Analysis of resolvins in fish cultured cells challenged with docosahexaenoic acid by liquid chromatography triple quadrupole mass spectrometry.

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Analysis of resolvins in fish cultured cells challenged with docosahexaenoic acid by liquid chromatography triple quadrupole mass spectrometry.

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List of Publications

- Araujo, P.; Iqbal, S.; Arnø, A.; Espe, M.; Holen, E. Validation of a Liquid–Liquid Extraction Method to Study the Temporal Production of D-Series Resolvins by Head Kidney Cells from Atlantic Salmon (Salmon salar) Exposed to Docosahexaenoic Acid. Molecules 2023, 28, 4728. https://doi.org/10.3390/molecules28124728

- Araujo, P.; Iqbal, S.; Arnø, A.; Espe, M.; Holen, E. Establishing appropriate levels of internal standards in quantitative targeted metabolomics research: profiling lipid mediators. 3rd Nordic Metabolomics Conference, October 2023, Trondheim, Norway. (Accepted)

Abbreviations

List of Abbreviations	
Arachidonic acid	ARA
Aspirin triggered lipoxin	ATL
Aspirin triggered resolvin	AT-Rv
Atmospheric pressure chemical ionization	APCI
Atomic absorption spectrometry	AAS
Chemokine-like receptor 1	CMKLR1
Coefficient of variation	CV
Cyclooxygenases	COX
Dihomo-γ-linolenic acid	DGLA
Docosahexaenoic acid	DHA
Docosapentaenoic acid	DPA
D-resolvin receptor	DRV
Eicosapentaenoic acid	EPA
Eicosatetraenoic acid	ETA
Electrospray ionization	ESI
Elongase of very-long fatty acid	ELOVL
Enzyme immunoassay	EIA
Formyl peptide receptor	FPR
Gas chromatography	GC
G-protein coupled receptors	GPCRs
High performance liquid chromatography	HPLC
Limit of detection	LOD
Limit of quantitation	LOQ
Linoleic acid	LA
Lipooxygenases	LOX
Liquid liquid extraction	LLE
Maresin 1	MaR1
Mass spectrometry	MS
Monounsaturated fatty acids	MUFAs
Polyunsaturated fatty acids	PUFAs
Principal component	PC
Protein precipitation	PP
Quadrupole linear ion trap	QLIT
Quadrupole time-of-flight	QToF
Resolvin D1	RvD1
Resolvin D2	RvD2
Resolvin D3	RvD3
Resolvin D4	RvD4
Resolvin D5	RvD5
Resolvin D6	RvD6
Response factor	RF

Solid phase extraction	SPE
Specialized pro-resolving mediators	SPM
Standard deviation	SD
Stearidonic acid	SDA
Thromboxane	TX
α-Linolenic acid	ALA
γ-linolenic acid	GLA

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Abstract

Resolvins are considered to be the active metabolites of ω-3 PUFA that aid in the resolution phase of acute inflammation. The extraction of D-series resolvins (RvD1, RvD2, RvD3, RvD4, and RvD5) released into Leibovitz's L-15 complete medium by head kidney cells from Atlantic salmon is proposed along with a method for the quick and easy measurement of liquid chromatography triple quadrupole mass spectrometry. To determine the best internal standard concentrations for evaluating performance parameters like linear range (0.1-50 ng mL-1), limits of detection and quantification (0.05 and 0.1 ng mL-1, respectively), and recovery values ranging from 96.9 to 99.8%, a three-level factorial design was proposed. The behavior of the relationship between analyte and internal standard of resolvins RvD1/RvD1-d_5, RvD2/RvD3-d_5, RvD3/RvD3-d_5, RvD4/RvD1-d_5 and RvD5/RvD1-d_5 were studied using validated mathematical models generated from the experimental points. The developed method was highly selective and gave reproducible recoveries. When head kidney cells exposed to docosahexaenoic acid were tested using the optimal approach, the results suggested that the production of resolvins may have been increased by circadian rhythms.

1. Introduction

1.1 Inflammation and Fatty Acids

Inflammation is a protective response against injury or infection. It promotes wound healing and tissue repair, whereas in cases of infections it builds memory to counter future invasions[1]. The chemical gradients required to produce signals to initiate inflammatory responses range from amine and peptides to lipid mediators[2]. These chemicals play an active role in onset, amplification, and resolution of inflammation. Each stage represents an opportunity to harness the inflammatory disease[1]. Uncontrolled acute inflammation leads to chronic prolongation of the condition resulting in scarring, loss of tissue function or repetitive forays by pathogens. Therefore, complete resolution of inflammation is the panacea of the ailment[3]. Resolution, once believed to be only a passive process, is now considered to be a highly choreographed process regulated by an intricate regulatory network of cells and mediators. Lipid mediators produced by the metabolism of necessary omega-3 unsaturated fatty acids have garnered the most interest among the mediators that regulate the resolution process[4]. Lipid mediators derived from fatty acids have been studied extensively to understand their role in alleviation of pain and swelling[3]. The cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P-450 mono-oxygenase metabolic system modulates inflammation by converting omega-6 (ω–6) fatty acids such as linoleic acid and arachidonic acid and omega-3 (ω–3) fatty acids such as alpha linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), into bioactive lipids[5]. Resolvins, maresins, protectins and lipoxins are hydroxylated fatty acids derived from EPA and DHA that are generally characterized for having inflammation resolving properties and are collectively known as "specialized pro-resolving mediators" (SPMs)[2].

1.2. Specialized Pro-resolving Mediators

Specialized Pro-resolving Mediators (SPMs) are produced during phagocytosis and foreign particle destruction such as bacteria. The fact that the normal inflammatory response tends to "resolve" over time following the removal of the initial debris, paving the way for healing and restoration, is a finding that has long perplexed doctors and researchers alike. Resolvins, protectins, maresins, and lipoxins are the four families of SPMs that play a key role in this self-regulation of

inflammation. The first three of these mediators are primarily in charge of preventing an endless extension of local immune responses since they are biochemically formed from long-chain omega-3 polyunsaturated fatty acids (ω-3 PUFAs)[6].

There are numerous reports linking a variety of human disorders to imbalances between specific proresolving and proinflammatory mediators that hinder resolution. Therefore, it is now well known that administering lipoxins, protectins, resolvins, and maresins in animal models (in vivo and in vitro) can aid the process of recovery from inflammation without compromising host defenses by causing immune suppression[7]. Additionally, there is a belief that promoting resolution rather than inhibiting inflammation may be a way forward in treating infections[8]. SPMs have reportedly been tested in a variety of experimental models, including those for peritonitis, periodontitis, colitis, arthritis, psoriasis, dry eyes, inflammatory pain, cardiovascular disease[9] (including atherosclerosis), depression, neurodegenerative disorders, and asthma and other lung conditions. It appears that SPMs have a significant potential for therapeutic intervention in acute inflammation or chronic inflammatory diseases. Age, gender, and ethnicity all have an impact on these outcomes[10].

SPMs show potential for neuroprotection by modifying the expression of pro-inflammatory genes and aiding in the resolution of neuroinflammation, and there is evidence that they can pass the blood-brain barrier[11]. Particularly, RVD1 and its epimer (one of two stereoisomers that differ in configuration at only one stereocenter) aspirin-triggered RvD1 (AT-RvD1), have been shown to have positive effects on a variety of neurological conditions, including experimental models of traumatic brain injuries, pain, depression, Alzheimer's disease, and Parkinson's disease, at nanomolar and micromolar concentrations. According to studies, SPMs reduce inflammatory biomarkers and lessen microglial activation in Parkinson's disease[12].

Each SPM is believed to interact with its own G-protein coupled receptors, some of which have yet to be found. These receptors provide fast intracellular signaling and long-term effects by controlling the expression of particular genes implicated in the reduction of inflammation. Each receptor can connect with more than one SPM depending on the cellular situation, therefore RvD1 interacts with DRV1 for homeostatic purposes and with ALX/FPR2 for anti-neutrophil activities in reducing inflammation. It is notable that SPMs can compete with each other to block pro-

inflammatory receptors.For instance, it has been demonstrated that resolvins (e.g., RvE1) and maresins (e.g., MaR1, and 22-OH-MaR1) antagonize with the pro-inflammatory leukotriene B4 receptor BLT1[13].

1.3. Fatty Acids

Fatty acids belong to the carboxylic acid family of organic acids. They contain an aliphatic chain consisting of ethylene (–CH2–CH2–), ethenyl (–CH=CH–) groups and one or more carboxyl groups (—C(=O) OH, —COOH, or —CO_2H) (Figure 1)[14].

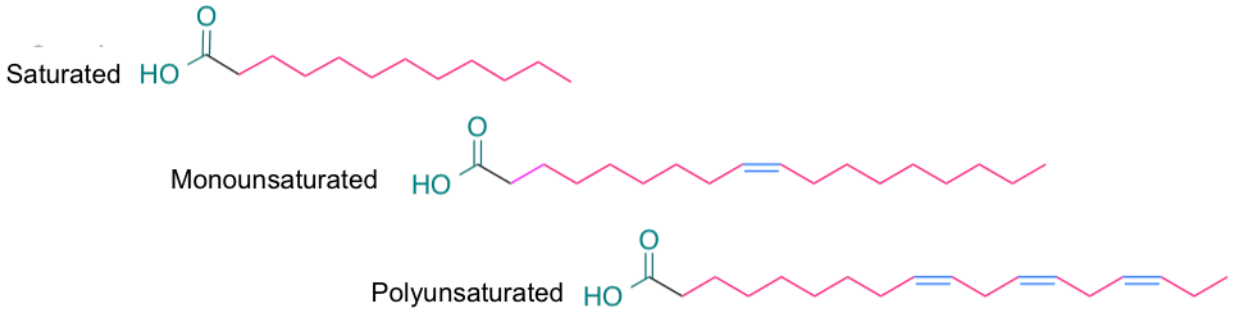

Figure 1 Types of fatty acids based on saturation.

Dietary fats, containing triglycerides and phospholipids, are major precursor to obtain fatty acids largely from vegetable and animal sources (e.g., oils, animals' products, fish)[15]. Naturally occurring fatty acids are usually categorized into short chain, middle chain and long chain fatty acids on the basis of unbranched chain of an even number of carbon atoms i.e., 4-28 carbons. However, there are few exceptions such as isopalmitic acid is branched, whereas as heptadecanoic acid has odd number of carbon atoms[16]. The fatty acids in which carbon chain is linked to a single bond are called saturated fatty acids and those linked to a double bond are termed as unsaturated fatty acids. Olefinic fatty acids with a single carbon-carbon double bond is called monounsaturated fatty acids (MUFA), commonly containing a chain of 16-22 carbons with a cis configured double bond. When a molecule has restricted rotation and each double-bonded carbon atom has two nonidentical groups, cis and trans isomers. Cis configuration has functional groups positioned on the same side of the double bond, whereas trans has on the opposite sides. Industrial processing of unsaturated oils often leads to the formation of trans isomers of MUFA which are more

thermodynamically stable with high melting points than cis fatty acids [14,16]. Polyunsaturated fatty acids (PUFAs) contain alternate methyl groups between double bonds in cis configuration and are mainly polyolefinic, a methylene group (—CH_2—) often separate the cis double bonds from each other such as linoleic acid (Figure 2).

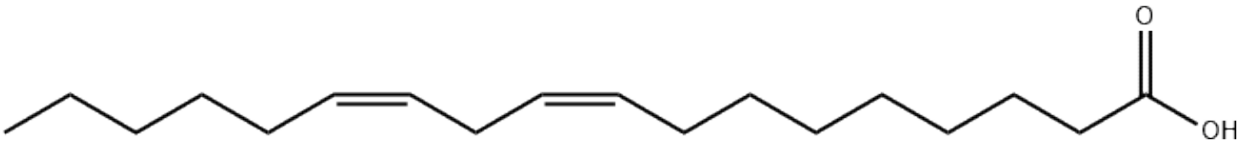

Figure 2 Chemical Structure of linoleic acid

Nearly all naturally occurring unsaturated fatty acids have double bonds that are in the cis configuration and are often found three, six, or nine carbon atoms away from the terminal methyl group. In a cis arrangement (Figure 3), the hydrogen atoms attached to the double bonds are all on the same side. When the hydrogen atoms lie on opposite sides, the arrangement is referred to as trans.

Almost all naturally occurring PUFAs have double bonds that are structured in a methylene-interrupted pattern, with each double bond being split by a single methylene group (CH_2). Starting with the four-carbon fatty acids found in dairy, there is a wide variety of chain lengths with up to 30 carbon long marine lipids [17–19].

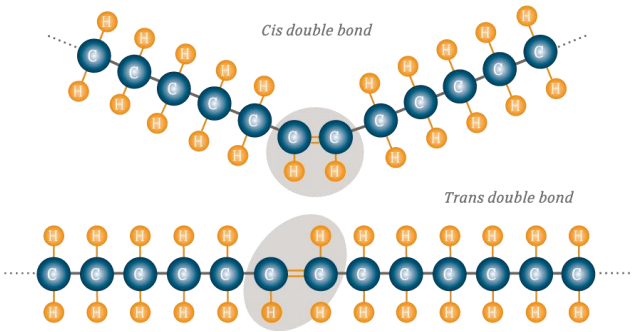

Figure 3 Cis and trans fatty acids[19]

1.3.1 Polyunstaurated Fatty Acids

Cellular membranes are highly dependent on PUFAs for absorptivity, plasticity, and suppleness. Numerous functions in plant and animals at varied levels are governed by PUFAs such as active transport, pathogen defense, development of chloroplast, neurogenesis, signaling functions, synaptic activity, production of pollen and enzymatic activities associated with membrane functions [20, 21]. The location of double bond from position ω-1 to ω-12 plays a crucial role in differentiating PUFA into 12 different families (as shown in Figure 4)[16].

