The effects of sous-vide cooking on the bio-functionality, nutritional value and health benefits of salmon lipids.

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Table of Contents

Abstract: .................................................................................................................................................. 4
Declaration ................................................................................................................................................... 5
Acknowledgements ......................................................................................................................................... 6
List of Tables .................................................................................................................................................. 7
List of Figures ................................................................................................................................................ 8
List of Abbreviation ....................................................................................................................................... 10
Chapter 1: Literature review .......................................................................................................................... 13
  1.1 The key role of Inflammation and platelet aggregation in Chronic Disorders with emphasis to cardiovascular diseases .................................................................................................................. 14
  1.2. The benefits of Healthy dietary patterns and fish intake ................................................................. 16
  1.3 Salmon lipid content and bio-functionality ....................................................................................... 20
  1.3.1 Classes of Lipids found in salmon: .............................................................................................. 21
  1.3.2 Fatty acid composition of salmon: Bioactive FA of salmon ...................................................... 22
  1.3.3 Salmon Polar Lipids ...................................................................................................................... 25
  1.4. Cooking and its effects on lipid content in fish in general ................................................................. 27
  1.4.1 Emerging Cooking Techniques i.e. sous-vide processing ......................................................... 32
  1.4.2 Benefits of sous-vide processing ................................................................................................. 33
  1.4.3 Negative Aspects of sous-vide processing ................................................................................... 34
  1.4.4. Sensory Analysis ......................................................................................................................... 34
  1.4.5 Aim of Study ................................................................................................................................... 36
Chapter 2: Materials and methods .................................................................................................................. 37
  2.1 Chemicals and Reagents ...................................................................................................................... 37
  2.2 Sous-vide process ................................................................................................................................. 37
  2.3 Extraction & separation of Bioactive lipid compounds from cooked salmon ............................... 40
  2.4 Platelet aggregometry bioassay ......................................................................................................... 41
  2.5 LC-MS Analysis ................................................................................................................................. 43
2.6 Sensory Analysis.................................................................44
2.7 Statistical Analysis.............................................................46
Chapter 3: Results ..................................................................47
3.1 Yield of extraction and separation of bioactive lipids from cooked salmon........47
3.2 The effect of several sous-vide cooking preparations on the bio-functionality of salmon lipids against platelet aggregation .................................................................49
3.3 The effect of several sous-vide cooking procedures on the Fatty acid content and structures of bioactive salmon lipids.................................................................56
3.4 Sensory Analysis..................................................................61
Chapter 4: Discussion ..............................................................65
4.1 Introduction to discussion ......................................................65
4.2 Effects of different sous-vide preparations on the yield of extraction and separation of bioactive salmon PLs.................................................................66
4.3 The effect of several sous-vide cooking preparations on the bio-functionality of salmon PLs against platelet aggregation .................................................................67
4.4 The effect of several sous-vide cooking preparations on the fatty acid content in relation to the bio-functionality of salmon PLs.................................................................74
4.5 Sensory Analysis..................................................................78
Chapter 5: Conclusions ..............................................................80
Appendices ...........................................................................81
Appendices I ...........................................................................82
Appendices II ...........................................................................90
Appendices III ...........................................................................91
References ............................................................................94
Abstract:

Cardiovascular diseases has become the major cause of death globally. Inflammation and platelet aggregation play crucial role in the development and progression of atherosclerosis and CVD. Inflammation, atherosclerosis, and endothelial dysfunction and ultimately cardiovascular events. Fish and especially oily fish such as Salmon are vital components of healthy dietary patterns with preventive properties against CVD. Raw salmon contain bioactive polar lipids (PLs) with strong antithrombotic bioactivities. However, salmon is mainly consumed cooked. The aim of the present study was to evaluate for the first time the effects of the emerging mild sous vide cooking processing on the antithrombotic properties, lipid content and sensory characteristics of salmon.

Total lipids (TLs) were extracted from brined and un-brined salmon sous vide preparations in several temperatures (52°C, 65°C and 80°C) and further separated into the Neutral Lipids (NLs) and PLs fractions. The antithrombotic properties of salmon PLs were tested in human platelets, while their fatty acid content was determined by LC-MS analysis. Sensory tests were performed using napping followed by check-all-that apply. Sous vide salmon preparations in all conditions did not affect the taste, whereas in 65°C and 80°C slight textural differences were observed. Mild sous vide cooking of brined or un-brined salmon in low temperatures (52-65°C) did not affect the potent inhibitory effect of salmon PLs against human platelet aggregation induced by the inflammatory and thrombotic mediators’ platelet-activating factor (PAF) and thrombin, but also by the well-established platelet agonists adenosine diphosphate (ADP) and collagen. Sous vide preparations of both brined and un-brined salmon in higher temperatures, such as those usually applied for pasteurization (80°C), resulted in reduction of these antithrombotic properties of salmon. This was accompanied by a reduction of the EPA and DHA content of the salmon in these conditions in the un-brined sous vide preparations. Nevertheless, the antithrombotic properties of salmon PLs were preserved in considerable potency even when high temperatures were applied for the sous vide salmon preparations. This outcome if combined with the observed low values of the n-6/n-3 ratio of the PUFA content in the salmon PLs of all sous vide preparations, further suggest a beneficial role for such a mild cooking procedure for preserving the cardio-protective properties of salmon.
Declaration:

I hereby declare that this is my own work and it has not been submitted for the award of degree at any other university

Signed: ________________________________
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List of Tables

Table 1.1. Fatty Acid composition of raw salmon ........................................23

Table 1.4. The effects of cooking on the lipid content of salmon using various conventional cooking techniques.................................................................29

Table 3.1. The Total lipid (TL), total neutral lipid (TNL) and the total polar lipid (TPL) expressed as g/100g of sample with TNL and TPL also being expressed as a percentage of TL ........................................................................................................49

Table 3.4. Multiple pairwise comparisons of products applying the McNemar test on the data from CATA.................................................................................................63

Table 5.1. The in vitro antithrombotic properties of the TPL of all samples against PAF/thrombin/collagen/ADP- induced platelet aggregation. The results are presented as mean IC$_{50}$ value in micrograms (µg) ± SD (n=3).........................................................90

Table 5.2. Fatty acid composition of the various different cooking preparations of the bioactive salmon lipids. Results are presented as percentage of the total fatty acid content, n=3)........................................................................................................91
List of Figures:

**Figure 1.** Flow diagram of the development of CVD and possible prevention by a healthy diet………………………………………………………………………………….16

**Figure 1.1.** An image demonstrating the role of PAF and PAF-R in response to inflammatory stimuli…………………………………………………………………………………..18

