 2016); while a few stress responses can be directly mediated by the stressor itself, for instance temperature-inducible molecular changes in heat shock proteins or stress-induced epigenetic changes (Petitjean *et al.*, 2019). Furthermore, the duration, intensity, and number of combined stressors is also of great importance to an organism's overall stress response as well as regaining homeostasis (Wendelaar Bonga, 1997; Balasch & Tort, 2019; Petitjean *et al.*, 2019). If the intensity of the stressor is overly severe and becomes chronic and long-lasting, the physiological response mechanisms may be compromised and there can be permanent and detrimental effects to the organism's overall health (Akhtar *et al.*, 2013a; Akhtar *et al.*, 2013b; Lushchak, 2015; Lange *et al.*, 2018). This type of stressor is associated with a state termed ''distress'' and is an important concern of environmental risk assessments, ecotoxicology, and aquaculturists (Flik &Wendelaar Bonga, 2001; Barton, 2002; Akhtar *et al.*, 2013a; Akhtar *et al.*, 2013b). In distress homeostatic regulations remain disturbed, and pathologies, impaired disease resistance, cancers, improper immunoregulation, reduced growth and compromised reproduction

will most likely occur (Flik *et al.,* 2006; Machado *et al.,* 2019). In addition, it must be commented on that due to the multiple anthropogenic activities and invertible global warming changes, most aquatic organisms are often exposed to several stressors all at once (Schinegger *et al.,* 2016; Schinegger *et al.,* 2018). Furthermore, due to the confounding nature of multiple stressors the majority of existing studies on stress biomarkers can only focus on the effect of one specific stressor event performed in isolation to ensure the measured response is to mediate the applied stress exposure (Hahn, 2011; Schinegger *et al.,* 2018; Petitjean *et al.,* 2019).

1.0 Combined effects of multi-stressors

Evaluating multi-stressors and chronic stress exposure remains a challenge due to the complex interactions that are elicited for each type of stress since different kinds of stressors trigger different physiological pathways that can lead to unexpected synergistic, antagonistic, and/or additive effects (Christensen *et al.,* 2006; Holmstrup *et al.,* 2010; Lange *et al.,* 2018; Petitjean *et al.,* 2019). Synergism happens when individuals exposed to a stressor have a lower resistance or tolerance from exposure to different stressor (Christensen *et al.,* 2006; Liess *et al.,* 2016; Lange *et al.,* 2018). For example, increases in temperature can amplify the deleterious effects of many environmental contaminants, such as pesticides, by increased metabolic rates and assimilation of contaminants in fish (Macrogliese *et al.,* 2005; Christensen *et al.,* 2006; Gandar *et al.,* 2017a; Gandar *et al.,* 2017b; Lange *et al.,* 2018; Petitjean *et al.,* 2019). In addition to synergism, threshold effects can also occur, such that interacting stressors may have little effect until a critical threshold is reached in one or more of the stressors (Holmstrup *et al.,* 2010; Hahn, 2011; Lange *et al.,* 2018; Schinegger *et al.,* 2018; Petitjean *et al.,* 2019). Other threshold effects are also possible, for example, stressors may act additively up to the organism's threshold, and then can act synergistically beyond it (Schinegger *et al.,* 2018; Petitjean *et al.,* 2019).

On the contrary, antagonism occurs when exposure to one stressor limits or decreases the effects of another stressor (Christensen *et al.,* 2006; Hahn, 2011; Holmstrup *et al.,* 2010; Schinegger *et al.,* 2016; Lange *et al.,* 2018; Schinegger *et al.,* 2018). Antagonism usually occurs when the physiological adaptations to one stressor are also beneficial against the exposure to additional stressors, resulting in a type of co-tolerance (Holmstrup *et al.,* 2010; Schinegger *et al.,* 2016; Schinegger *et al.,* 2018). For example, some families of Atlantic salmon (*Salmo salar*) that are tolerant to higher temperatures are also more tolerant to hypoxia, due to the increased heart ventricle size and higher myoglobin levels (Anttila *et al.,* 2013; McBryan *et al.,* 2013). In every changing ecosystem this type of higher temperature acclimation will aid in certain species continued survival (McBryan *et al.,* 2013).

Fish are also impacted by natural/biotic stressors, such as climate change, invasive predators, parasites and emerging diseases that can all play a detrimental part in the overall health of aquatic organisms (Holmstrup *et al.,* 2010). For instance, a growing number of studies showed that fish exposed to contaminants are more susceptible to predation due to neurotoxic effects and disruption of social interactions by pollution (Hahn, 2011; Schinegger *et al.,* 2016; Lange *et al.,* 2018; Schinegger *et al.,* 2018). In addition, fish exposed to contaminants can be more susceptible to parasitism due to reduced immune resistance (Macrogliese *et al.,* 2005). For example, the occurrence and negative effects of the emerging proliferative kidney disease on brown trout (*Salmo trutta*) resulting from parasitism are increased by climate change and water warming which has resulted in severe consequences for wild and aqua cultured European salmonid populations (Bruneaux *et al.,* 2017). The combined effects of multiple biotic and abiotic stressors on fish and aquatic ecosystems are still poorly known (Christensen *et al.,* 2006; Schinegger *et al.,* 2016; Lange *et al.,* 2018). This highlights the need for multiscale studies as well as biomarkers for the combined stress response to better understand the effects multi-stressors have on

overall aquatic organisms' health, acclimation, and ultimately adaptation/acclimation to environmental changes (Christensen *et al.,* 2006; Holmstrup *et al.,* 2010; Petitjean *et al.,* 2019).

1.1 Stress responses in fish

Teleost fish were the first vertebrates that developed stress responses due to the intimate and direct contact with their aqueous environments via the delicate epithelium of their gills (Wendelaar Bonga, 1997; Balasch & Tort, 2019; Petitjean *et al.,* 2019). The stress responses of teleost fish consist of a cascade of neuroendocrine signaling that is regulated by immune responses and involves the immediate release of catecholamines, such as epinephrine and norepinephrine, from chromaffin tissue in the head kidney and adrenergic nerves (Pankhurst, 2011; Conde-Sieira *et al.,* 2018). When fish are exposed to acute stress, the circulatory levels of catecholamines increase quickly and recover the basal values within minutes (Pankhurst, 2011; Kumar *et al.,* 2014; Conde-Sieira *et al.,* 2018; Kumar *et al.,* 2018; Balasch and Tort, 2019). Synthesis of cortisol under stress is directly controlled by the activation of hypothalamic-pituitary-interrenal (HPI) axis (Kumar *et al.,* 2014; Conde-Sieira *et al.,* 2018; Kumar *et al.,* 2018; Balasch and Tort, 2019). After recognition of stress exposure by the central nervous system (CNS), the corticotrophin releasing factor is released from hypothalamus and stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) in the corticotropes cells of the anterior pituitary gland (Pankhurst, 2011; Conde-Sieira *et al.,* 2018). The ACTH is released into the blood stream to reach the steroidogenic cells in the interrenal tissue inducing the activation of the melanocortin 2 receptor, thereby stimulating the signaling cascade for the synthesis and release of glucocorticoids (mainly cortisol) into circulation (Pankhurst, 2011; Conde-Sieira *et al.,* 2018; Kumar *et al.,* 2018; Balasch and Tort, 2019). These changes in the sympatho-chromaffin axis and the HPI axis in fish allow for rapid stress response and the energy requirements to meet the situation (Winberg *et al.,* 1997; Tort, 2011; Winberg & Thörnqvist, 2016; Yada

& Tort, 2016; Balasch and Tort, 2019). This response also includes activation of the cardio-respiratory system, increased blood perfusion to the gills and muscles, and increased glucose supply to critical tissues (Winberg *et al.*, 1997; Tort, 2011; Winberg & Thörnqvist, 2016; Yada & Tort, 2016). The metabolic process is triggered by the release of corticosteroids through glycolysis and gluconeogenesis to the critical tissues, however, this process comes at a cost and results in the suppression of long term and high-cost energy processes, such as immunoregulation and disease resistance (Mommsen *et al.*, 2004; Coccaro *et al.*, 2016; Yada & Tort, 2016; Winberg & Thörnqvist, 2016; Balasch and Tort, 2019; Sadoul & Geffroy, 2019). In general, autonomic, endocrine, and behavioral stress responses are well conserved and similarities between other vertebrates and fish are prominent (Wendelaar Bonga, 1997; Flik *et al.*, 2006; Backström & Winberg, (2017). Although cortisol is universally recognized as a critical component of the endocrine response to stress, it must be mentioned that there are other hormones that are also involved in the stress response, such as arginine vasotocin, isotocin, urotensins, dopamine, serotonin (5-hydroxytryptamine, 5-HT), or β-endorphin (Tort, 2011; Yada & Tort, 2016; Kalamarz-Kubiak, 2017; Grayfer *et al.*, 2018).

Evolution has shown that stress responses have major adaptive and acclimatisation value because they support the organism developing ways to deal with difficult processes and challenges that are appraised as stressful (Puglisi-Allegra & Andolina, 2015; Winberg & Thörnqvist, 2016; Balasch and Tort, 2019; Sadoul & Geffroy, 2019). However, stressful responses cannot be sustained indefinitely and as such the organism must develop effective coping strategies if it wants to survive (Balasch and Tort, 2019). These, in turn, require major physiological and psychological changes (Puglisi-Allegra & Andolina, 2015). In other words, a stressful experience invariably leads to adaptation, which can be either healthy or pathogenic (Puglisi-Allegra & Andolina, 2015; Balasch and Tort, 2019). Adaptation to stressful exposure

and events involves physiological allostasis and ultimately some type of learning (Puglisi-Allegra & Andolina, 2015). The more often an organism is exposed to the same stressors the quicker it is able to learn how to cope and deal with the stress event (Puglisi-Allegra & Andolina, 2015 Winberg & Thörnqvist, 2016; Balasch and Tort, 2019; Sadoul & Geffroy, 2019).

1.2 Cortisol and stress in fish

In fish, just like in mammals, cortisol is the major corticosteroid produced by the HPI axis whereas, in reptiles and birds, it is corticosterone (Sadoul & Geffroy, 2019). In fish, cortisol is produced by inter-renal cells sparsely distributed within the head kidney and released into the blood to allow for the organism's rapid stress response (Barton, 2002; Balasch & Tort, 2019; Sadoul & Geffroy, 2019). Measuring cortisol has long been an excellent approach to assess the effect of a given stressor on fishes (Sadoul *et al.,* 2016). The only physiologically active form of cortisol in plasma is considered to be unbound cortisol (Breuner & Orchinik, 2002; Balasch & Tort, 2019; Sadoul & Geffroy, 2019). Produced primarily in the liver, corticosteroid binding globulins (CBG) is a plasma glycoprotein that binds up to 90% of all circulating glucocorticoids, and regulates the delivery of the hormones to target tissues (Breuner & Orchinik, 2002; Hill *et al.,* 2016). No CBG *per se* has been detected in any fish species, unlike in many other vertebrates and mammals (Breuner & Orchinik, 2002; Balasch & Tort, 2019; Sadoul & Geffroy, 2019). However, there are reports of binding molecules that reduce the bioavailability of cortisol in fish, but their exact purpose is still not understood (Balasch & Tort, 2019; Sadoul & Geffroy, 2019). In salmonid fish, like rainbow trout, it was estimated that 30–55% of cortisol is bound in plasma, with the percentage being significantly higher in females (45%) when compared with males (37%) (Breuner & Orchinik, 2002; Balasch & Tort, 2019; Sadoul & Geffroy, 2019). The percentage of cortisol bound in plasma also varies throughout the different developmental/life stages, reproductive cycles, social status

and seasonal variations (Breuner & Orchinik, 2002; Balasch & Tort, 2019; Sadoul & Geffroy, 2019). In addition, food intake can affect cortisol synthesis, with fasting animals generally producing more cortisol (Breuner & Orchinik, 2002; Hill *et al.*, 2016; Sadoul & Geffroy, 2019). Diet composition can also influence the ability to respond to a stressor (Sadoul *et al.*, 2016).

Catabolism of cortisol in fish occurs in different organs such as the head kidney and liver and occurs by the 11β-oxidation resulting in the release of cortisone, the biologically inactive form of the metabolite (Mommsen et al. 2004; Balasch & Tort, 2019). Due to the high lipophilic nature of the molecule, uptake by cells occurs by passive diffusion (Scott & Ellis, 2007). Once cortisol reaches the cells of target tissues it binds to glucocorticoid and mineralocorticoid receptors (Balasch & Tort, 2019; Sadoul & Geffroy, 2019). Cortisol is then metabolized and inactivated, primarily through the hepato-biliary–faecal route (Mommsen et al.,2004; Sadoul & Geffroy, 2019). In the liver and the bile, cortisol is inactivated by means of reduction and conjugation to a glucuronide or sulphate (Barton, 2002; Sadoul & Geffroy, 2019). Both inactivated metabolites are then released into the surrounding environment through the urine and faeces, respectively, which is comparable to other steroids (Breuner & Orchinik, 2002; Scott & Ellis, 2007). Moreover, due to the passive 'leakage' of steroid, the free form of cortisol, diffuses out of the fish into water in microgram quantities, across the delicate epithelium of the gills (Scott & Ellis, 2007). On this basis, the concentration of cortisol in the water equates to the concentration of 'physiologically active' unbound form of cortisol in the plasma (Scott & Ellis, 2007).

Cortisol has shown a central role in fish physiology, behaviour, and even evolution highlighting major roles in stress responses and mediation, as well as immunoregulation (Balasch & Tort, 2019; Sadoul *et al.*, 2018), optimal growth (Sadoul & Vijayan, 2016), and sex determination or sex-change (Goikoetxea *et*

al., 2017). Cortisol regulates the immune response in fish and modulates tissue inflammatory responses through inhibitory effects on cytokines production (Kumar *et al.,* 2014; Lepage *et al.,* 2003; Wu *et al.,* 2015). Furthermore, increases in cortisol affects a wide range of other metabolic effects including increases in protein turnover, regulation of amino acids metabolism, ammonia output and increased lipolysis (Kumar *et al.*, 2014; Wu *et al.*, 2015; Sadoul & Geffroy, 2019). Measuring cortisol has been used extensively as a biomarker of a given stressor on fish for several decades (Barton, 2002; Wu *et al.,* 2015; Balasch & Tort, 2019; Sadoul & Geffroy, 2019) and is also an important tool in characterising coping abilities and acclimation of fishes as well as investigating how environmental change can disturb this (Barton, 2002; Wu *et al.,* 2015; Balasch & Tort, 2019; Sadoul & Geffroy, 2019). Even though cortisol levels are a well-accepted indicator of acute stressors, they cannot fully reflect exposure to chronic long-term stressors including exposures to pollutants and contaminants in the aquatic ecosystem (Lepage *et al.*, 2003; Wu *et al.,* 2015; Sadoul & Geffroy, 2019). This is because chronic stress enables species-specific adaptive responses that are able to cause permanent alterations to the metabolic homeostasis (Lepage *et al.*, 2003; Cabanillas-Gámez *et al.,* 2017; Javed *et al.*, 2017; Balasch & Tort, 2019). These alterations can result in detrimental consequences including decreased resistance to infection, decreased growth, and impaired reproduction (Balasch and Tort, 2019). Moreover, cortisol is ineffective as a biomarker for chronic stress exposure because of HPI desensitisation, which occurs as a consequence of allostatic overload (Aerts *et al.*, 2015; Wu *et al.,* 2015).

Clearly, other biomarkers of stress would be invaluable to help monitor overall fish health, optimal growth and reproduction, as well as acclimation to ever-changing ecosystems. Past and recent studies on measuring cortisol in fish include different fish sampling matrices, such as mucus, faeces, urine, surrounding water, plasma, scales, fins, whole body, and eggs (Barton, 2002; Salinas, 2015; Wu *et al.*,

2015; Balasch & Tort, 2019; Sadoul & Geffroy, 2019). The most invasive and lethal sample types are whole body, fins, and eggs (Sadoul & Geffroy, 2019). Sampling plasma and scales are not lethal; however, they are still moderately invasive and can cause additional stress from sampling (Sadoul & Geffroy, 2019). Due to the desire to measure total environmental stress effects without causing additional stress from sampling has led to the development of non-invasive methods to collect the stress hormones from mucus, faeces, urine, surrounding water (Bulloch *et al.*, 2019). However, non-lethal methods are usually less sensitive and accurate to analyze due to the difficult nature of collecting non-invasive samples (Salinas, 2015; Sadoul & Geffroy, 2019).

1.3 Metabolism of tryptophan in fish

In mammals and fish, tryptophan (TRP) is an essential amino acid with crucial roles in protein synthesis and as a precursor for several bioactive metabolites, such as serotonin (5-HT), melatonin, and kynurenine (KYN) (Kumar *et al.*, 2014; Ciji *et al.*, 2015; Kumar *et al.*, 2018; Hoseini, 2019; Machado *et al.*, 2019; Cabanilla-Gaméz *et al.*, 2020). Typically, fish requirements for amino acids are generally established by means of optimal growth, life stage, season, and/or reproduction stage (Kumar *et al.*, 2014; Ciji *et al.*, 2015; Kumar *et al.*, 2018; Hoseini, 2019; Machado *et al.*, 2019; Cabanilla-Gaméz *et al.*, 2020). However, the accumulating demands of physiological challenges require extra amino acids for metabolic cost processes such as stress, thereby increasing the demand of certain nutrients, such as TRP (Kumar *et al.*, 2014; Ciji *et al.*, 2015; Kumar *et al.*, 2018; Cabanilla-Gaméz *et al.*, 2020).

Numerous studies in human and animal diseases have shown TRP requirements increase as a result of stress exposure, inflammation, and infection (Ciji *et al.*, 2015; Machado *et al.*, 2019; Cabanilla-Gaméz *et al.*, 2020). Available nutrients are known to influence several aspects of the immune system, and some of

the immune mechanisms can be modulated and strengthened through nutritional strategies (Machado *et al.*, 2019; Sadoul & Geffroy, 2019). Just like in mammals, TRP in fish have shown a range of effects in the modulation of stress response, antioxidant system, behavioural response, and the immune system (Möller *et al.*, 2012; Ciji *et al.*, 2015; Machado *et al.*, 2019; Hoseini *et al.*, 2019). All vertebrates are incapable of synthesizing TRP and must obtain it from their dietary sources, unlike some bacteria, fungi, and plants (Yin, 2017). TRP produces 5-HT in the central nervous system and in the gastrointestinal tract and its synthesis controls the ACTH release, thereby regulating cortisol production (Mommen *et al.*, 2004; Pankhurst, 2011; Machado *et al.*, 2019; Jamshed *et al.*, 2021). Both immune and endocrine cells share common receptors while different hormones and cytokines are involved in the same mechanisms (Dhabhar, 2009; Khansari *et al.*, 2017). Glucocorticoids are a clear example of this interaction, as they modulate the secretion of cytokines and has become evident that most immune cell types can be affected by them (Stolte *et al.*, 2008). In particular, cortisol can have a clear effect on multiple characteristics of the immune defence mechanism in teleosts, as reviewed by Hoseini, et al, 2019. For example, cortisol is able to inhibit the expression of pro-inflammatory cytokines and nitric oxide upon lipopolysaccharides induction (Stolte *et al.*, 2008; Verburg-van Kemenade *et al.*, 2011; Machado *et al.*, 2019) or induction of apoptosis and inhibition of immune cells proliferation in fish (Verburg-van Kemenade *et al.*, 2011; Machado *et al.*, 2019). Therefore, an endocrine-immune perspective ought to be considered when evaluating TRP immune-nutrition, since it has roles in both systems. Together with 5-HT and KYN, the TRP metabolites; melatonin and N-acetyl serotonin, appear to enhance host immunity by reducing the production of superoxides, scavenging free radicals, and attenuation of the production of pro-inflammatory cytokines (Machado *et al.*, 2019; Jamshed *et al.*, 2021).

Tryptophan is transformed in mammals and fish into the monoaminergic neurotransmitter 5-HT in only approximately 1-2% of its total body weight (Winberg *et al.,* 1997; Winberg & Thörnqvist, 2016; Höglund *et al.,* 2019). The 5-HT metabolic pathway is initiated by TRP being hydroxylated to the intermediate 5-hydroxytryptophan (5-HTP), which is sequentially decarboxylated to become 5-HT (Backström & Winberg, 2017; Höglund *et al.,* 2019). Tissue levels of 5-HTP are usually very minimal since it is rapidly decarboxylated by the enzyme aromatic amino acid decarboxylase (Winberg & Thörnqvist, 2016). Thus, the rate limiting step in the biosynthesis of 5-HT is the hydroxylation of TRP catalyzed by the enzyme tryptophan hydroxylase (TPH), as shown in Figure 1.1 (Winberg *et al.,* 1997; Winberg & Thörnqvist, 2016; Höglund *et al.,* 2019). This enzyme is only specific for 5-HT producing cells (Winberg *et al.,* 1997; Winberg & Thörnqvist, 2016; Höglund *et al.,* 2019).

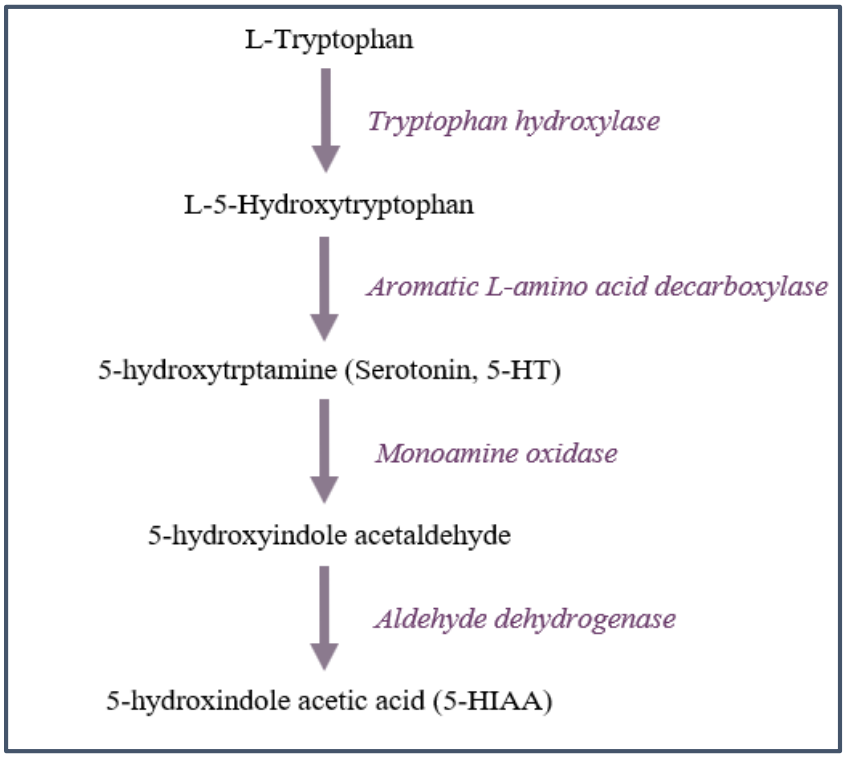


Figure 1.1. Tryptophan metabolism via the serotonin pathway.

Tryptophan is able to cross the blood brain barrier and enters the brain in competition with other large neutral amino acids (LNNAs); such as, valine, isoleucine, leucine, tyrosine, phenylalanine and methionine, all through a common transporter protein (Winberg *et al.,* 1997; Winberg & Thörnqvist, 2016; Backström & Winberg, 2017; Höglund *et al.,* 2019). Therefore, the amount of TRP entering the brain depends on the plasma concentrations of TRP in relation to the other LNAAs. This differential amino acid uptake is caused by the fact that TRP in blood plasma is bound to albumin whereas other LNAA are not (Backström & Winberg, 2017). Tryptophan influx to the brain is then promoted by the common LNAA transporter protein in the blood brain barrier having a much higher affinity for TRP compared to albumin (Winberg *et al.,* 1997; Winberg & Thörnqvist, 2016; Höglund *et al.,* 2019).