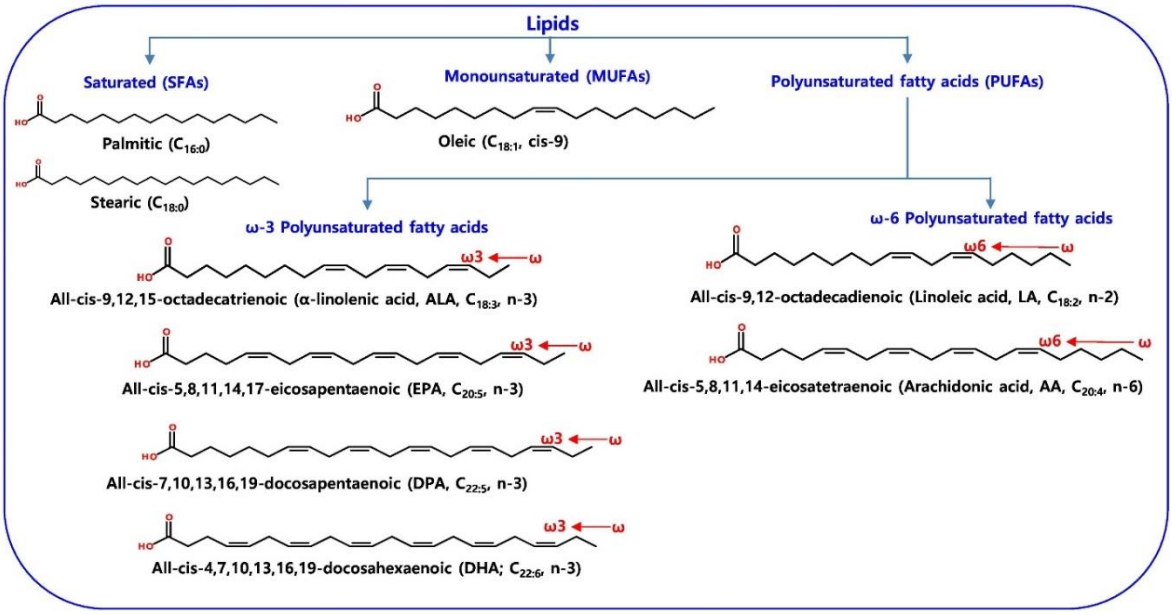

Figure 4 Chemical structures of major Fatty Acids found in animals and plants.[18]

Ralph T. Holman[22] proposed the omega nomenclature, in which the Greek letter omega (ω) represents the terminal methyl group of fatty acids which is farthest from the α-carbon. It denotes the change in direction of counting as the length of structure is indicated by the number that comes after. When a terminal structure has a 6-carbon saturation, the double bond that is closest to the terminal methyl group is referred to as the omega 6 (ω-6) position. Omega 3 (ω-3) refers to a

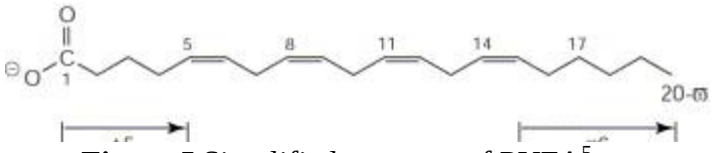

Figure 5 Simplified structure of PUFA[5]

terminal structure that has three saturated carbons. These terminal structures (Figure 5) have an impact on the physical properties of the membranes where they reside [22]. Omega 3 and 6 PUFA are considered essential fatty acids, whereas the non-essential fatty acids belong to omega-9 family which could be either mono-unsaturated or polyunsaturated (Figure 6) [23].

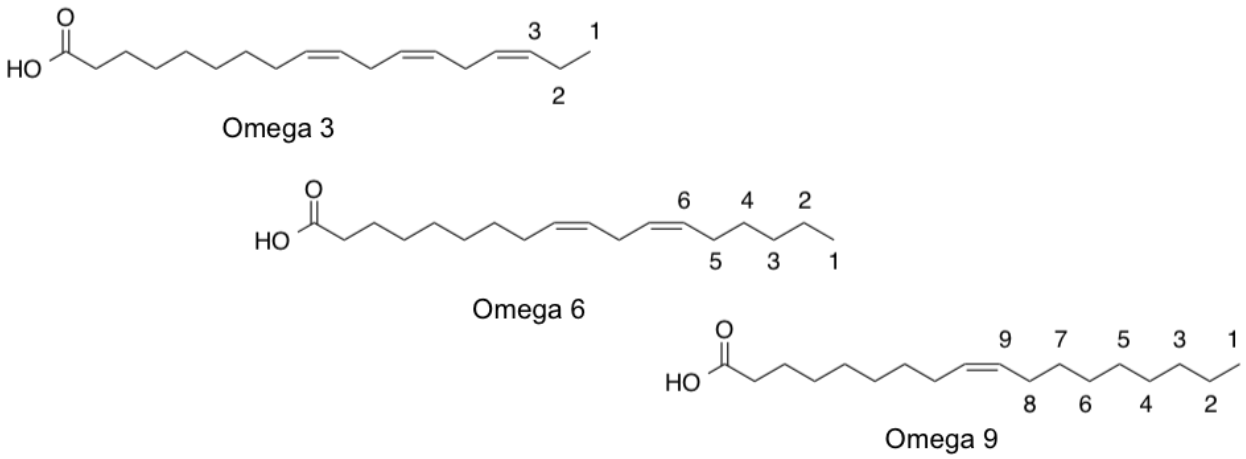

Figure 6 Chemical configuration of omega 3, 6 and 9 Fatty Acids[5]

1.3.1.1 Omega-3 and Omega-6 Fatty Acids

Omega-3 fatty acids are not synthesized in the human body and are obtained through diet. Plant sources like nuts and vegetable oils contain a high concentration of parent omega-3 fatty acid known as alpha-linoleic acid (ALA), which has 18 carbon molecules and three double bonds (C18:3 n-3). Marine organisms like fish produce long chain omega-3 fatty acids that have at least 20 to 22 carbon atoms and five or more double bonds known as eicosapentaenoic (20:5ω-3, EPA) and docosahexenoic (22:6ω-3, DHA) acids, respectively [17, 24, 25].

Omega-6 fatty acids are also essential for human health and can be found in a variety of foods such as vegetable oils, nuts, and seeds. Omega-6 fatty acids have also been linked to a number of health benefits, including reducing inflammation and the risk of heart disease[26]. Linoleic acid (LA), containing 18 Carbon atoms and 2 double bonds, is the precursor of omega-6 fatty acids. It is important to note that while both ω-3 and ω-6 fatty acids are important for human health, maintaining a balance between these two types of PUFAs is crucial. Excessive intake of ω-6 fatty acids can lead to inflammation, while inadequate intake of ω-3 fatty acids can lead to a deficiency

in these essential fatty acids leading to poor wound healing, increase susceptibility to infections and growth restriction in children[27]. In Western diet, there is a large imbalance between ω-6 and ω-3, with an excess of ω-6 because it occurs in almost every dietary fat most commonly in vegetable oils [17]. Therefore, it is recommended to increase the intake of ω-3 rich foods, such as fatty fish and flaxseed, and decrease the intake of ω-6 rich food, such as vegetable oil.

1.3.1.1.1. Biosynthesis

Biosynthesis of PUFA (Figure 7) occurs in endoplasmic reticulum of the liver cells [18], however due to competition between the substrates and product inhibition, an abundance of LA may hinder the metabolism of ALA [28]. Desaturation is a crucial step in conversion of LA and ALA to pro- and anti-inflammatory metabolites. Fatty acid desaturase 2 encoded by the FADS2 gene is associated with desaturase enzyme. FADS1 and FADS2 appear to be responsible for all ω-3 and ω-6 PUFA desaturation in mammals, with FADS1 having 5-desaturase activity and FADS2 initially classified as a 6-desaturase but later revealed to also have 4- and 8-desaturase activities [29]. LA is converted to γ-linolenic acid (GLA, C18:3n-6) and ALA is converted to stearidonic acid (SDA, C18:4n-3) by the action of the delta 6 desaturase (Δ6-desaturase) [18]. After desaturation, the elongase enzyme encoded by *ELOVL5 gene* (elongase of very-long fatty acid 5) catalyzes carbon chain extension of SDA into eicosatetraenoic acid (ETA, 20:4n-3), which on further desaturation by Δ5-desaturase is converted to eicosapentaenoic acid (EPA, 20:5n-3) [20]. Docosapentaenoic acid (DPA, 22:5n-3) is synthesized from EPA by elongation, and the addition of a double bond by the Δ4-desaturase leads to the formation of DHA. EPA acts as a precursor in the production of prostaglandin 3 series (PG3), thromboxane (TX), leukotrienes5(LTs-5). Conversion of EPA to DHA has been documented as irreversible process upon supplementation of dietary EPA[30]. GLA is converted to dihomo-γ-linolenic acid (DGLA, C20:ωn-6) by the action of elongase *ELOVL5*. DGLA undergoes desaturation by Δ5-desaturase to synthesize arachidonic Acid (ARA, 20:4n-6)[29]. DGLA is a precursor to series 1 PGs, whereas ARA aids in the synthesis of PGs 2, thromboxane (TX), leukotrienes 4 (LTs- 4) and anti-inflammatory lipoxins. EPA is a major anti-inflammatory and antiaggregatory agent. As previously stated, ARA is a precursor to a variety of proinflammatory and pro-aggregatory mediators, and EPA competes with ARA for the major COX and LOX

enzymes, resulting in less proinflammatory products. As a result, the EPA:ARA ratio may be a measure of chronic inflammation, with a lower ratio indicating higher levels of inflammation [31].

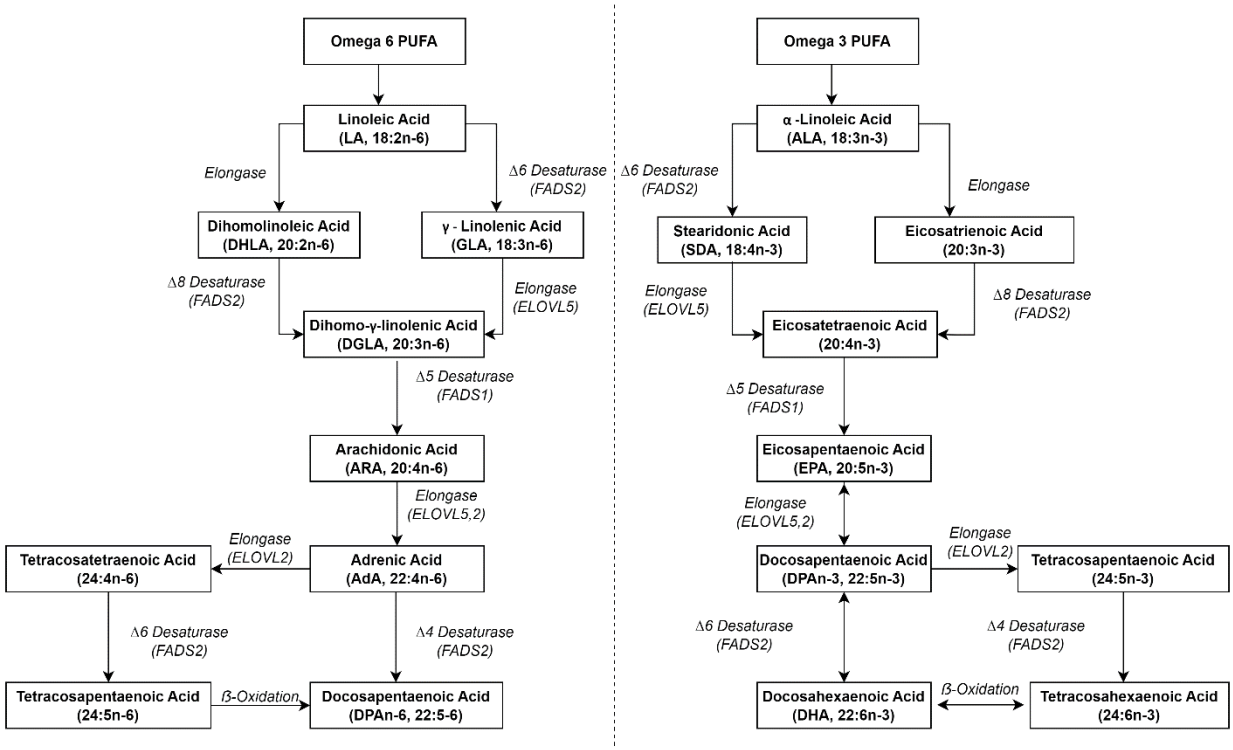

Figure 7 Omega-3 and omega-6 PUFA biosynthesis. Longer-chain omega-3 and omega-6 PUFAs are synthesized through a sequence of alternating position-specific desaturation and elongation processes from ALA and LA, respectively. FADS1 and FADS2 appear to be responsible for all omega-3 and omega-6 PUFA desaturation in mammals, with FADS1 having 5-desaturase activity and FADS2 initially classified as a 6-desaturase but later revealed to also have 4- and 8-desaturase activities. Octadecanoids are lipid mediators generated from C18 PUFAs like ALA or LA, eicosanoids from C20 PUFAs like DGLA, ARA, or EPA, and docosanoids from C22 PUFAs like DPAn-3 and DHA[29]. Further information can be found in the text.

1.4 Resolvins

Alternative therapeutic strategies that concentrate on treating acute inflammation and preventing the development of chronic inflammation have emerged as a result of the awareness of the proactive character of inflammation resolution. Several endogenous lipid mediators have been found to work in this way, suggesting a lipid mediator class switching from the proinflammatory lipid mediators leukotriene and prostaglandins, which were initially active, to the anti-inflammatory and pro-resolving activities of lipoxins, resolvins, protectins, and maresins [32]. In 2002, the Serhan laboratory at Harvard Medical School discovered resolvins while exploring the potential endogenous bioactive molecules generated from ω-3 fatty acids [33]. This new family of bioactive chemicals has been given the name "resolvins" (resolution phase interaction products) because they have potent (at submicromolar-nanomolar levels) anti-inflammatory, immunoregulatory, neuroprotective, and pro-resolving properties[34].