**Figure 1.2** (A) Structural representation of a triglyceride molecule. (B) Structural representation of a glycerol-based polar lipid molecule; the polar lipid structure bears two hydrophobic, hydrocarbon chains esterified to the glycerol backbone in the sn-1 and sn-2 position, with a charged phosphate group at the sn-3 position. (C) Classic sphingosine-based polar lipid (D) Alkyl-acyl-glycerol based polar lipids (Image modified with permission from Dr. Tsoupras (Lordan et al., 2017))……………………………………………………………………………………………………….21

**Figure 1.3.** (A) Typical structure of a PAF molecule. (B) Image representing biologically active phospholipids. (C) Image representing biologically active salmon PL bearing EPA and DHA within their structures. Reproduced with modifications after permission from Dr. Tsoupras (Tsoupras et al., 2019a)……………………………………………………………………………………………………….26

**Figure 2.1.** The sous-vide cooking process using brined/un-brined salmon fillets and various cooking temperatures……………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………...
Figure 3.2. Antithrombotic activities of TPL extracts from various salmon samples (raw and various sous-vide preparations) against thrombin-induced aggregation of human platelets………………………………………………………………………………………………52

Figure 3.3. Antithrombotic activities of TPL extracts from various salmon samples (raw and various sous-vide preparations) against ADP-induced aggregation of human platelets………………………………………………………………………………………………53

Figure 3.4. Antithrombotic activities of TPL extracts from various salmon samples (raw and various sous-vide preparations) against collagen-induced aggregation of human platelets…………………………………………………………………………………………54

Figure 3.5. Antithrombotic activities of TPL extracts from salmon heads (raw and different sous-vide preparations) against platelet-activating factor (PAF)/thrombin/ADP/collagen-induced aggregation of human platelets………………..56

Figure 3.6. Representative chromatograms of the LC-Ms analysis of the saponified salmon TPL from all sous vide preparations…………………………………………………………58

Figure 3.7. The fatty acid composition of the various different cooking preparations of the bioactive salmon lipids………………………………………………………………………………59

Figure 3.8. Bi-plots (PCA) from multiple factor analysis on the napping data……………………………………………………………………………………………………………………………………………………………………………….61

Figure 3.9. Symmetric plot of products as revealed from the principle coordinate analysis on the CATA data………………………………………………………………………………62
List of Abbreviations:

AA - Arachidonic acid
AC - Adenylate cyclase
ACS - Acute coronary syndrome
Akt - Protein kinase B
ADP- Adenosine diphosphate
ATP- Adenosine triphosphoric acid
BMI- Body Mass Index
BSA - Bovine serum albumin
cAMP- cyclic adenosine monophosphate
CATA – Check-all-that-apply
CDP - Cytidine diphosphate
CHD - Coronary heart disease
CL - Cardiolipin
CNS - Central nervous system
COX – Cyclooxygenase
cPLA₂ – cytosolic phospholipase A₂
CVD - Cardiovascular disease
DAG - Diacylglycerol
DASH - Dietary approaches to stop hypertension
DHA- Docosahexaenoic acid
DPA - Docosapentaenoic acid
eNOS - Endothelial nitric oxide synthase
ERK - Extracellular signal-regulated kinases
EPA - Eicosapentaenoic acid
FAME – Fatty acid methyl ester
LC-MS – Liquid chromatography-mass spectrometry
HCAs – Heterocyclic aromatic amines
IL - Interleukin
iNOS - Nitric oxide synthase
LDLc – Low-density lipoprotein cholesterol
L-PC- Lyso-phosphatidylcholine
LPCAT - Acetyl-CoA: lyso-PAF acetyltransferases
MAPK – mitogen activated protein kinase
MFA – Multiple Factor Analysis
MMP- metalloproteinase
mTOR- mechanistic target of rapamycin
MUFA – Monounsaturated fatty acid
NADPO – nicotinamide adenine dinucleotide phosphate oxidase
NF-kB - Nuclear factor-kappa light-chain-enhancer of activated B cells
PAF - Platelet-activating factor
PAF-R - Platelet-activating factor receptor
PC - Phosphatidylcholine
PE – Phosphatidylethanolamine
P.E- Petroleum ether
PI – Phosphatidylinositol
PI3K - phosphatidylinositol 3-kinase
PKC - protein kinase C
PKA - protein kinase A
PLC - phospholipase C
PLs – polar lipids
PPP – Platelet-poor plasma
PRP – Platelet-rich plasma
PUFA- Polyunsaturated fatty acid
RNS - Reactive nitrogen species
ROS - Reactive oxygen species
SD – Standard deviation
SF- Salmon fillets
SH- Salmon heads
SFA- Saturated fatty acid
SM – Sphingomyelin
TFA – Trans fatty acid
TG - Triglyceride
TL – Total lipids
TNF-α - tumour necrosis factor
TNL – total neutral lipids
TPL – Total polar lipids
VEGF – vascular endothelial growth factor
XO – xanthine oxidase
Chapter 1:

Literature review
1. Introduction

1.1 The key role of Inflammation and platelet aggregation in Chronic Disorders, with emphasis to cardiovascular diseases

The global burden of systemic chronic diseases such as obesity and type 2 diabetes mellitus, cardiovascular disease (CVD), cancer, etc. is constantly rising, resulting in significant losses of life and disability adjusted life years in both developing and developed countries (Mathers and Loncar, 2006). The World Health organisation (WHO 2017) has estimated that one in three global deaths is because of CVD related events such as myocardial infarction and stroke (WHO). In 2017, WHO released figures stating that approximately 17.7 million global deaths were due to CVD-related events (WHO). According to Irelands Health Service Executive, approximately 10,000 Irish people die each year as a result of CVD, including coronary heart disease (CHD), stroke and other circulatory diseases (Tsoupras et al., 2018a). According to the American Heart Association, a similar worldwide trend exists. CVD globally accounts for more than 17.3 million deaths annually which is predicted to rise even further to 23.6 million by the year 2030 (WHO 2017). Looking at these numbers, it is evident that the development and effects of CVD is of major concern worldwide.

It is now widely regarded that one of the underlying mechanisms at cellular and molecular level, which is related to a common mechanistic pathway of the initiation and progression of several chronic diseases is chronic inflammation and in particular, that affecting the endothelium (Tsoupras et al., 2019a).

Inflammation and platelet aggregation represent a physiological reaction of the innate immune system in order to maintain and protect a constant internal milieu while being exposed to continuously changing environmental pressures, irrespective of whether the initial causes originate from microbial infection, traumatic injury or metabolic dysfunction. The inflammatory response aims to reduce the agent that causes tissue injury (and/or minimise these effects) to induce appropriate wound healing and repair programs while restoring tissue homeostasis.