Studies in rainbow trout (*Oncorhynchus mykiss*) show that the amino acid composition of trout albumin differs from that of mammalians and lacks the binding site for indoles (Fernstrom, 1990; Höglund *et al.,* 2019). Thus, in rainbow trout, the majority of plasma TRP is in its free non-protein bound state (Breuner & Orchinik, 2002). Numerous studies have shown that elevated dietary TRP has suppressive effects on aggressive behavior and post-stress plasma cortisol concentrations, as well as increased immune status, inflammatory response and disease resistance in teleosts, higher vertebrates, and mammals (Mommen *et al.,* 2004; Machado *et al.,* 2019). Although it was once hypothesized to be mediated by the brain serotonergic system alone, research has now shown the kynurenine pathway (KP) metabolites also play a large role in immunoregulation, immune status, inflammatory response and disease resistance. However, all the mechanisms involved are not well understood and further research is required. The remaining 95% portion of TRP that is not being utilized for protein synthesis is metabolized by the KP, leading to the synthesis of several KYN metabolites, each of which can be either ‘excito-toxic’ or ‘neuro-protective’ (Yin, 2011; Perez-Gonzalez *et al.,* 2015; Coccaro *et al.,* 2016).

1.4 Kynurenine pathway in fish

Historically, the significance of the KP was accredited to the synthesis of the coenzymes nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP) (Ng *et al.,* 1997; Perez-Gonzalez *et al.,* 2014). Nicotinamide adenine dinucleotide and NADP are cofactors that are essential for electron transfer reactions (i.e., redox reactions) are involved in a plethora of cellular energy metabolism reactions occurring in all living cells, such as glycolysis, fatty acid β-oxidation, and the tricarboxylic acid cycle (Ng *et al.,* 1997; Fang *et al.,* 2017). Beyond the importance of the NAD^+, the KP has received notable attention with the discovery of the 'excito-toxic' and 'neuro-protective' metabolites that are produced, which modulate glutamatergic neurotransmission (Barone, 2019; Joisten *et al.,* 2021). The kynurenine pathway exists mainly in the liver, which contains all the enzymes necessary for NAD^+ synthesis from TRP and is responsible for ~90% of overall TRP degradation under normal physiologic conditions (Badawy, 2017a; Badway, 2017b; Badawy & Guillemin, 2019). The kynurenine pathway also exists extrahepatically, but its contribution to TRP degradation is normally minimal (5%-10%) but then becomes quantitatively more significant under conditions of immune activation and stress responses (Badawy, 2017a; Badway, 2017b; Badawy & Guillemin, 2019).

The two main fundamental steps through the KP are: a) formation of KYN from TRY, and b) post-KYN metabolism via three routes competing for KYN as their initial substrate (Oxenkrug, 2010; Coccaro *et al.,* 2016; Cabanillas-Gámez *et al.,* 2020). The first catabolic step of the KP is the bottleneck of the entire pathway and it is mediated by two rate-limiting enzymes, namely indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) that metabolize TRP into KYN, an aryl hydrocarbon receptor ligand (Le Floc'h *et al.,* 2011; Yeung *et al.,* 2015; Barone, 2019; Mor *et al.,* 2021). The kynurenine pathway in fish is shown in Figure 1.2. During homeostasis most TRP metabolism occurs through the KP in the liver

and is mediated by the enzyme TDO (Dhabhar, 2009; Munn & Mellor, 2013; Cortés *et al.,* 2017; Hoseini *et al.,* 2019). The heme-enzyme TDO (EC1.13.11.11) opens the pyrrole ring of TRP, but also of D-tryptophan, 5-hydroxytryptophan, tryptamine, and serotonin (5-hydroxytryptamine (5-HT)). Tryptophan 2,3-dioxygenase (also known as tryptophan oxygenase or tryptophan pyrrolase) has a short half-life *in vivo* (of the order of 2 h) and is subject to regulation by three mechanisms: stabilization by its heme cofactor, glucocorticoid hormonal induction, and feedback inhibition and repression by high concentrations of NADP (Yuasa *et al.,* 2015).

Under immune activation and inflammation, the same pathway of TRP catabolism is present in macrophages and is mediated by the IDO enzyme (Mommen *et al.,* 2004; Hoseini *et al.,* 2019; Machado *et al.,* 2019). Indoleamine-pyrrole 2,3-dioxygenase (EC 1.13.11.52) is also a heme enzyme that catalyzes the O_2-dependent oxidation of L-tryptophan to N-formyl kynurenine (Munn & Mellor, 2016). Indoleamine 2,3-dioxygenase is an important part of the immune system and plays a large role in natural defense against various pathogens and toxins (Munn & Mellor, 2016). It is produced by cells in response to inflammation and has an immunosuppressive function because of its ability to limit T-cells and natural killer (NK) cells and generating T-regulators and myeloid-derived suppressor cells (Prendergast *et al.,* 2014). Research has shown IDO plays a crucial part of carcinogenesis since it is activated during tumor development by helping malignant cells escape eradication by the immune system (Munn & Mellor, 2013; Prendergast *et al.,* 2014; Munn & Mellor, 2016). The IDO enzyme allows tumor cells to escape the immune system by two main mechanisms. The first mechanism is based on TRP depletion from the tumor microenvironment while producing the catabolic products of the KP called kynurenines, that are both cytotoxic for T lymphocytes and NK cells (Munn & Mellor, 2013; Prendergast *et al.,* 2014; Munn & Mellor, 2016). Therefore, inhibition of IDO could increase the effects of chemotherapy drugs as well as

other immunotherapeutic inventions (Munn & Mellor, 2013; Prendergast *et al.,* 2014; Munn & Mellor, 2016).

Indoleamine 2,3-dioxygenase relies on TRP availability from the flux of un-bound TRP in plasma as well as its induction by inflammatory stimuli such as interferon-γ (INF-γ), cytokines, and nitric acid (Badawy, 2017a; Badway, 2017b; Badawy & Guillemin, 2019; Mor *et al.,* 2021). The pro-inflammatory cytokine IFN-γ induces both the enzyme IDO to degrade TRP and the enzyme GTP-cyclohydrolase I to form neopterin (Lanser *et al.,* 2020). As such, IDO is increasingly recognized as an important link between the immune system and the KP which has heralded a new era in immunological research (Cortés *et al.,* 2017; Lovelace *et al.,* 2017). Recently, the IDO gene was identified in several groups of vertebrates like chickens, reptiles, and Osteichthyes such as zebra-fish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss),* and grass carp (*Ctenopharyngodon idella*) (Cortes *et al.,* 2010; Cui *et al.,* 2019; Li et al, 2021). In fishes IDO also seems to be present in the same organs than mammals as liver, kidney, and mucosal organs (Cortes *et al.,* 2010; Cui *et al.,* 2019).

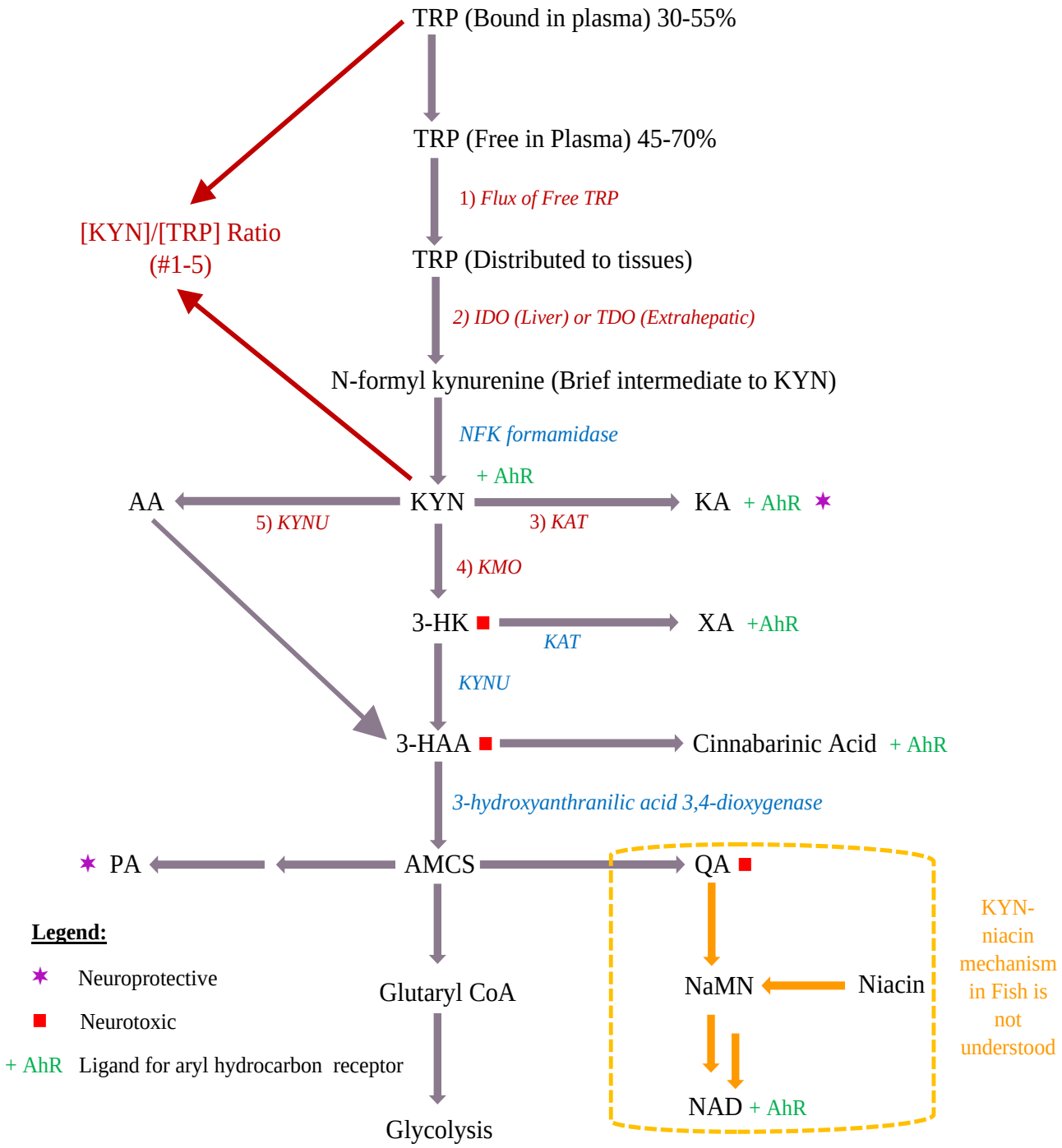
Table 1.1 summarizes the characteristics of the two KP rate limiting enzymes: TDO and IDO. Once TRP is converted to brief intermediate N-formyl kynurenine by either TDO or IDO it is rapidly converted to KYN by the enzyme N-formyl kynurenine formamidase (Le Floc'h *et al.,* 2004; Le Floc'h & Seve, 2007; Castro-Portuguez & Sutphin, 2020). At this point TRP metabolism becomes more complicated resulting in KYN being partly metabolized to kynurenic acid and anthranilic acid via KYN aminotransferase and kynureninase, respectively (Khansari *et al.,* 2017; Marszalek-Grabska *et al.,* 2021). Additionally, KYN is partly converted into 3-hydroxykynurenine, which is further transaminated to xanthurenic acid (Munn & Mellor, 2013; Joisten *et al.,* 2021). At this point in the KP kynureninase catalyzes the hydrolysis of 3-

hydroxykynurenine to form 3-hydroxyanthranilic acid (3-HAA) which can spontaneously transform to nicotinic acid via the enzyme 3-hydroxyanthranilate 3,4-dioxygenase (3-HAAO) or alternatively can be completely oxidized to carbon dioxide (Davis & Liu, 2015; Barone, 2019; Marszalek-Grabska *et al.*, 2021). Initially, 3-HAAO converts 3-HAA to the unstable intermediate 2-amino-3-carboxymuconic semialdehyde, which spontaneously rearranges to quinolinic acid, or is further converted into picolinic acid after enzymatic decarboxylation via 2-amino-3-carboxymuconate semialdehyde decarboxylase (Mandi & Vecsei, 2012; Perez-Gonzalez *et al.*, 2014; Davis & Liu, 2015; Khansari *et al.*, 2017). Quinolinic acid is then decarboxylated to form nicotinic acid, the NAD^+ coenzyme precursor (Dhabhar, 2009; Le Floc'h *et al.*, 2011; Coccaro et al, 2016).

Table 1.1. Comparison of tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase characteristics.

	Tryptophan 2,3-dioxygenase	**Indoleamine 2,3-dioxygenase**
Enzyme Commission Number	EC. 1.13.11.11	EC. 1.13.11.52
Substrates	L-tryptophan	L- and D-tryptophan 5-hydroxytryptophan 5-hydroxytryptamine (serotonin (5-HT))
Cofactors	Molecular oxygen (heme)	Molecular oxygen (heme) & Superoxide anion
Tissue distribution	Primarily liver	Ubiquitous
Functions	Degradation of tryptophan in excess	Immune regulation
Regulations	Tryptophan and glucocorticoids	IFN-γ, LPS, NO, virus, and bacteria

IFN-γ: interferon-γ, LPS: lipopolysaccharide, NO: nitric oxide



Abbreviations: AA, anthranilic acid; 3-HAA, 3-hydroxyanthranilic acid; 3-HK; 3-hydroxykynurenine; KA, Kynurenic acid; IDO, indoleamine 2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine monooxygenase; KYNU, kynureninase; QA, Quinolinic acid; TDO, tryptophan 2,3-dioxygenase; TRP, tryptophan; XA, xanthurenic acid..

Figure 1.2. Tryptophan metabolism via the kynurenine pathway in fish indicating the factors and enzymes that influence the upregulation of the [KYN]/[TRP] ratio.

In mammals and other vertebrates including teleosts, the KP is evolutionary well conserved (Yuasa *et al.,* 2015; Hoseini *et al.,* 2019). Cortes *et al.,* 2010 provided the first evidence for the expression of IDO in teleost fish, namely the rainbow trout (*Oncorhynchus mykiss*), which has recently been confirmed to mediate the fish's immunoregulation responses, stress response, and inflammation (Yuasa *et al.,* 2015; Cortés *et al.,* 2017; Grayfer *et al.,* 2018). Moreover, interferon-γ (INF-γ) induces IDO and it controls the induction of downstream enzymes along the KP (Perez-Gonzalez *et al.,* 2014; Perez-Gonzalez *et al.,* 2015; Hoseini *et al.,* 2019). Interferon-γ also stimulates the formation of neopterin in macrophages (Fuertig *et al.,* 2018). The [Kyn]/[Trp] ratio (KTR) is a marker for both the upregulation of IDO and downregulation of TDO activities as well as the IFN-γ-mediated immune activation (Perez-Gonzalez *et al.,* 2015; Badway, 2017; Lefèvre *et al.,* 2019). Furthermore, neopterin is often referred to as a biomarker of IFN-γ activity. Increases of neopterin formation has been correlated with KTR increases (Fuertig *et al.,* 2018; Badawy & Guillemin, 2019). High neopterin concentrations as well as an increased KTR in the blood of cancer patients and heart diseases patients are predictive for a worse outcome (Lanser *et al.,* 2020). Additionally, IDO is the only enzyme that can employ superoxide as a substrate, other than superoxide dismutase, which also implicates it in oxidative stress responses (Badawy, 2017a; Badawy & Guillemin, 2019; Mor *et al.,* 2021).

Most of the components in the mammalian KP are known to be present in fish and the general catabolic routes through KYN are assumed to be similar to those in mammals (Cortes *et al.,* 2010; Hoseini *et al.,* 2019). However, unlike mammals, in fish it is still not understood how quinolinic acid is decarboxylated to form nicotinic acid (also known as niacin or Vitamin B_3), which is the key precursor to NAD synthesis (Cortés *et al.,* 2017; Hoseini et al, 2019). Figure 1.2 outlines the metabolism of TRP down the KP in fish, as well as the portion on the pathway that is not fully understood in fish (Cortés *et al.,* 2017; Hoseini *et*

al., 2019). As a result, the KYN–niacin pathway in fish is thought to mainly direct the removal excess TRP, aiding in regulating the immune and stress responses as well as, producing sexual pheromones (Yambe *et al.*, 2015; Cortés *et al.*, 2017; Hoseini *et al.*, 2019).

1.5 Factors that influence the [Kyn]/[Trp] ratio

Monitoring the [Kyn]/[Trp] ratio (KTR) in individual organs is necessary to measure the enzymatic activity since it is free from interference or participation of in vivo 'external' modulating factors, such as supply of nutrients, the presence of multiple cell types, complex structural and functional tissue arrangements, the extracellular matrix, and hormonal, cytokine, and paracrine interactions (Badawy *et al.*, 2017a; Badawy, 2017b; Badawy & Guillemin, 2019). To date it has not been possible to extrapolate cell culture to the whole organism without controlling the prevailing *in vivo* factors (Cortés *et al.*, 2017; Hoseini *et al.*, 2019). However, a wide array of in vitro studies allows for monitoring KYN and TRP in a 'closed' system, such as in isolated tissues or organs and/or culture media and thus justifies the use of the KTR to reflect changes in IDO activity and establish beyond a doubt the role of IDO in controlling the KTR (Mandi & Vecsei, 2012; Perez-Gonzalez *et al.*, 2014; Davis & Liu, 2015; Badawy, 2017a; Badawy, 2017b; Badawy & Guillemin, 2019). This extrapolation method has been adopted by many researchers, because either they did not have the means of direct measurement of IDO activity, or such activity was hardly detectable under basal conditions (Mor *et al.*, 2021). It should also be noted as an alternative other authors attempted to measure the IDO gene expression, however, the increased mRNA expression was not synonymous with increased IDO activity (Yeung *et al.*, 2015; Badawy & Guillemin, 2019).

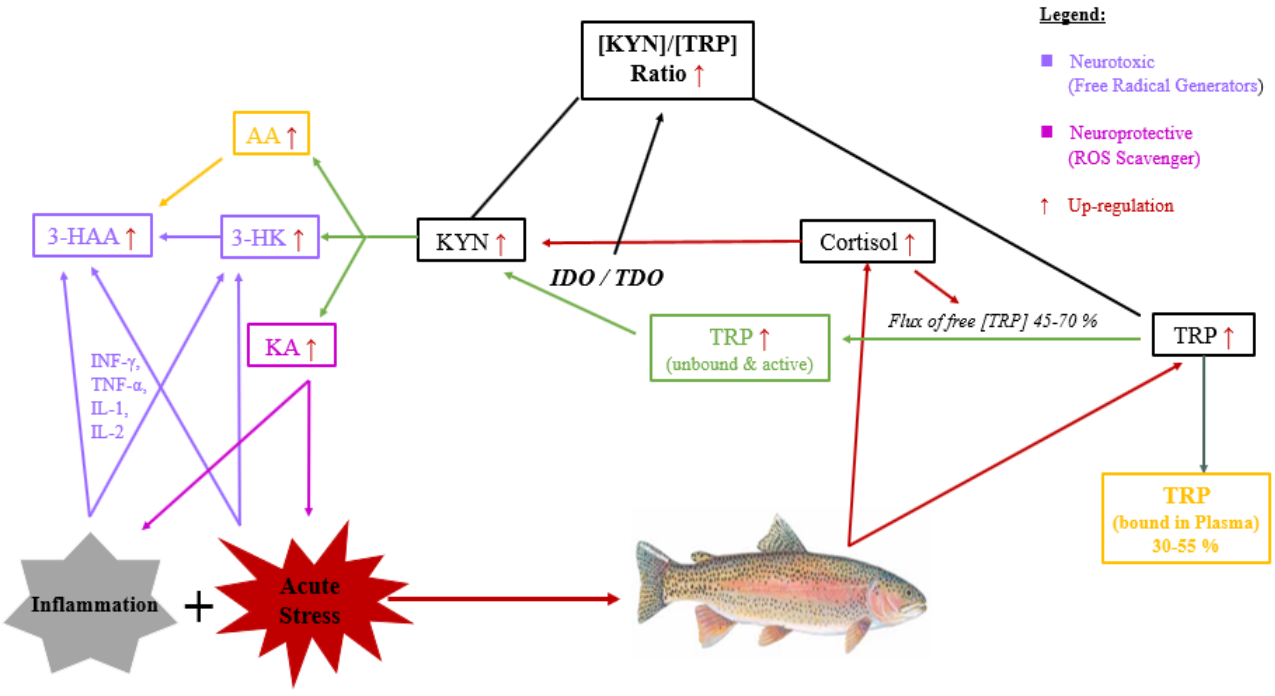
An increase in the [Kyn]/[Trp] ratio could be due to an increase in [KYN], a decrease in [TRP], and/or both (Badawy, 2017a; Badawy, 2017b). In most of the KTR studies, a significant increase in [KYN] is

very apparent (Badawy & Guillemin, 2019; Mor *et al.*, 2021). However, there are studies that do show the increase in KTR is solely attributed to a decrease in [TRP] (Badawy & Guillemin, 2019; Barone, 2019; Hoseini *et al.*, 2019, Mor *et al.*, 2021). Cortisol induction in isolated rat liver showed a ~50% decrease in [TRP] that was accompanied by a ~4-fold increase in [KYN], resulting is a dramatic increase in the KTR (Badawy & Guillemin, 2019). Kynurenine can also be influenced by the KP enzymes kynurenine monooxygenase (KMO) also known as kynurenine hydroxylase, kynureninase (KYNU), and to a partial extent kynurenine aminotransferase (KAT). These three KP enzymes determine which of the three divisions KYN will be metabolized to, where KMO converts KYN to 3-hydroxykynurenine, where KYNU converts KYN to anthranilic acid and then 3-hydroxyanthranilic acid, and KAT converts KYN to kynurenic acid (Badawy & Guillemin, 2019; Barone, 2019; Hoseini *et al.*, 2019) as outlined in Figure 1.2. The first two branches produce the 'excito-toxic' KYN metabolites and lead to an increase in the KTR with stress mediation (Barone, 2019; Badawy & Guillemin, 2019). Whereas the KAT enzyme produces the 'neuro-protective' KP metabolite kynurenic acid, showing the two divisions in the KP that ultimately determine the KTR (Barone, 2019; Badawy & Guillemin, 2019). The upregulation of KMO and KYNU leads to a decrease in circulating and tissue concentration of KYN thus lowering the KTR, whereas their downregulation or inhibition can have the opposite effect (Badawy & Guillemin, 2019). With kynurenine aminotransferase (KAT), the situation is less certain, because its catalytic activity on upregulation is determined by an increase in KYN availability through TDO/IDO induction or the downregulation of KMO or KYNU. However, the downregulation of KAT is unlikely to influence the KTR, but more research is needed to ascertain the exact role of KAT influence on the KTR (Barone, 2019; Badawy & Guillemin, 2019). Table 1.3 outlines the up- and down-regulation of the five enzymes that influence the KTR, as well as the increase or decreases in the KP metabolites. Figure 1.2 shows how stress affects the KTR in fish, as well as factors that influence increases in the enzymatic ratio.

Table 1.2. Expected changes in tryptophan in plasma and kynurenine metabolites after up- or down-regulation of the five kynurenine pathways enzymes that affected [Kyn]/[Trp] ratio.

Enzyme	TRP	KYN	[KYN]/[TRP]	KA	AA	3-HK	XA	3-HAA	QA
TDO↑	↓	↑	↑	↑	↑	↑	↑	↑	↑
↓	↑	↓	↓	↓	↓	↓	↓	↓	↓
IDO↑	↓?	↑	↑	↑	↑	↑	↑	↑	↑
↓	-	-	-	-	-	-	-	-	-
KMO↑	-	↓	↓	↓	↓	↑	↑	↑	↑
↓	-	↑	↑	↑	↑	↓	↓	↓	↓
KAT↑	-	↑	↑	↑	↑	-	↑	-	-
↓	-	?	?	↓	?	?	↓	?	?
KYNU↑	-	-	-	-	-	-	-	↑	↑
↓	-	↑	↑	↑?	↓	↑	↑	↓	↓

Abbreviations: AA, anthranilic acid; 3-HAA, 3-hydroxyanthranilic acid; 3-HK; 3-hydroxykynurenine; KA, kynurenic acid; IDO, indoleamine 2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine monooxygenase; KYNU, kynureninase; QA, quinolinic acid; TDO, tryptophan 2,3-dioxygenase; TRP, tryptophan; XA, xanthurenic acid.



Symbols used: ↑, increase; ↓, decrease; -, no change; ?, unknown. Reproduced with permission: Badawy & Guillemin, 2019

Abbreviations: AA, anthranilic acid; 3-HAA, 3-hydroxyanthranilic acid; 3-HK; 3-hydroxykynurenine; INF-γ, interferon-Gamma; IL-1, interleukin-1; IL-2, interleukin-2; KA, kynurenic acid; IDO, indoleamine 2,3-dioxygenase; KYN, Kynurenine; THF- α, tumour Necrosis Factor alpha; TDO, tryptophan 2,3-dioxygenase; TRP, tryptophan.