Resolvins are classified into several subclasses, including resolvin E and resolvin D on the basis of their derivatization from EPA or DHA, respectively. The chemical structure of resolvins includes a unique combination of EPA or DHA in various configurations [35]. It is worth noting that both E-series resolvins (such as RvE1 and RvE2), which are bioactive oxygenated lipid products of EPA, and D-series resolvins (such as RvD1, RvD2, RvD3, and RvD5), which are DHA derivatives, exert their proresolving action via transmembrane G-protein-coupled receptors (GPCRs). The four resolvin receptors that have been found thus far are ALX/FPR2, D-resolvin receptor 1 (DRV1)/GPR32, D-resolvin receptor 2 (DRV2)/GPR18, and chemokine-like receptor 1 (CMKLR1), also known as ChemR23 or ERV1. Similar to the of aspirin triggered lipoxins (ATL), there are aspirin-triggered versions of resolvins as well (AT-Rv) [36,9]. Aspirin-acetylated COX2 first converts DHA to 17(R)-HpDHA, which can then be used as a substrate for 5-LOX-mediated transformation to epimeric resolvins, thereby generating the AT-resolvins [37]. Resolvins derived from DHA have shown promising therapeutic benefits in reducing cell injury, inhibiting oxidative stress, and suppressing tumor growth[35].

1.4.1 Resolvin D

DHA upon lipo-oxygenation by 15-LOX is converted to 17S-hydro(peroxy)-DHA (17S-H(p)DHA) majorly in human blood, leucocytes and glial cells[38]. This intermediary compound undergoes multiple de-oxygenation and reduction process through 5-LOX and peroxidase enzymes to produce different types of resolvin D, as shown in Figure 8.

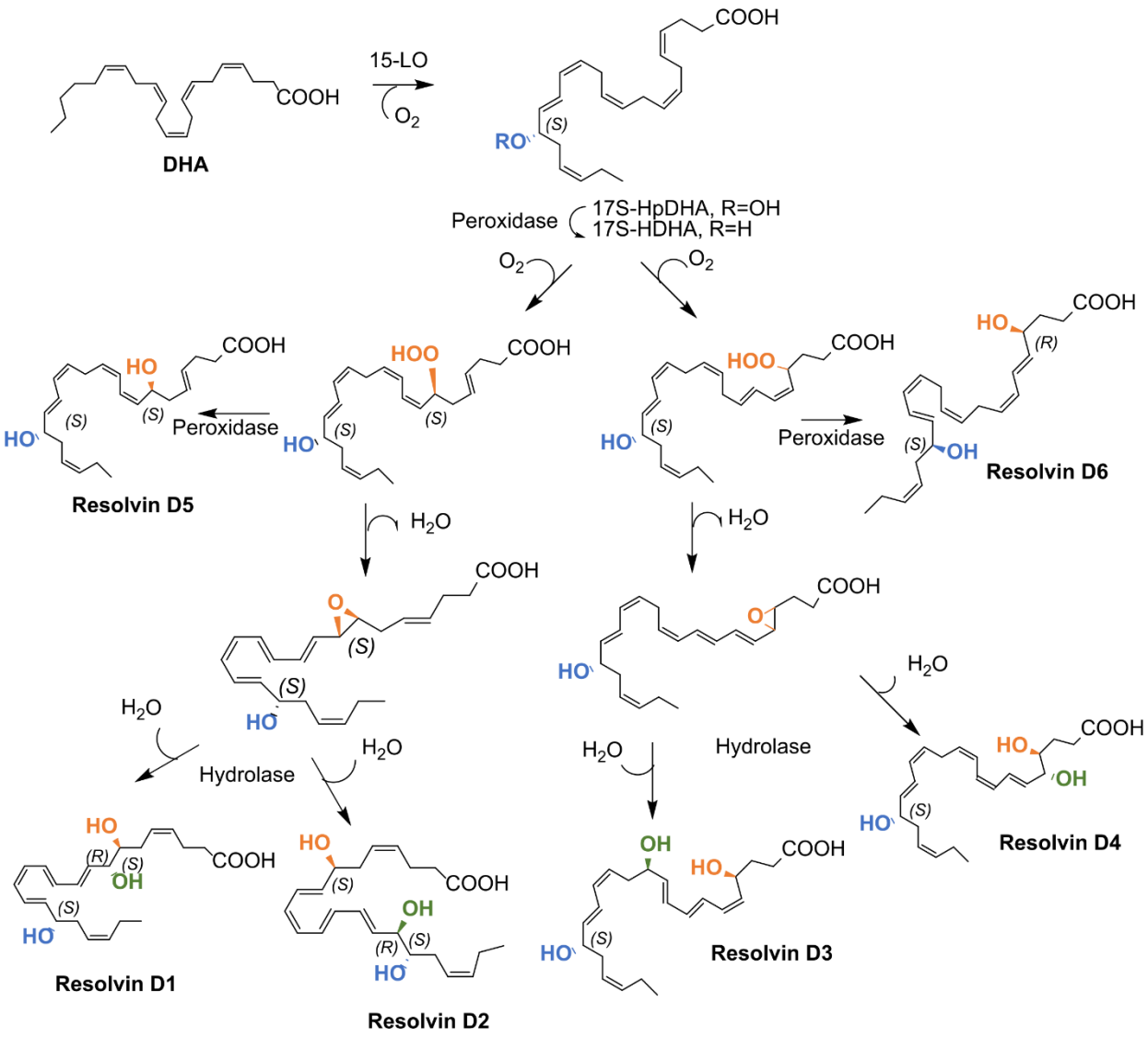

Figure 8 Biosynthesis of Resolvins[15]

1.4.1.1 Resolvin D1

Resolvin D1 (RvD1) at picomolar and nanomolar levels regulates phagocytosis in humans. Multiple studies have given evidence of therapeutic benefits of RvD1 such as decrease in progression of osteoarthritis in joints, prevention of neuronal dysfunction in Parkinson's disease and increased efferocytosis in aging [39].

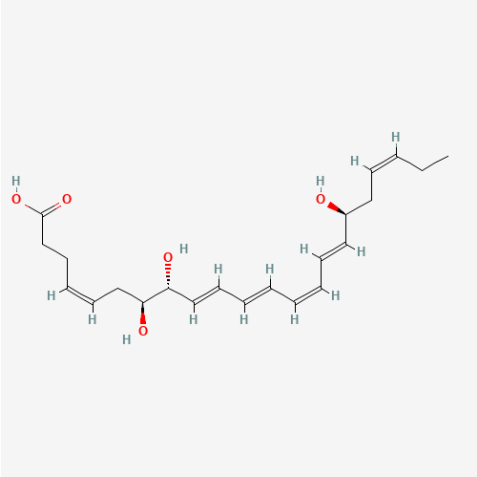

Figure 9 Chemical structure of Resolvin D1

The substrate used for the biosynthesis of RvD1 is found to be produced by mechanisms involving cellular exudates and membrane phospholipids[40]. RvD1 is synthesized after the conversion of the intermediate 17S-hydroperoxy-4Z, 7Z, 10Z, 13Z, 15E, 19Z-docosahexaenoic acid (17S-HpDHA) into 7-hydroperoxy 17-S-H(p)DHA by the action of 5-LOX. Epoxidation leads to the formation of 7(8) epoxy-17-S-H(p)DHA which upon enzymatic hydrolase action forms 7S,8R,17S-trihydroxy-DHA (RvD1)[40 33].

1.4.1.2 Resolvin D2

Resolvin D2 (RvD2) promotes specific cellular repair, regenerative, and protective actions such as keratinocyte restoration, muscle regeneration and restriction of tissue necrosis in burn wounds. In mice RvD2 has shown promising results in suppressing tumor growth and clearance of cellular debris [29,39]. RvD2 upon administration of parenteral peritoneal preparations indirectly decreases bacteria in blood and peritoneal cavity by increasing phagocytosis, reduces pro-inflammatory mediators like PGE_2 and LTB_4, and lowers cytokine levels of interleukin 6 (IL-6), IL-1ß, IL-23 and tumor necrosis factor alpha (TNF-α)[32].

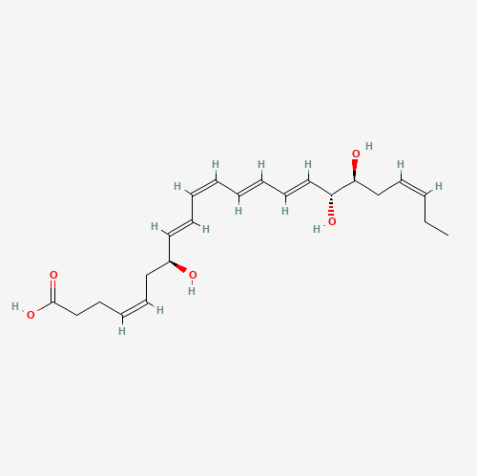

Figure 10 Chemical structure of Resolvin D2

RvD2 biosynthesis is similar to the formation of RvD1, changing at enzymatic transformation of 7(8) epoxy - 17-S-H(p)DHA to 7S,16R,17S-trihydroxy-DHA (RvD2)[41]. In human leukocytes, 17-lipoxygenation of DHA results in 17S-HpDHA, which is enzymatically converted to a 7(8) epoxide-containing intermediate. 5-lipoxygenase (5-LOX) and its epoxide-generating activity are involved in this metabolic process. These processes might take place within a single cell type or through transcellular biosynthesis. Eosinophils, for example, which are high in 15-LOX, can convert DHA to 17-HpDHA, which polymorphonuclear neutrophils (PMNs) can then convert to RvD2 [42].

1.4.1.3 Resolvin D3

Resolvin D3 (RvD3) has potent anti-inflammatory actions with leukocytes and also reduces the levels of pro-inflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1), IL-6 and keratinocyte chemoattractant (KC). Systemic administration of RvD3 significantly reduces some pro-inflammatory eicosanoids such as LTB_4 (80%), PGD_2 (67%), and TxB_2 (50%), while promoting the production of PGE_2. Compared to RvD1 and RvD2, the RvD3 emerges later in vivo and enhances proresolving effects both in vitro and in vivo. It blocks transmigration of neutrophils and promotes macrophage uptake of microbial particles [7].

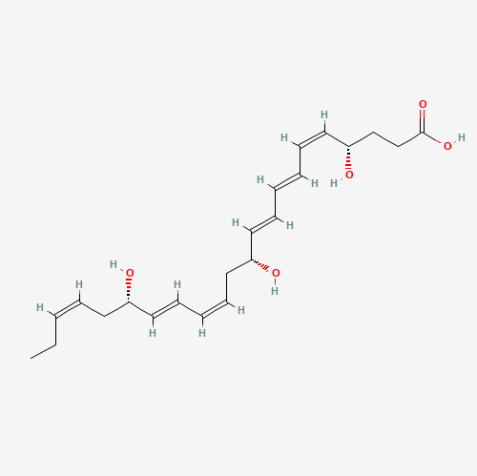

Figure 11 Chemical structure of Resolvin D3

The biosynthesis of RvD3 results from sequential lipoxygenase reactions. The process starts with insertion of molecular oxygen at the C-17 position DHA in the presence of 15-LOX producing 17S-HpDHA. This reduced hydroxy product (17S-HDHA) undergoes a second lipoxygenation by 5-LOX that results in a new hydroperoxide at the C-4 position. This hydroperoxide is then transformed enzymatically into the 4S,5S epoxide, which then undergoes enzymatic hydrolysis to generate RvD3 [43].

1.4.1.4 Resolvin D4

Resolvin D4 (RvD4) plays a prominent role in neutrophilic infiltration, inhibition of cytokine production in glial cells and regulation of leucocyte diapedesis. It has organ protective action as observed in ischemic kidney injury [39,44]. In mice RvD4 has shown promising anti-inflammatory actions by regulating LTB_4 and PGD_2 and at sub nanomolar concentrations it promotes the phagocytosis of *S. aureus* by macrophages. By encouraging the removal of apoptotic PMNs, synthetic RvD4 dramatically improved efferocytosis (removal of apoptotic cells by phagocytes) in human fibroblasts [44].

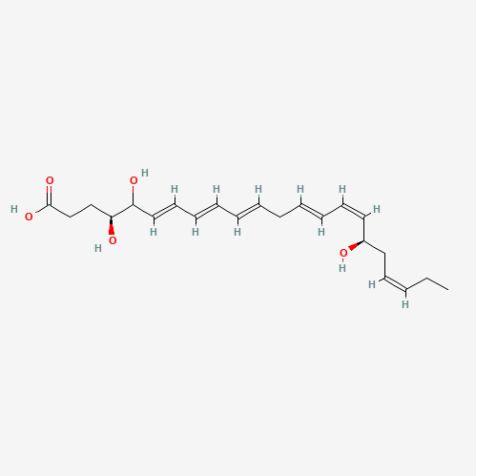

Figure 12 Chemical structure of Resolvin D4

The biosynthetic process begins with the conversion of DHA to 17S-HpDHA, which is catalyzed by 15-LOX. The hydroperoxyl intermediate can also be converted to the hydroxyl product, which results in 17S-HDHA. Both 17S-HpDHA and 17S-HDHA undergo a second lipoxygenation via 5-LOX, resulting in a new C-4 hydroperoxide. This peroxide is subsequently converted to a transient intermediate called 4S,5S-epoxy-17S-HDHA8 by enzymatic hydrolysis, which results in the production of RvD4 [45].