The inflammatory response occurs when the innate immune cells such as blood leukocytes, mast and dendrite cells are activated. Activated immune cells produce various toxic agents such as reactive oxygen species (ROS), reactive nitrogen species
(RNS), elastases, cathepsins, eicosanoids, proteinases, but also inflammatory and thrombotic mediators such as platelet activating factor (PAF) and thrombin and platelet agonists such as collagen and ADP (Adenosine diphosphate). All the above can induce or suppress inflammation, depending on the initial stimuli and of the phase of inflammatory injury (Li et al., 2010, Tsopanoglou and Maragoudakis, 2009). Provided that the immune response succeeds in eliminating the infectious agents or repairing the initial tissue injury, the inflammatory process will be terminated at an appropriate time and therefore only marginally affect tissue function.

However, in cases where the inflammation fails to resolve, for example, due to persistence of a pathogen or the unsuccessful repairing of the initiating injury and tissue dysfunction, a sustained underlying inflammatory process develops, leading to tissue dysfunctions and detrimental consequences for the established chronic inflammatory condition (Chen et al., 2017). Chronic and unresolved inflammatory manifestations that accumulate in the walls of arteries trigger the onset of atherosclerosis, a chronic developing vascular disease (Rafieian-Kopaei et al., 2014, Tsoupras et al., 2018b, Tsoupras et al., 2019b) Upon progression, this disease may lead to ensuing major cardiovascular events (Demopoulos et al., 2003; Tsoupras et al., 2018b).

Atherosclerosis is widely regarded as the principal cause of CVD-related events that lead to morbidity and mortality. It is apparent that inflammation, thrombosis and platelet activation-aggregation play vital role in all stages of the formation of vascular lesions perpetuated and intensified by risk factors such as diet, lifestyle, smoking, autoimmune diseases, hypertension etc (Demopoulos et al., 2003, Tsoupras et al., 2018b). Therefore, healthy dietary patterns have been proposed for both Primordial and Primary prevention to prevent inflammation, atherosclerosis, and endothelial dysfunction from taking hold, and thus reduce risk and ultimately cardiovascular events (Yu et al., 2018).
Figure 1. Flow diagram of the Development of CVD and Possible Prevention by a healthy diet (Yu et al., 2018).

1.2. The benefits of Healthy dietary patterns and fish intake

Nutrition notably should be regarded as a lifestyle issue and implemented as a vital biochemical tool against the formation of such chronic diseases as CVD (Tierney et al., 2019, Tsoupras et al., 2018b). The cross-talk of pathways of several inflammatory and thrombotic mediators, but also of platelet aggregation agonists such as PAF, thrombin, collagen and ADP has been implicated in these chronic disorders (Tsoupras et al., 2018b). Diet has been found to influence these pathways either detrimentally (Westernised Diet) or beneficial (Mediterranean Diet) (Tierney et al., 2019, Tsoupras et al., 2018b).

Over the last 5 years, the beneficial outcomes of the adoption of the Mediterranean Diet (Med-diet) have come to prominence (Tierney et al., 2019). The Med-diet and its
cardioprotective attributes has been attributed to several of its pleiotropic protective effects (Tierney et al., 2019, Tsoupras et al., 2018b). The beneficial effects of the Med-diet include reduction in insulin resistance, reduction of lipid levels, lowering of BMI and blood pressure and the improvement of HDL-cholesterol functionality (Tierney et al., 2019, Tsoupras et al., 2018b). Notably, the primary beneficial effect of the Med-diet is on the amelioration of endothelial functionality and the decrease of the inflammatory milieu, inflammatory-related mediators, biomarkers such as platelet-activation factor (PAF), and several other (Tsoupras et al., 2018b). Moreover, it has been proposed that there are beneficial effects on oxidative stress, with lower concentrations of oxidised LDL and improved apolipoprotein profiles, and, in conclusion, evidence exists of the beneficial effects against platelet aggregation and blood coagulation (Tierney et al., 2019, Tsoupras et al., 2018b). It is now well established that several micro-constituents of the Med-Diet affect platelet aggregation and activation of inflammatory pathways and cells by their effects on the receptors and/or on the metabolism of the mediators involved in these pathways, such as the PAF-pathway (Figure 1).

Fish is one of the main animal-origin constituents of such healthy dietary patterns. The nutritional guidelines of healthy dietary patterns favours fish-consumption twice per week or once a week for oily fish such as salmon and sardines (Goel et al., 2018). For example, in the ATTICA study fish consumption exhibited favourable effects against CVD (Zampelas et al., 2005). More specifically, the dietary pattern in this study that was mainly characterized by cereals, small fish, hardtack and olive oil intake, was associated with lower CVD risk (Zampelas et al., 2005). In addition, meta-analysis of observational studies investigating fish consumption and the correlation with incidences of coronary heart disease mortality concluded that individuals whom consume fish had a 15% reduced risk in comparison to individuals who did not consume fish (He et al., 2004). Moreover, the consumption of fish and fish oils is also associated with an improvement of platelet functionality in CVD and several other inflammation related disorders (Mori, 2017, Goel et al., 2018, Tsoupras et al., 2019c, Tsoupras et al., 2018c, Lordan et al., 2017).
Figure 1.1. An image demonstrating the role of PAF and PAF-R in response to inflammatory stimuli.

(A1) Pro-inflammatory stimuli stimulate the syntheses of PAF and the expression of PAF-R on the surface of cells which can result in the amplification of certain pro-inflammatory stimuli. (A2) The binding of PAF to PAF-R signals the initiation of several inflammatory responses such as the release of PAF from the activated cells and the production of more arachidonic acid within the body to be used as a substrate in the formation of more PAF. (A3) Further activation of PAF-R on the surface of cells signals the release of
chemokines, expression of adhesion molecules on endothelial cells, and release/synthesis of PAF, further amplifying the inflammatory response. This results in the cellular signalling for the recruitment of lymphocytes to the site of inflammation where lymphatic cells bind to the adhesion molecules expressed on the surface of the damaged endothelial cells. (B) The inflammatory cascade leads to an increase in the concentration of PAF at the site of inflammation and so promotes a broad spectrum of effects caused by the increased levels of PAF and other pro-inflammatory mediators circulating the blood. The increased levels of PAF, chemokines, and other inflammatory mediators in the bloodstream causes further activation of PAF-R. This promotes the aggregation of platelets and leukocytes and subsequent damage and activation of other endothelial cells downstream. These damaged endothelial cells promote leukocyte adherence, invasion, migration and subsequent endothelial dysfunction, thus stimulating the development of an inflammatory-related chronic disorders as seen in this image. (C) Demonstrates how PAF antagonists, such as microconstituents of the Mediterranean diet, can act to disrupt the binding of PAF to the PAF-R. This disruption of PAF, possibly occurs by competitive displacement and, could aid in lowering PAF concentrations in the blood (Image reproduced with permission from (Tsoupras et al., 2018b).