Figure 1.3. Factors that effect the [KYN]/[TRP] ratio in fish from acute stress exposure.

1.6 Kynurenine-tryptophan ratio as a biomarker for acute stress

Tryptophan metabolites are involved in a multitude of physiological functions for the growth and proliferation of all types of cells, in all types of organism from bacteria to mammals. Tryptophan metabolism is involved in several essential life functions from inflammation, aging, reproduction, and disease (Wang *et al.*, 2018; Kaiser *et al.*, 2019). Disruptions of TRP metabolism are associated with many pathologies such as cancer, infections, autoimmune disorders, like HIV, herpes, inflammatory bowel disease, irritable bowel syndrome, metabolic disorders (diabetes, obesity, non-alcoholic fatty liver disease, insulin resistance and atherosclerosis) and neuropsychiatric affections, including anxiety, depression and autism (Lefèvre *et al.*, 2019). A large body of research shows the KTR reflects the IDO activity and is therefore often used in research to monitor the TRP-KYN metabolism (Badawy & Guillemin, 2019; Barone, 2019; Hoseini *et al.*, 2019, Mor *et al.*, 2021). The expression of IDO is induced during inflammation, immune-activation, transplantation, pregnancy, and infectious diseases by proinflammatory stimuli and T-helper-cell and cytokines such as INF-γ (de Jong *et al.*, 2009). Interferon-Gamma also stimulates the formation of reactive oxygen species, implicating the IDO enzyme with oxidative stress. Furthermore, IDO appears to play a role in immunoregulation and communication between the immune and nervous systems and is suggested to mediate the tumor immune escape (Wang *et al.*, 2018; Kaiser *et al.*, 2019).

1.7 Concurrent quantitation of TRP, KYN, 5-HT and cortisol

Methods capable of quantifying all the catabolites of TRP in different key physiological compartments should make it possible to identify, confirm and validate key biomarkers resulting from disruption in TRP and KYN metabolism. To date, several methods for detection and quantification of TRP and its metabolites have been developed, mostly based on liquid or gas chromatography (LC or GC) with various

detection modalities, such as mass spectrometry (MS), UV absorbance, and fluorescent, electrochemical, and evaporative light scattering. Analysis by LC–MS or GC–MS typically involves strategies such as chemical derivatization and micro-extractions with evaporation steps that have been used for pre-treatment of the TRP metabolites. Furthermore, synthesis of deuterated standards, somewhat complicated and time-consuming sample preparation procedures, and large sample volumes that are required further chemical derivatization complicated analysis. In most cases, the methods had various analytical problems, such as derivative instability, the possibility of side reactions, low recoveries, complicated matrix effects, and expensive and time-consuming preparations. Therefore, to overcome the above problems, the development of an underivatized method with less pre-treatment extraction steps was the basis of my work.

1.8 Thesis Hypothesis

My alternate thesis hypothesis is that the [Kyn]/[Trp] ratio can be used to as a biomarker to assess acute stress in fish.

1.9 Hypothesis Testing

To test my hypothesis, laboratory held rainbow trout (*Oncorhynchus mykiss*) were subjected to an acute physical stress that included handling, vigorous net chasing, and hypoxic stress for a total of 5 minutes. The liver and brain were excised from the fish that were subjected to the external stress at two time points: 4- and 48-hrs. We also collected tissues from fish prior to the start of the induced stress experiment (t = 0hrs) to account for baseline levels. Two fish per tank were chosen from the four experimental groups to represent baseline levels. Fish from separate tanks were not stressed (i.e., control groups) and were sampled at 4- and 48-hrs. In addition to measuring TRP and KYN in fish tissues, I also measured 5-HT

and cortisol. The extraction and detection of the four target analytes were based on methods I developed and validated using liquid-solid extraction and high-pressure liquid chromatography tandem mass spectrometry (HPLC/MS/MS).

1.10 Thesis Structure

My thesis is divided into six (6) chapters. *Chapter 1* introduces the mechanisms of stress exposure and stress response in fish, multi-stress effects, TRP metabolism in fish, and use of the KTR as a measure of IDO activity and quantitation of TRP, KYN, 5-HT, and cortisol, as well as highlighting my thesis hypothesis and how I tested my hypothesis. *Chapter 2* discusses the methodology adopted for sample processing and analysis of my target analytes. This chapter serves as an introduction to the background on sample processing and the analytical instrumentation utilized namely high-performance liquid chromatography, hydrophilic interaction chromatography, mass spectrometry and ionization techniques. *Chapter 3* provides the materials and experimental methods section outlining the chemical and reagents used, the acute stress exposure design and fish tissue sampling, fish liver and brain sample preparation and extraction, separation of TRP, KYN, 5-HT and cortisol, optimization parameter by tandem MS, and the method validation parameters utilized. *Chapter 4* presents the method validation results of a concurrent quantitative method for analysis of TRP, KYN, 5-HT, and cortisol in rainbow trout liver and brain using HPLC-MS/MS. The criteria of the Eurachem guidelines were meticulously followed to prove the validity of the extraction and quantitation method that was developed. *Chapter 5* shows the application and results of the extraction, separation, detection and quantitation method of fish liver and brain sample extracts from the acute stress exposure study performed in the University of Saskatchewan laboratory study. My study was recently published in the journal *Chemosphere* (impact factor: 7.1) (Wish *et al.,* 2022). *Chapter*

6 provides the conclusion of the study as well as suggestions and recommendations for future research directions.

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Chapter 2. Sample analysis and analytical instrumentation

2.0 Background on sample processing and instrumental techniques

This chapter will describe theoretical aspects of the sample processing and instrumental techniques that I used in my research. A simplified workflow is shown in Figure 2.1.

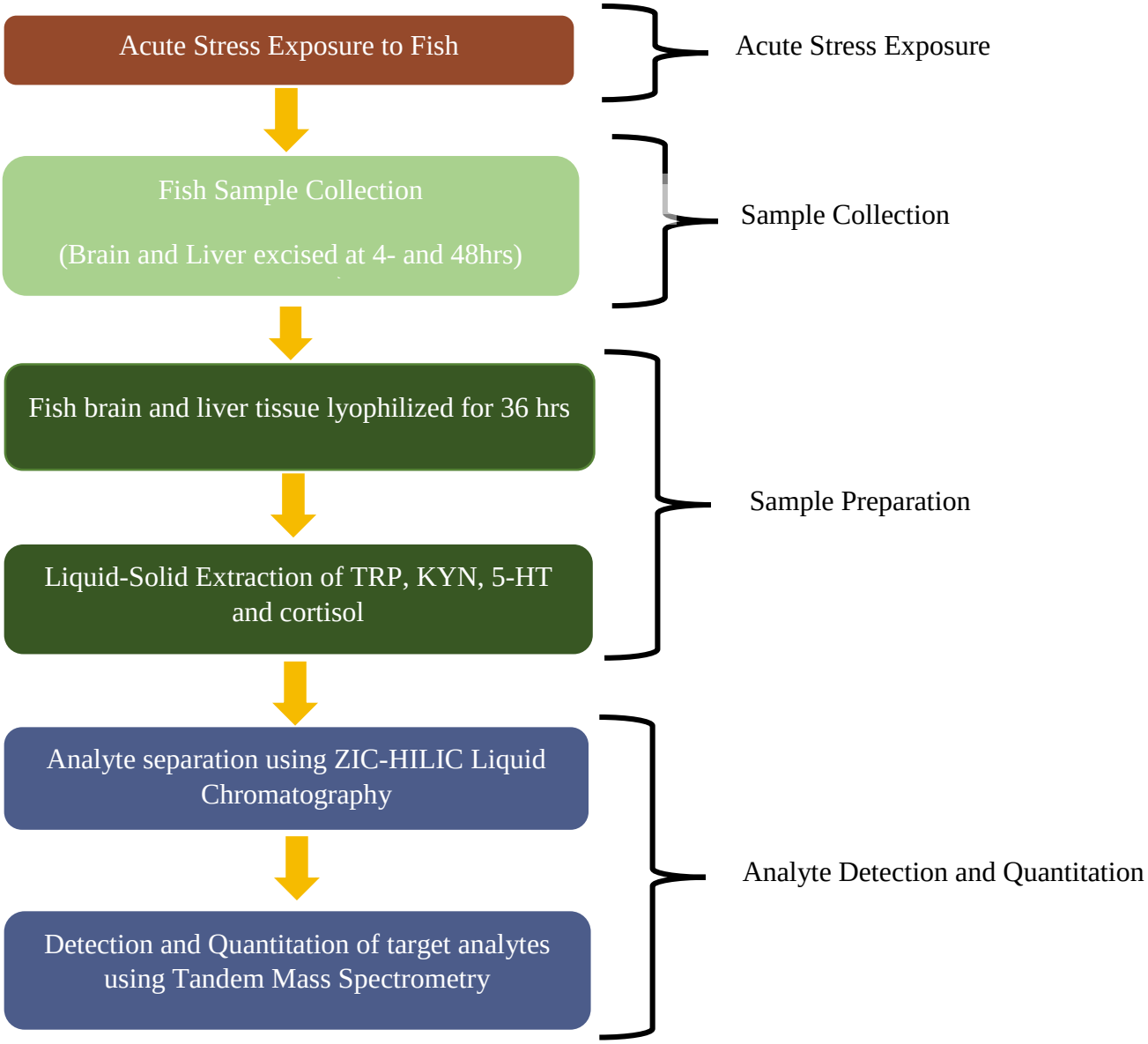

Figure 2.1. Experimental workflow for tryptophan, kynurenine, serotonin and cortisol extraction, separation, detection and quantitation.

2.1 Sample preparation

The main goal of sample preparation is to isolate one or several target analytes from the other components of the sample mixture (matrix) (Henion *et al.,* 1998). This may involve sub-sampling, pulverizing, dilution, dissolution, filtration, extraction and chemical digestion to name a few steps (Henion *et al.,* 1998). Depending on their chemical nature and concentration levels, co-components of the sample matrix can influence the quantitation of target analyte(s) during subsequent LC-MS or tandem LC-MS/MS experiments if not removed prior to analysis (Vogeser *et al.,* 2007). The development of new LC-MS/MS methods for small molecules in biological samples is becoming increasingly more challenging, because of the need to continuously achieve higher sensitivity and better assay robustness in complex biofluids such as serum, plasma, urine, faeces, mucus, or cerebrospinal fluid (Bylda *et al.,* 2014). In addition, because of the very low concentration levels of metabolic targets, samples often need to be pre-concentrated before analysis (Bylda *et al.,* 2014). However, this does not only increase the concentration of the desired compound in the sample extract but also often increases the amounts of interfering components (Vogeser *et al.,* 2007). Sample preparation is important because often the least complex of samples can rarely be utilized in their raw state either due to excessive dilution or concentration of target analytes and/or incompatibility with analytical instruments. However, sample preparation can sometimes constitute the principal source of error to acquired data and still remains one of the most time-consuming and costly steps in the analytical process (Gorityala *et al.,* 2021). As a result, very specific and effective sample clean-up procedures are required for sensitive and selective HPLC-MS/MS assays today, despite on advances in analytical instrumentation (Bylda *et al.,* 2014).

2.2 Analyte extraction

Analyte extraction involves transferring the target analytes into a liquid phase through partitioning between the sample matrix and an extracting solvent. The most common extraction techniques in use are liquid-liquid extraction (LLE), liquid-solid extraction (LSE), and solid-phase extraction (SPE) (Hemstrom & Igrum, 2006). My research involved the use of LSE and theoretical aspects of this technique will now be briefly described. Liquid-solid extraction involves the disruption of the analyte-matrix interaction(s) by agitation (i.e., sonication, vortexing, ball-milling) and an extracting solvent that has a high affinity for the analytes of interest. In this way, analytes partition from the matrix and into the extracting solvent. Naturally, it is prudent to select an extracting solvent that has similar chemical properties to the analytes of interest. Temperature, solvent pH and pressure are just some of the variables that can affect analytes recoveries (Hemstrom & Igrum, 2006). After extraction, a centrifugation and concentration step are normally used followed by reconstitution into an appropriate solvent, namely the accompanying HPLC mobile phase for HPLC-MS analysis. One of the major advantages of LSE is its ease of use and cost effectiveness (Appelblad *et al.,* 2009). By judiciously selecting the appropriate extracting solvent and by carefully controlling the external variables that affect recoveries, LSE can result in high selectivity and repeatability for analytes of interest. (Appelblad *et al.,* 2009).

2.3 Hydrophilic interaction chromatography

It is known that analyses of underivatized amino acids can be challenging. This is because these compounds are zwitterionic, hydrophobic and/or hydrophilic molecules largely UV-inactive (Hemstrom & Igrum, 2006). Numerous reverse phase (RP) chromatography methods have been published for the individual analysis of TRY, KYN, 5-HT and cortisol as well as all their respective metabolites (Möller *et al.,* 2012; Lesniak *et al.,* 2013; Chen *et al.,* 2018; Yılmaz & Gökmem, 2018; Fuertig *et al.,* 2019; Lefèvre

et al., 2019; Sadok *et al.*, 2019). Hydrophilic interaction chromatography (HILIC) was first suggested by Alpert in 1990 (Alpert, 1990) and is more suitable than RP chromatography for separating small polar ionisable molecules, providing reasonable retention without using costly and time-consuming sample preparation, chemical derivatization methods and/or ion pair reagents (Hemstrom & Igrum, 2006; Boersema *et al.*, 2008; Appelblad *et al.*, 2009; Spagou *et al.*, 2010; Kawachi *et al.*, 2011; Buszewski & Noga, 2012; Kathriorachchia *et al.*, 2018; Garcia *et al.*, 2019; Werneth *et al.*, 2019). HILIC separations are performed either in isocratic mode with a high percentage of organic solvent or with gradients starting with a high percentage of organic solvent and ending with a high proportion of aqueous solvent (Buszewski & Noga, 2012). In HILIC separations, the mobile phase forms a water-rich layer on the surface of the polar stationary phase vs. the water-deficient mobile phase, creating a liquid/liquid extraction system. The analyte is then distributed between these two layers (Buszewski & Noga, 2012).

Figure 2. 2 illustrates a comparison of HILIC and RP-HPLC for the separation of peptides under similar chromatography conditions using water: acetonitrile as their respective mobile phases (Appelblad *et al.*, 2009; Walker *et al.*, 2012). Hydrophilic interaction chromatography peptide separation results in higher selectivity, sensitivity, and better resolved peaks than the RP-LC peptide separation (Appelblad *et al.*, 2009; Walker *et al.*, 2012). Furthermore, by switching to HILIC from RP-LC, common disadvantages including peak fronting and tailing, column bleed, irreversible sorption, and slow equilibration times can be avoided (Walker *et al.*, 2012).

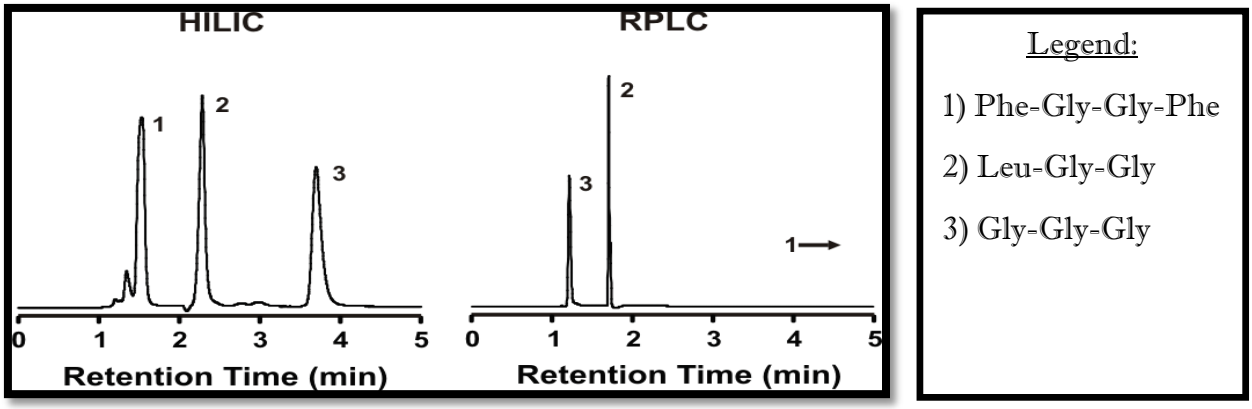

Figure 2.20. Comparison of the HILIC and RP-HPLC chromatograms for the separation of peptides under similar chromatographic eluents; (HILIC) 60:40 acetonitrile/10 mM ammonium acetate, pH 7, (RPLC) 5:95 acetonitrile/10 mM ammonium acetate, pH 7. Taken with permission from Appelblad *et al.,* 2009.

In the past decade there has been an increase in the use of HILIC promoted by the need to analyze difficult polar compounds at low concentrations in increasingly complex biological and environmental matrixes (Hemstrom & Igrum, 2006; Boersema *et al.*, 2008; Appelblad *et al.,* 2009; Spagou *et al.,* 2010; Kawachi *et al.*, 2011; Buszewski & Noga, 2012; Dell'mour *et al.*, 2012; Tang *et al.*, 2016; Kathriorachchia *et al.*, 2018; Garcia *et al.*, 2019; Werneth *et al.,* 2019). Hydrophilic interaction chromatography mass spectrometry-based metabonomic studies has highlighted the potential for using HILIC as a complement to RP chromatography for difficult to retain, metabolites, like TRP, KYN and 5-HT. Additionally, hydrophilic interaction chromatography has established itself as the separation mode of choice for uncharged highly hydrophilic and amphiphilic compounds, that are too polar to be well retained in RP-HPLC, as well as molecule that tends to exist as a zwitterion, like TRP, KYN and 5-HT. However, HILIC has insufficient charge to allow effective electrostatic retention that is required in ion-exchange chromatography (Hemstrom & Igrum, 2006; Boersema *et al.*, 2008; Appelblad *et al.*, 2009; Spagou *et al.*,

2010; Kawachi *et al.,* 2011; Buszewski & Noga, 2012; Dell'mour *et al.*, 2012; Tang *et al.,* 2016; Kathriorachchia *et al.,* 2018; Garcia *et al.,* 2019; Werneth *et al.,* 2019).

Hydrophilic interaction chromatography has shown many advantages over the conventional used RP-HPLC, it is suitable for analyzing complicated and problematic compounds in complex systems that always elute near the void volume in RP chromatography (Appelblad *et al.,* 2009; Spagou *et al.,* 2010; Kawachi *et al.,* 2011; Buszewski & Noga, 2012; Dell'mour *et al.,* 2012; Tang *et al.,* 2016). The use of expensive ion pair reagents is not required in HILIC, and it can be conveniently coupled to mass spectrometry (MS) like RP chromatography, especially in the electrospray ionization (ESI) mode. Mass spectrometry sensitivity is known to be significantly improved using HILIC because of the better desolvation of the highly organic mobile phases (low surface tension of acetonitrile versus water) (Hemstrom & Igrum, 2006; Boersema *et al.,* 2008; Appelblad *et al.,* 2009; Spagou *et al.,* 2010; Kawachi *et al.,* 2011; Buszewski & Noga, 2012; Dell'mour *et al.,* 2012; Tang *et al.,* 2016; Kathriorachchia *et al.,* 2018; Garcia *et al.,* 2019; Werneth *et al.,* 2019). In our case, better desolvation is further aided by the ESI source being equipped with a custom designed hot source induced de-solvation source with orthogonal injection. Another advantage of HILIC is the lower back pressure and the improvement in ESI-MS sensitivity due to the higher proportion of organic modifier eluting the analyte (Buszewski & Noga, 2012; Dell'mour *et al.,* 2012; Tang *et al.,* 2016; Garcia *et al.,* 2019). A gain in sensitivity with a factor up to ten in HILIC–ESI-MS versus reversed-phase LC–ESI-MS has been reported in previous studies (Ahn *et al.,* 2010; Buszewski & Noga, 2012; Dell'mour *et al.,* 2012; Tang *et al.,* 2016; Kathriorachchia *et al.,* 2018; Garcia *et al.,* 2019; Werneth *et al.,* 2019). In contrast to RP chromatography, gradient elution HILIC begins with a low-polarity organic solvent and elutes polar analytes by increasing the polar aqueous

content (Buszewski & Noga, 2012; Dell'mour *et al.*, 2012; Tang *et al.*, 2016; Kathriorachchia *et al.*, 2018; Garcia *et al.*, 2019; Werneth *et al.*, 2019).

A major disadvantage of HILIC is its reliance on acetonitrile as the mobile phases (Boersema *et al.*, 2008; Jandera, 2011; Periat *et al.*, 2015; Jandera & Jan, 2017). Additionally, HILIC is more sensitive to the solvent strength mismatch between the sample solvent and the mobile phase in comparison to RP-LC chromatography. Moreover, the high organic solvent poses environmental issues with extensive use and costly disposal (Hemstrom & Igrum, 2006; Boersema *et al.*, 2008; Appelblad *et al.*, 2009; Spagou *et al.*, 2010; Kawachi *et al.*, 2011; Buszewski & Noga, 2012; Kathriorachchia *et al.*, 2018; Garcia *et al.*, 2019; Werneth *et al.*, 2019).

The retention mechanisms of HILIC are much more complex than that of RP-HPLC. The retention mechanisms of HILIC utilizes the principles of a hydrophilic stationary phase and a hydrophobic organic mobile phase. The HILIC retention mechanism is mostly based on the hydrophilic partitioning of polar or zwitterionic compounds between the bulk organic solvent and partially immobilized water-rich layer, present at the surface of the stationary phase (Dell'mour *et al.*, 2012 Buszewski *et al.*, 2012; Jandera & Jan, 2017). For this reason, HILIC requires the use of an aprotic solvent such as acetonitrile or acetone that interacts poorly with the stationary phase through hydrogen bonding. Consequently, the mobile phase must include at least 3% water to produce the hydrophilic partitioning mechanism (Boersema *et al.*, 2008; Jandera, 2011; Dell'mour *et al.*, 2012 Buszewski *et al.*, 2012; Periat *et al.*, 2015; Jandera & Jan, 2017). However, additional interaction mechanisms like hydrogen-bonding, ionic interactions, and dipole–dipole interactions also take place, as illustrated in Figure 2. 3 (Periat *et al.*, 2015). Hydrogen bonding depends on Lewis acidity/basicity and dipole–dipole interactions depend on dipole moments and the polarizability

of analyte molecules (Buszewski & Noga, 2012; Dell'mour *et al.*, 2012; Tang *et al.*, 2016; Kathriorachchia *et al.*, 2018; Garcia *et al.*, 2019; Werneth *et al.*, 2019). Electrostatic interaction is also a strong contributor to HILIC retention which explains why ionizable compounds can also be retained under HILIC conditions (Boersema *et al.*, 2008; Jandera, 2011; Dell'mour *et al.*, 2012 Buszewski *et al.*, 2012; Periat *et al.*, 2015; Jandera & Jan, 2017). The nature of the bonding, be it amino, bare silica, zwitterionic, or another bonding type, should be selected on the basis of how the analyte is being retained. In protein and peptide analysis, hydrogen bonding and electrostatic interactions play a more significant role (Boersema *et al.*, 2008; Jandera, 2011; Periat *et al.*, 2015; Jandera & Jan, 2017).