1.4.1.5 Resolvin D5

Like all other resolvins, resolvin D5 (RvD5) has been actively involved in enhancing phagocytosis by increasing the migration of neutrophils and macrophages. Reportedly, blood, synovial fluid and exudates released after hemorrhage have shown a strong presence of RvD5 where it regulates the TNF-α and nuclear factor kappa β (NF-κ β). Production of RvD5 in the late stages of coagulation prolongs the agglutination process decreasing the chances of hemorrhage. It is a prominent resolvin in containing *E. coli* [46].

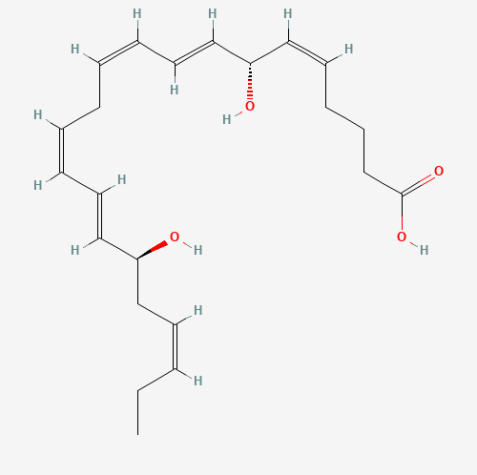

Figure 13 Chemical structure of Resolvin D5

The action of peroxidase on 7-hydroperoxy 17-S-H(p)DHA leads to the formation of RvD5. Similar to the formation of 7S,14S-diHDHA, the sequential reaction of two LOXs is postulated to produce RvD5, which appears to require two oxygenation events. It can also activate the RvD1 receptor DRV1/GPR32 [39].

1.4.1.6 Resolvin D6

Resolvin D6 (RvD6) with a molecular weight of 360 exerts potent anti-inflammatory activities specially on corneal system [33,47]. In 2020, Pham and co-workers found a novel RvD6 stereoisomer(s) in mice tears after topical treatment of damaged corneas with pigment epithelium-derived factor (PEDF) and DHA[48].

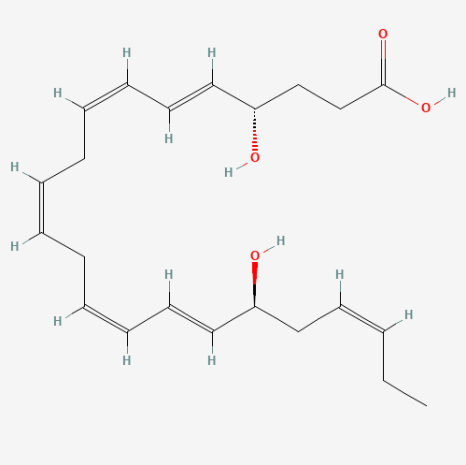

Figure 14 Chemical structure of Resolvin D6

Chiral liquid chromatography (separation of optical isomer or enantiomers) and tandem mass spectrometry methods revealed that these RvD6 stereoisomer(s) produces fragmented ions similar to the diagnostic ions of the RvD6 reference standard with a shorter retention time. The presence of a conjugated diene moiety was confirmed by the compound(s) showing a UV maximum absorbance in methanol at $\lambda_{max}\approx$ 237 nm, similar to RvD6. Through the stimulation of gene expression in the trigeminal ganglia (TG), RVD6 isomer elicited powerful biological effects, including the promotion of corneal innervation and the induction of wound healing [47].

RvD6 production does not entail the creation of epoxide intermediates, as with other forms of resolvins. Two distinct oxygenation processes of DHA at the C-17 site are involved in biosynthesis. 15 LOX first oxygenates to make 17S-HpDHA, then reduces it with peroxidase to produce 17S-HDHA. The oxygenation is then carried out at C-4 position by 5 LOX, followed by another round of peroxidase reduction, resulting in the creation of RvD6[47].

1.5 Immune system of Atlantic Salmon

The majority of the lymphoid organs seen in mammals, with the exception of lymphatic nodules and bone marrow, are also present in fish, making the immune systems of fish and mammals similar. Immune cells are present throughout the kidney, however the anterior kidney, also known as head kidney, has the highest amount of growing B lymphoid cells and the least amount of antibody-secreting cells (ASC)[49]. In contrast to higher vertebrates, the head kidney performs hemopoietic functions and is the main immune organ in charge of phagocytosis, antigen processing, and the production of IgM and immunological memory through melano macrophagic centers. The head kidney, also known as the pronephros, is the active immunological component and is made up of two Y-shaped arms that extend below the gills. Similar to the mammalian adrenal glands, the head kidney of fish is a highly innervated organ that plays a significant endocrine role by secreting corticosteroids and other hormones.[50–52] Innate and adaptive immunity of salmon is affected by the amount of fatty acids in nutrition. Studies have demonstrated that dietary long-chain polyunsaturated fatty acids (LC-PUFA), such as EPA and DHA, affect the immunological responses of Atlantic salmon[53–55]. Ionic regulation, cellular synthesis and development of neural system are some of the major functions where EPA and DHA play a significant role. Adjusting the levels of fatty acids in fish has shown reduction in cardiac and skeletal muscle inflammation[56]. EPA and DHA can only be partially biosynthesized by salmonids from their precursor, alpha-linolenic acid (ALA). To maintain fish growth and health, these crucial biomolecules must be given through aquafeeds.

2 Objectives

The aim of the study is to develop a novel and convenient method for analysis for resolvins in fish cultured cells by liquid chromatography triple quadrupole mass spectrometry. Specific objectives are:

- Determination of the optimal concentrations of internal standards by using a factorial design and model a relationship between resolvins and internal standards.
- Computation of a model with constant response factors over a broad analytical range.
- Validation of the proposed method for resolvin analysis with emphasis on selectivity, linearity, precision, limit of detection, limit of quantification, and recovery.
- Simultaneous quantification of five resolvins excreted in Lebovitz L-15 media by head kidney cells exposed to DHA.

3 Significance

In general, conventional solid phase extraction and liquid chromatography mass sepectrometry (LCMS) are the preferred techniques to analyze resolvins by using external standards. The process is expensive, time consuming and requires meticulous standard solutions preparation of varied concentrations to formulate a calibration curve. Ultimately, sample preparation for multiple compound analyses (e.g., resolvins) is the biggest bottleneck in analytical laboratories. Therefore, it is convenient to propose a method which is less complex and time and cost effective. This study proposes the use of a simple liquid-liquid extraction protocol and the internal standard addition method for the quantification of five resolvins (RvD1, RvD2, RvD3, RvD4, RvD5) released into Leibovitz's L-15 complete media by Atlantic salmon head kidney cells by means of liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS).

Finding suitable biomarkers for assessing the inflammatory reactions of the cells exposed to various stimuli or chemicals depends heavily on the examination of pro- and anti-inflammatory prostaglandins and resolvins in activated cells. The dynamics of ω-3 and ω-6 PUFAs and their mechanisms of action in cellular models might be greatly understood with the effective application of the devised approach on culture media. Resolvins must be quantified to determine the pathophysiological state, and monitoring these biomarkers is crucial to comprehend the

mechanisms that control their synthesis and cell signaling. These studies can pave way for the development of novel targeted therapies to counter inflammation.

4 Analytical Method

4.1 Instrumental Techniques

The present section describes the commonly used techniques used for the analysis of resolvins in different biological samples.

4.1.1 Enzyme Immunoassay (EIA)

Enzyme immunoassay (EIA) kits have been used for determining the production of RvD_1 in human and fish plasma with high sensitivity. Despite EIA is one of the most extensively used methods for estimating resolvins in biological samples, it has several limitations due to its lack of specificity and inability to detect many analytes in a single analysis. The approach requires the use of a unique antibody for each resolvin to be examined. EIA is prone to cross-reactivity, which leads to an overestimation of the individual resolvin levels. Furthermore, EIA are limited to only one type of resolvin per commercial kit. For instance, the analysis of RvD1 and RvD2 requires to purchase two different kits for every resolvin, making the technique prohibitively expensive when different resolvins are considered [57,58].

4.1.2 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS was proposed as a suitable alternative for resolvin analysis to overcome the drawbacks of EIA. Its ability to detect several analytes in a single run gives it a distinct edge over EIA. Although GC-MS has higher sensitivity and selectivity for resolvin analysis, it involves chemical derivatization processes, which limit its utility because the analyzed compounds must be both volatile and thermally stable in order to undertake GC-MS based investigations [59].

4.1.3 Liquid Chromatography Mass Spectrometry (LC-MS)

LC-MS is an effective analytical approach for separating, identifying, and quantifying resolvins in biological samples. This technique involves employing liquid chromatography to separate chemicals depending on their physicochemical qualities, followed by mass spectrometry to detect and quantify the molecules [33]. The combination of chromatography and spectrometry provides high selectivity, sensitivity and accuracy in identifying unknown peaks and complex compound separation. In LC-MS the samples are ionized during chromatographic separation and their m/z ratios are then utilized to identify or quantify the samples. There are many different MS devices and MS procedures available.

Electrospray-ionization (ESI) and atmospheric pressure chemical ionization are the two ionization techniques most frequently employ in metabolomic research [60]. ESI is a sensitive ionization method for analytes in the LC eluent that exist as ions. A solvent spray is created in ESI by applying a high voltage potential between a stainless steel capillary and the instrument orifice. Usually, the process takes places under the influence of axial flow of a nebulizing gas such as nitrogen. Spray solvent droplets evaporate in the mass spectrometer's ion source, releasing ions into the gas phase for examination in the mass spectrometer. Heat is used in some ESI sources to improve desolvation efficiency. While ESI is commonly utilized, it is susceptible to matrix effects, especially ion suppression, which must be considered during method development[61]. APCI is comparable to ESI in that ionization occurs at atmospheric pressure, involves nebulization and desolvation, and employs the same ion extraction cone design as ESI. The primary distinction is in the method of ionization. No high voltage is provided to the inlet capillary in APCI. Instead, the separation device's mobile phase evaporates, and the vapor passes through a needle with an applied current[62].

The most often utilized methodologies in resolvin analysis are based on liquid chromatography - tandem mass spectrometry (LC-MS/MS). LC isolates resolvins according on their physical and chemical properties in this case. The tandem mass spectrometer's first mass analyzer can then isolate a specific ion. In the following stage, this ion is fragmented in vacuum via collision-induced dissociation (CID). The second mass spectrometer can then isolate a specific fragment ion. The structure of the precursor molecule determines the amount of fragment ions. As a result, fragmentation patterns are specific to the analyte and can be used for identification and quantification [63]. Single reaction monitoring (SRM), a strategy that is frequently employed in lipidomic and which bases the study of lipids on the selection of distinctive precursor-product ion pairs, is based on this idea. The benefit of LC-MS/MS is the ability to perform multiple reaction monitoring (MRM) where several precursor-product ion pairs are examined in a single run, as well as multiple product ions for a single precursor ion[64]. The use of SRM or MRM can provide a substantially lower limit of detection (LOD).

The last 20 years have seen a notable breakthrough in LC-MS/MS technology. There has been a significant market need for increased resolution and lower run times for an increasing variety of applications. Mass analyzers and hybrid devices like QTOF, QLIT, and Orbitrap are growing in popularity because of their ability to provide precise mass measurements and obtain crucial

qualitative data in the form of full-scan spectra. Another noteworthy development is the progressive transition from parent compound analysis to metabolite and transformation product analysis which helps greatly in analysing thermolabile compounds otherwise not detectable by GC-MS [65,66].

4.2 Extraction techniques

The initial step in analyzing docosanoids (e.g., resolvins, maresin, etc) is to obtain biological samples from human or animal subjects. These samples can be solid (tissue) or biofluids (e.g., plasma, serum, urine)[67]. Because additional disruption and homogenization processes of tissues or cells are required prior to docosanoids extraction, the sample-preparation protocol for tissues is more labor intensive and complex than for biofluids. Commonly, the tissues after excision from animal are flash-frozen by using liquid nitrogen and then stored at extremely low temperatures (-80°C). This procedure aids in reducing the rate of oxidation, peroxidation, and hydrolytic breakdown of lipids, as well as inhibiting enzymatic activity.

In biological fluids and organs, such as peripheral blood, cerebral fluid, placenta, synovial fluids, urine, sputum, spleen, lymph nodes, cell cultures, and others, resolvins are frequently present at incredibly low amounts [68]. Therefore, efficient extraction is crucial to wash and clean up the sample and subsequent drying and reconstitution in small volume of solvent. Various extraction strategies are employed in resolvin research ranging from solid phase extraction (SPE), liquid-liquid extraction (LLE) to protein precipitation [59,69,70].

4.2.1 Solid Phase Extraction

The extraction of analytes from a complex matrix is frequently done in analytical laboratories using the sample preparation technique known as solid-phase extraction (SPE). Prior to measurement, analytes can be extracted, cleaned up, and concentrated using this sample preparation process. Frequently, SPE requires the use of additional sample preparation steps like dilution or pH adjustment. The earliest contemporary use of SPE in the colloquial sense probably employed animal charcoal to remove colors from chemical reaction mixtures. In such cases, the charcoal was filtered out of the mixture and discarded along with the substances it had absorbed [71,72].

In SPE the compounds are separated in accordance with their physical and chemical properties by using an adsorbent material and a combination of solvents. SPE cartridges are commonly used for this purpose. Conditioning of cartridges, sample loading, washing with solvents and collection of fractions are some of the basic tasks involved in SPE [73] as shown in figure 15. It has a major benefit of preventing degradation of analytes that have been sorbed onto the SPE cartridge/column/disc, allowing long-term storage without any alteration in concentration [72].

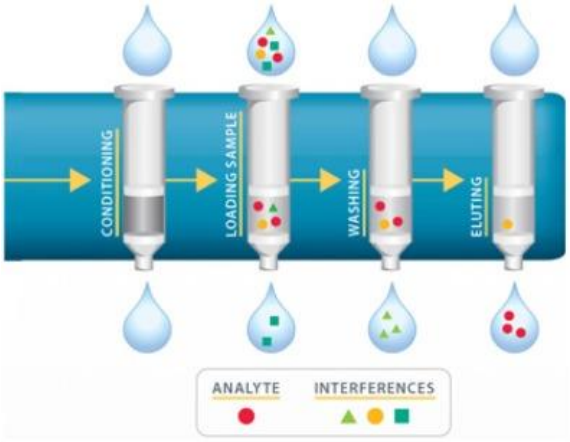

Figure 15 Schematic representation of a typical SPE process.