Therefore, several dietary guidelines (i.e. The US Dietary Guidelines) and healthy dietary patterns (i.e. the Mediterranean diet) recommend fish intake at least twice weekly (Lordan et al., 2017, Megson et al., 2016, Raatz et al., 2016, Tsoupras et al., 2018a). In particular, intake of farmed Atlantic salmon twice weekly influences lipoprotein particle size, decreases serum concentrations of triglycerides, and increases HDL cholesterol in a manner associated with CVD risk reduction (Zhang et al., 2012b, Hagen et al., 2016, Tsoupras et al., 2018a). It has also been proposed that salmon may possess natural resistance mechanisms to reduce its own risk of atherosclerosis and cardiovascular risk, which corresponds well with its high investment in its own lipid metabolism and lipid profile (Tsoupras et al., 2018a, Dalum et al., 2016). Epidemiologic studies have shown that high dietary consumption of salmon and other oily fish, such as herring and trout, is associated with reduced rates of myocardial infarction, atherosclerosis, and other ischemic pathologies (See Appendix I). In addition, dietary fish oil induces changes in cardiac function that might contribute to cardiovascular health benefits in humans and does so by modifying cardiac membranes within a dose range achievable in the human diet (Gladysev et al., 2006).
Although fish such as salmon can be served raw in preparations such as sushi and ceviche, fish is generally consumed using a cooking technique. This cooking technique can lead to conformational changes in the composition of the food stuff which could affect the nutrient content and therefore the health benefits of consumption of fish such as salmon. Even though the health benefits of fish-consumption and their lipid contents are universally recognized today by medical professionals, there is limited reviewing on the effects of cooking and emerging mild cooking techniques such as the sous vide. Especially for salmon, this mild cooking technique has recently gained a lot of attention, and thus the study of the effects of such a sous-vide cooking process on salmon lipid content, activities and bio-functionality is significant for elucidating the benefits of applying mild cooking techniques that can preserve the bio-functionality of fish.

1.3 Salmon lipid content and bio-functionality

Salmon has been characterised as an oily fish (Gil and Gil, 2015). It is rich in bioactive lipids and thus it has been proposed as a classic beneficial fish intake in several dietary guidelines. Consumption of fish such as salmon regularly has been shown to have cardio-protective effects as its lipid content displaces intakes of pro-atherosclerotic foods higher in saturated fatty acids and trans fatty acids in the diet (Burri et al., 2012, Tsoupras et al., 2018b, Tsoupras et al., 2018a). In addition, the beneficial effects of fish such as salmon consumption and of related marine oils, were primarily attributed to their high content of n-3 polyunsaturated fatty acids (PUFA), such as the eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids) and their eicosanoid-related anti-inflammatory and antiplatelet effects (Mori et al., 1997). Thus, significant research has focused on n-3 fatty acid EPA and DHA, which have been shown to demonstrate several beneficial properties for human health.

However, apart from the n-3 content of fish, several other studies have proposed that the beneficial effect of fish intake on cerebrovascular risk is likely to be mediated through the interplay of a wide range of lipid nutrients abundant in fish (Goel et al., 2018, Chowdhury et al., 2012, Lordan et al., 2017). Therefore, it is important to classify the lipid content of salmon and its health impact, detrimental or beneficial.
1.3.1. Classes of Lipids found in salmon

Lipids are classed as an extremely diverse class of biomolecules possessing a vast array of functions and structures. They can be divided generally into two important subclasses: neutral lipids (such as triacylglycerols, fatty acids, waxes, terpenes etc.) and polar lipids (such as glycolipids, phospholipids etc.) that contain both a hydrophobic hydrocarbon residue and also a polar hydrophilic group ie. a carbohydrate group, or a phosphate head group which possesses a hydrophilic residue within its structure (Figure 1.2). Salmon contains several of these lipid classes, with highly bioactive being mostly its fatty acid composition and polar lipid content (Tsoupras et al., 2018a, Tsoupras et al., 2019a) Tsoupras et al. (2018) reported total lipid value of Irish organic farmed salmon of 5.51±1.90g of lipids per 100g of salmon fillet which is quite higher than other fish species but being classified as an oily fish this is in agreement with the literature (Tsoupras et al., 2018a). Total lipid was further separated into total neutral lipid (TNL) and total polar lipid (TPL). The TNL value was 4.66±0.28g of lipids per 100g of salmon fillet whereas the TPL value 0.86±0.36g.

![Figure 1.2](image-url) 

**Figure 1.2** (A) Structural representation of a triglyceride molecule. (B) Structural representation of a glycerol-based polar lipid molecule; the polar lipid structure bears two hydrophobic, hydrocarbon chains esterified to the glycerol backbone in the sn-1 and sn-2 position, with a charged phosphate group at the sn-3 position. (C) Classic sphingosine-based polar lipid. (D) Alkyl-acyl-glycerol based polar lipids (Image modified with permission from Dr Tsoupras (Lordan et al., 2017)).
1.3.2 Fatty acid composition of salmon; Bioactive FA in salmon

Knowledge of the FA composition of commercially important fish species such as salmon is required owing to the post mortem deterioration and changes of the nutritional value of fish. The fish lipid composition also has applications in food, health care, pharmaceutical products and as ingredients in feed in agriculture and the aquaculture industry (Moroney et al., 2015).

The fatty acid composition of salmon as reviewed by several research teams is presented in Table 1.1. More specifically, it was reported that in raw King Salmon, the most abundant fatty acids were MUFA which accounted for 43.8% of total fatty acids followed by PUFA accounting for 28.23% and SFA 27.97% of total fatty acid. In the raw salmon samples, oleic acid was present in the highest quantity (32.36%). The dominant PUFA was linoleic acid being the most abundant n-6 fatty acid (9.54%). DHA (7.36%) was the most dominant n-3 fatty acid present followed by EPA (5.7%). Palmitic acid (17.9%) and stearic acid (5.26%) were the most dominant SFA present in the raw salmon samples (Larsen et al., 2010). The composition of the fatty acids in raw salmon in this article were similar with results obtained by other authors (Bastias et al., 2017, Şengör et al., 2013). Larsen et al., 2010 furthermore reputed a n-6/n-3 of 0.68 which was in accordance with other authors (Bastias et al., 2017) but lower than the findings of (Şengör et al., 2013, Al-Saghir et al., 2004).

Table 1.1. Fatty acid composition of raw salmon.