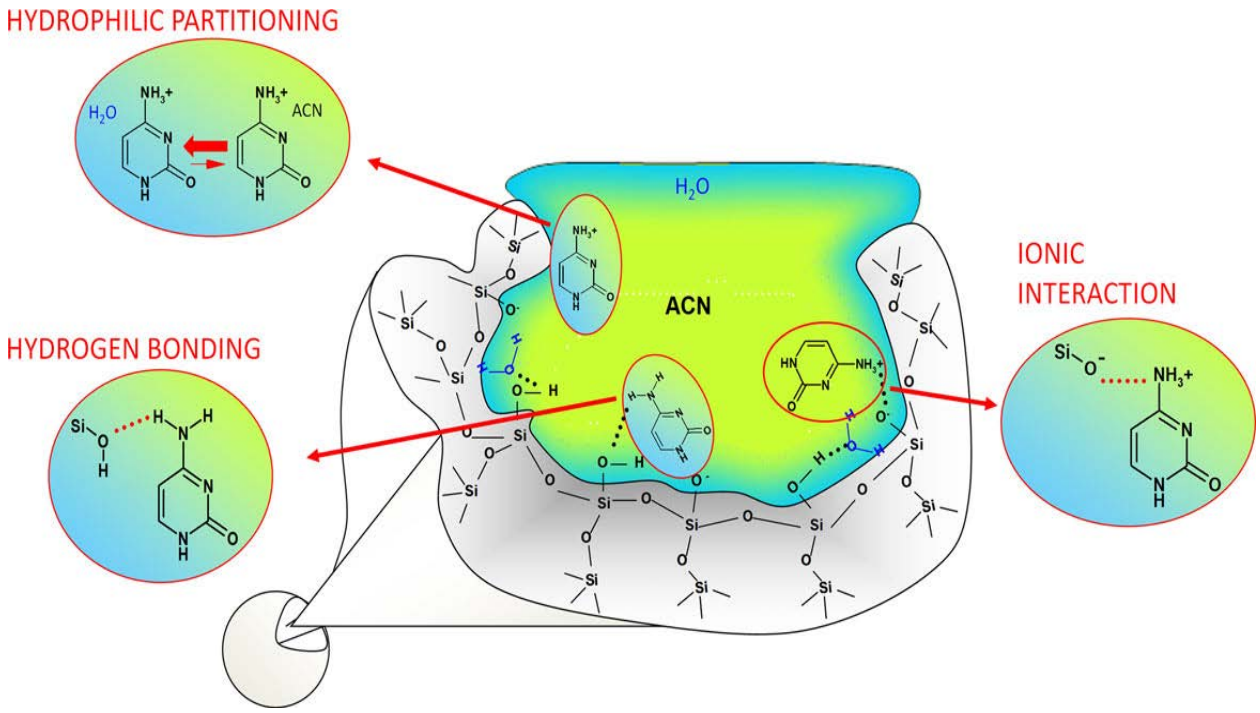

Figure 2.3. Schematic illustration of the retention mechanisms in hydrophilic interaction chromatography. Taken with permission from Periat et al 2015.

The hydrophilic interaction chromatography stationary phases can be divided into three different groups: neutral (no electrostatic interactions), charged (strong electrostatic interactions) and zwitterionic (weak electrostatic interactions) (Dell'mour *et al.,* 2012 Buszewski *et al.,* 2012 and Jandera & Jan, 2017). Recently, several stationary phases have emerged that are specifically made for HILIC approaches, like zwitterionic hydrophilic interaction liquid chromatography (ZIC®-HILIC) and ZIC-pHILIC® columns (Boersema *et al.,* 2008; Jandera, 2011; Periat *et al.,* 2015; Jandera & Jan, 2017). Some of the more popular HILIC stationary phases are shown in Figure 2. 4, they include underivatized silica stationary phases that contain varying functional groups such as siloxanes, silanols with (or without) a small quantity of metals derivatized silica, such as the cation exchanger polysulfoethyl A, the weak cation exchanger Polycat A, the weak anion exchanger PolyWAX, and TSKgel amide 80 (Boersema *et al.,* 2008; Jandera, 2011; Periat *et al.,* 2015; Jandera & Jan, 2017). Each type of stationary phases has different retention characteristics, and the separation of analytes require distinct mobile phase and buffer constitutions for optimal selectivity and results (Boersema *et al.,* 2008; Jandera, 2011; Periat *et al.,* 2015; Jandera & Jan, 2017).

As previously discussed, HILIC utilizes RP eluents, the ideal mobile phase for HILIC as well as the most used solvent is acetonitrile. However, other mobile phases can be used in the place of acetonitrile, like acetone or isopropanol. Hydrophilic interaction chromatography must simply contain a high degree of organic solvent and a typical gradient in HILIC would involve altering the aqueous composition between only 5 and 30%, a minimum of 3% aqueous is required (Buszewski & Noga, 2012; Dell'mour *et al.,* 2012; Tang *et al.,* 2016; Kathriorachchia *et al.,* 2018; Garcia *et al.,* 2019; Werneth *et al.,* 2019).

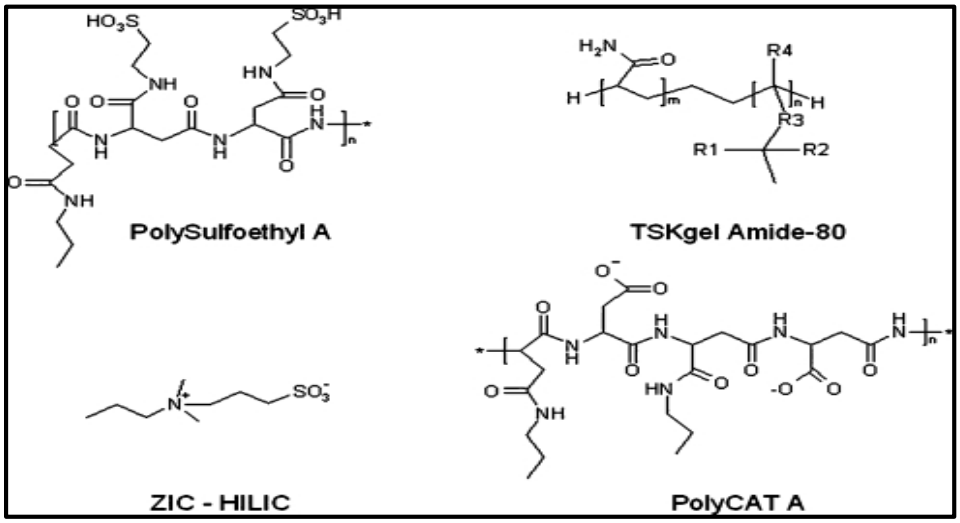

Figure 2.4. Molecular structures of typical stationary phases used in hydrophilic interaction liquid chromatography.

Furthermore, in HILIC chromatography changes in eluent buffer ion concentration can have a profound effect on the analyte ionization as well as the polarity of the eluent (Boersema *et al.*, 2008; Jandera, 2011; Periat *et al.*, 2015; Jandera & Jan, 2017). Having a sufficiently high ionic strength act as a counter ion is sometimes essential to achieving good peak shape and reproducible retention in HILIC chromatography for certain types of analytes (Jandera, 2011; Periat *et al.*, 2015; Jandera & Jan, 2017). A HILIC buffer must have a minimum of 70% acetonitrile, methanol or isopropanol otherwise it will result in poor chromatography or no analyte retention (Boersema *et al.*, 2008; Jandera, 2011). It is believed that the using HILIC buffers helps the hydrophilic stationary phase by enriching the water from the buffer, generating a larger/stronger aqueous layer (Periat *et al.*, 2015; Jandera & Jan, 2017). Nonetheless, an elution buffer is not necessary for this analysis of TRY, KYN and 5-HT because ZIC-HILIC chromatography use zwitterion capabilities which is sufficient to enhance the HILIC stationary phase for this analysis.

Zwitterionic hydrophilic interaction liquid chromatography has shown better selectivity for the test analytes than any of the other HILIC stationary phases used for the detection and separation of amino acid zwitterionic ligands. The ZIC®-HILIC column comprises of a permanent positive charged group and a permanent negative charged group. These phases are particularly hydrophilic but at the same time they also have modest electrostatic ion-exchange properties. For those qualities the ZIC®-HILIC column is employed for the quantitation of TRP, KYN, 5-HT and their respective deuterated internal standards in rainbow trout liver and brain.

2.4 Mass spectrometry

John B. Fenn, the co-inventor of ESI for biomolecules and the 2002 Nobel Laureate in Chemistry, defines MS as the art of measuring atoms and molecules to determine their molecular weight (Siuzdak, 2004; Pitt, 2009). Mass spectrometers operate by converting the analyte molecules to a charged (ionized) state, with subsequent analysis of the ions and any fragment ions that are produced during the ionization process, on the basis of their mass to charge ratio (*m/z*) (Siuzdak, 2004; Pitt, 2009). Several different technologies are available for both ionization and ion analysis, resulting in many different types of mass spectrometers with different combinations of these two processes. In practice, some configurations are far more common and versatile than others and the following descriptions focus on the instrument that I used as part of my research which was a triple quadrupole MS.

2.4.1 Ionization techniques

Prior to detection, neutral analyte molecules eluting off the HPLC column are ionized in an ion source. Ionization modes can either produce either positive or negative ions (Siuzdak, 2004; Pitt, 2009). Electrospray ionization was used as a fundamental part of my research. Liquid samples are pumped

through a metal capillary maintained at 3 to 5 kV and nebulised at the tip of the capillary to form a fine spray of charged droplets (Siuzdak, 2004; Pitt, 2009). The capillary is usually orthogonal to, or off-axis from, the entrance to the MS in order to minimise contamination (Kebarle, 2000; Ho *et al.*, 2003; Rosenberg, 2003). The EIS source uses a stream of air or nitrogen, heat, a vacuum, or a solvent sheath (often nitrogen) to facilitate rapid desolvation of the droplets and the residual electrical charge on the droplets is transferred to the analytes (Rosenberg, 2003; Siuzdak, 2004; Pitt, 2009). After the ions form (at atmospheric pressure), they are electrostatically directed into the mass analyzer and then transferred into the high vacuum of the MS via a series of small apertures and focusing voltages (Rosenberg, 2003; Siuzdak, 2004; Pitt, 2009). The ion source and subsequent ion optics can be operated to detect positive or negative ions and switching between these two modes within one analytical run, if desired. Figure 2.5 illustrates the ion formation from EIS source in HPLC-MS analysis.

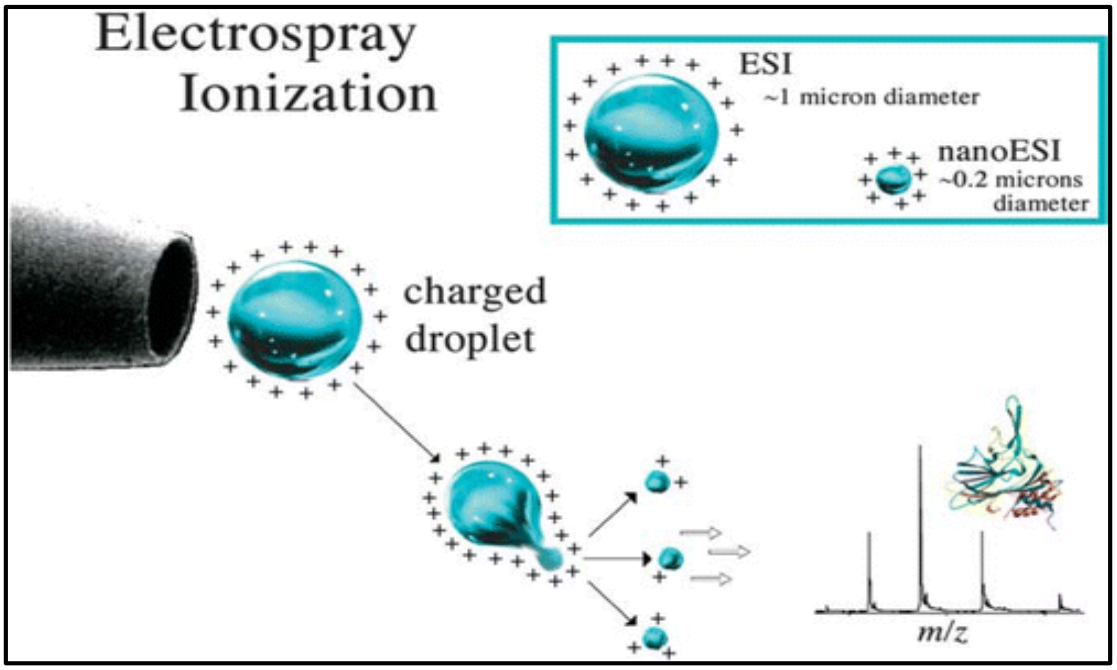

Figure 2.4.1. Ion formation from electrospray ionization source in mass spectrometry. Taken with permission from Siuzdak 2004.

Under normal conditions, ESI is considered as a "soft" ionisation source, unlike 'hard' ionization sources such as electron ionization (EI) and chemical ionization (CI) (Kebarle, 2000). Soft ionization sources mean that relatively little energy is imparted to the target analyte, and therefore only a limited amount of fragmentation occurs (Rosenberg, 2003; Siuzdak, 2004; Pitt, 2009). This is in contrast to other hard MS ion sources, such as EI source commonly used in GC-MS, which tends to cause extensive fragmentation (Kebarle, 2000).

Small molecules (<500 Da), such as TRP, KYN, 5-HT and cortisol with a single functional group are capable of carrying electrical charge to give predominantly singly charged ions (Kebarle, 2000; Pitt, 2009). This can involve the addition of a proton to the analyte $[M+H^+]$ when the ion source is operated in positive ion mode or the loss of a proton $[M-H^-]$ when operated in negative ion mode. Adduction of cations (for example, $[M+NH]^{4+,}$ $[M+Na]^+$, $[M+K]^+$) and anions such as, $[M+formate]^-$ and $[M+acetate]^-$ can also occur when salts are present in buffer (Rosenberg, 2003; Siuzdak, 2004; Pitt, 2009). Larger molecules and molecules with several charge-carrying functional groups such as proteins and peptides can exhibit multiple charging, resulting in ions such as $[M+2H]^{2+,}$ $[M+3H]^{3+}$ etc.

2.4.2 Mass Analyzers

A quadrupole mass analyzer also known as a quadrupole MS can separate ions using a combination of constant and varying (radio frequency) voltages which allows the transmission of a narrow band of *m/z* values along the axis of the rods (Kebarle, 2000; Rosenberg, 2003; Siuzdak, 2004; Pitt, 2019). By varying the voltages with time, it is possible to scan across a wide range of *m/z* values, resulting in a mass spectrum. Most quadrupole analysers operate at <4000 *m/z* and scan speeds up to 1000 *m/z* per sec or more are

common (Kebarle, 2000; Rosenberg, 2003; Siuzdak, 2004; Pitt, 2019). They usually operate at unit mass resolution meaning that the mass accuracy is seldom better than 0.1 *m/z*.

A triple quadrupole mass spectrometer consists of three quadrupoles where the first (Q1) and third (Q3) quadrupoles serve as mass filters while the second quadrupole (q2) is used as a collision cell. This combination is called a triple quadrupole mass spectrometer and is an example of tandem MS in which two or more stages of mass analysis are independently applied (Kebarle, 2000; Rosenberg, 2003; Siuzdak, 2004). The collision cell employs the use of inert gas such as argon, helium, or nitrogen to induce dissociation of parent ions(s) exiting Q1, resulting in a process called collision induced dissociation (Pitt, 2019). Q1 is usually set to filter a known mass (parent/precursor ions), where the fragments of ions produced after a collision in q2 are being monitored for in Q3. The fragment ions are essential for more reliable compound identification from sample extracts. A single and triple quadrupole system are capable of running in different detection modes such as, single (or selected) ion monitoring (SIM) and/or multiple reaction monitoring (MRM) (Kebarle, 2000; Rosenberg, 2003; Siuzdak, 2004). Multiple reaction monitoring is a process when the first and third quadrupoles can be simultaneously stepped to different *m/z* values (Pitt, 2019).

Selected ion monitoring monitors the ion current at prescribed masses that are characteristic of the target analytes in an expected retention time (RT) window. In this mode, the mass spectrometer does not spend time scanning the entire mass range, but rapidly changes between *m/z* values for which characteristic ions are expected (Siuzdak, 2004). With modern instruments, the data system can be programmed to examine different ions in multiple RT windows (Pitt, 2019). The advantage of this method is that both high sensitivity and high specificity are achieved (Kebarle, 2000; Rosenberg, 2003; Siuzdak, 2004).

As previously discussed, MRM makes use of the first and the third quadrupoles to scan for specific precursor/product ion pairs (Pitt, 2019). The production of the fragment ions from their precursor ion is referred to as ion transitions. In SIM, a single ion transition is done which detects only the precursor ion while MRM involves multiple transitions detecting precursor ions and daughter/product ions (Kebarle, 2000; Rosenberg, 2003; Siuzdak, 2004; Pitt, 2019). This dual filter approach makes MRM a better choice compared to SIM. Numerous compounds have the same precursor ions, but identical product ions are limited, thereby making the identification of analytes more certain with MRM. These product ions can serve as fingerprints for specific compounds in an analysis (Kebarle, 2000; Rosenberg, 2003; Siuzdak, 2004; Pitt, 2019).

The analysis of TRP, KYN, 5-HT and cortisol utilizes the combined attributes of ZIC-HILIC separation, followed by EIS ionization where better desolvation is further aided by the custom designed hot source induced de-solvation source with orthogonal injection. Detection was performed in positive mode using the dual filter approach of MRM. The mass spectrums provided an excellent fingerprint for each of the analytes and is provided in the subsequent thesis chapters.

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Whiley L., Nye L. C., Grant I., Andreas N., Chappell K. E., Sarafian M. H., Misra R., Plumb R. S., Lewis M. R., Nicholson J. K., Holmes E., Swann J. R., Wilson I. D., (2019) Ultrahigh-Performance Liquid Chromatography Tandem Mass Spectrometry with Electrospray Ionization Quantification of Tryptophan Metabolites and Markers of Gut Health in Serum and Plasma-Application to Clinical and Epidemiology Cohorts. *Analytical Chemistry.* 91 (8): 5207-5216. https://doi:10.1021/acs.analchem.8b05884.

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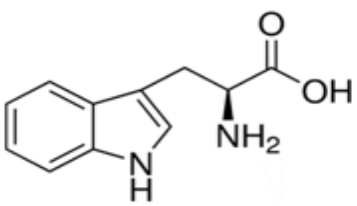
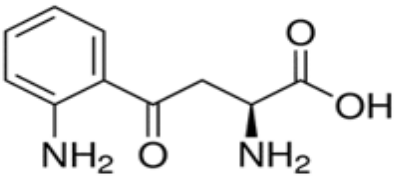
Chapter 3. Materials and experimental methods

3.0 Chemicals and reagents

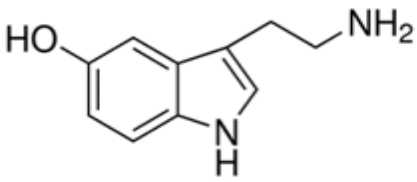
HPLC grade L-tryptophan (TRP), ≥98% (HPLC), L-kynurenine (KYN), ≥98% (HPLC), cortisol, acetonitrile (HPLC grade), and formic acid, were all purchased from Sigma-Aldrich Canada Co. (Oakville, ON, CA). Tryptophan, KYN, and acetonitrile are all stored at room temperature while formic acid was stored at 4 °C in the refrigerator and cortisol was stored at -20 °C in the freezer. Serotonin (5-hydroxytryptamine) (5-HT) was purchased from Toronto Research chemicals (TRC) (North York, ON, CA) and it was stored at -20 °C. The molecular structures of the TRP, KYN, 5-HT, and cortisol are shown in Figure 3.1. Mass labeled L-tryptophan-2',4',5',6',7'-d_5 (indole-d_5) (98% Atom D), L-kynurenine-d_4 [4-(2-aminophenyl-3,5-d2); 3,3-d2], (99% Atom D), serotonin-d_4 HCL (99% Atom D), and cortisol-9,11,12,12-d4 (98%) were all purchased from CDN Isotopes (Pointe-Claire, QB, CA). The tryptophan-d_5 and kynurenine-d_4 standards were stored at 4 °C and serotonin-d_4 and cortisol-d_4 were stored at -20 °C. Their deuterated internal standards structures are shown in Figures 3.2. All standards are >98% purity. In-house TRP, KYN, and 5-HT and cortisol standards were mixed in 35:65 Milli-Q water:acetonitrile (pH 7) and were prepared with varying concentration as required for analysis and method validation. The suite of isotope labeled compounds are the internal standards (IS) and are used as recovery internal standards (RIS) for the method validation. For MS tuning and calibration an MS Chemical Kit 1, with low-high standard concentrations of polypropylene glycol polymers (PPGs) (Positive PPG 1 x 10^{-4}, 1 x 10^{-5}, 1 x 10^{-6} M) was purchased from AB Sciex (Redwood City, CA, USA).



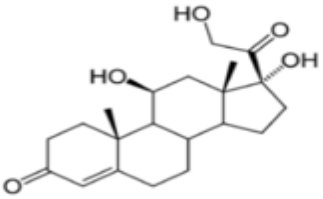
L-Tryptophan
MW: 204.229 g/mol
CAS: 73-22-3



L-Kynurenine
MW: 208.217 g/mol
CAS: 2922-83-0

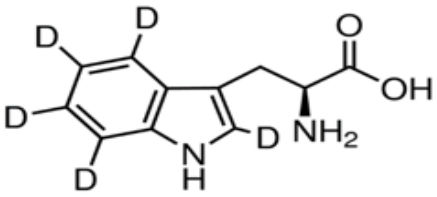


Serotonin
MW: 176.215 g/mol
CAS: 50-67-9

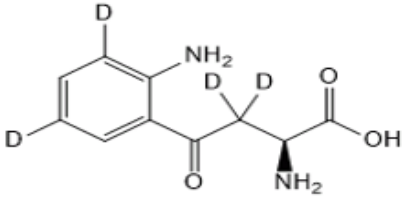


Cortisol
MW: 362.460 g/mol
CAS: 50-23-7

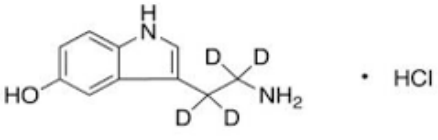
Figure 3.1. Chemical structures and molecular weights (MW) of tryptophan, kynurenine, serotonin, and cortisol.



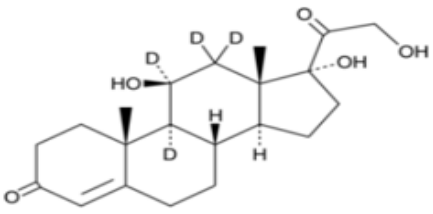
L-Tryptophan-d5
MW: 209.229
CAS: 62595-11-3



L-Kynurenine-d4
MW: 212.217
CAS: 2922-83-0



Serotonin-d4 · HCL
MW: 180.215
CAS: 153-98-0



Cortisol-d4
MW: 366.460 g/mol
CAS: 73565-87-4

Figure 3.2. Molecular structures and molecular weights of L-tryptophan-d_5, L-kynurenine-d_4, serotonin-d_4, and cortisol-d_4.

3.1 Acute stress exposure design and fish tissue sampling

All animal experiments were conducted in accordance with the standards established by the Canadian Council on Animal Care. The acute stress exposure and sampling of rainbow trout were performed in the Aquatic Toxicology Research Facility, Toxicology Centre, University of Saskatchewan (AUP #F16 018 and 20090115). Forty rainbow trout *(Oncorhynchus mykiss)* (*n=10* per tank) (157 ± 32 g) were randomly assigned to four 700 L rectangular tanks (Frigid Units Inc, model MT 700) and acclimated for 10 days. Each tank was equipped with a continuous flow system that operated at a water flow of 7 L/min. A water temperature of 13 °C and an 8:16 hrs light/darkness was maintained throughout the experiment and fish were fed a total of 2% their body weight daily of a commercial salmon diet (CoRey Aquafeeds (Fredericton, NB, Canada). Since TRP cannot be synthesized with the organisms and it can only be obtained from the diet and decreases significantly after 24-hrs; feeding ended on the day before the start of our laboratory experiment to remove any confounding exogenous TRP from food intake during the stress exposure experiment (Johnston and Glanville, 1994). At baseline (t=0-hrs), two fish from each of the four tanks (*n=8* total) were sacrificed and TRP, KYN, 5-HT and cortisol were measured in liver and brain tissue using the procedures described below. Subsequently, fish in two tanks (*n=8* each) were vigorously chased with a net, caught, briefly lifted out of the water and then released over a total period of 5 mins. A plastic divider was placed into these two tanks to corral the fish into a smaller area (~one-quarter of the total tank) to further ensure that all the fish were being stressed. This experimental protocol has been shown to cause stress in rainbow trout (Aluru and Vijayan, 2006). Fish in the other two tanks were not chased or handled and they are the control fish groups. At 4- and 48-hrs after the imposed stress exposure, the 8-remaining fish from each of the four tanks were sampled. Figure 3. 3 outlines the acute stress exposure study experimental design and sampling outline.