To enhance the effective surface area and decrease interferences, solid-phase materials are conditioned by flowing organic solvents or water through the column. The interactions between analytes and the solid-phase material are broken by flushing tiny volumes of organic solvents after drying the sorbent and possibly removing interferences. This causes the target analytes to desorb from the solid phase[74]. More than 30 years ago, SPE disposable cartridges were initially launched (the earliest cartridges date from 1978, syringe-format kinds from 1979, and columns for on-line coupling with liquid chromatography (LC) from the early 1980s)[75]. SPE cartridges are expensive, harmful for the environment and there is a possibility of low recovery. Costs can add up rapidly if several commercially manufactured SPE cartridges are frequently needed because the cartridges are single-use and specifically coated. To get the best analyte extraction, method development involves fine-tuning of volumes, flow rates, and pressure. When working with fresh samples and their associated matrices, this might consume a lot of time and resources [76,77].

An overview of the literature indicated that solid phase extraction (SPE) is arguably the most popular extraction method for the analysis of resolvins in different kind of samples and LC-MS/MS the preferred quantitative technique for its outstanding sensitivity. For the quantification of resolvins in human keratinocyte cell lysates, a recent article proposed a laborious methodology that combines liquid-liquid extraction (LLE) with chloroform and acetate-water buffered at pH 4, followed by -SPE with methanol-water buffered at pH 4, and a final LC-MS/MS analysis to obtain recoveries at around 42 and 64% [78]. Unfortunately, SPE alone (not to mention when combined with LLE) is a time-consuming and complicated method that requires multiple steps and different solvents prior to LC-MS/MS analysis.

4.2.2 Liquid-Liquid Extraction

Liquid-liquid extraction (LLE) is generally utilized as a separation method where an immiscible or partially immiscible solute is transferred from one solvent to another. Besides separation it is also a crucial enrichment technique. The distribution of an analyte between two substantially immiscible solvents is the foundation of LLE. The ratio of an analyte's total concentration in the organic phase to that in the aqueous phase at equilibrium is known as the distribution ratio. The higher the recovery of the organic compound and the higher the enrichment factor, when the desired organic compound is extracted, the larger the distribution ratio of the analyte and the smaller that of the matrix. The reverse is needed for successful enrichment after matrix removal by extraction. The enrichment factor is improved after the extraction by back washing matrix components selectively from the organic phase into the aqueous phase. In LLE normally the extract is reduced to dryness and reconstituted in a small volume of solvent to ensure high analyte enrichment. It is a good sample clean-up method for salts and biological macromolecules, however a wide variety of additional chemicals may be co-extracted during the process [79].

The outstanding capacity of liquid-liquid extraction to selectively separate and purify components from complicated mixtures is one of its key advantages. This selectivity is especially useful in industries such as pharmaceuticals and petrochemicals, where it is critical to separate specific compounds from multi-component mixtures. Liquid-liquid extraction, for example, is used in pharmaceutical manufacture to isolate and purify active pharmaceutical components from reaction mixtures, ensuring that the final medicine product meets high quality criteria[80,81].

Liquid-liquid extraction is also very flexible to different operational sizes. It can be used in laboratories, pilot plants, and large-scale industrial processes. This scalability enables rapid process development and optimization, guaranteeing that the benefits of liquid-liquid extraction may be realized in a variety of applications. Liquid-liquid extraction is also used in a wide number of industries, indicating the breadth of its potential uses. It is employed in the environmental industry to remove toxins and pollutants from water and wastewater streams. Liquid-liquid extraction is used in the food business to draw tastes, fragrances, and essential oils from natural sources. It is also essential for the recovery and purification of priceless metals in mining and metallurgy, as well as for the reprocessing of nuclear fuel[81].

4.2.3 Protein Precipitation

One of the most popular sample extraction methods in bioanalysis is the protein precipitation technique (PPT). This method is reasonably easy to use and offers speedy sample cleanup, particularly for whole blood, plasma, and serum. The working theory is based on the sample being added to an organic solvent, acid, salt or metal ion [82].

Protein precipitation with organic solvents is presently one of the most popular sample preparation procedures for proteomics analysis. Various solvents are used for this task, including acetone, methanol, ethanol, acetonitrile and chloroform[83]. Protein's intermolecular electrostatic interactions are scaled by their dielectric constants, which vary depending on protein size and composition[84]. When an organic solvent is added to a sample such as plasma, whole blood or serum, it lowers the dielectric constant of the sample. As a result, water molecules are driven away from the hydrophilic regions of the protein surface, leading to increase in electrostatic attraction between those charged protein molecules, and decreases hydrophobic interactions, allowing the protein crystals to precipitate[83]. The use of acidic reagents such as hydrogen chloride is another approach that facilitates PPT. The reaction with the positively charged amino groups of the proteins produces an insoluble salt when the acidic reagent is added at pH values lower than their isoelectric points, thereby separating proteins. The final PPT approach involves adding excessive amounts of salt, such as zinc sulfate, which is frequently employed in bioanalysis. The addition of this salt causes the hydrophobic protein surfaces to lose water, which then permits protein aggregation through hydrophobic interactions. Metal ion binding decreases protein solubility by altering its isoelectric point[82].

PPT has several drawbacks, including difficulties when concentrating analytes, severe ion suppression brought on mostly by serum phospholipids and lack of robustness. Phospholipids present in serum are only marginally eliminated by PPT methods, and are infamous for suppressing ions during electrospray ionization[77]. Therefore, a combination with other extraction techniques is often required to enhance sample preparation. Many studies have used LLE and SPE after protein precipitation method to improve separation of resolvins from human spleen, lymph nodes and blood samples and sample clean up prior to LCMS-MS analysis [85,86].

4.3 Method Validation

Validation comes from the Latin root word "Validus," which means "effective, strong, active, powerful" and "sufficiently supported by facts or authority, well-grounded. Its meaning was first recorded in the 1640s [87]. Validation is the procedure used to determine whether an analytical procedure's performance characteristics meet the needs of the application for which they are intended. Development of a new method requires confirmation that the new technique is reliable, precise, sensitive, reproducible [88]. It is essential to demonstrate that the analytical method used is accurate, sensitive, specific, precise, and reproducible.

The statistical validation of the model helps in determining if the hypothesized relationship of the data is acceptable as description of the data. By separating the residual error into two components—pure error, which describes the variation of the experimental replicates around their mean value, and lack of fit error, which describes the variation of the mean values around the model prediction—it is possible to assess the suitability of the model. By dividing the size of the two components by the corresponding degrees of freedom, the variances of the two components are computed. To assess how well a model fits the data, we used the variance ratio of the lack of fit error to pure error also known as the "Fisher-test".

4.3.1 Selectivity

The ability of a method to differentiate a specific analyte in a complex mixture without interference from other components in the mixture is referred to as its selectivity [89]. In chromatography the compounds are separated and eluted at different retention times which helps in guaranteeing selectivity. However, in some cases the analytes have the same retention times, therefore in such

cases mass spectra are useful to distinguish between different compounds and enhancing selectivity. Selectivity is assessed in term of Resolution [90], given as

$$Rs = \frac{\Delta t}{\frac{1}{2}(W_A - W_B)} \qquad \text{Equation 1}$$

Δt = The time difference between separation of two peaks
W = Width of the chromatographic peak at base

4.3.2 LOD and LOQ

The lowest amount of analyte in a sample that can be accurately identified but not necessarily quantitated by a specific analytical method is known as the limit of detection (LOD). Whereas limit of quantification (LOQ) is the lowest concentration or amount of analyte that can be determined with an acceptable level of precision and accuracy [88,89]. Both LOD and LOQ can be determined by following approaches:

- Visual evaluation: Samples of known analytical concentration are prepared, and the minimal level at which the analyte can be measured with an acceptable level of uncertainty is determined.
- Signal/noise ratio: The signals of sample with known analytical concentration are compared to those of blank samples up to an analytical concentration that provides a signal 10 times the standard deviation of the blank sample (10 x σ_{blank}).
- Calibration curve: Calculations based on the standard deviations of signals and slope of calibration curve. It uses a series of samples with known or unknown analyte concentrations and standard solutions for calibration curves [89].

$$LOD = \left(3.3 \times \frac{\sigma}{\varphi}\right) \qquad \text{Equation 2}$$

$$LOQ = \left(10 \times \frac{\sigma}{\varphi}\right) \qquad \text{Equation 3}$$

4.3.3 Linearity

The ability of an analytical process to produce test results that are directly proportional to the concentration of analyte in the sample within a specified range is known as linearity. On the basis of the notion that a linear calibration function almost always produces a high correlation coefficient, the correlation coefficient, abbreviated r, has frequently been used as a rudimentary test for linearity. However, a correlation coefficient that is close to unity does not always denote a linear calibration function. Also, it is challenging to interpret the numerical value of r in terms of linearity deviation. As a result, while testing for linearity, the correlation coefficient is misleading. The F-test is a dependable method for determining if any calibration function is linear, according to the Analytical Method Committee [91]. Both the regression coefficients (R^2) and the Fisher test, which is calculated as the ratio between the lack-of-fit and the pure error variances, provided information about the degree of linearity of the calibrations.

A statistical method for assessing the linearity of analytical results and analyte concentrations is the error sum of the square[89]. It can be used to figure out the regression line's standard error. The residual error sum of squares, pure experimental error sum of squares, and lack of fit sum of squares are used to demonstrate that the calibration curve of the test result is linear.

4.3.4 Recovery

Recovery is the percentage of the analyte content that is extracted and measured from the test material's analytical component or that is added to it. It is assessed by comparing the degree of agreement between the experimental and nominal concentrations, (Eq 4)

$$Recovery = 100 \times \frac{[A]_{found}}{[A]_{nominal}} \qquad \text{Equation 4}$$

4.4 Principal Component Analysis

Principal component analysis (PCA) is a mathematical approach that decreases data dimensionality while keeping the majority of the data set's variation. This is accomplished by determining directions, known as principal components, along which the variation in the data is greatest. Each sample can be represented by a few numbers rather than values for hundreds of

variables by employing a few components. Samples can then be plotted, allowing for a visual assessment of similarities and differences between samples and determining if they can be categorized. PCA aims to (*i*) identify the most crucial patterns/correlations between samples from a given data set, (*ii*) reduce the size of the data set by retaining just this crucial data, (*iii*) streamline the data set's description, and (*iv*) assess the composition of the observations and variables. PCA computes new variables that are produced as linear combinations of the original variables to accomplish these objectives. Since the first principal component must include the greatest amount of variance, it will "explain" or "extract" the greatest amount of inertia from the data table. The second component must have the most inertia and be orthogonal to the first component in order to be computed. The calculations for the other factors are similar[92].

5 Experimental Design

Screening is an important function of experimental design. A wide range of factors influence most analytical methods. An extraction, for example, may involve 20 steps. It is critical for the experimenter to understand which steps are critical to extraction efficiency. Unexplained changes in extraction efficiency occur frequently when monitoring processes. These could be caused by column variability, pH and temperature changes, a change in reagent quality, sample homogeneity issues, and so on. The most important aspects are not usually visible at first glance. Similarly, a range of factors influence the optimization of analytical procedures such as high-performance liquid chromatography (HPLC) and atomic absorption spectrometry (AAS). It is critical to first determine which elements are significant before narrowing down the final optimization to three or four significant factors utilizing screening tools such as factorial designs[93].

5.1 Three-Level Factorial Design (3^k)

The most economically sound data collection strategy to understand the link between response variables and predictor factors is provided by factorial designs. They are able to estimate each effect on the response as linear, which allows them to analyze only two levels of each predictor variable. Synergistic and antagonistic interactions between the input variables frequently occur in complex industrial processes. Unless all active interactions in addition to the major impacts of each variable have been recognized, we are unable to adequately quantify the effects of input variables on our answers. The aim of factorial experiments is to evaluate the effect of all the variables and their potential interactions. The three-level design is written in the form of a 3^k factorial design. It denotes that k components are taken into account, each at three levels. They are (often) classified as low, moderate, and high level, numerically expressed as -1, 0 and +1. The three-level designs were introduced in order to handle the case of nominal factors at three levels and to reflect any curvature in the response function. Investigation of a quadratic relationship between the response and each of the components is made easier by the addition of a third level for continuous factors.

5.2 Selection of optimal concentration of internal standards

To aid quantification in LC/MS systems, an internal standard having comparable chemical structure and characteristics to the analyte of interest is commonly used [94]. This entails preparing

a solution of known concentration of analyte [A] spiked with known concentration of internal standard [IS], then determining their signal ratio (S_A/S_{IS}) and response factor (RF), which are calculated as follows:

$$RF = \frac{S_A}{S_{IS}} \times \frac{[IS]}{[A]} \qquad \text{Equation 5}$$

As a result, after determining RF at a certain known concentration of spiking IS, the unknown concentration of the analyte can be derived from their response signals, providing that the two components ([A] and [IS]) exhibit a linear relationship towards the detector over the studied concentration range. Details on detector linearity are often supplied for the analyte alone or in combination with a specific amount of internal standard, with no description of how to estimate the appropriate level of internal standard provided [94,95].

The degree of ionization of the internal standard in the electrospray ion source and the interaction between analyte and internal standard may cause the RF of the internal standard to fluctuate significantly over the analytical range. In order to guarantee constant RF values throughout the analytical ranges, it is necessary to optimize both the concentration ranges of the analytes and the internal standards. Some academics have noted the necessity for more thorough investigation on the effects of simultaneous changes in the analyte and internal standard on the response factor RF and, consequently, the quantification process [96].