<table>
<thead>
<tr>
<th>Raw Salmon</th>
<th>Larsen et al. 2010*</th>
<th>Bastias et al. 2017*</th>
<th>Sengor et al. 2014*</th>
<th>Al-Saighir et al. 2004*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>27.97</td>
<td>26.58</td>
<td>17.49</td>
<td>15.90</td>
</tr>
<tr>
<td>MUFA</td>
<td>43.80</td>
<td>44.7</td>
<td>39.64</td>
<td>53.70</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>32.36</td>
<td>36.44</td>
<td>31.39</td>
<td>23.00</td>
</tr>
<tr>
<td>PUFA</td>
<td>28.23</td>
<td>26.74</td>
<td>28.47</td>
<td>30.20</td>
</tr>
<tr>
<td>n-3</td>
<td>15.91</td>
<td>15.9</td>
<td>14.66</td>
<td>17.10</td>
</tr>
<tr>
<td>EPA</td>
<td>5.70</td>
<td>5.83</td>
<td>4.81</td>
<td>4.30</td>
</tr>
<tr>
<td>DHA</td>
<td>7.36</td>
<td>7.64</td>
<td>5.74</td>
<td>6.70</td>
</tr>
<tr>
<td>n-6</td>
<td>10.89</td>
<td>10.84</td>
<td>12.56</td>
<td>9.30</td>
</tr>
<tr>
<td>n-6/n-3†</td>
<td>0.68</td>
<td>0.68</td>
<td>0.85</td>
<td>1.26</td>
</tr>
</tbody>
</table>

*Results are expressed as expressed as a percentage of the total fatty acid. † The ratio of the total amount of n-6 fatty acids divided by the total amount of n-3 fatty acids.
Consumption of oily fish such as salmon that is rich in n-3 PUFA such as DHA and EPA has been associated with several health benefits, such as improved platelet functionality and cardiovascular health (Mori et al., 1997, Mori, 2018, Goel et al., 2018) while a low value of the ratio of n-6/n-3 PUFA in a diet seems also to provide several beneficial health outcomes in CVD and other chronic disorders (Simopoulos, 2002). These effects were primarily attributed to their high content of n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) acid, and their eicosanoid-related antiplatelet effects (Mori, 2018).

A significant number of studies on the health benefits of n-3 PUFAs have been assessed (Ferraro et al., 2013, Bhatt et al., 2019a, Bhatt et al., 2019b). These fatty acids are classed as “essential fatty acids” as they must be obtained via the diet and in particular, the consumption of fish and fish oils rich in such fatty acids. The consumption of the aforementioned fatty acids is essential in the maintenance of homeostasis. It is reported that EPA is an initial substrate for resolving and eicosanoid synthesis.

The initial studies on the health benefits of n-3 PUFAs originates in the early 1970’s when Dyerberg and Bang observed the effects of fish consumption amongst the Greenland Eskimos and its correlation with incidences of atherosclerosis and thrombosis (Dyerberg et al., 1975). Further studies have demonstrated the importance of n-3 PUFAs at cellular levels for maintaining membrane homeostasis, its influence on gene expression and its vital importance for an optimal balance with n-6 FA to regulate the inflammatory response (Tierney et al., 2019).

More specifically, EPA has universally-accepted antiplatelet effects (Thorngren and Gustafson, 1981), whereas DHA is a structural fatty acid in nervous tissues such as the brain and the retina (Tierney et al., 2019, Goel et al., 2018, Ferraro et al., 2013). Recent studies have also shown that supplements of EPA at high doses (4 g/day) reduce both major adverse cardiovascular events (MACE) and hard endpoints in the REDUCE-IT clinical trial (Reduction of Cardiovascular Events With Icosapent Ethyl–Intervention Trial) (Bhatt et al., 2019b), while EPA reduced both first events and recurrent events (Bhatt et al., 2019b). Interestingly, the protective effects of n-3 fatty acids are more evident in patients with low fish consumption (as shown in the VITAL clinical trial (Vitamin D and Omega-3 Trial) (Manson et al., 2019).
On the other hand, there are several recent reviews and meta-analyses highlighting that marine oil n-3 PUFA supplements (as purified fatty acids or esters and triglycerides) do not affect the risk of major adverse cardiac events, cancer and all-cause death (Goel et al., 2018, Manson et al., 2019, Rizos et al., 2012, Enns et al., 2014, Walz et al., 2016, Kwak et al., 2012, Chowdhury et al., 2012, Lordan et al., 2017). Furthermore, some of these studies suggest that the observed beneficial effects of fish and fish oils are likely to be mediated through the interplay of other beneficial lipid nutrients (Lordan et al., 2017, Goel et al., 2018, Chowdhury et al., 2012).

The therapeutic dose of n-3 fatty acids depends not only on the degree of disease severity, but also on the form that these essential lipids are consumed, with different digestion mechanisms and bioavailability in cell membranes and lipoproteins (Goel et al., 2018, Lordan et al., 2017, Burri et al., 2012, Davidson et al., 2012).

Marine DHA or EPA themselves are usually moieties of lipid molecules such as esters, triglycerides (TG), or polar lipids (e.g., phospholipids and glycolipids). Although the most abundant DHA- or EPA-rich lipid class in most marine by-products is TG, some of the fisheries by-product sources or poorly-used marine resources are rich in DHA- or EPA-containing phospholipids that are often called marine phospholipids (Vázquez et al., 2019). TG and esters are typical hydrophobic compounds, while on the other hand, phospholipids are amphiphilic compounds. For this reason, the phospholipid form of DHA and EPA are considered to be much more bioactive and bioavailable than those of TG and esters, but also more effective in delivering the desired PUFA to the desired tissue when administrated, especially in difficult to reach tissues such as the brain because they can surpass the blood–brain barrier (Lordan et al., 2017, Tsoupras et al., 2019a, Tsoupras et al., 2018a, Burri et al., 2012, Takahashi and Inoue, 2012).

Within this concept, promising outcomes have been attributed to polar lipids (PLs) of marine origin (Lordan et al., 2017), especially to those bearing ω3 PUFA within their structure (Lordan et al., 2017, Tsoupras et al., 2019a, Tsoupras et al., 2018a, Burri et al., 2012, Davidson et al., 2012, Bjorndal et al., 2014), with strong anti-inflammatory, antithrombotic, and cardioprotective activities potentially mediated through anti-PAF and anti-thrombin effects (Tsoupras et al., 2018b, Lordan et al., 2017, Tsoupras et al., 2019a, Tsoupras et al., 2018a, Nasopoulou et al., 2011, Kalogeropoulos et al., 2008, Antonopoulou et al., 2005).
1.3.3 Salmon Polar Lipids

The classes of PLs of importance are sphingoPLs, ether glycerolipids and glycerolPLs. Glycerolipids can be further separated into different subgroups defined by the head group located in the PL. The head group can comprise of serine, inositol, choline, glycerol or ethanolamine. The most common PLs in marine sources are phosphatidycholine (PC), which consists of a choline head group and phosphatidylethanolamine (PE), which contains an ethanolamine head group. Phosphatidylinositol, lysophosphatidylcholine, phosphatidylserine and sphingomyelin are found to lesser extents in marine sources. Generally, oily fish such as mackerel, herring and salmon are the most predominant sources of marine PLs (Lordan et al., 2017).