At the time of sampling, fish were netted and anaesthetized in a pH buffered MS-222 (0.4 g/ L) bath until opercular movement ceased. Once anaesthetized, fish were measured and weighed, and their weight and length (total and fork) were recorded for the determination of Fulton condition factor (CF) index provided in Table A1. (Stevenson *et al.*, 2006; Dekić *et al.*, 2016). Statistical differences in CF indices amongst experimental groups are provided in Table A2. Sex was determined visually using external characteristics. Tissues of liver and brain were dispensed into small individual pre-rinsed tie sampling bags and stored on dry-ice and shipped to the COGRAD laboratory. The liver samples were weighed to determine the hepatosomatic index (HSI) (Sadekarpawar *et al.*, 2013) which is provided in Table A3. Statistical differences in hepatosomatic indices amongst experimental groups are provided in Table A4.

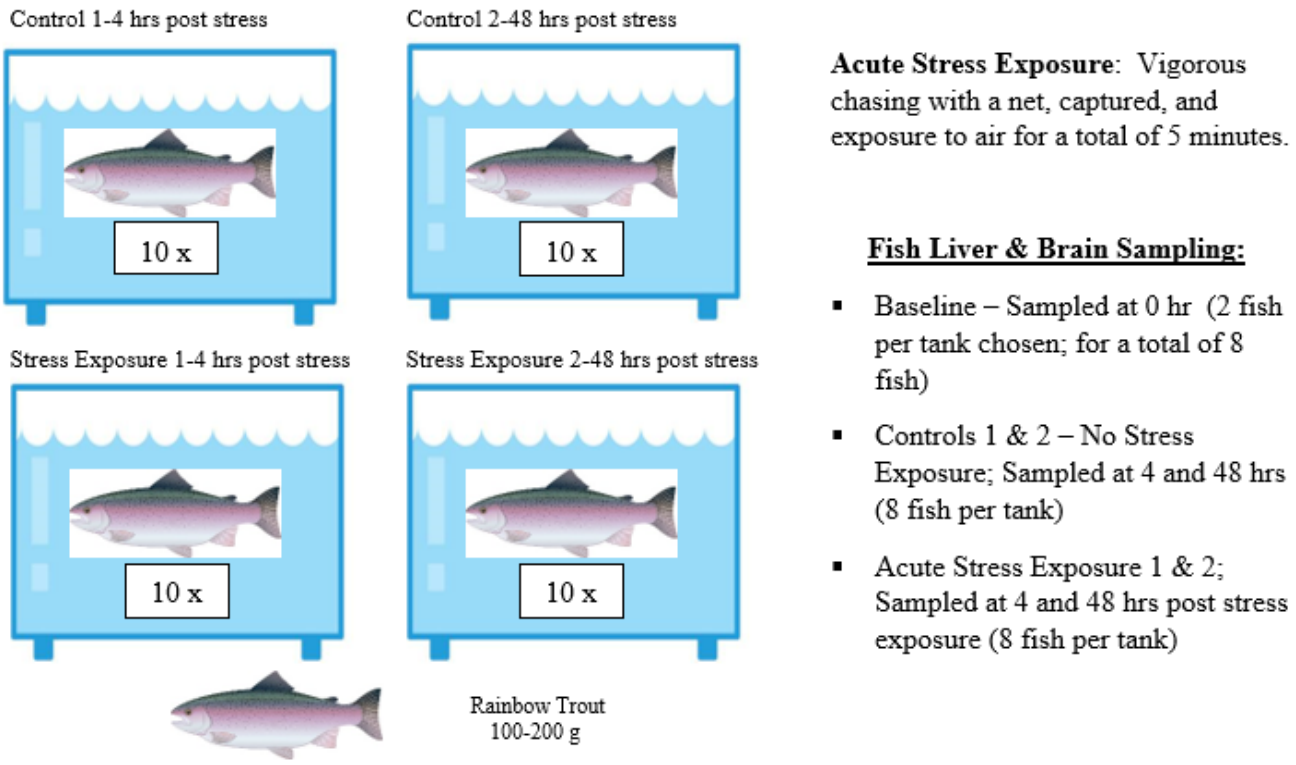


Figure 3.3. Acute stress exposure study laboratory experimental design and sampling outline.

3.2 Fish liver and brain sample preparation and extraction

Rainbow trout (*O. mykiss*) and walleye (*Sander vitrues*) livers collected by a local angler were used for our method validation. Liver collected from fish in the field were placed into separate pre-rinsed Whirl-pak bags and stored on ice during transportation to the laboratory. Once in the laboratory, tissue was stored at -80 °C until sample processing. For sample preparation, all our samples (liver and brain) were removed from the freezer and thawed at room temperature prior to liquid-solid extraction. Tissues were lyophilized separately for 48-hrs then ground using a mortar and pestle and 0.02 g was transferred to a 1.5 mL microcentrifuge test tubes. Mass labelled IS of TRP-d_5, KYN-d_4, and cortisol-d_4 (5000 pg/µL, 50 µL) were spiked directly into the tube and 1.35 mL of cold 35:65 Milli-Q water:acetonitrile (maintained at 4ºC, pH 7) was used to simultaneously extract our suite of target analytes. Samples were first sonicated in ice water for 12-mins followed by 1-min of vortexing, centrifuged at 14,800 rpm for 15-mins and the supernatant was transferred to 12 mL volumetric glass test tubes. The remaining pellet was disturbed using a sterile Pasteur pipet and extracted a second time using the identical protocol. The combined supernatant was evaporated to 0.5 mL under a steady stream of UHP nitrogen gas and analyzed for cortisol. After cortisol analysis, extracts were diluted to 2.5 mL for TRP, KYN and 5-HT determination. A flow chart for fish brain and liver sample preparation is outlined in Figure 3.4.

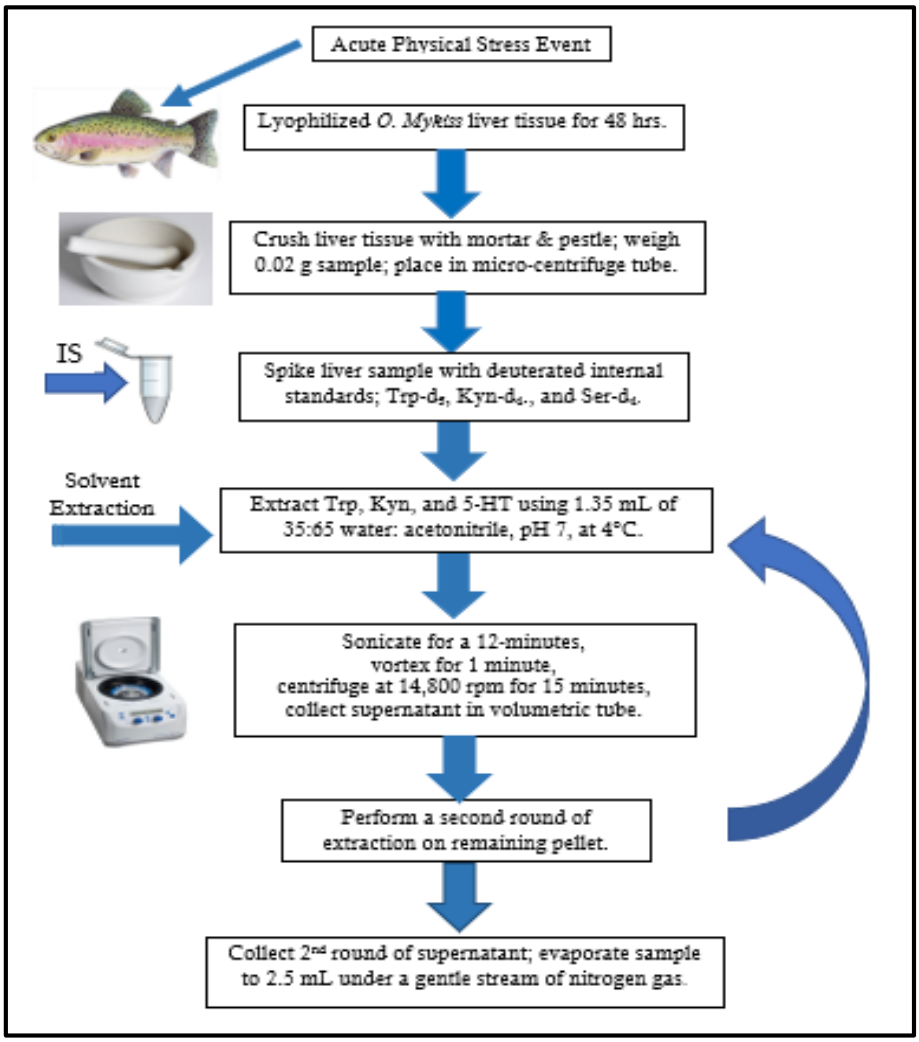


Figure 3.4. Flow chart of fish liver and brain sample preparation for tryptophan, kynurenine, serotonin, and cortisol analysis.

3.3 Separation of tryptophan, kynurenine, serotonin and cortisol by HPLC

The HPLC system consisted of an Agilent 1100 HPLC (Palo Alto, CA) with a binary gradient pump and CTC PAL autosampler. A volume of 10 µL of sample extract was injected onto the injection loop (10 µL) using the CTC PAL autosampler. HPLC separation for TRP, KYN and 5-HT was performed using a SEQUANT ZIC®HILIC PEEK coated HPLC column (4.6 mm x 100 mm, 3.5 µm particle size, 200Å

pore size) (Merck, Darmstadt, Germany). Cortisol analysis was performed using a Zorbax Eclipse XBD-C_{18} (4.6 mm x 150 mm, 5 microns) HPLC column (Agilent Technologies, Santa Clara, CA, United States). The column oven temperature was maintained at 40 °C for all subsequent analysis. The mobile phase consisted of Milli-Q water containing 0.1% formic acid (A) and acetonitrile (B). A flow rate of 300 mL/min was utilized for all analyses. The TRP, KYN and 5-HT separation was performed using a solvent gradient of: 0-0.5 min, 20% A; 0.5-2.0 min linear ramp gradient increasing to 35% A; 2-9 min remain constant at 35% A; 9-12 min linear ramp decreasing to 20% B; 11-14.98 min 20% A was used. The cortisol analysis also employed a gradient elution of: 0-0.5 min, 70% A; 0.5-5.0 min linear ramp gradient increasing to 100% B; 5.0-10 min remain constant at 100% B; 10-12 min linear ramp decreasing to 30% B; 12-15 min 30%B.

3.4 Optimization parameters of tryptophan, kynurenine, serotonin, and cortisol by tandem MS

The HPLC was coupled to a Sciex API 365 triple quadrupole mass spectrometer (QqQ-MS) equipped with an electrospray ionization (ESI) source. The QqQ-MS was tuned and calibrated with AB Sciex MS Chemical Kit 1, with low-high standard concentrations of polypropylene glycol polymers (PPGs) (Positive PPG 1 $x10^{-5}$ M). The source was a custom designed hot source induced desolvation source with orthogonal injection and was operated at 200 °C. The QqQ-MS instrument was always operated in positive ionization mode. The ion spray voltage was constantly maintained at 5500 V. Nitrogen was used as the drying and nebulizing gas. The fragmentor and skimmer voltages were set at 180 and 80 V, respectively. The gas temperature was 350 °C, dry gas 10 L/min, and nebulizer 40 psi. Direct flow injection analysis of the final mobile phase composition was then used to optimize final ion source parameters. The full scan analysis was performed using the target analytes at a concentration of 5 ng/μL, prepared in cold 35:65

Milli-Q water:acetonitrile (4ºC, pH 7). These standards were then run in full-scan mode from *m/z* 50 to 400 to select the precursor ions for each analyte. Step-by-step ion source parameters including declustering potential (DP), focusing potential (FP), collision energy (CE), and cell exit potential (CXP) were optimized individually for each of the eight chemical standards. All samples were analyzed using multiple reaction monitoring (MRM) mode. Each analyte was monitored using two MRM transitions; one for quantitation (Q) and one for confirmation (C). Precursor ions were selected based on the most abundant peaks and second most abundant peak, respectively. For all of the analytes, the base peak was the molecular ion $[M]^+$. The ion transitions used for quantitation were as follows: TRP: *m/z* 205.1 → 188.2; KYN: *m/z* 209.4 → 94.1; 5-HT: *m/z* 177.1 → 160.3; cortisol: *m/z* 363.3 → 160.3; TRP-d_5: *m/z* 210.2 → 192.4; KYN-d_4: *m/z* 213.3 → 196.2; SER-d_4: *m/z* 181.3 → 164.3; and cortisol-d_4: *m/z* 367.4 → 121.9. The final MS/MS optimization parameters for all our compounds are summarized in Table 3.1, as well as the ions that were used for confirmation.

Table 3.1. MS/MS optimization parameters for tryptophan, kynurenine, serotonin, cortisol, and the deuterated internal standards.

Analyte ID	**Q1 Mass (Da)**	**Q3 Mass (Da)**	**Declustering Potential (DP) (Volts)**	**Focusing Potential (FP) (Volts)**	**Collision Energy (CE) (Volts)**	**Cell Exit Potential (CXP) (Volts)**
Analytes						
TRP-Q	205.10	188.20	19.76	1.77	13.77	17.10
TRP-C	205.10	146.10	19.76	1.77	24.06	11.91
KYN-Q	209.40	94.10	18.90	11.95	20.52	5.24
KYN-C	209.40	146.20	18.90	11.95	22.95	11.86
SER-Q	177.30	160.30	17.99	12.77	12.58	10.48
SER-C	177.30	148.80	17.99	12.77	13.86	17.60
Cort-Q	363.30	121.10	19.38	29.78	42.84	13.98
Cort-C	363.30	327.40	19.38	29.78	26.50	25.18
Internal Standards						
TRP-d_5-Q	210.20	192.40	8.79	4.09	14.10	16.85
TRP-d_5-C	210.20	150.10	8.79	4.09	24.12	11.15
KYN-d_4-Q	213.30	196.20	17.60	1.50	14.59	22.93
KYN-d_4-C	213.30	95.90	17.60	1.50	32.05	12.97
SER-d_4-Q	181.20	164.30	22.77	5.60	12.98	13.11
SER-d_4-C	181.20	122.90	22.77	5.60	14.15	22.93
Cort-d_4-Q	367.20	121.90	20.30	28.27	12.98	13.11
Cort-d_4-C	367.20	96.40	20.30	28.27	14.15	22.93

3.5 Method validation parameters and data analysis

Method parameters were validated according to the Eurachem guidelines for analytical methods (Magnusson & Örnemark, 2014). The matrix effects, accuracy, limits of detection (LOD), limits of quantitation (LOQ), linear dynamic range, precision, repeatability, selectivity and robustness were all assessed to ensure the method characteristics fell within acceptable Eurachem guidelines values (Magnusson & Örnemark, 2014). Wild rainbow trout and walleye liver (*Sander vitreus)* tissue were used for method development and validation. Since native target analytes are endogenously present in our wild fish liver samples, mass labeled compounds were used to assess the performance characteristics of our method. HPLC-MS/MS data acquisition and processing were carried out using Analyst® software Version 1.6.1. Microsoft Office Excel 2010 was used to calculate the intra- and inter- assay means, standard deviation (SD), relative standard deviations (RSD) or coefficient of variance (CV). The calibration curve regression parameters of slope, intercept, and correlation coefficient were calculated by weight ($1/x^2$) linear regression. The baseline, control and experimental data were compared using the independent Student t-tests, a $p \leq 0.05$ was considered to be statistically different.

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Stevenson R. D. and Woods Jr W. A, (2006). Condition indices for conservation: new uses for evolving tools. *Integrative and Comparative Biology* 46 (6): 1169–1190. https://doi.org/10.1093/icb/icl052.

Virág D., Király M., Drahos L., Édes A. E., Gecse K., Bagdy G., Juhász G., Antal I., Klebovich I., Dalmadi Kiss D. B., Ludányi K., (2020). Development, validation and application of LC–MS/MS method for quantification of amino acids, kynurenine and serotonin in human plasma, *Journal of Pharmaceutical and Biomedical Analysis*, 180. https://doi.org/10.1016/j.jpba.2019.113018

Chapter 4. Results of method validation

4.0 Selectivity

The most abundant transition for each analyte was used as the quantitative ion (Q) and the second most abundant ion was used as the confirmation ion (C). No interfering peaks were observed for TRP, KYN, 5-HT and cortisol. The selectivity of the method was evaluated by comparing the theoretical ion intensities of the Q ion to the C ion from the standards to those observed in the samples. In all cases this ratio fell within our threshold limit of ± 20% implying that there were no measurable interferences and that our method is selective for TRP, KYN, 5-HT, and cortisol.

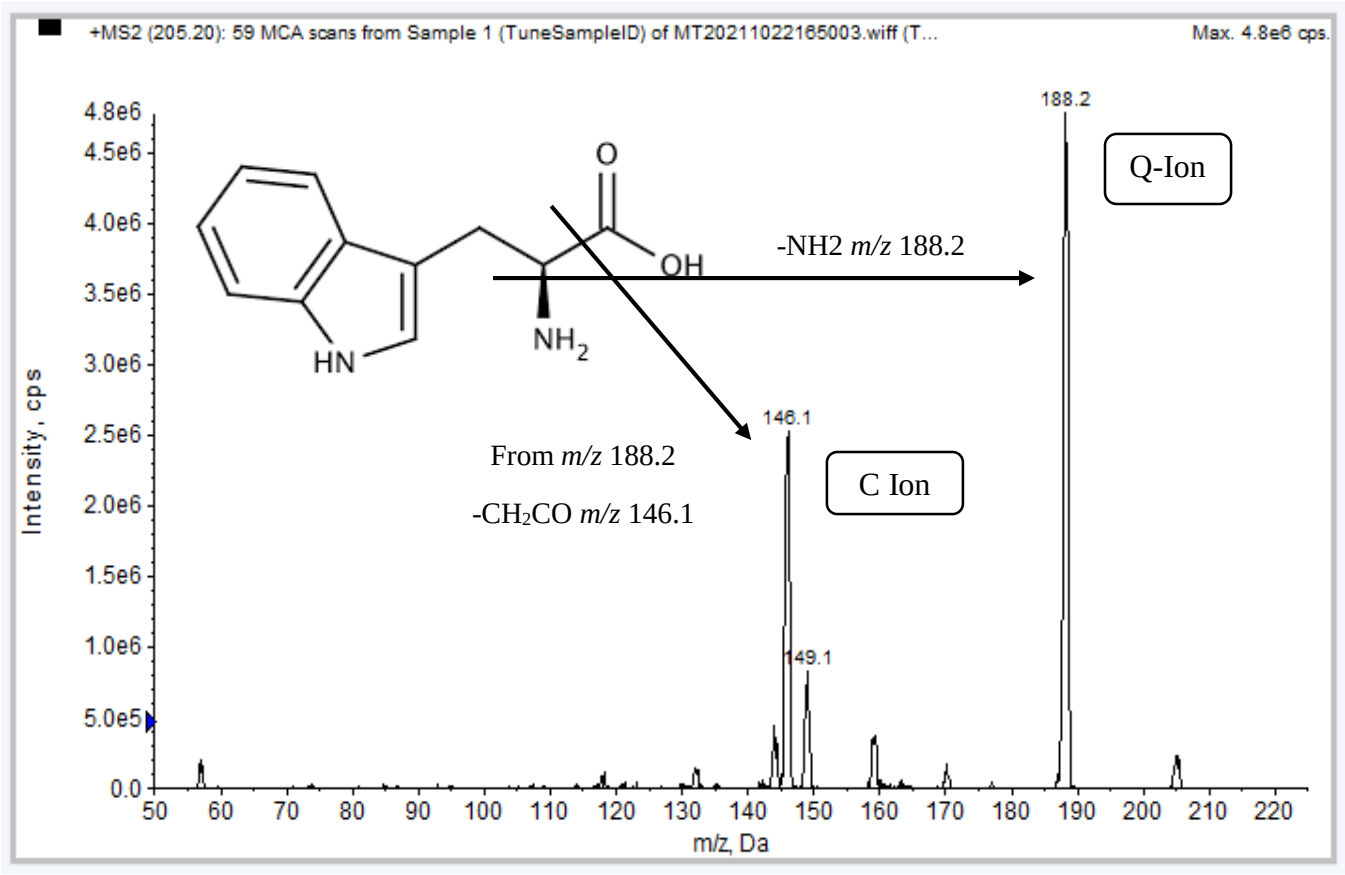

Figure 4.1. The chemical structure and mass spectra for tryptophan using HPLC-MS/MS, showing the ions used for quantitation and confirmation.

The ESI positive ion mass spectrum of TRP is shown in Figure 4.1. Tryptophan's ($C_{11}H_{12}N_2O_2$) (MW: 204.23 g/mol) most abundant ion is observed at *m/z* 188.1 resulting from the loss of the amino radical (NH_2) attached to the carboxylic acid end (COOH) of the TRP molecule, which gives the fragment at the *m/z* 188.1 ($C_{11}H_9NO_2$). The peak at *m/z* 146.1 ($C_{10}H_7N$) is the second most abundant ion in the mass spectrum. This latter ion at *m/z* 146.1 ($C_{10}H_7N$) is the result of a loss of carboxylic acid group (CH_2CO) from the *m/z* 188.1 ($C_{11}H_9NO_2$). Both these ions are very common and expected ion fragmentation patterns for TRP and has been used by others in the quantitation of TRP (Vazquez *et al.*, 2001; Vogeser *et al.*, 2007; Hu *et al.*, 2017; Tong *et al.*, 2018; de Jong *et al.*, 2019; Möller *et al.*, 2012; Lesniak *et al.*, 2013; Chen *et al.*, 2018; Yılmaz & Gökmem, 2018; Fuertig *et al.*, 2019; Lefèvre *et al.*, 2019; Sadok *et al.*, 2019; Parker *et al.*, 2019; Sadok *et al.*, 2017; Chawdhury *et al.*, 2021). The fourth most abundant ion observed is that of the molecular/parent ion, at *m/z* 205.1 ($C_{11}H_{13}N_2O_2$).

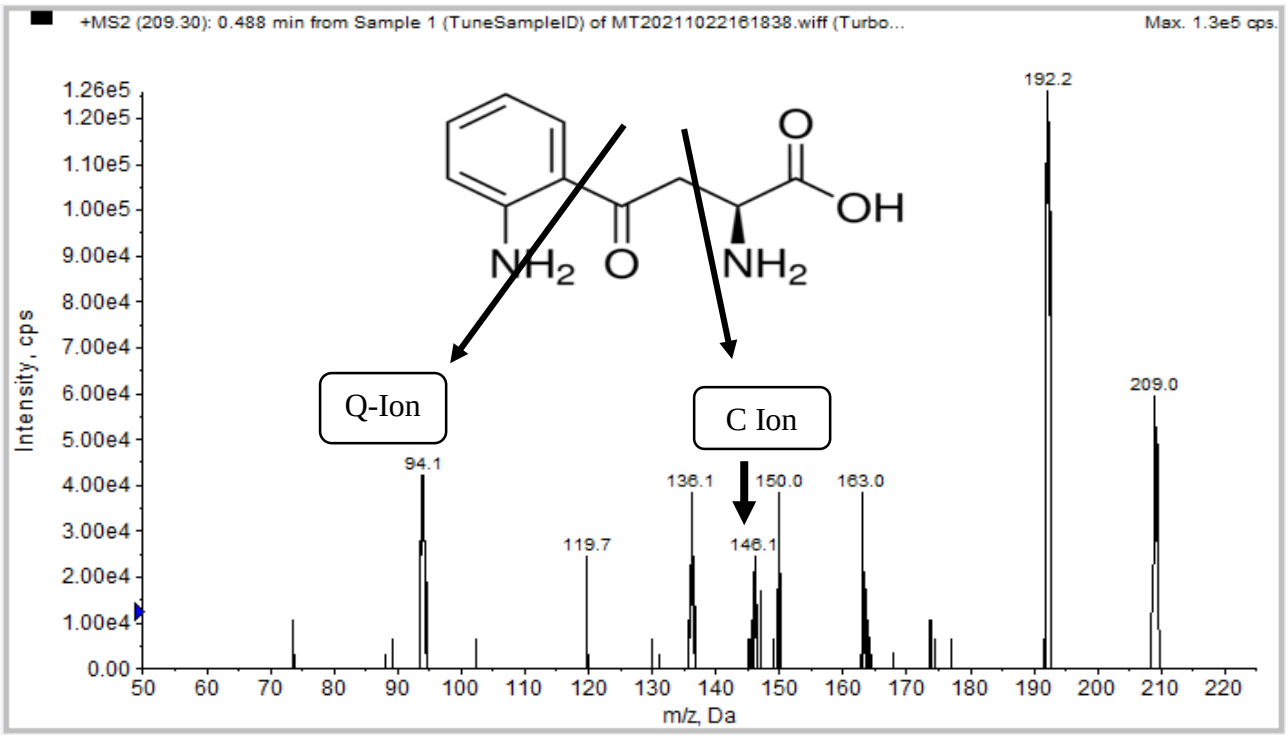

Figure 4.2. The chemical structure and mass spectra for kynurenine using HPLC-MS/MS, showing the quantitation and confirmation ions and fragmentation patterns.