6 Experimental Method

6.1 Reagents and Chemicals

Resolvin D1 (RvD1, 95%), resolvin D2 (RvD_2, 95%), resolvin D3 (RvD_3, 95%), resolvin D4 (RvD4, 95%), resolvin D5 (RvD5, 95%), deuterated resolvin D1 (RvD1-d_5, 95%), deuterated resolvin D2 (RvD2-d_5, 95%) and deuterated resolvin D3 (RvD3-d_5, 95%) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile (99.8%), methanol (99.8%) and formic acid (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-propanol (HPLC grade, 99.9%) chloroform (HPLC grade, 99.8%) were obtained from Merck (Darmstadt, Germany). A Millipore Milli-Q system was used to produce ultra-pure water 18 MΩ (Millipore, Milford, CT, USA). Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA, ≥98%), Cis-5,8,11,14-eicosatetraenoic acid (ARA, 85%) and cis-5,8,11,14,17-eicosapentaenoic acid (EPA, 99%), were purchased from Sigma–Aldrich (Oslo, Norway). Leibovitz's L-15 medium was from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS, cat# 14-801F) was from BioWhittaker (Petit Rechain, Belgium). The glutaMaxTM 100× (Gibco-BRL, cat# 35056) was from Gibco-BRL (Cergy-Pontoise, France). The penicillin-streptomycin mixture (cat#17-602E) and the trypan blue solution (cat#17-942E) were from Lonza (Falun, Sweden). EDTA buffer was purchased from Sigma–Aldrich (Oslo, Norway).

6.2 Cell Culture

6.2.1 Isolation of head kidney cells

As previously discussed, head kidney cells, being crucial in innate and adaptive immune regulation, exhibit a wide array of heterogeneity and secrete elevated amounts of eicosanoids (prostaglandins, leukotrienes) and docosanoids (resolvin D, protectins) lipid mediators. Therefore, in order to study the production of resolvins, we use head kidney cells in this study. The process of isolation uses percoll, which is a colloidal suspension of uniformly sized, 17 nm-diameter silica particles covered with polyvinyl pyrrolidone[97]. The amount of erythrocytes in the isolated cells is greatly reduced but not entirely eliminated by the percoll gradient used for cell purification. Percoll is frequently employed in investigations using the density gradient because it has low viscosity, is nontoxic to cells, and has a low osmolarity[98].

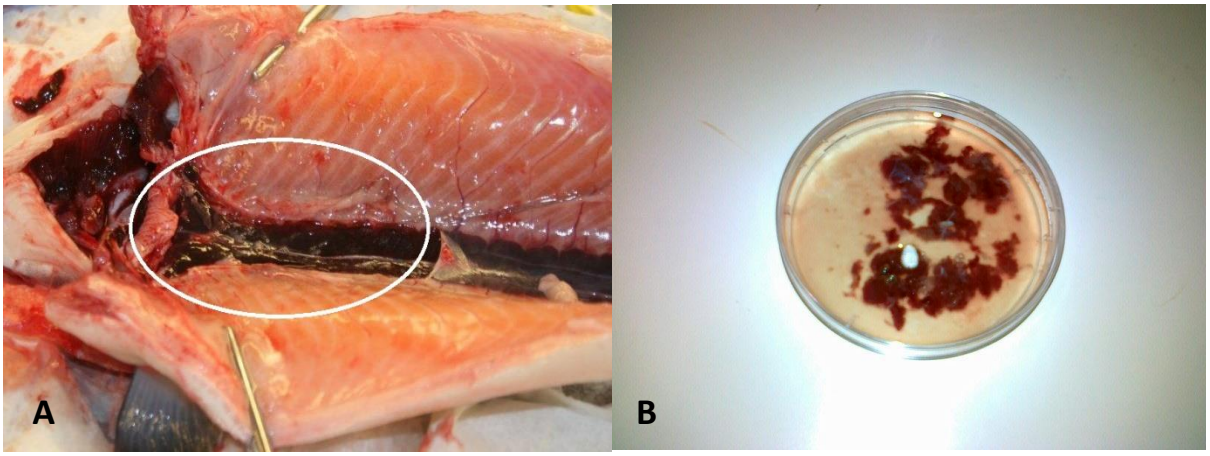

Figure 16 (A)Dissection and collection (B) of head kidney cells

Head kidney from six Atlantic salmon (200-300g) were excised from the fish with the help of a scalpel and placed into a petri dish with sterile EDTA buffer (Figure 16 a and b). PBS at 5 °C was added to the head kidney and then cut with scissors and squeezed through a 40 µM Falcon cell strainer into an Eppendorf tube. The contents of tube were centrifuged at 400 x g for 5 min at 4°C (Hettich Universal zentrifugen 320R, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). The cell pellets at the bottom of the tube were resuspended in PBS and layered carefully on top of equal amounts of diluted Percoll in a density of 1.08 g/mL. The tubes were centrifuged at 800× g for 30 min at 4 °C. The cell layer in the interface containing the head kidney leukocytes was collected and the cells were pelleted by centrifugation, 400× g for 5 min at 4 °C. An additional washing step in PBS was performed. Cell layers were pipetted into four new centrifugation tubes and washed with EDTA buffer at 400 x g for 5 min which resulted in the formation of a cell pellet which was resuspended in EDTA buffer and again centrifuged at 400 x g for 5 min. Cell pellets were then resuspended in complete media (c-15) consisting of L-15 medium (Sigma, USA, L 5520, Leibovitz), added 1% 2 mM GlutaMAX (Gibco, USA, A12860 , 100x), 1% 50 U /mL Pen Strep Amphotericin B (BioWhittaker, USA, 17-745 E) and 10% FBS. The number of cells was counted after mixing the four tubes. The cells were counted after mixing the four tubes.

6.2.2 Cell Count

Determining the leukocyte concentration was a crucial step in ensuring that the number of cells in each well was equal. In this experiment, a cell culture plate with 6 wells was used, and the optimal

cell density was $10x10^6$ cells/well. Using a Bürker chamber (Figure 18 a & b) and 0.4% trypan blue solution, the number of cells were counted. The counting procedure involves mixing one part leukocyte solution with four parts tryphan blue (0.4%) and added to the counting chamber. A Burker counting chamber consists of a total of 18 A-routes, the cells within a range of one A-box (equivalent to 4 x 4 smaller squares) were counted. The viability of the cells was determined by counting the total number of cells, which included both live and dead cells. Tryphan blue can permeate broken cell membranes and stain the cells blue, whereas the membrane is not damaged in living cells and so is not stained. Clear distinction can be made between live and dead cells using a light microscope. The viability obtained was above 85%, which is optimal to obtain reproducible results as a higher viability above 75% is preferred.

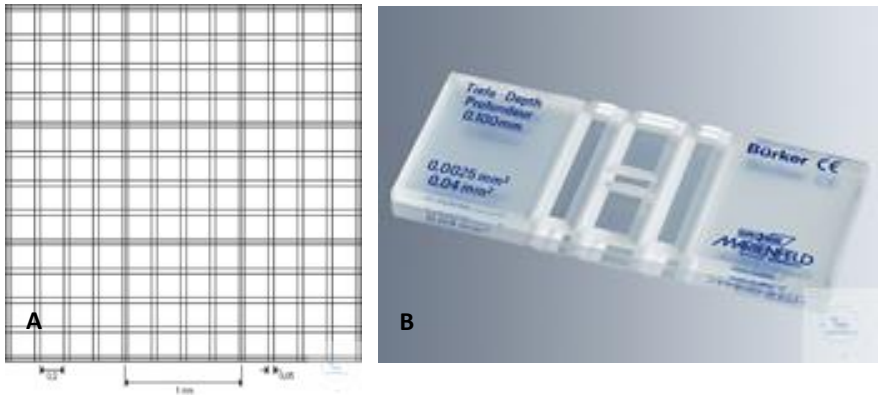

Figure 17 (a & b) Bruker counting chamber

6.2.3 Treatment and harvesting leukocytes

A total of eighteen cell culture plates were prepared in three sets of 6 wells, containing 2mL cL-15 media and $10x10^6$ leukocytes were incubated at 9ºC for 24 hours at normal atmosphere. Each well was supplemented with DHA in a concentration of 50 µM. Controls were untreated cultures. Each set of 6 wells was incubated for 6, 12 and 24 hours, respectively at 9ºC. The cells were harvested after each incubation period and centrifuged at 50 × g for 5 min at 4◦C to separate cell pallet from supernatant. The supernatants were removed from each sample for docosanoid analysis and were stored at -80 ºC.

6.3.3 Optimal Concentrations of the Internal Standards

A 3^k factorial design, where 3 represents the number of concentration levels (low, medium, high) and k the number of factors (analyte and internal standard), was used to study variations in the response factor (RF) when the concentrations of both the internal standards (IS) and the analytical resolvins varied between 15 and 45 ng/mL.along with 4 blanks of L-15 media. The optimal IS concentrations should yield a stable RF over the explored analytical range.

In this study the experiments are carried out using a 3^2 full-factorial design with three replicates, resulting in a total of 27 experimental runs with various combinations of input parameters. Replication is performed to reduce model variability and estimate error.

Table 1 Proposed 3^2-factorial design to select optimal concentration of internal standards. -1 denotes the low level as 15 ng/mL, 0 denotes medium level as 30 ng/mL and +1 denotes high level as 45 ng/mL, respectively.

Experiment	**Resolvin [A] (ng/mL)**	**Internal Standard [IS] (ng/mL)**
1	-1	-1
2	0	-1
3	+1	-1
4	-1	0
5	0	0
6	+1	0
7	-1	+1
8	0	+1
9	+1	+1

6.3 Extraction

The extraction protocol has been described elsewhere for the determination of arachidonic and eicosapentaenoic acid metabolites from the LOX pathway [99], with some minor modifications.

Briefly, two successive aliquots of acetonitrile (500 µL) containing the mixture of internal standards at the concentration level of 15 ng/mL and chloroform (500 µL) were added successively into an Eppendorf tube containing 200 µL of aliquot from supernatant media. The Eppendorf tube was vortex-mixed for 30 s (Bandelin RK 100 ultra mixer, Berlin, Germany), the top phase was removed, and the extraction procedure repeated in the remaining phase using acetonitrile without internal standards, and chloroform. The contents of each tube were filtered using 1ml Henke-Ject syringe equipped with Millex- HV 0.45 µm filter and vacuum-dried at room temperature (Labconco vacuum drier system, Kansas, MO, USA) for 2 hours. After drying the samples were redissolved with 50 µL of methanol, centrifuged at 1620× g for 3 min (Eppendorf AG centrifuge, Hamburg, Germany) and transferred to an autosampler vial, and submitted to LC-MS/MS analysis.

6.4 HPLC MS/MS Analysis

An Agilent ultra-high performance liquid chromatography (UHPLC), coupled to a 6495 QQQ triple quadrupole (Agilent Technologies, Waldbronn, Germany) with an electrospray ionization (ESI) interface and iFunnel ionization, was used to quantify the eicosanoids. The UHPLC system was equipped with a Zorbax RRHD Eclipse Plus C18, 95Å, 2.1 × 50 mm, 1.8 µm chromatographic column. Figure 18 represents schematic illustration of the experimental process. The mobile phase delivered at 0.4 mL/min in gradient mode consisted of ultra-pure water with 0.1% formic acid (solution A) and an equal-volume mixture of acetonitrile and methanol with 0.1% formic acid (solution B). The solvent gradient was as follows: solution A was reduced from 60 to 5% from 0.00 to 4.00 min, kept at 5% between 4.00 and 5.50 min, increased to 60% between 5.50 and 5.51 min and kept at 60% between 5.51 and 10.00 min. Mass spectrometric detection was performed by multiple reactions monitoring (MRM) in negative mode. The monitored transitions in percentage of ion counts (%) are given in Table 2.

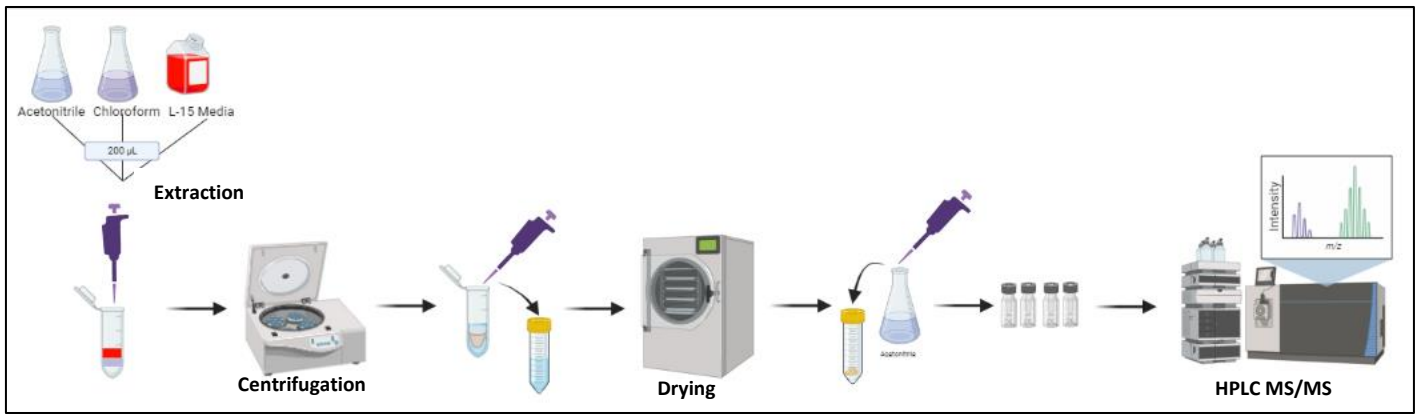

Figure 18 Schematic representation of LLE and LCMS/MS method (Created with BioRender.com)

Table 2 Fragmentation pattern for various resolvins.