Salmon contain between 0.5-1.3g of polar lipids per 100g of fillet tissue (Tsoupras et al., 2018a). The most abundant polar lipids present in fatty oily fish such as herring and salmon are derivatives of phosphatidylcholine (PC), the second most predominant been phosphatidylethanolamine (PE) followed by phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatidylcholine (lyso-PC) and sphingomyelin are also present but in lesser quantities (Lordan et al., 2017) Notably, the principal PLs class of oily fish such as salmon derived PLs is PC, typically binding with unsaturated omega-3 PUFAs, predominantly EPA and DHA and to a lesser extent stearidonic acid and docosapentarnoic acid (Tsoupras et al., 2018a, Burri et al., 2012) (Figure 1.3).
Figure 1.3. (A) Typical structure of a PAF molecule. (B) Image representing biologically active phospholipids. (C) Image representing biologically active salmon PL bearing EPA and DHA within their structures. Reproduced with modifications after permission from Dr Tsoupras (Tsoupras et al., 2019a).

Unlike n-3 PUFAs, salmon phospholipids are not so susceptible to oxidation. This is possibly due to the natural presence of antioxidants such as astaxanthin which is extracted with various other lipids and PLs (Raatz et al., 2016).

Marine PLs, including those derived from salmon, especially those bearing n-3 PUFA within their structure (Lordan et al., 2017, Tsoupras et al., 2019a, Tsoupras et al., 2018a, Burri et al., 2012), have exhibited promising outcomes against inflammation, thrombosis, platelet aggregation and related chronic disorders (Lordan et al., 2017). Such marine PL possess a plethora of beneficial bioactivities against inflammation-related disorders and high bioavailability of their bioactive n-3 PUFA into plasma lipoproteins, cell membranes, and several tissues, including those with difficult accessibility such as the brain (Tsoupras et al., 2018b, Lordan et al., 2017, Tsoupras et al., 2019a, Tsoupras et al., 2018a, Tierney et al., 2019, Burri et al., 2012, Davidson et al., 2012, Bjorndal et al., 2014). Marine PLs also possess strong anti-inflammatory, antithrombotic, and cardioprotective activities against platelet-activating factor (PAF)-related pathways and metabolism (Tsoupras et al., 2018b, Lordan et al., 2017, Tsoupras et al., 2019a, Tsoupras et al., 2018a, Nasopoulou et al., 2011), but also against the thrombin pathways (Tsoupras et al., 2019a, Tsoupras et al., 2018a, Kalogeropoulos et al., 2008, Antonopoulou et al., 2005). Recently salmon PLs have also been found to possess strong anti-platelet effects against well renowned platelet agonists too, such as collagen and ADP (Tsoupras et al., 2019c) and unpublished data communicated by Dr Alexandros Tsoupras.
However, to our knowledge there aren’t any reports studying the effects of cooking on the lipid content and bio-functionality and lipid content of salmon PLs and marine PLs in general.

1.4 Cooking and its effects on lipid content in fish

It is generally accepted that due to consumers requirements, fish is generally consumed cooked. During cooking, physical and chemical reactions take place that may impair or improve the nutritional value of the foodstuff. Cooking induces the loss of water from the food, which in turn, increases the lipid content in the majority of cases, but in some instances, fat is lost in foods with a high fat content. Traditional cooking methods such as frying, steaming and pan-frying are used worldwide but an emerging cooking method has gained popularity over the past decade: sous vide. Harsh cooking methods such as the traditional cooking methods tend to degrade EPA and DHA due to the high temperatures generated which consequently leads to the breaking of double bonds leading to oxidation (Adili et al., 2018). The generation of such high temperatures generated during these cooking processes tends to increase the formation of free radicals and reactive oxygen species (ROS) and consequently results in the release of peroxides (Adili et al., 2018) Aldehydes and various alcohol-derived compounds of the hydroperoxide primary metabolites are released upon further oxidation. One such product are the isoprostanes which are prostaglandin-like compounds formed upon the free radical catalysed peroxidation of arachidonic acid. The formation of such compounds are generally regarded as biomarkers for oxidative stress leading to various diseases (Adili et al., 2018).

The effect of various common consumer cooking techniques (raw, poached, steamed, oven -baked, and pan-fried with no added oil) was investigated on New Zealand King Salmon and the effects on the fatty acid profile. The fatty acid profile showed only minor differences between the different cooking methods (Larsen et al., 2010). In addition, oven baking salmon does not decrease the n-3 PUFA contents, indicating that baking salmon is an acceptable means of preparation that does not alter the potential health benefits of high n-3 seafood consumption (Raatz et al., 2011, Tsoupras et al., 2018a). However, the pan-fried and oven baked King Salmon had higher % yields of extractable lipids than the control sample (no fat was added in either thermal
procedure). It was hypothesised that the increase in the extractable lipid was due to
greater moisture loss during cooking.

It was observed that significant increases in the fatty acid content and fat content was
due to the dehydration during thermal treatment (Al-Saghir et al., 2004). They also
reported no significant changes in total amounts of SFA, MUFA or PUFA. During
thermal treatments, fatty acids behave differently. In general, SFA are reasonably heat
stable below temperatures of 150ºC but once in excess of this temperature and in the
presence of oxygen, oxidation tends to occur (Sioen et al., 2006)

Unsaturated fatty acids tend to be more heat labile. However, an increase in the degree
of unsaturation leads to less stability, therefore PUFAs are the less stable fatty acid class
(Sioen et al., 2006). Fish lipids possess significant quantities of long chain PUFAs. The
application of heat, including cooking, has been reported to increase the susceptibility
of n-3 PUFA oxidation (Regulska-Ilow and Ilow, 2002). Larsen et al. (2010) reported
that after all thermal treatments (steamed, oven-baked and pan-fried), MUFAs were the
most abundant fatty acids and was dominated by oleic acid. No significant differences
in MUFA content was observed between the control and cooked samples. In relation to
PUFA content, similar levels were observed between cooked and the control (raw)
salmon. SFA levels were similar in both cooked and the control samples. No significant
differences in percentages of DHA, EPA or DPA was observed across the thermal
treated samples.
Table: 1.4. The effects of cooking on the lipid content of salmon using various conventional cooking techniques. The results are presented as %s of Total Fatty Acids.