The ESI positive ion mass spectrum of KYN ($C_{10}H_{12}N_2O_3$) is shown in Figure 4.2. The most abundant ion was observed at *m/z* 192.1 arising from the loss of the amino radical (NH_2) group first, and then the concomitant loss of a water group (H_2O) and carbon monoxide (CO) group, resulting in the 8-hydroxyquinoline (indole-3-carboxaldehyde) (C_9H_8NO) ion at *m/z* 146.1, which is subsequently used as the confirmation ion. The second most abundant peak for was observed to be the parent ion at *m/z* 209.1 ($C_{10}H_{12}N_2O_3$). The second most abundant fragment occurred at 94.1 (C_6NH_7), which arises from the *m/z* 146.1 (C_9H_8NO) fragment further losing the CCCOH from the benzene end of the KYN molecule. Similar to TRP, the *m/z* 94.1 and 146.1 ions are both very common and used by others to quantify KYN (Vazquez *et al.*, 2001; Vogeser *et al.*, 2007; Hu *et al.*, 2017; Tong *et al.*, 2018; de Jong *et al.*, 2019; Möller *et al.*, 2012; Lesniak *et al.*, 2013; Chen *et al.*, 2018; Yılmaz & Gökmem, 2018; Fuertig *et al.*, 2019; Lefèvre *et al.*, 2019; Sadok *et al.*, 2019; Parker *et al.*, 2019; Sadok *et al.*, 2017; Chawdhury *et al.*, 2021).

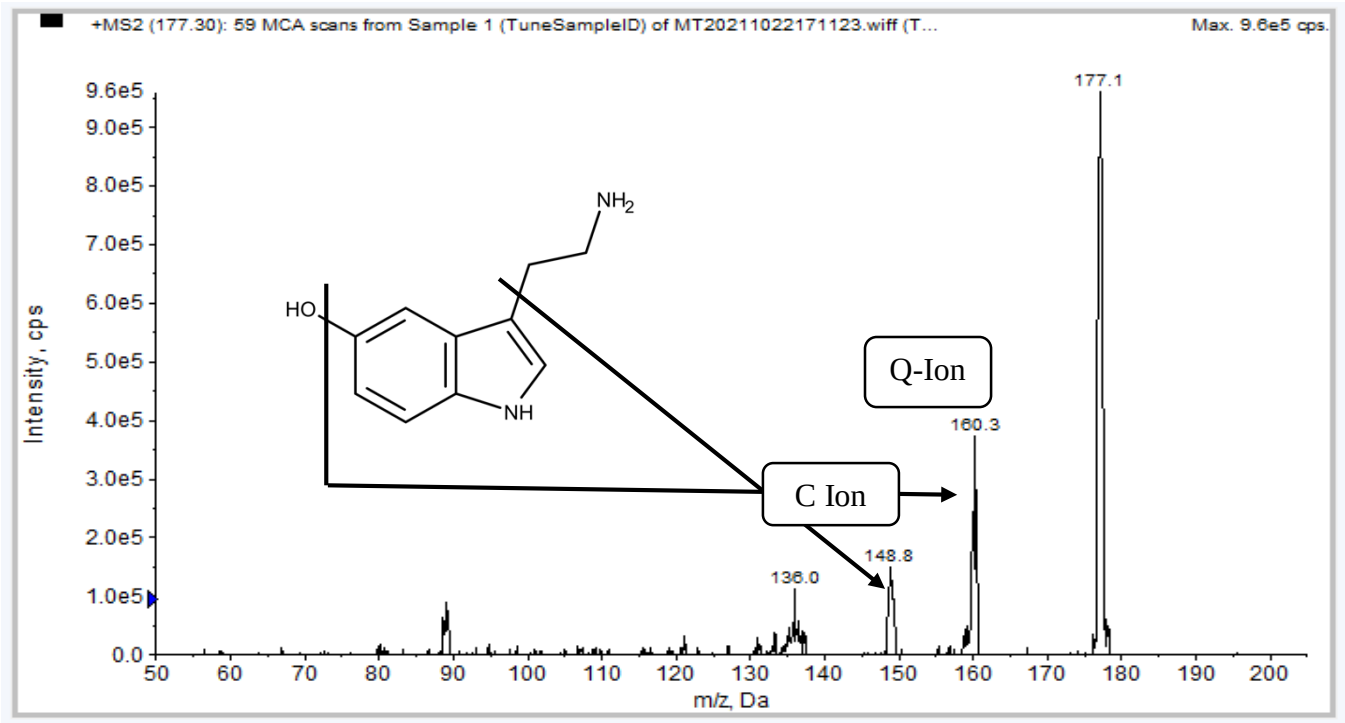

Figure 4.3. The chemical structure and mass spectra for serotonin using HPLC-MS/MS, showing the quantitation and confirmation ions and fragmentation patterns.

The ESI positive ion mass spectrum of 5-HT ($C_{10}H_{12}N_2O$) is shown in Figure 4.3. The most abundant ion, *m/z* 160.1 ($C_{10}H_{10}NO$) arises from the loss of amino radical group (NH_2) group from the end of the alkane chain portion of the molecule. The second most abundant ion was observed at *m/z* 148.8 ($C_9H_{10}NO$) which is formed from by a loss of carbon (C^-) atom from *m/z* 160 ($C_{10}H_{10}NO$). Similar to TRP and KYN, these two ions have been used by other research and analysis to quantify 5-HT (Vazquez *et al.*, 2001; Vogeser *et al.*, 2007; Hu *et al.*, 2017; Möller *et al.*, 2012; Lesniak *et al.*, 2013; Chen *et al.*, 2018; Yılmaz & Gökmem, 2018; Fuertig *et al.*, 2019; Lefèvre *et al.*, 2019; Sadok *et al.*, 2019; Parker *et al.*, 2019; Sadok *et al.*, 2017; Chawdhury *et al.*, 2021).

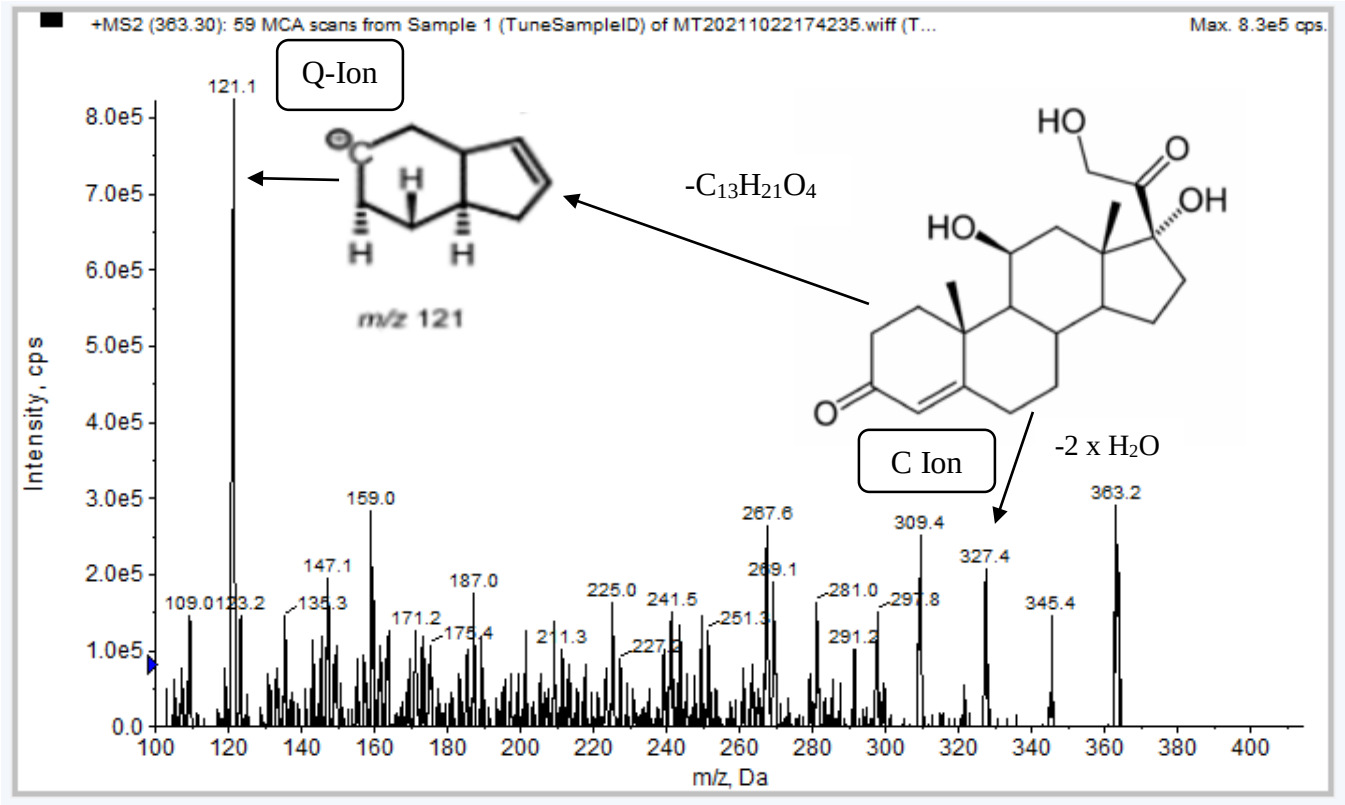

Figure 4.4. The chemical structure and mass spectra for cortisol using HPLC-MS/MS, showing the quantitation and confirmation ions and fragmentation patterns.

The ESI positive ion mass spectrum of cortisol ($C_{21}H_{30}O_5$) is shown in Figure 4.4. The most abundant ion for cortisol is by far at *m/z* 121.2 (C_8H_9O) arising from a loss of $C_{13}H_{21}O_4$ from the parent molecule of cortisol + H^+ ($C_{21}H_{31}O_5$). The second most abundant ion was observed at *m/z* 363.2 ($C_{21}H_{30}O_5$), which is the parent ion. The confirmation ion at *m/z* 327.4 results from the loss of two water groups (H_2O) from the right side of the parent molecule with carboxylic acid end (Szeitz *et al.*, 2014). Similar to the previous analytes, the cortisol ions chosen are often used for detection and quantitation of cortisol Szeitz *et al.*, 2014).

4.1 Linear dynamic range

The linear dynamic range for each analyte is shown in Table 4.1 and was determined by injecting calibration standards prepared in Milli-Q H_2O 35:65 ACN at pH 7. The calibration curves were calculated using $1/x^2$ weighted least squares regression equations. All analytes and IS showed linearity within this concentration range with a coefficient of determination (R^2) value > 0.99. In a similar manner, we determined the linearity of our matrix-matched external standard (MMES) by intentionally fortifying known amounts of our mass labeled IS into rainbow trout and walleye liver extracts. Irrespective of the fish species, the linearity of the mass labeled IS in our MMES was similar to that of their corresponding native analogs in solvent. Linearity was observed over this range for concentrations of each of the MMES with R_2 > 0.99 for all the analytes as shown in Table 4.1.

4.2 Limits of detection and quantitation

Mass labeled compounds were used to establish the limits of detection and quantitation (LOD and LOQ) of our method. Freeze dried fish liver (*n=8*) were fortified with TRP-d_5, KYN-d_4, SER-d_4 and cortisol-d_4 (resulting in a final concentration of 10 pg/µL) and extracted according to procedures described earlier.

Four unfortified samples of liver tissue ($n_{blk}=4$) were also extracted. Limits of detection were determined according to the equation below:

$$s_0' = s_0 \sqrt{\frac{1}{n} + \frac{1}{n_{blk}}} \qquad \ldots(1)$$

where s_0 is the estimated standard deviation of the 8 replicate fortified samples, s_0' is the standard deviation used to calculate LOD and LOQ, n is the number of replicate observations and n_{blk} is the number of blanks. Limits of detection and LOQ were then calculated as $3 \times s_0'$ and $10 \times s_0'$, respectively. A summary of the LODs and LOQs is shown in Table 4.1. Low LODs and LOQs were found for all four analytes. The LODs ranged between 1.20 - 1.79 pg/µL and the LOQs ranged between 3.99 and 5.95 pg/µL. Cortisol showed the lowest concentrations for both the LOD and LOQ for all four analytes, using the C18 column. While TRP showed the lower LOD and LOQs with respect to KYN and 5-HT, using the ZIC®HILIC column. The low values obtained can be attributed to the effective extraction method and the sensitivity and selectivity of the triple quadruple system in MRM mode.

Table 4.1. Linearity dynamic ranges, R^2 values, LODs and LOQs for TRP, KYN, 5-HT and cortisol.

	Tryptophan	**Kynurenine**	**Serotonin**	**Cortisol**
Linearity Range (ng/mL)	2.5-5000.0	2.5-750.0	2.5-750.0	1.0-1000.0
R^2 Value	0.999	0.999	0.999	0.998
LOD (ng/mL)	1.198	1.786	1.528	0.678
LOQ (ng/mL)	3.994	5.952	5.094	1.145

4.3 Accuracy and recovery

The accuracy of our method was determined by intentionally fortifying freeze-dried liver samples with known amounts of mass labeled internal standards (IS) followed by extraction and clean-up method described earlier. Quantitation of the IS in the fortified liver samples was achieved using the MMES. Accuracy was assessed at four fortification levels: 10, 200, 400, 700 pg/µL, and the recoveries are summarized in Table 4.2 The concentration of IS in the extracted sample was then calculated using the equation:

$$Extract\ Conc_{RIS} = \left[\frac{(Area_{RIS})}{\left(\frac{Area_{MMES}}{Conc\ Spike_{MMES}}\right)}\right] \qquad \ldots (2)$$

where,

$$Conc\ Spike_{MMES} = \frac{Initial\ Spike\ Amount_{MMES}\ (pg)}{Extract\ Volume\ (\mu L)} \qquad \ldots(3)$$

The percent recovery is then calculated by dividing the Extract $Conc_{IS}$ by the spiked MMES concentration times 100. The accuracies were assessed at four fortification concentrations of: 10, 200, 400, 700 pg/µL for TRP, KYN, and 5-HT. The extraction accuracy of cortisol was only assessed at one fortified concentration level of 100 pg/µL. With the exception of Kyn-d_4 at the lowest concentration, recoveries of all the compounds were greater than 70.60 % as shown in Table 4.2. In general, SER-d_4 showed a lower percent recovery for all four fortified concentrations levels, averaging 72.14 ± 1.96 %. Student t-tests were performed to determine if there were any significant statistical differences in analyte percent recovery at the four concentrations tested. All four concentrations for each IS showed no statistically significant differences, and the respective p-values were all greater than 0.05 as outlined in Table 4.3.

Table 4.2. The relative standard deviation for trytopahn-d_5, kynurenune-d_4 and serotonin-d_4, and cortisol-d_4 representing for repeatability and accuracy of the extraction and analysis method.

Analyte (*n = 8*)	**Spiked Concentration (pg/µL)**	**Measured Concentration (pg/µL) (mean ± SD)**	**% Recovery**	**Relative Standard Deviation**
Trp-d_5	10.00	7.57 ± 1.02	75.68	13.44
	200.00	174.30 ± 23.37	87.45	13.41
	400.00	407.13 ± 76.29	102.13	15.29
	700.00	492.58 ± 55.58	70.61	11.28
Kyn-d_4	10.00	5.18 ± 0.73	52.04	14.15
	200.00	190.43 ± 18.03	95.64	9.47
	400.00	486.57 ± 74.89	122.18	15.40
	700.00	500.71 ± 59.54	71.85	11.90
Ser-d_4	10.00	7.26 ± 0.05	72.55	6.61
	200.00	148.22 ± 15.67	73.68	10.57
	400.00	295.76 ± 33.61	73.52	11.36
	700.00	466.19 ± 15.53	66.83	3.33
Cort-d_4	100.00	118.98 ± 12.55	118.92	10.55

Table 4.3. Student t-test probabilities for statistical differences in the four fortified concentration levels of 10, 200, 400, and 700 pg/µL and internal standard percent recovery.

Control Group	**Trp-d5 vs. Kyn-d4**	**Trp-d5 vs. Ser-d4**	**Kyn-d4 vs. Ser-d4**
P-value =	0.8819	0.7908	0.6845
Is P-value < 0.05	FALSE	FALSE	FALSE

4.4 Repeatability

Rainbow trout livers were fortified with our IS to give final concentrations of 10, 100, 200 and 400 pg/µL (*n=8*) and processed according to our extraction protocol described above. The same-day repeatability is shown above in Table 11 and are represented as relative standard deviation percent (RSD %). In all cases, the same-day repeatability was <17% is below the Eurachem guideline limit of 20% (Magnusson, 2014). The intra- and inter-day repeatability were also measured using rainbow trout livers that were fortified with the ISs to give final concentrations of 10, 200 and 400 pg/µL (*n=4* for intra-day and *n=16* for inter-day repeatability). Day-to-day repeatability tests were carried out for 3 consecutive days. There were no statistical differences in the inter- or intra-day repeatability of our IS at any of the spiking levels and the RSD % was <15% A summary intra- and inter-day RSD % for TRP-d_5, KYN-d_4, and SER-d_4 is provided in Table 4.4. The variability associated with the peak area ratios of our quantitation: confirmation (Q/C) ions during and after batch analysis was also assessed. The resulting fragmentation patterns of stable molecules will remain constant during analyses if conditions are unchanged. The average Q/C ratios of TRP-d_5, KYN-d_4, and SER-d_4, and cortisol remained relatively constant (within or below < 10%) throughout the entirety of the method validation process. This result is characteristic of the analytes that show no degradation over the analysis period. Lastly, since the RSD in Q/C ratios for TRP-d_5, KYN-d_4, SER-d_4, cortisol-d_4 was small (≤ 10%) it is implying that the instrument variability was also negligible.

Table 4.4. The relative standard deviation (RSD) for trytopahn-d_5, kynurenune-d_4 and serotonin-d_4 representing the intra- and inter-day repeatability.

	TRP-d5	**SER-d4**	**KYN-d4**
(Intra-day n = 4) (Inter-day n = 16)			
Low-Intra-10pg-Day 1	13.05	11.60	12.60
Low-Intra-10pg-Day 2	7.46	11.55	12.87
Low-Intra-10pg-Day 3	7.90	17.64	18.94
Low-Inter-10pg-Day 1-3	8.11	5.58	8.06
Mid-Intra-100pg-Day 1	7.16	11.01	8.27
Mid-Intra-100pg-Day 2	6.52	11.64	9.29
Mid-Intra-100pg-Day 3	7.53	16.66	12.69
Mid-Inter-100pg-Day 1-3	9.25	6.97	12.06
Med-Intra-200pg-Day 1	6.32	9.63	7.52
Med-Intra-200pg-Day 2	6.39	10.91	6.76
Med-Intra-200pg-Day 3	6.76	14.35	7.82
Med Inter-200pg-Day 1-3	11.29	12.21	15.22
High-Intra-400pg-Day 1	15.70	15.55	12.72
High-Intra-400pg-Day 2	13.38	14.59	8.14
High-Intra-400pg-Day 3	17.16	13.12	16.79
High Inter-400pg-Day 1-3	10.97	10.02	10.54

4.5 Matrix effects

The presence of co-extracted biogenic material can have an effect on the ionization efficiency of target analytes, i.e., matrix effects. This can result in the suppression or enhancement of the ion signals. It is important, therefore, to determine the extent of matrix effects of our tissue extracts on our target analytes (de Jong *et al.*, 2009; Hu *et al.*, 2017; Tong *et al.*, 2018; Parker *et al.*, 2019). The absolute matrix effect may be quantitatively assessed by comparing the response of the analyte spiked into the extracted blank

matrix (MMES) with the response of the analyte spiked into a matrix-free reconstitution solution, such as pure solvent. This ensures that the analyte of interest faces same conditions with respect to matrix effects and extraction recoveries in the validation samples as well as the experimental samples (Yamada *et al.*, 2008; Wang *et al.*, 2018; Virága *et al.*, 2020).

The MMES were prepared by spiking the labeled IS into liver tissue extracts to give final concentrations of 10, 25, 50, 100, 200, 400, 500, and 700 pg/µL and were injected in triplicate. We then compared the slopes of the calibration curves of each analyte of interest in the MMES to that of the analytes prepared in 100% pure ACN that were also run-in triplicate, to see if there were statistically different. The calibration curves for TRP-d_5, KYN-d_4, and SER-d_4 are provided in Figure 4.5, Figure 4.6, and Figure 4.7, respectively. To determine if there is a statistical difference, the standard error (SE) of the two graphs were used to calculate an x-value using the equation below:

$$x = \sqrt{(SE_1)^2 + (SE_2)^2} \quad \ldots(4)$$

where SE_1 and SE_2 are the standard errors in the slope of the calibration curve for the isotope labeled compounds in the MMES and in the pure acetonitrile, respectively. The x-value along with the absolute slope values were used in the equation below:

$$y = \frac{Slope\ 1 - Slope\ 2}{x} \quad \ldots(5)$$

where Slopes 1 and 2 are the respective slopes of the calibration curve for the labeled compound in the MMES and in the standard solvent. Matrix effects are implied if y-value is greater than z-value 2.326 (at the 99% confidence interval). Calculated y-values were 6.254, 6.682 and 11.790 for TRP-d_5, KYN-d_4, and SER-d_4, respectively. These results are implying that there were statistically differences in the slopes of these compounds in the MMES and in the pure solvent and that matrix effects are not

negligible, as shown in Table 4.5. However, the results of our accuracy study implies that our ISs are effective at mitigating the pernicious effects of the matrix.

Table 4.5. Matrix effect calibration curve equations of tryptophan, kynurenine and serotonin in rainbow trout liver tissue extracts [a].

Analyte	Curve Equation of MMES	Curve Equation of Solvent Standards	% Difference in the Matrix
Tryptophan	y = 49.349x + 914.85	y = 675.26x + 24935	7.30
Kynurenine	y = 16.093x + 583.77	y = 166.86x + 7822.3	9.64
Serotonin	y = 253.54x + 13369	y = 1072.40x + 30358	23.64

[a] Matrix effect is determined by dividing the slopes of a matrix-matched standard calibration curve and a standard curve from analytes prepared in a pure solvent, acetonitrile.