Compound	**Parent ion (m/z)**	**Precursor ion (m/z)**
Resolvin D1	375	141
Resolvin D2	375	141
Resolvin D3	375	147
Resolvin D4	375	101
Resolvin D5	359	199
Resolvin D1-d_5	380	141
Resolvin D2-d_5	380	141
Resolvin D3-d_5	380	147

The ESI parameters were gas temperature (120 °C), gas flow rate (19 L/min), nebulizer pressure (20 psi), sheath gas temperature (300 °C), sheath gas flow (10 L/min), capillary voltage (3500 V) and nozzle voltage (2000 V). The integration of the chromatograms was performed using the Mass Hunter Qualitative Navigator software (version 10.0). The levels of resolvins were estimated by means of the internal standards and expressed in ng/mL units.

6.4 Statistical Software

Statgraphics Centurion XV Version 15.2.11 (StatPoint Technologies, Inc., Warrenton, VA, USA) and Microsoft® Excel® for Microsoft 365 MSO (Version 2308 Build 16.0.16731.20052) was used for the statistical analyses.

7 Results and Discussion

7.1 Determination of concentrations of internal standards

7.1.1 Factorial Design

The three-level factorial design (3^2) proposed nine combinations of analytes and internal standards that were made by dissolving the analytical resolvins and internal standards in the L-15 medium. For instance, experiment 3 in Table 2 uses an L-15 media solution containing all the analytes (RvD1, RvD2, RvD3, RvD4, RvD5) at a concentration level of 45 ng/mL and subjected to the extraction technique by employing a combination of deuterated internal standards (RvD1-d5, RvD2-d5, and RvD3-d5) at a concentration level of 15 ng/mL each. Additionally, four blanks were prepared using L-15 medium and internal standards at concentrations of 0, 15, 30, and 45 ng/mL. The experimental solutions in Table 2 were prepared in triplicate and were run in random order.

At each experimental point in Table 2, the RF for each resolvin at the various IS concentrations was computed using Equation 1. Modelling the RF for selecting the optimal concentration of RvD2-d_5 for extracting RvD1 and RvD2 from fish cells using a SPE-based method and subsequent measurement using an LCMS ion-trap device [100]. The results after implementing the factorial design are presented in Table 3.

Table 3 *Calculated response factors (RF), after implementation of the 3^2-factorial design, that were used to select an optimal concentration of deuterated internal standard for the liquid-liquid extraction of resolvins from L-15*

[IS]	*RvD1-d_5*	*RvD2-d_5*	*RvD3-d_5*	*RvD1-d_5*	*RvD1-d_5*	*RvD1-d_5*	*RvD2-d_5*	*RvD3-d_5*	*RvD1-d_5*	*RvD1-d_5*	*RvD1-d_5*	*RvD2-d_5*	*RvD3-d_5*	*RvD1-d_5*	*RvD1-d_5*
	15 ng/mL					**30 ng/mL**					**45 ng/mL**				
[A] (ng/mL)	**RvD1**	**RvD2**	**RvD3**	**RvD4**	**RvD5**	**RvD1**	**RvD2**	**RvD3**	**RvD4**	**RvD5**	**RvD1**	**RvD2**	**RvD3**	**RvD4**	**RvD5**
15	2.70	12.41	33.74	0.79	1.00	2.85	12.81	34.63	0.83	0.95	3.32	16.68	39.22	1.00	1.16
15	2.57	13.78	31.24	0.79	0.92	2.77	14.39	32.76	0.87	0.93	3.35	16.73	38.37	0.99	1.15
15	3.04	15.80	36.96	0.93	1.10	2.85	14.05	34.03	0.87	0.96	3.27	16.00	39.58	1.01	1.22
30	3.06	15.07	36.22	0.85	1.07	3.03	14.97	32.95	0.89	1.03	3.12	15.41	35.83	0.95	1.25
30	2.94	14.01	32.97	0.80	1.08	3.24	16.35	37.58	0.98	1.14	3.23	15.43	35.06	0.91	1.26
30	2.98	15.17	33.63	0.86	1.26	3.09	15.24	32.57	0.95	1.00	3.18	16.22	38.42	0.94	1.32
45	2.92	14.63	34.31	0.84	1.11	2.82	14.53	32.44	0.82	1.01	2.92	14.15	34.87	0.83	1.16
45	2.97	14.58	33.71	0.84	1.11	2.87	14.33	34.57	0.87	0.98	2.92	14.86	32.89	0.90	1.09
45	2.96	14.80	36.16	0.82	1.24	2.60	13.35	31.01	0.80	1.05	2.97	14.57	32.29	0.82	1.14
AVG	2.90±0.05	14.43±0.33	34.10±0.61	0.84±0.01	1.08±0.04	2.94±0.06	14.58±0.35	33.94±0.63	0.89±0.02	1.00±0.02	3.17±0.06	15.69±0.31	36.78±0.91	0.94±0.02	1.20±0.02
%CV	1.86	2.27	1.78	1.75	3.25	2.14	2.37	1.85	2.21	2.14	1.79	1.95	2.47	2.48	2.01

7.1.2 Modeling of RF as a function of analyte and internal standard

Regression models for various RFs are generated in MS Excel as functions of analyte concentration and internal standards, and the F test was used to assess the models' fitness. The calculated RF was obtained by using the following polynomial model:

$$RF = b_0 + b_1[A] + b_2[IS] + b_{12}[A] \times [IS] + b_{11}[A]^2 + b_{22}[IS]^2 \qquad \text{Equation 5}$$

The term b_0 is the intercept, b_1 and b_2 are the coefficients for variable [A] and [IS] respectively, b_{12} the coefficient for the interaction between [A] and [IS].

The experimental RF values at the various levels of concentrations of RvD1 and RvD1-d_5 were modeled successfully by reducing to four-parameters the model in Equation 5. The computed mathematical given by Equation 6 is as follows:

$$RF = 0 + 0.077[RvD1] + 0.093[RvD1 - d_4] - 0.0024[A][IS] \qquad \text{Equation 6}$$

The statistical acceptability was checked by using F-test as shown in Table 3. It is evident from the data that RF remains fairly constant below the concentration of 20 ng/mL of IS. Similar regression models were derived for RvD2, RvD3, RvD4 and RvD5 where RF values were expressed as functions of analytes and their internal standard and the interaction between them.

Response factors should be determined under controlled and consistent experimental conditions to ensure accurate and reproducible results. Calibration curves are often used to establish the relationship between concentration and response, and they can be used to validate the accuracy of the response factor calculation. Below we can see the contour plots for calibration which shows the accuracy of response factor calculations. In order to identify significant differences (p 0.05) between the computed RF values, a multiple range test was used. The results showed that, aside from RvD5, the RF remained constant for IS concentrations of 15 and 30 ng/mL with coefficients of variation of roughly 2%. Therefore, the optimal concentration level to be used in conjunction

with the suggested extraction protocol and for additional quantitative analysis using LC-MS/MS was chosen as 15 ng/mL of RvD1-d_5 (for RvD1, RvD4, and RvD5), RvD2-d_5 (for RvD2), and RvD3-d_5 (for RvD3), which yields average RFs of 2.90 ± 0.05, 14.47 ± 0.33, 34 ± 0.61, 0.84 ± 0.01 and 1.01 ± 0.04. Although there were statistically significant differences in the RF values for RvD5 (1.10 ± 0.04; 1.01 ± 0.02; and 1.19 ± 0.02 at 15, 30, and 45 ng/mL of IS, respectively), the RF for 15 ng/mL was between 30 and 45 ng/mL, with a coefficient of variation (3.2%) comparable to that reported elsewhere (4-13% at 1 ng/mL IS) for cell experiments using LCMS [101].

7.2 Evaluation of Method Validation Characteristics

7.2.1 Selectivity

The selectivity of the procedure was assessed in this investigation by comparing the chromatograms obtained after injecting L-15 medium samples with and without analytes. The resolvin calibration curves in L-15 medium were generated between 0 and 45 ng/mL and extracted using the appropriate concentration of internal standards determined by the 3^k factorial design. RvD1 and RvD2, which contain the same precursor (m/z 375) and product (m/z 141) ions, were separated chromatographically based on their retention times. RvD3 (2.55 min), RvD2 (2.59 min), RvD1 (2.77 min), RvD4 (3.12 min), and RvD5 (3.55 min) were the chromatographic elution orders. The extracted ion chromatograms (EIC) demonstrated that the suggested extraction process detects resolvins definitively with little background interferences (Figure 19).

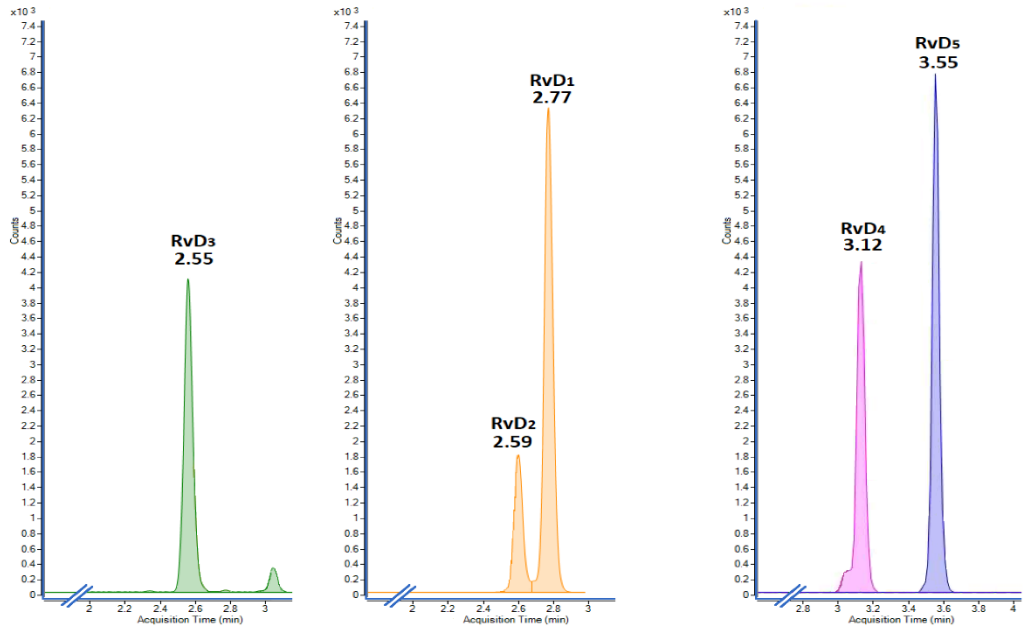

Figure 19 Extracted ion chromatograms, in increasing order of retention times, to indicate the selectivity of the analysis towards the five analyzed resolvins, after implementing the proposed LLE.

7.2.1 Limit of Detection (LOD) and Limit of Quantification (LOQ)

In this study the third approach, defined by Equations 2 and 3, is utilized to calculate LOD and LOQ as ratio of the standard deviation (σ) to the slope (φ) of the regression curves for every resolvin. The chromatographic peak area ratios RvD1/RvD1-d_5, RvD2/RvD2-d_5, RvD3/RvD3-d_5, RvD4/RvD1-d_5 and RvD5/RvD1-d_5 were calculated and plotted against the analytical concentrations to compute the function $yA/yIS=\varphi[A]+\beta$ and obtain the various calibration parameters displayed in Table 4.

Table 4 Analytical performance parameters. The linearity is judged by considering simultaneously the closeness of R2 to the unity and the comparison of $F_{experimental}$ against the tabulated $F_{critical}$ = 2.958 for 5 and 14 degrees of freedom at the 95% confidence level

Resolvin (0-50 ng/mL)	Slope (φ)	Intercept (β)	R^2	$F_{experimental}$	LOD	LOQ	Recovery (%)
RvD_1	0.197	-0.063	0.997	0.526	0.042	0.127	98.2±1.3
RvD_2	0.982	-0.235	0.999	0.342	0.028	0.086	99.5±1.1
RvD_3	2.304	-0.318	0.998	0.142	0.032	0.097	99.5±0.9
RvD_4	0.055	0.002	0.999	0.123	0.024	0.074	99.8±1.2
RvD_5	0.077	-0.048	0.994	0.624	0.059	0.180	96.9±2.3

The LOD and LOQ were deemed suitable for all resolvin species since they fell within the range of previously published values for cell cultures[102]. The LOQ for resolvins in L-15 complete medium measured in the current work using an Agilent 6495 triple quadrupole are comparable to (and in some cases better than) those published for pure standards measured using a Sciex QTRAP 6500[103,104]. The referenced Quadrupole/QTRAP (present/[[104]]) values, for instance, are 0.127/0.05, 0.5/0.086, 0.097/0.05, 0.074/0.1, and 0.180/0.1, respectively, for RvD1, RvD2, RvD3, RvD4, and RvD5. The LOQ values of the current research can be regarded as outstanding based on the matrix complexity, specifically the present L-15 medium vs the pure standard [24]. Additionally, given the general consensus that QTRAP provides superior data to quadrupole systems, the proposed extraction methodology in conjunction with the triple quadrupole spectrometer is an excellent strategy[104].

7.2.2 Linearity

Both the regression coefficients (R^2) and the Fisher test, which is calculated as the ratio between the lack-of-fit and the pure error variances ($F_{experimental}$ in Table 4), provided information about the degree of linearity of the calibrations. R^2 values (between 0.994 and 0.999) show that a significant portion of the variance of the calculated yA/yIS signals is explained by the analytical concentrations [A] in the suggested regression models. In general, the five resolvins were linear over the studied range of concentrations. The $F_{experimental}$ values for the five calibration models, which were less than the crucial value of 2.958 for 5 and 14 degrees of freedom at the 95% confidence level, further corroborate this result.