<table>
<thead>
<tr>
<th>Cooking method of salmon</th>
<th>ΣPUFA</th>
<th>Σn-3</th>
<th>Σn-6</th>
<th>ΣMUFA</th>
<th>ΣSFA</th>
<th>Σn-3/n-6</th>
<th>ΣOleic acid</th>
<th>ΣEPA</th>
<th>ΣDHA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven-Baked</td>
<td>28.39</td>
<td>17.78</td>
<td>10.61</td>
<td>43.51</td>
<td>24.29</td>
<td>1.68</td>
<td>35.56</td>
<td>6.17</td>
<td>8.60</td>
<td>Bastias et al. 2017</td>
</tr>
<tr>
<td>Oven-Baked</td>
<td>27.65</td>
<td>15.85</td>
<td>10.09</td>
<td>44.51</td>
<td>27.84</td>
<td>1.37</td>
<td>33.03</td>
<td>5.48</td>
<td>6.74</td>
<td>Larsen et al. 2010</td>
</tr>
<tr>
<td>Oven-Baked</td>
<td>28.47</td>
<td>15.03</td>
<td>11.73</td>
<td>39.15</td>
<td>17.25</td>
<td>1.28</td>
<td>30.48</td>
<td>5.05</td>
<td>6.04</td>
<td>Sengor et al. 2014</td>
</tr>
<tr>
<td>Pan-fried</td>
<td>27.19</td>
<td>15.50</td>
<td>10.98</td>
<td>44.86</td>
<td>27.95</td>
<td>1.33</td>
<td>32.88</td>
<td>5.26</td>
<td>6.57</td>
<td>Larsen et al. 2010</td>
</tr>
<tr>
<td>Pan-fried</td>
<td>28.40</td>
<td>14.60</td>
<td>8.1</td>
<td>54.6</td>
<td>16.9</td>
<td>1.8</td>
<td>25.5</td>
<td>3.4</td>
<td>5.8</td>
<td>Al-Saighir et al. 2004</td>
</tr>
<tr>
<td>Steamed</td>
<td>31.44</td>
<td>21.20</td>
<td>10.34</td>
<td>42.30</td>
<td>26.08</td>
<td>2.04</td>
<td>33.45</td>
<td>7.46</td>
<td>10.85</td>
<td>Bastias et al. 2017</td>
</tr>
<tr>
<td>Steamed</td>
<td>28.25</td>
<td>16.50</td>
<td>11.44</td>
<td>44.55</td>
<td>27.19</td>
<td>1.42</td>
<td>32.35</td>
<td>5.61</td>
<td>7.1</td>
<td>Larsen et al. 2010</td>
</tr>
<tr>
<td>Steamed</td>
<td>29.50</td>
<td>16.90</td>
<td>9.5</td>
<td>54.20</td>
<td>16.1</td>
<td>1.6</td>
<td>23</td>
<td>4.2</td>
<td>6.7</td>
<td>Al-Saighir et al. 2014</td>
</tr>
</tbody>
</table>

- All values are expressed as % of Total Fatty Acids

The effects of steamed and oven-baked cooking on the fatty acid profile of salmon were examined and it was concluded that no significant difference was observed when compared to raw salmon. However, omega-3 fatty acids exhibited significant changes in oven-baked (17.78% total fatty acids) and steamed (21.1% total fatty acids) samples when compared with the control (15.9% total fatty acids) sample (Bastias et al., 2017).

The effect of using two different cooking oils (extra virgin olive oil and sunflower oil) in pan-frying of farmed salmon was investigated by another research team. Extra virgin
olive oil led to a higher fat absorption rate than sunflower oil. No significant difference was observed in the lipid profile of the farmed salmon. Frying in extra virgin olive oil, slightly increased oleic acid (from 27.59g/100g to 30.37g/100g fatty acids). The n-6/n-3 ratio increased from 0.38 (raw) to 0.39-0.58 in the pan-fried salmon. The quantity of EPA+DHA decreased slightly but no significantly in the pan-fried sample (Ansorena et al., 2010).

Sengor et al., (2013) reviewed the effects of different cooking methods (baking, steaming and grilling) on the fatty acid composition on Atlantic salmon (Salmo salar) and reported no significant change (p > 0.05) in the various cooking methods when compared to the control (raw salmon). There was a significant difference in the cholesterol content (p<0.05) of the cooked salmon. EPA and DHA, the essential n-3 PUFAs, increased in the grilled and oven-baked salmon samples. Gladyshev et al., (2006) concluded that thermal treatment (boiling, frying or grilling) generally did not affect the EPA and DHA content in humpback salmon, with the exception of a slight reduction when pan-fried. ∑n-3 PUFA content decreased in steam cooked and oven-baked samples, whilst ∑n-3 PUFA content decreased in the steamed sample only.

Similar findings were reported upon the analysis of grilled salmon, steamed and roasted salmon when compared to raw salmon. Differences were due to the fat variations observed using different thermal treatments. The highest fatty acid content was observed in the grilled salmon (Costa et al., 2015).

Al-Saighir et al., (2004) examined the effect of different cooking procedures on lipid quality and cholesterol oxidation of farmed salmon fish (Salmo salar). The changes in the fatty acid content after the thermal treatments (pan-fried without oil, with olive oil, with corn oil, with partially hydrogenated plant oil and steamed) were not significant, in particular with emphasis on EPA and DHA. Changes in the total amounts of SFA, MUFA and PUFA after thermal treatment were marginal upon comparison to the control sample. Insignificant increases were observed in the SFA content of the pan-fried samples using different frying oils when compared to the raw salmon and the steamed salmon. The use of different frying oils was attributed to the changes of total MUFA and PUFA content. As a consequence of these results, it was hypothesised by Al-Saighir et al., (2004) that pan-frying does not have an influence on the high content of n-3 fatty acids significantly, but a detectable yet marginal exchange of fats occurs between the salmon sample and the pan-frying medium. The use of partially
hydrogenated plant oil, which initially contains 2.2% TFA, did not increase the TFA content of the treated samples.

The effects of the oxidation process affecting fatty acids and cholesterol in fried and roasted salmon was examined. Salmon samples were fried using different oils (olive oil and soya oil) and roasted. No significant differences were observed in the fatty acid profile of the treated samples. DHA exhibited a significant increase when sample was roasted. Samples that were fried in olive oil exhibited higher contents of MUFAs than the samples fried in soya oil. All of the thermally treated samples displayed an increase in MUFA content when compared to the control samples. An increase in the n-6/n-3 ratio was also observed. Roasted salmon exhibited the lowest n-3 content (Echarte et al., 2001).