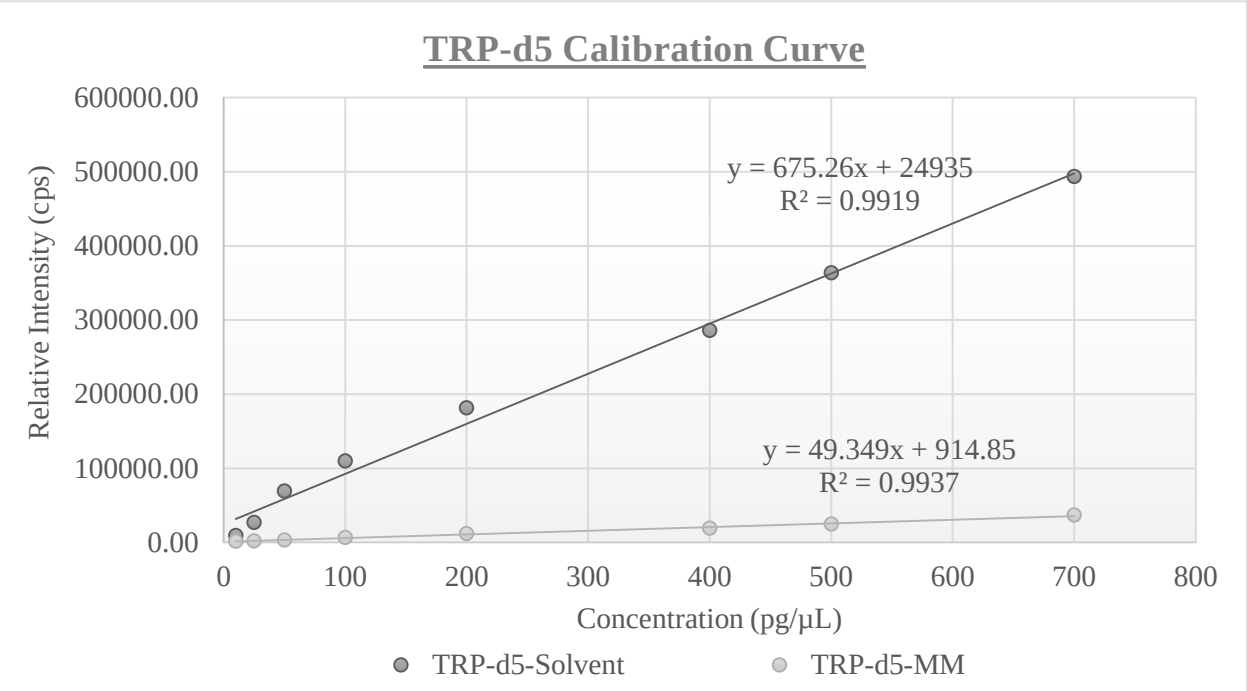

Figure 4.5. Calibration curves of tryptophan-d_5 for matrix matched standards and standards prepared in solvent for matrix effects determinations.

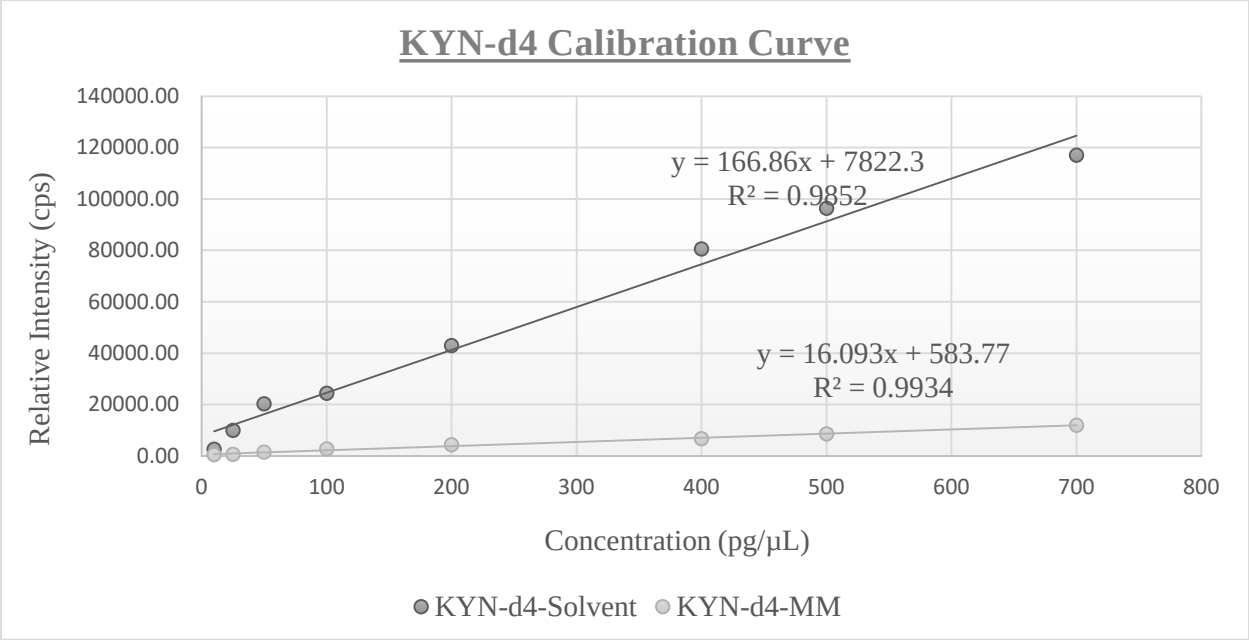

Figure 4.6. Calibration curves of kynurenine-d_4 for matrix matched standards and standards prepared in solvent for matrix effects determinations.

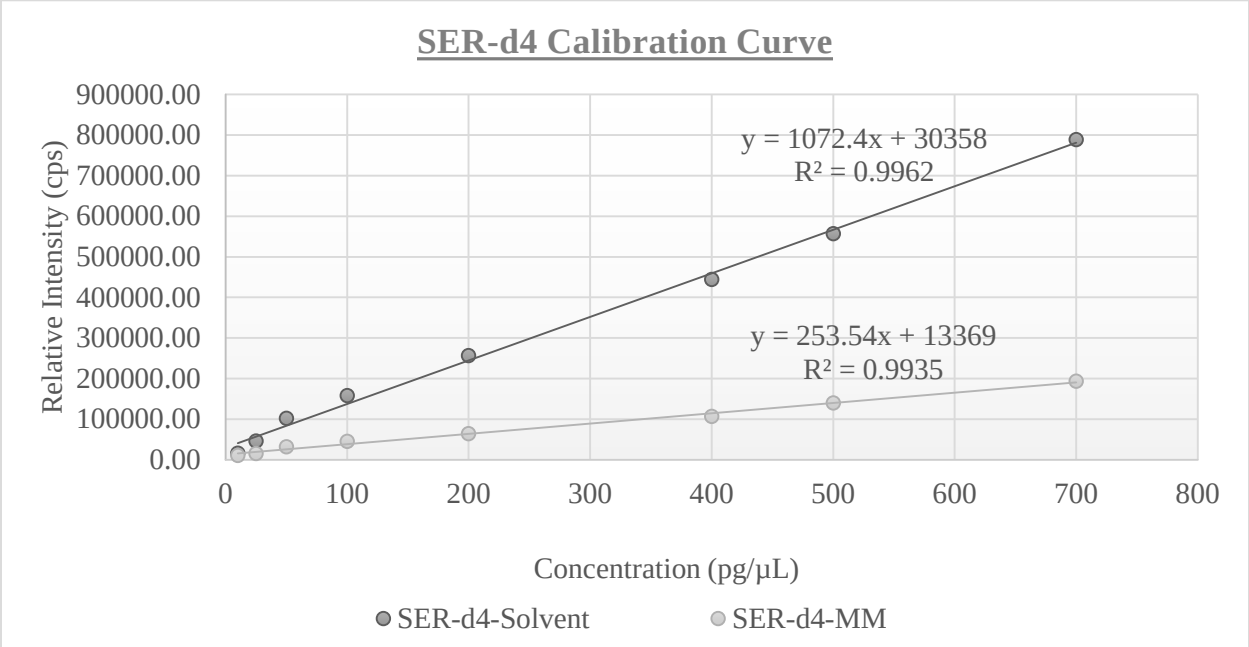

Figure 4.7. Calibration curves of serotonin-d_4 for matrix matched standards and standards prepared in solvent for matrix effects determinations.

4.6 Robustness/Ruggedness

Ruggedness/robustness of the method was assessed based on the method's capacity to remain unaffected by deliberately introducing small variations in method parameters. The test parameters of the method were experimented using two different types of fish liver, to show the method can be applied to most common teleost species found in freshwater. Liver from yellow perch (*Perca flaveceans*) and white crappie (*Pomoxis annularis*) was spiked with TRP-d_5, KYN-d_4, and SER-d_4 resulting in a final concentration of 200 pg/µL. Both the yellow perch and white crappie showed very similar precision and accuracy for the IS recoveries compared to the rainbow trout liver extracts, as shown in Table 4.6. The white crappie showed higher recoveries overall than the yellow perch liver, however, Student t-tests showed there was no statistical differences between the liver extracts of the three fish species, and all IS recoveries were > than 80 %. These results indicated this method is suitable for use in other freshwater species.

Table 4.6. Yellow perch and white crappie internal standard recoveries for trytopahn-d_5, kynurenune-d_4 and serotonin-d_4 in liver tissue extracts at 200 pg/µL.

	Tryptophan-d_5	**Serotonin-d_4**	**Kynurenine-d_4**
	Yellow Perch Liver Tissue		
% IS Recovery:	83.64	69.27	79.84
Precision (Std Dev.)	5.96	6.30	5.56
Accuracy (RSD %)	7.12	9.09	6.96
	White Crappie Liver Tissue		
% IS Recovery:	91.23	66.96	90.75
Precision (Std Dev.)	4.52	9.14	4.42
Accuracy (RSD %)	4.95	13.65	4.87

Our method validation results for the extraction of TRP, KYN, 5-HT and cortisol in fish brain and liver extracts were validated according to the Eurachem guidelines for analytical methods (Magnusson & Örnemark, 2014). Method parameters of selectivity, accuracy, limits of detection (LOD), limits of quantitation (LOQ), linear dynamic range, precision, repeatability, matrix effects and robustness were all assessed to ensure they fell within acceptable Eurachem guidelines values (Magnusson & Örnemark, 2014). Each method characteristics all fell within the necessary boundaries of method validation, thereby establishing the extraction, detection, and quantitation methods as a viable alternative to longer sample preparation methods previously published.

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Chapter 5. Results of acute stress laboratory exposure study

5.0 The Fulton Condition Factor

The Fulton Condition Factor (K) is a measure of the health index of fish and uses the relationship between body weight and length (Steveson & Woods Jr, 2006; Barrilli *et al.,* 2015). The factor is calculated using the Fulton formula, where K = 100 × weight (g) /length^3 (cm). The weight of the fish is divided by the length cubed. The resulting number is multiplied by 100 and a value close to 1 indicates a "healthy" fish. (Steveson & Woods Jr, 2006; Barrilli *et al.,* 2015). The K provides direct information about how the animal takes advantage of the available resources in its surrounding aquatic ecosystem (Barrilli *et al.,* 2015). It is an important tool that demonstrates the changes in the condition of fish over a certain period of time that can be used to indicate the overall nutritional health and status, environmental/pollution changes, reproductive periods, dietary changes, fat accumulation, and parasitic infections (Steveson & Woods Jr, 2006; Araújoa *et al.,* 2018). The K allows researchers to compare teleost populations that are subjected to different environmental conditions, such as changes in water temperature, salinity, dissolved oxygen, pH, as well as many other types of environmental contaminants/pollutants and stressors (Araújoa *et al.,* 2018). As mentioned, K factors depend on the physiological and health status of fish, as well as their overall fatty tissue condition. Fish with higher percent fatty tissue are generally regarded as being healthier than lean body fishes. The K values for the fish in the acute stress exposure study is presented in the Table A1. Rainbow trout generally have K values from 0.8 – 2.0 (Dekić *et al.,* 2016). The experimental results agree with this and range from 0.843-1.341, with the overall condition of the test fish being classified as poor after the stress exposure, since fish with K values below 1.0 are generally regarded as being long and thin (Dekić *et al.,* 2016). On the other hand, the fish sampled at baseline, showed the highest K values overall. Whereas fish with K values from 1.2-1.4 are considered to be in good overall health (Barrilli *et al.,* 2015). Under typical/normal homeostatic conditions, the theoretically value of K

will be at its highest, whereas any change in resources or stressful event that interferes with the health or welfare of the fish can produce variations in K (Barrilli *et al.*, 2015).

The baseline and exposure groups at 4- and 48- post stress exposure showed significant statistical differences in K values, as outlined in Table A2. The 48-hrs control and post exposure stress group also showed significant statistical differences, but the 4-hrs control showed no statistical differences from their respective exposure group. Moreover, the baseline group at 0-hrs and the control groups showed their K values were also statistically different from one another. These results indicated that the overall condition and health of the fish were affected from the acute stress exposure.

5.1 The Hepatosomatic Index

The hepatosomatic index (HSI) is widely known as bioindicator of contaminant/pollution exposure (Sadekarpawar and Parikh, 2013). The liver plays a large role in detoxification and exposure to contaminants can lead to an increase in liver size from hypertrophy (an increase in size) (Dekić *et al.*, 2016), hyperplasia (an increase in number) of hepatocytes or both (Dekić *et al.*, 2016; Araújoa *et al.*, 2018). The liver is also an energy reservoir which plays an important role in the metabolism (Dekić *et al.*, 2016). Studies evaluating fish toxicology from contaminated sites and last disturbed sites often utilize the HSI, which expresses liver size as a percentage of total body weight (Steveson & Woods Jr, 2006; Barrilli *et al.*, 2015). The HSI values for baseline, control and exposure groups chosen for the acute stress experimental groups is outlined in Table A3. Changes in fish liver size and weight are directly related to the oxidative stressors that come from their surrounding aquatic ecosystems (Dekić *et al.*, 2016). The HSI values in the fish sampled for the acute stress exposure study was determined for the baseline, control, and exposure groups, and the respective P-values are summarized in Table A4. The baseline and exposure

groups at 4- and 48-hrs post stress exposure showed significant statistical differences in HSI. Moreover, the baseline group at 0-hrs and the control groups also showed their HSI values were statistically different from one another. These results indicate the health and condition of the fish degraded and the liver size is increased as time continued throughout the exposure and sampling study.

5.2 Cortisol in fish liver and brain extracts

As examples, the total ion chromatograms (TIC) of cortisol (native and mass labeled) in rainbow trout liver sample extracts from our post-stress group at 48-hrs is shown in Figure 5.1. Cortisol was fully baseline resolved and eluded at a retention time (r_t) of 9.40 minutes.

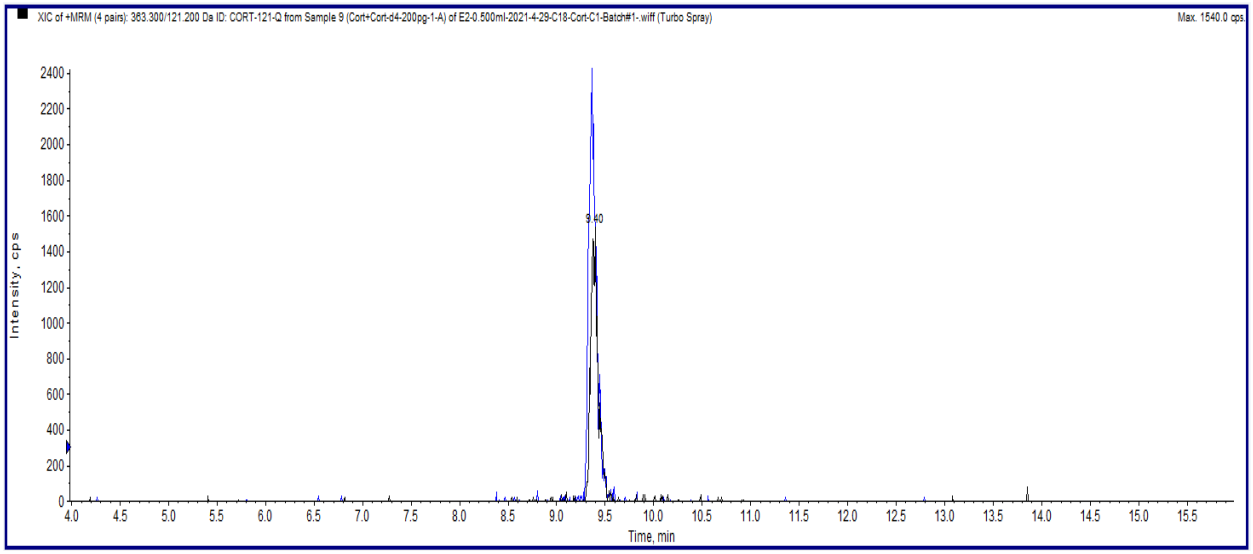

Figure 5.1. TIC chromatogram using an XBD-C18 column of cortisol and cortisol-d_4 (r_t = 9.40 min) in rainbow trout liver extracts stress exposed group sampled at 48-hrs post-stress.

In both the brain and liver fish tissues, cortisol was significantly induced following the acute stress-exposure at both the 4- and 48-hrs time points as shown in Figure 5.2. Interestingly, cortisol concentrations peaked at 4-hrs and then decreased significantly ($p < 0.05$) by approximately 1.5x by the 48-hrs time point in both the liver and brain tissue extracts. The highest overall cortisol concentrations (0.99 ± 0.26 ng/g) were found in the fish brain extracts at 4-hrs post stress. Cortisol concentrations in the liver extracts ranged from 0.05 ± 0.02 ng/g to 0.17 ± 0.03 ng/g and were statistically different ($p < 0.05$) between control and stress-exposure at both time points (Figure 5.2). These results are consistent with other studies in fish which have shown an increase in plasma cortisol concentrations within 0.5–1 hr after a stressful disturbance (Kumar *et al.,* 2014; Lepage *et al.,* 2003; Wu *et al.,* 2015). There were no statistical differences ($p > 0.05$) in the concentrations of cortisol in brain tissue amongst the fish in the baseline group (t=0 hr) and the fish in either of the control groups, indicating the controls did not show a stress-response. The decrease in cortisol induction between 4- and 48-hrs stress exposure groups is consistent with the literature as other studies have shown that plasma cortisol returns to baseline values approximately 4-hrs post stress exposure (Lepage *et al.,* 2003; Cabanillas-Gámez *et al.,* 2017; Javed *et al.,* 2017; Balasch & Tort, 2019; Bulloch *et al.,* 2020). The time difference between the peak plasma cortisol levels and peak tissue concentration is likely due the time needed to distribute and regulate cortisol throughout the rainbow trout tissues, as well as the amount of the available and active cortisol (unbound cortisol) present in the circulating plasma. Furthermore, there may have been some confounding unknown chronic stressors present that affected cortisol concentrations in both 4- and 48-hrs post stress treatment groups since cortisol levels did not return to baseline or control levels even after 48-hrs post-acute stress exposure.

Cortisol induction in plasma is one of the primary stress responses of fish that is intermittently raised to supply adequate energy to meet the immediate stress demands (Lepage *et al.,* 2003; Cabanillas-Gámez *et*

al., 2017; Javed *et al.*, 2017; Balasch & Tort, 2019; Bulloch *et al.*, 2020). As such, levels should regain baseline concentrations 48-hrs post-acute stress exposure. To definitely determine the temporal relationship between cortisol concentrations in plasma versus liver and brain tissue extracts, more sampling times would need to be included in future studies.

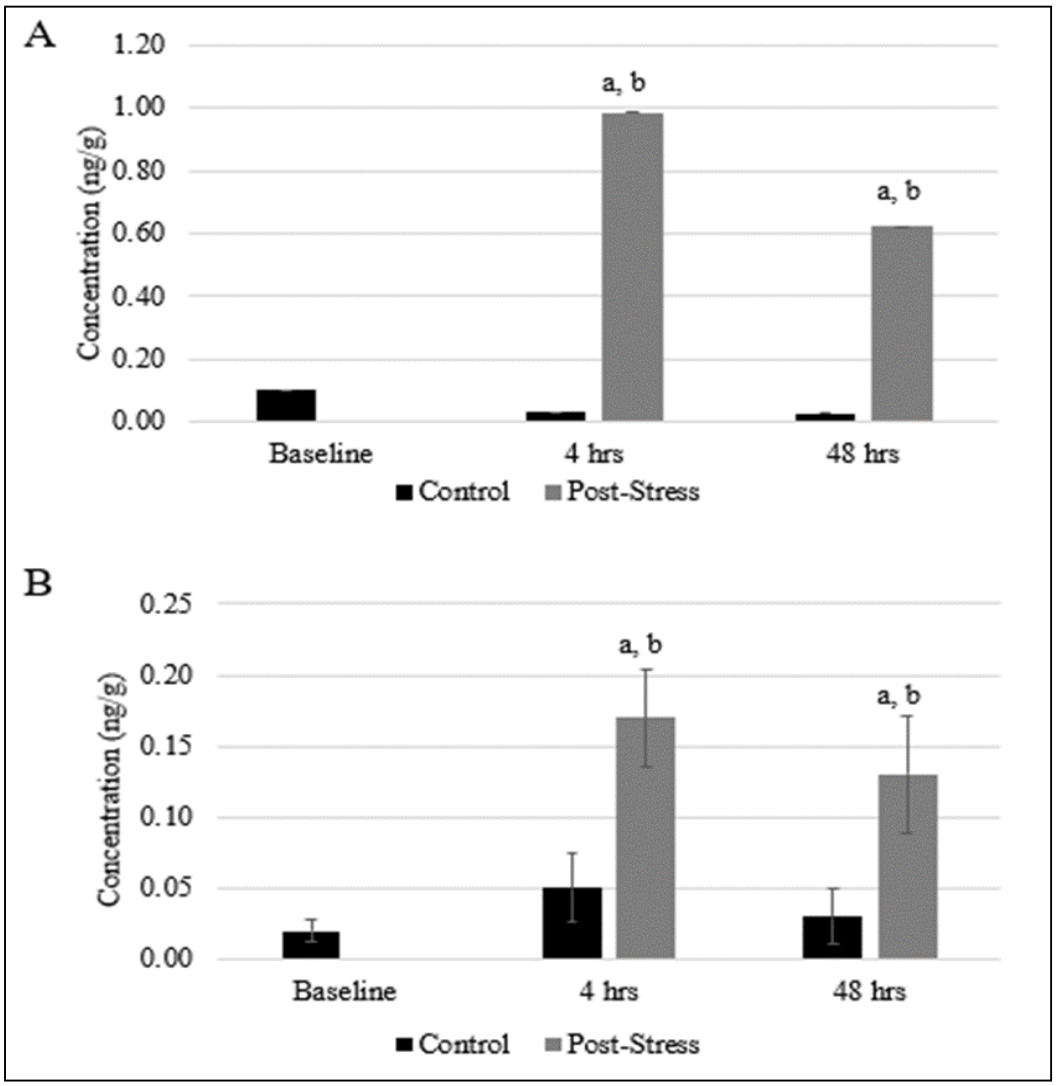

Figure 5.2. Cortisol concentration in rainbow trout (A) brain and (B) liver following exposure to an acute stress event. Values are shown as mean ± SD. Values with letter a or b denote significantly differences versus control and baseline groups (T-test; $p < 0.05$), respectively.

5.3 Tryptophan, kynurenine and serotonin in fish liver and brain

The total ion chromatogram (TIC) of TRP, KYN, and 5-HT (native and mass labeled) separated using the ZIC-HILIC column in rainbow trout liver sample extracts from our 48-hrs post-stress group is shown in Figures 5. 3.

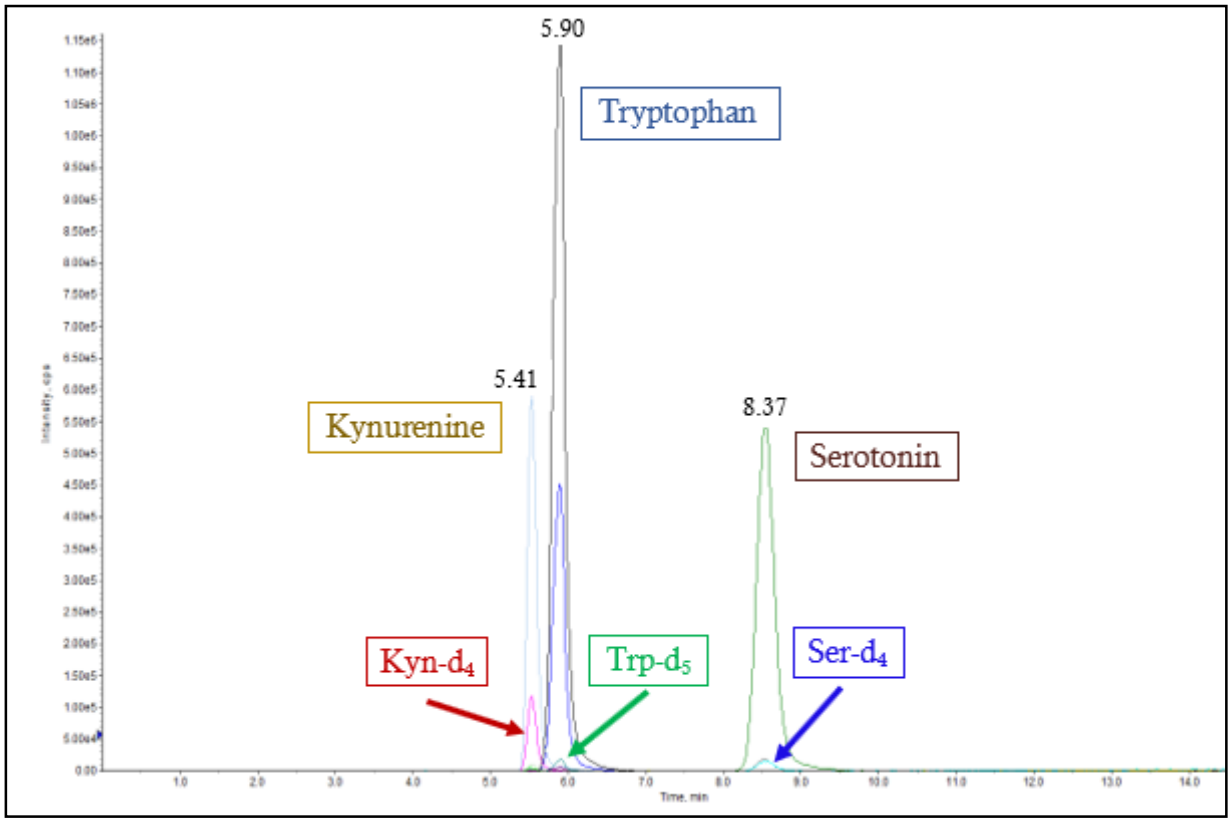

Figure 5.3. TIC chromatogram using an ZIC-HILIC column of TRP, KYN, and 5-HT (r_t -KYN = 5.41 min; r_t -TRP = 5.90 min; and r_t -5-HT = 8.37 mins) in rainbow trout liver extracts stress exposed group 2 sampled at 48-hrs post-stress.