7.2.3 Recovery

The method's accuracy was evaluated in this study based on the recovery of the analyte spiked in the blank cell culture media. The analyte recovery is measured across the entire concentration range studied. In triplicate measurements, L-15 cell culture media is spiked with RvD1, RvD2, RvD3, RvD4 and RvD5 solution and a constant amount of IS at concentrations ranging from 15, 30 and 45 ng/ml. The percentage of recovery is computed by dividing the estimated concentration by the theoretical (spiked) concentration and multiplying by 100. The recovery of the method was higher than 95% for all resolvins as expressed in Table 4.

7.3 Quantification of Released Resolvins in L-15 Media by Head Kidney Cells

In order to investigate production of resolvins by salmon head kidney cells with and without exposure to exogenous DHA the proposed LLE methodology and further LC-MS/MS quantification were put into practice. At 6, 12, and 24 hours, the production of resolvins was measured and expressed in ng/mL in control (CTL) and DHA groups, as shown in Table 5.

Table 5 Temporal production of resolvins by head kidney cells from Atlantic salmon (Salmon salar) exposed to DHA.

Group	***RvD_1***	***RvD_2***	***RvD_3***	***RvD_4***	***RvD_5***
CTL-6	0.127 ± 0.022	0.190 ± 0.109	0.186 ± 0.055	0.601 ± 0.090	0.055 ± 0.018
CTL-12	0.074 ± 0.009	0.238 ± 0.141	0.119 ± 0.049	0.382 ± 0.082	0.069 ± 0.009
CTL-24	0.162 ± 0.010	0.093 ± 0.033	0.312 ± 0.073	0.587 ± 0.049	0.097 ± 0.022
DHA-6	3.92 ± 1.80	4.41 ± 1.39	6.85 ± 3.16	10.23 ± 6.34	3.70 ± 2.02
DHA-12	1.39 ± 0.40	2.57 ± 1.14	5.21 ± 2.33	4.74 ± 1.1	1.39 ± 0.36
DHA-24	4.39 ± 2.10	8.09 ± 4.13	22.92 ± 15.66	10.441 ± 5.12	10.92± 7.96

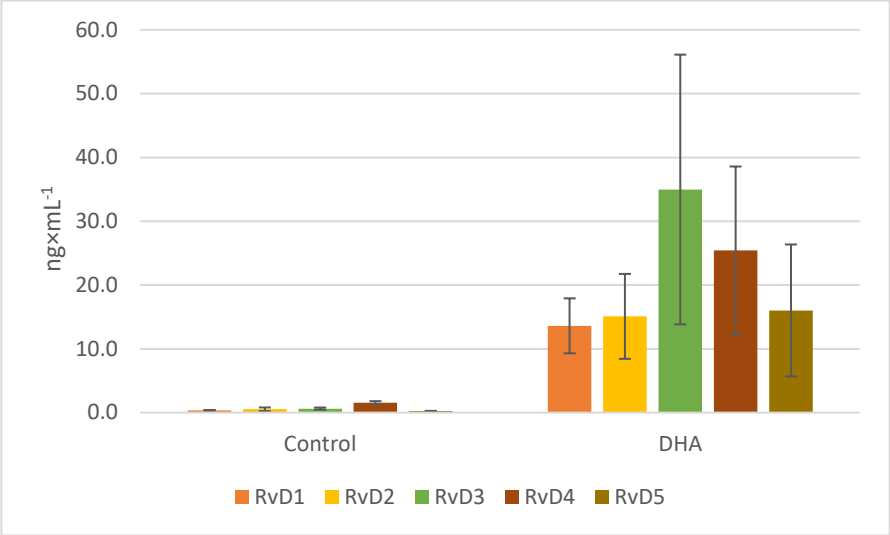

Figure 20 Graphical representation of resolvin production in head kidney cells with and without DHA.

The results presented in Table 5 and Figure 20 give a clear indication that production of resolvins is directly influenced by the presence of DHA as concluded by multiple researchers previously. It is also noteworthy that the levels of RvD3 are highest among the others. In decreasing order of concentration resolvins yield are in following order: RvD4 > RvD3 > RvD2 > RvD1 > RvD5 in control and RvD3 > RvD4 > RvD5 > RvD2 > RvD1 in DHA, respectively. These levels are consistent with previously published findings that estimated the production of RvD1 and RvD2 by salmon liver cells using SPE and LC-MS/MS. It was also suggested that after subjecting the cells to various polyunsaturated fatty acids, including DHA, the production of RvD4 was preferred over RvD1 and RvD2[100].

According to a principal component analysis (PCA), the concentrations of resolvins in the control and DHA groups described 76.00% of the overall data variability, while the different time frames explained 16.24% (Figure 21).

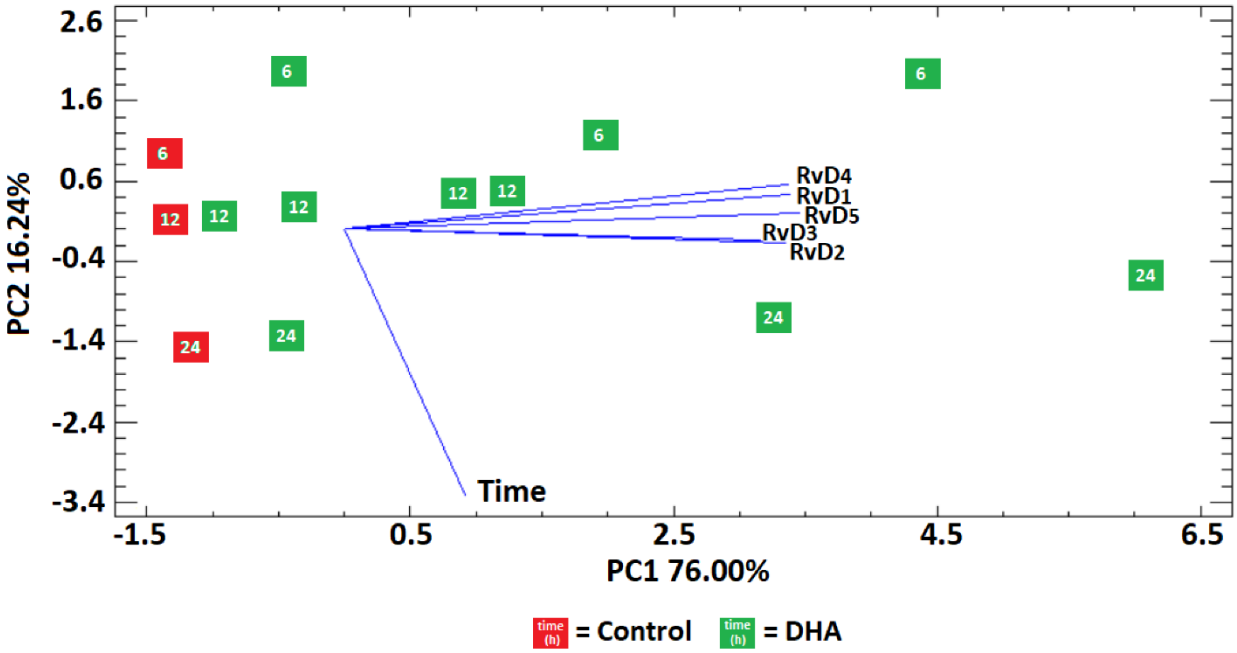

Figure 21 Principal component analysis of the released resolvins in L-15 media by head kidney cells with and without exposure to DHA, after implementing the proposed LLE protocol.

The PC1 axis allowed for easy differentiation between the control and DHA groups, which were distinguished by negative and positive scores, respectively. Within the DHA group as a whole, there was more overlap than there was within the control group. For instance, the scores for the control at 6, 12, and 24 h showed up as three separate clusters along PC2 (red squares), while the DHA's time clusters were separated along PC2 (green squares), but they were widely dispersed along PC1, showing a larger degree of dispersion in the DHA data. The PCA also revealed that the largest amounts of resolvins were associated with the DHA group, implying that exogenous DHA stimulated the formation of resolvins. The variations in RvD1, RvD2, RvD3, RvD4, and RvD5 concentrations were independent of time because the vectors of time and resolvin concentrations were orthogonal. The lack of a correlation between time and concentration was confirmed by examining the within and between variances for each resolvin at the three specified

times. The predicted p-values for any sort of response were not significant ($p > 0.05$) in either the control or DHA groups.

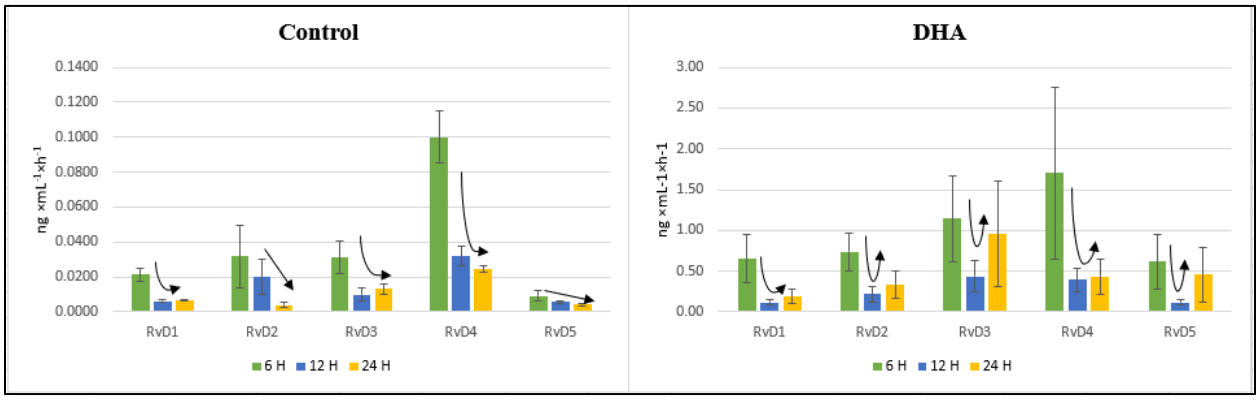

Figure 22 Concentration/time ratio for the different resolvins in the control and DHA group. The former group shows a continuous decrease in production and the latter a plausible regulated production by a circadian clock.

A relationship between concentration and time was computed from Table 5 for DHA and control which shows that over the time period from 6 to 24 hours there is a constant decrease in the concentration of resolvins in control samples (Figure 22). In contrast, subjective production of resolvins at midnight (24 h) than midday (12 h) suggests the presence of a circadian clock that may impose a 24 h rhythmicity on the head kidney cells to process the production of resolvins from the added 50 μM of DHA. It is not known clearly how the circadian rhythms affect resolvin synthesis. Results from a number of recent investigations show that circadian rhythm proteins regulate numerous metabolic processes[105].

The circadian release of glucocorticoids is well-known, and their connection to the downstream anti-inflammatory and pro-resolution mediator annexin A1 facilitates the return to homeostasis[106]. However, the direct impact of circadian rhythms on SPM and endogenous mechanisms in the resolution of inflammation remains largely unexplored. In keeping with this view, prostaglandin 15d-PGJ2 has recently been found to be an entrainment factor that is in tune with circadian oscillations and has the ability to exhibit anti-inflammatory characteristics[107]. Colas and colleagues investigated diurnal production of docosapentanoic acid induced resolvins $RvD_{n\text{-}3DPA}$. They

concluded that in patients at risk for myocardial infarction, the production and diurnal regulation of these mediators were significantly altered. These alterations were connected to decreased 5-lipoxygenase expression and activity as well as elevated levels of systemic adenosine[108]. In the light of the current findings, it is conceivable that an immunological clock that regulates resolvin/15-LOX activity drives the generation of resolvins from endogenous DHA.

8 Conclusion and Future Perspectives

For the past decade a great deal of work has been done in identification and quantification of resolvins. However, not all researchers explored the aspects explored in this study specifically the cell culture analysis of resolvins in Fish. The current study gives insights into developing the best strategy for extraction of resolvins from L-15 media with high precision and accuracy.

The use of a factorial experimental design to estimate the optimal concentration of multiple internal standards for the simultaneous analysis of a wide variety of resolvins is simple and convenient. Moreover, preference of LLE over SPE saved not only a lot of time but also prevented incurrence of high costs and is environment friendly, since disposable SPE cartridges are expensive and a menace to the environment.

The proposed LLE method, that requires three solvents, is an important analytical tool for the analysis of resolvins, considering that the popular SPE (not to mention combined with LLE) is a time-consuming and complicated method that requires multiple steps and different solvents prior to LC.MS/MS analysis. For instance, the current SPE protocol for the analysis of resolvins at the Institute of Marine Research requires a total of seven different solvents (six solvents for SPE and one solvent for final reconstitution) making it material/time consuming, expensive, in addition to the amount of generated waste to the environment (solvents plus SPE cartdriges).

The method validation reveals that the method is highly selective and produces LOD (0.028-0.059 ng/mL) and LOQ (0.074-0.180 ng/mL) values within the previously reported ranges for cell cultures[102]. It requires a very small amount of solvent and media for sample extraction. Multiple analytes can be analyzed simultaneously. The sample manipulation is minimal thereby reducing the risk of degradation, contamination, analytes loss and exposure to infectious biological materials. The method is reproducible with high recoveries, making it easier to extend the research with reduced errors and ambiguity.

Knowledge of the involvement of a circadian clock in the production of resolvins and the present analytical approach are valuable tools in understanding the production of resolvins by human cells in-vitro and in-vivo in order to potentially open new windows in inflammation therapies. The *in-vivo* and *in-vitro* trials can be conducted in collaboration with other institutes and hospitals to understand and monitor the circadian production of resolvins in humans. Many diseases in humans

like hypertension, arthritis, atherosclerosis, depression, diabetes mellitus, myocardial infarction, thrombosis, and several malignancies respond well to DHA supplementation. Furthering the exploration of current research in humans by exposing cells to DHA may pave ways to new therapeutic avenues. The circadian production of resolvins by DHA will aid in designing dosage regimen of DHA to aid in treatment of various diseases.

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