While KYN and TRP are not fully baseline resolved both compounds are easily mass resolvable using their distinct MRM transitions. The mass labeled IS all co-elute with their respective native analogs which are mass resolved due to their unique respective MRM transitions. No interfering peaks were observed in any of our brain or liver extracts.

Concentrations of our TRP, KYN, and 5-HT in each of our experimental groups are shown in Figure 5.4A-F; for the brain and liver tissues, respectively. In the liver extracts, KYN concentrations ranged from 10.60 ± 0.96 ng/g to 22.21 ± 11.78 ng/g, while in the brain, the range was much smaller and from 0.58 ± 0.14 ng/g to 0.80 ± 0.12 ng/g. Although KYN levels in the livers of stress-induced fish at 4-hrs were not significantly different from control fish ($p = 0.579$; Figure 5.4B), we did observe a statistically significant increase in KYN levels in the stress-induced fish livers at the 48-hrs post stress time point ($p = 0.0025$; Figure 5.4B). Similar results were shown in brain tissues of control and stress-exposed fish, while KYN levels at 4-hrs post-stress were near significance ($p = 0.0938$, Figure 5.4A), KYN was significantly increased in the stressed group at 48-hrs post-stress ($p = 0.0014$, Figure 5.4A). This data strongly suggests that there is a time-lag in which elevated levels of KYN are observed after a stressful exposure. This time-lag might represent the amount of time required to upregulate TRP metabolism and KYN accumulation to regulate the demands of the stress event (Hoseini *et al.*, 2019).

TRP concentrations in the liver ranged from 59.85 ± 9.69 ng/g to 70.56 ± 12.77 ng/g, while TRP concentrations in the brain were much lower and ranged from 17.80 ± 5.41 ng/g to 21.34 ± 3.91 ng/g. Comparing between control and exposure groups at each time point, TRP concentrations in liver did not significantly differ between control and stress-exposure at either time point sampled ($p = 0.283$; $p = 0.723$, 4 and 48-hrs respectively, Figure 5.4D). These results are consistent with results from LePage *et al.*, 2003

who did not observe any changes in plasma TRP levels between stressed and non-stressed rainbow trout maintained on a standard TRP enriched diet. Similarly, in the brain, TRP concentrations did not significantly differ between control or stress-exposed fish, at either time point (Figures 5. 4C). Baseline and control TRP concentrations at 48-hrs in both the brain and liver extracts were both not significantly different from one another ($p = 0.152$, Figure 5.4D and $p = 0.153$, Figure 5.4C). In addition, brain and liver TRP concentrations in controls at 48-hrs were smaller compared to those at baseline. This observation was not unexpected as TRP is obtained from the diet and feeding was terminated 24-hrs prior to acute stress exposures. As such, in the 48-hrs post stress group it had been a total of 72-hrs since the last TRP replenishment from their diets.

Serotonin concentrations in the liver ranged from 0.51 ± 0.14 ng/g to 1.46 ± 0.32 ng/g, while 5-HT concentrations in the brain were much higher and ranged from 13.65 ± 4.78 ng/g to 42.34 ± 8.68 ng/g. Comparing between control and exposure groups at each time point, 5-HT concentrations in liver did not significantly differ between control and stress-exposure at the 4-hr sampling time point sampled, however at the 48-hrs sampling time 5-HT levels were significantly decreased ($p = 0.936$; $p = 1.037E^{-06}$, 4- and 48-hrs respectively, Figure 5.4 F). Conversely, in the brain, 5-HT concentrations did significantly differ between control or stress-exposed fish, at the 4-hr sampling time point (Figures 5.4E) and increased back to control levels by the 48-hr sampling time point ($p = 9.575E^{-05}$; $p = 0.346$, 4- and 48-hrs respectively, Figure 5.4E). Baseline and control 5-HT concentrations at 48-hrs in both the brain and liver extracts were both significantly different from one another ($p = 0.027$, Figure 5.4E and $p = 0.0002$, Figure 5.4F). In addition, liver 5-HT concentrations in controls at 4-hrs were significantly smaller compared to those at baseline ($p= 0.027$, Figure 5.4F). Figure 5.4E & F also show the levels of 5-HT decreased with imposed acute stress, illustrating its fundamental and crucial role in stress mediation and immune activation.

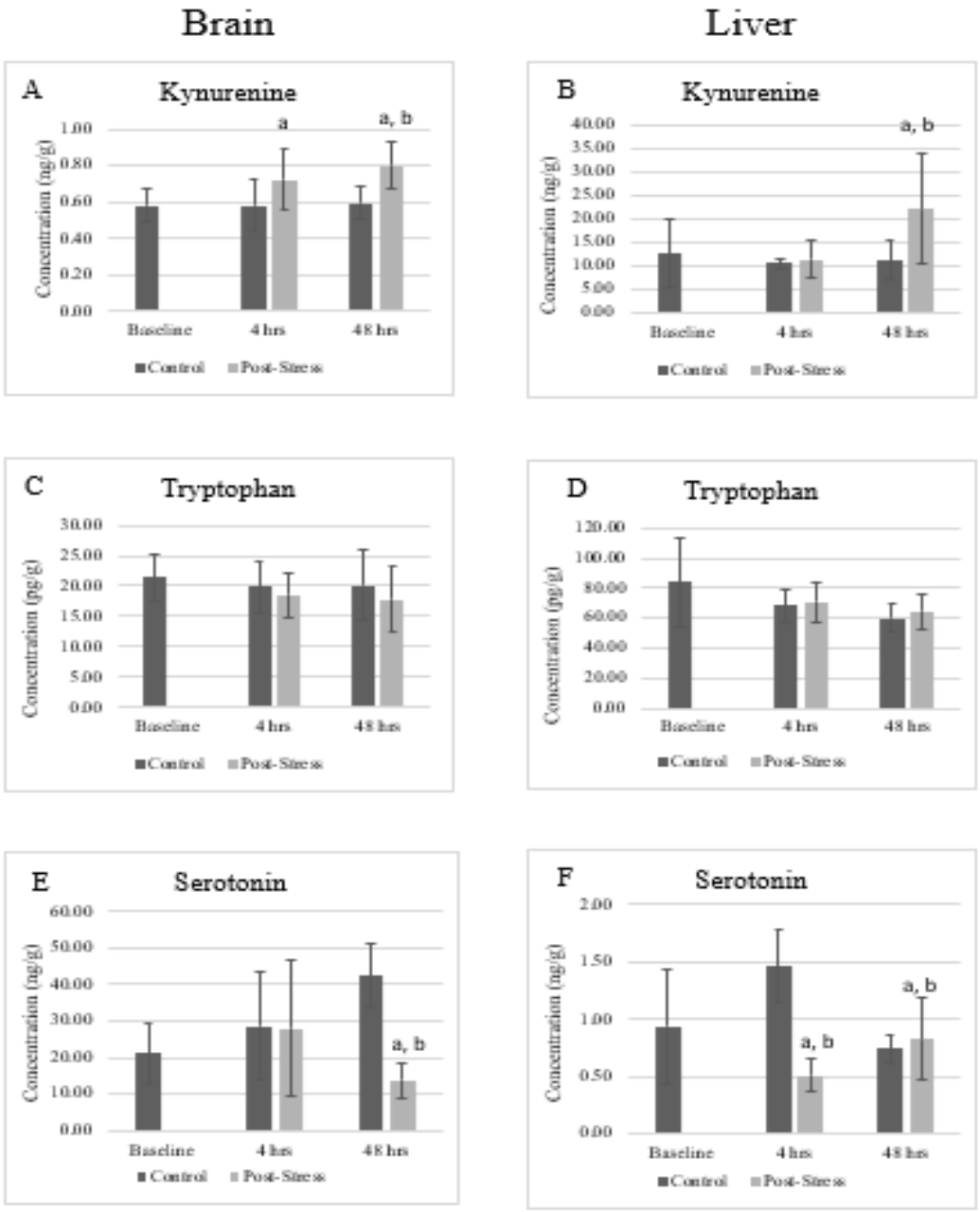

Figure 5.4. Detected concentrations of (A,B) Kynurenine, (C,D) Tryptophan and (E,F) Serotonin in rainbow trout brain and liver tissues following exposure to an acute stress event. Values are shown as mean ± SD. Values with letter a or b denote significantly differences versus control and baseline groups (T-test; $p < 0.05$), respectively**.**

We also chose to examine the temporal change in KYN and TRP levels to see if they can be related to the imposed acute stressful event. We did so by calculating the KTR in liver and brain of fish from each of the stressed experimental groups. The observed increases in KYN levels were paralleled by changes in the KTR (Figure 5.4A, 5.4B, 5.5A, and 5.5B). Although KTR levels in the livers and brains of stress-induced fish at 4-hrs were not significant ($p = 0.723$ and $p = 0.065$, respectively; Figures 5.5A and 5.5B), 48-hrs post-stress there was a significant increase in the KTR in both tissues ($p = 0.0017$, and $p = 0.0013$, respectively; Figures 5.5A and 5.5B). Furthermore, when the stress exposure is compared to the baseline as opposed to the control groups, both types of tissues show increased KTRs at 48-hrs post-stress event ($p = 0.0014$, and $p = 0.0030$, respectively; Figures 5.5A and 5.5B). Moreover, the brain tissue shows a statistical difference in the KTR at 4-hrs post stress when compared to baseline levels ($p = 0.0266$, Figure 5.5A). This suggests that the KTR is reflective of the alteration in KYN levels, and not a depletion in TRP in the fish brain and liver tissues.

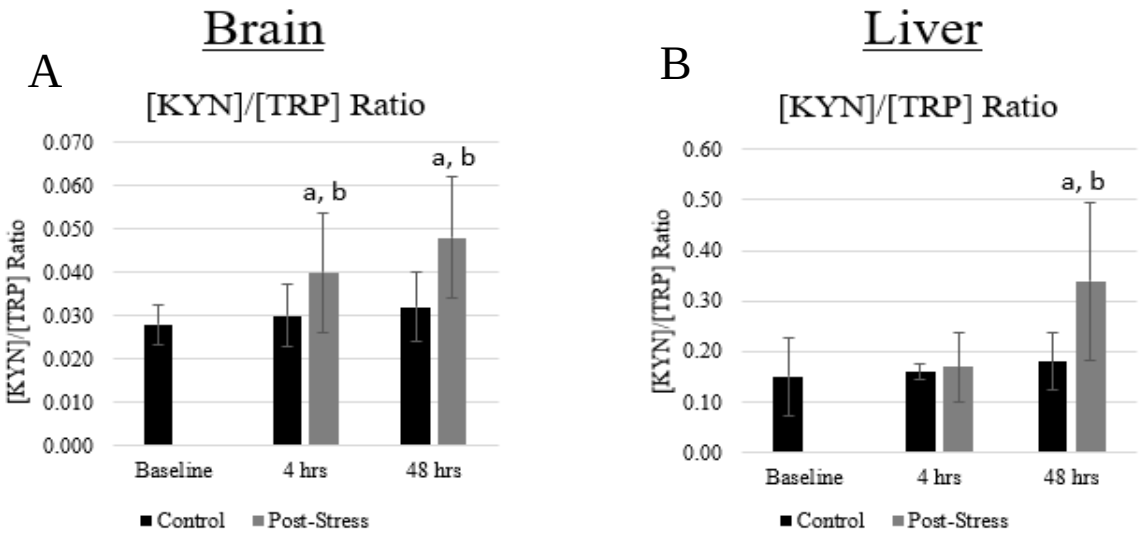

Figure 5.5. Kynurenine-Tryptophan Ratio in rainbow trout brain (A) and liver (B) tissues following exposure to an acute stress event. Values are shown as mean ± SD. Values with letter a or b denote significantly differences versus control and baseline groups (T-test; $p < 0.05$), respectively.

In general, the temporal trends of our analytes in brain tissues were similar to those observed in liver, where 4-hrs post-stress event showed no alterations in TRP, KYN or KTR, but both KYN and KTR showed statistically significant alterations at 48-hrs post-stress. This temporal response may follow the peak-induction of cortisol observed at 4-hrs, confirming the time-lag in the delayed alterations to KYN levels, and thus the delayed changes to the KTR. Additionally, it is important to note the variation in brain and liver tissues, where the alterations in KYN and KTR levels in the brain are more pronounced than those observed in the liver. This variation is also paralleled by the changes in cortisol, with cortisol concentrations in the brain being more pronounced than those observed in the liver. Furthermore, there were no statistical differences between the KTR of fish liver or brain from the baseline group to either of the two control groups, again confirming the control groups were free of stress induced cortisol regulation and KYN metabolism. Taken together, this effect may be attributed to the temporal response that teleosts have to stress, with the induction of neuroendocrine organs releasing the necessary stress remedying hormones as the initial stage, followed by changes in the cardio-metabolic organs, such as the liver (Lepage *et al.*, 2003; Cabanillas-Gámez *et al.*, 2017; Balasch & Tort, 2019).

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Chapter 6. Conclusion and future research directions

My research has shown that rainbow trout exposed to acute stress showed an increase in the [Kyn]/[Trp] ratio (KTR). This evidence supports the KTR can effectively measure IDO induction in the kynurenine pathway as well as confirming the stress response in fish after acute stress exposure. My validated analytical method was able to accurately extract, separate, detect and quantify TRP, KYN, 5-HT, and cortisol concentrations in teleost brain and liver tissue extracts. The acute imposed-stress, a well-established model for cortisol induction, caused an increase in KYN concentrations measurable at 48-hrs post stress event, in both fish liver and brain. In line with this, the KTR was elevated 48-hrs post-stress exposure, combining to provide evidence that KTR can be used as a reliable biomarker of acute stress in fish. However, my exposure study clearly lacks the time resolution necessary to provide more meaningful statements pertaining the time-lag for which changes to the KTR becomes measurable. Further research is needed to ascertain the exact point of the KYN increases, by sampling more time points closer to the acute stress exposure time. Value could also be added by including some of the neurotoxic KYN metabolites, such as 3-hydroxykynurenine and 3-hydroxyanthranilic acid, and the neuro-protective kynurenic acid as well as neopterin due to its direct association with the IDO, KTR and stress exposure and mediation (Badawy, 2017b; Badawy & Guillemin, 2019).

The universal nature of TRP, could also allow the KTR to be used as a biomarker for stress in many different species, depending on the complexity of the organism's metabolism and immune regulation. Furthermore, the KTR biomarker in fish could also be used to assess ecosystem and aquaculture health, since any stressors that causes distress will likely increase the KTR. Optimal TRP requirements for growth and performance, as well as increased immune resistance and stress modulation can also be determined using continuous measurements of the KTR. Since numerous studies have shown that elevated dietary

TRP has suppressive effects on aggressive behavior and post-stress plasma cortisol concentrations, as well as increased immune status, inflammatory response and disease resistance in teleost's, higher vertebrates, and mammals (Mommen *et al.*, 2004; Machado *et al.*, 2019). This biomarker could prove to be invaluable asset in ecotoxicology, environmental risk assessments, and in aquaculture activities. Work is ongoing in our group in this area as well as determining the usefulness of KTR as a biomarker for exposure of fish to aquatic contaminants and other environmental stressors especially in samples that can be collected via non-lethal means e.g., mucus, fins, scales, urine, faeces, and/or surrounding water.

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APPENDIX

Table A1. Fulton Condition Factor Index (K) for salmonid fish (*O. mykiss*) sampled for acute stress exposure study.

Sample Code	Weight of Fish (g)	Length of Fish (mm)	Fulton Condition Index (K)	Overall Quality
Baseline-0hr-1	167.5	232	1.341	Good to Fair
Baseline-0hr-2	191.5	245	1.302	Good to Fair
Baseline-0hr-3	141.0	227	1.205	Fair
Baseline-0hr-4	145.5	217	1.424	Good
Baseline-0hr-5	121.5	222	1.111	Poor
Baseline-0hr-6	173.5	250	1.110	Poor
Baseline-0hr-7	127.5	222	1.165	Fair
Baseline-0hr-8	157.5	247	1.045	Poor
Baseline-0hr-Avg.			**1.213**	
Baseline-0hr-Std Dev.			**0.131**	
Baseline-0hr-CV (RSD)			**10.814**	
C1-4 hrs-3	140.5	241	1.004	Poor
C1-4 hrs-4	173.0	245	1.176	Fair
C1-4 hrs-5	94.5	215	0.951	Poor
C1-4 hrs-6	105.0	225	0.922	Poor
C1-4 hrs-7	154.0	235	1.187	Fair
C1-4 hrs-8	171.5	255	1.034	Poor
C1-4 hrs-9	143.0	249	0.926	Poor
C1-4 hrs-10	182.0	247	1.208	Fair
C1-4 hrs-Avg.			**1.051**	
C1-4 hrs-Std Dev.			**0.122**	
C1-4 hrs-CV (RSD)			**11.572**	
C2-48 hrs-3	207.5	260	1.181	Fair
C2-48 hrs-4	157.5	247	1.045	Poor
C2-48 hrs-5	190.0	250	1.216	Fair
C2-48 hrs-6	196.5	260	1.118	Fair
C2-48 hrs-7	119.0	221	1.102	Fair
C2-48 hrs-8	172.5	247	1.145	Fair

C2-48 hrs-9	172.5	245	1.173	Fair
C2-48 hrs-10	189.0	270	0.960	Poor
C2-48 hrs-Avg.			**1.118**	
C2-48 hrs-Std Dev.			**0.083**	
C2-48hrs-CV (RSD)			**7.400**	
E1-4 hrs-3	138.0	255	0.832	Extremely Poor
E1-4 hrs-4	140.5	245	0.955	Poor
E1--4 hrs-5	189.0	262	1.051	Poor
E1--4 hrs-6	133.5	233	1.055	Poor
E1--4 hrs-7	198.0	261	1.114	Poor
E1--4 hrs-8	173.5	259	0.999	Poor
E1--4 hrs-9	139.0	235	1.071	Poor
E1--4 hrs-10	154.0	248	1.010	Poor
E1-4 hrs-Avg.			**1.011**	
E1-4 hrs-Std Dev.			**0.087**	
E1-4 hrs-CV (RSD)			**8.613**	
E2-48 hrs-3	94.0	213	0.973	Poor
E2-48 hrs-4	126.0	222	1.152	Fair
E2-48 hrs-5	159.0	245	1.081	Poor
E2-48 hrs-6	167.0	250	1.069	Poor
E2-48 hrs-7	159.5	248	1.046	Poor
E2-48 hrs-8	111.5	211	1.187	Fair
E2-48 hrs-9	166.0	258	0.967	Poor
E2-48 hrs-10	146.5	259	0.843	Extremely Poor
E2-48 hrs-Avg.			**1.040**	
E2-48 hrs-Std Dev.			**0.110**	
E2-48hrs-CV (RSD)			**10.619**	

Table A2. Student t-test P-values in control and stress exposure groups for Fulton Condition Index.

Exposure Groups	**4 hrs Post Stress**	**48 hrs Post Stress**
Control	1.051	1.118
Exposure	1.011	1.040
P-Value	0.69445	0.29685

where P-value < 0.05 are statistically different.

Table A3. Hepatosomatic index (HSI) values of *O. mykiss* fish sampled for acute stress exposure study.

Sample Code	Weight of Fish (g)	Weight of Liver (g)	HSI Values
Baseline-0hr-1	167.5	2.573	**1.536**
Baseline-0hr-2	191.5	1.740	**0.909**
Baseline-0hr-3	141.0	1.793	**1.272**
Baseline-0hr-4	145.5	1.983	**1.363**
Baseline-0hr-5	121.5	1.854	**1.526**
Baseline-0hr-6	173.5	1.754	**1.011**
Baseline-0hr-7	127.5	1.367	**1.072**
Baseline-0hr-8	157.5	2.245	**1.425**
Baseline-0hr-Avg.			**1.2642**
Baseline-0hr-Std Dev.			**0.2407**
Baseline-0hr-CV (RSD)			**19.0424**
C1-4 hrs-3	140.5	3.278	**2.333**
C1-4 hrs-4	173.0	2.308	**1.334**
C1-4 hrs-5	94.5	1.482	**1.568**
C1-4 hrs-6	105.0	1.480	**1.410**
C1-4 hrs-7	154.0	1.779	**1.155**
C1-4 hrs-8	171.5	2.148	**1.252**
C1-4 hrs-9	143.0	1.675	**1.171**
C1-4 hrs-10	182.0	3.214	**1.766**
C1-4 hrs-Avg.			**1.4987**
C1-4 hrs-Std Dev.			**0.3952**
C1-4 hrs-CV (RSD)			**26.3691**
C2-48 hrs-3	207.5	2.213	**1.066**
C2-48 hrs-4	157.5	2.153	**1.367**
C2-48 hrs-5	190.0	2.947	**1.551**
C2-48 hrs-6	196.5	4.134	**2.104**
C2-48 hrs-7	119.0	1.976	**1.661**
C2-48 hrs-8	172.5	3.488	**2.022**
C2-48 hrs-9	172.5	2.902	**1.682**
C2-48 hrs-10	189.0	2.749	**1.454**
C2-48 hrs-Avg.			**1.6134**
C2-48 hrs-Std Dev.			**0.3388**
C2-48 hrs-CV (RSD)			**21.0002**

E1-4 hrs-3	138.0	2.578	**1.868**
E1-4 hrs-4	140.5	2.536	**1.805**
E1-4 hrs-5	189.0	2.701	**1.429**
E1-4 hrs-6	133.5	1.774	**1.329**
E1-4 hrs-7	198.0	2.011	**1.016**
E1-4 hrs-8	173.5	2.106	**1.214**
E1-4 hrs-9	139.0	2.323	**1.671**
E1-4 hrs-10	154.0	2.349	**1.525**
E1-4 hrs-Avg.			**1.4821**
E1-4 hrs-Std Dev.			**0.2944**
E1-4 hrs-CV (RSD)			**19.8638**
E2-48 hrs-3	94.0	1.803	**1.918**
E2-48 hrs-4	126.0	2.789	**2.213**
E2-48 hrs-5	159.0	2.960	**1.862**
E2-48 hrs-6	167.0	2.519	**1.508**
E2-48 hrs-7	159.5	2.455	**1.539**
E2-48 hrs-8	111.5	1.673	**1.500**
E2-48 hrs-9	166.0	2.678	**1.613**
E2-48 hrs-10	146.5	2.315	**1.580**
E2-48 hrs-Avg.			**1.7168**
E2-48 hrs-Std Dev.			**0.2562**
E2-48 hrs-CV (RSD)			**14.9212**

Table A4. Student t-test P-values for the stress exposure and control groups for Hepatosomatic index.

Experimental Groups	**4 hrs Post Stress**	**48 hrs Post Stress**
Control	1.4987	1.6134
Exposure	1.4821	1.7168
P-Value	0.72592	0.98097

where P-value < 0.05 are statistically different.