Different cooking methods did not significantly affect the concentration of PUFAs such as arachidonic acid, adrenic acid, EPA and DHA. Although salmon that was either baked or fried showed a significant change in some PUFA levels (Echarte et al., 2001) the principle fatty acids in salmon were palmitic, oleic, EPA and DHA. Lauric, arachidic and behenic acid levels did not significantly change during frying or baking process. DHA showed a significant increase during roasting. Further observation concluded that unsaturated fatty acids which are prone to oxidation did not oxidise significantly (Echarte et al., 2001). A study on deep-frying fish concluded the lipid content of salmon was not significantly altered (Candela et al., 1998). Al-Saighir et al., 2004 reported no significant differences in the total amount of SFA, MUFA and PUFA in pan-fried salmon with oil, pan-fried salmon without oil or steamed salmon. D. Larsen et al., 2010 reported no significant differences in % of DHA, EPA or DPA in poached, pan-fried, microwaved, oven-baked steamed salmon except for the deep-fried sample which had significantly lower levels of DHA, EPA and DPA when compared to the all other methods. This is presumably due to the absorption of fatty acids from the cooking oil. The proportions of linolenic acid and oleic acid increased and consequently reduced the proportions of the LC n-3 FAs. Omega-6 FA content was significantly higher in the deep-fried sample. Poached and microwaved salmon had the lowest amount of DHA, EPA and DPA.

Changes to the MUFAs and the PUFAs levels was attributed to the different oils used in the various preparations. Pan-frying in different oils and frying in different oils marginally increased the SFA content as opposed to the initial raw salmon and steamed
salmon. Bastias et al., 2017 reported no significant differences in PUFA content for all heat treatments compared to the control. Omega-3 FA levels increased in steamed salmon compared to the control. Ansorena et al., 2010 concluded that frying with olive oil slightly increased oleic acid to 30.4% whereas frying with sunflower oil increased linoleic acid up to 11.6%.

1.4.1 Emerging Cooking Techniques namely the sous-vide cooking process

The modern-day consumer markets are showing a demand for high quality pre-cooked chilled ready-to-eat meals. Sous vide cookery is an emerging cooking technique which has been proven to produce chilled foods with an extended shelf-life of about 4 weeks when stored at the appropriate temperatures (1-5°C) (Schellekens, 1996). Sous vide is a French term meaning “under vacuum” and the foodstuff is cooked using sous vide can be defined as “raw materials or raw materials with intermediate foods cooked using precise temperatures and times using heat-stable vacuumized pouches” (Schellekens, 1996). Sous vide is an emerging cooking technique having come to prominence in the 1990s in due to its ability to extend the shelf life of minimally processed foods without affecting the nutritional content of the food stuff. Sous vide cookery differs from conventional cookery methods in two fundamental areas: the use of vacuum sealed heat stable pouches that contains the food stuff to be cooked and also the use of precise cooking temperatures. The use of vacuum sealing has several benefits including: heat is transferred efficiently from the water (or steam) to the food; the food stuffs shelf life is extended due to the elimination of contamination risk during storage; oxidation is prevented thus off-flavours are inhibited; food remains nutritious and flavourful since moisture and flavour volatiles are retained within the vacuum-sealed pouches used in the process and this also reduces aerobic bacterial growth (Baldwin, 2012). During conventional cooking methods such as grilling and roasting, some reactions occur that can alter the nutritional value of food positively (enhancement of the digestibility through denaturation of proteins in the food) or negatively (alteration of the fatty acid composition, especially a decrease in PUFAs due to lipid oxidation and the loss of heat labile water or fat-soluble vitamins and minerals. Consequently, some harmful chemical compounds are produced during cooking of protein-rich food such as fish. Among these chemical compounds, heterocyclic aromatic amines (HCA) have attracted significant
attention over the past decade. Several epidemiological studies have demonstrated a positive correlation between fish and meat consumption and several forms of cancer (Lichtenstein et al., 2006). HCAs generally occur when protein-rich foods such as fish and meat are grilled or fried at high temperatures. It has been reported that most HCAs are mutagenic (Sugimura et al., 2004), and in 1993, the International Agency for Research on Cancer concluded that almost all HCAs were carcinogenic. Sous-vide cooking possesses advantages over other conventional cooking methods due to the relatively mild cooking temperatures required.

Lipids in fish and seafood tend to undergo oxidation quite easily due to the abundance of PUFA present. This lipid oxidation significantly lowers the nutrition and acceptability of the products (Cropotova et al., 2019b). The sous vide cooking process consists of lower heating temperatures and longer cooking times compared to conventional cooking methods which is followed by the rapid cool down to temperatures between 0-4ºC and chilled storage. This process is beneficial to obtaining the flavour of the food product and also improve the nutritional, texture and palatability characteristics (Cropotova et al., 2019a, Cropotova et al., 2019b).

A study performed to exhibit the effects of boiling, steaming and the sous-vide cooking process on the lipid and volatile profile of fish was carried out, and especially the European sea bass (Dicentrarchus labrax) and it was reported that none of the above cooking processes effected the sea bass lipids, including vitamin A, cholesterol and phospholipids. Steaming and the sous-vide process actually enriched the sea bass headspace (Nieva-Echevarria et al., 2017).

1.4.2 Benefits of Sous-vide processing

As previously mentioned, sous vide processing present advantages from both a technological and sensory analysis point of view. Regarding the technological aspect, sous vide provides a very efficient and consistent transfer of heat from water to food product. This efficiency allows for a greater degree of reproducibility in the process. The sous-vide process has been shown to increase the shelf life of products due to the absence of oxygen in the vacuum sealed pouches, therefore inhibition of lipid oxidation occurs. From the sensory analysis point of view, the sous-vide process is beneficial as it conserves the food stuffs volatile compounds and due to the anaerobic environment of the foodstuff, oxidative deterioration is avoided which is widely regarded as the
principal cause of off-flavours and odours. The benefits of sous-vide from a human health perspective have been widely documented. The conservation of the nutritional value of the foodstuff is the principal health benefit that sous-vide cookery has when compared to conventional cooking methods such as boiling and frying. This phenomenon is due to the use of low cooking temperatures which in turn inhibit the loss of compounds such as vitamins and anti-oxidants through solubilisation and volatilisation (Baldwin, 2012, Iborra-Bernad et al., 2013, Kosewski et al., 2018).

1.4.3 Negative aspects of sous-vide processing

Whilst sous-vide processing has many positive attributes, the process gives rise to issues from a microbiological aspect. The anaerobic conditions within the sous vide pouches can lead to botulism if toxinogenic strains of Clostridium botulinum are present and the temperature abuse of foodstuff that contains the microorganism occurs (Schellekens, 1996). Garcia- Linares et al., 2004 postulated that the three main factors that determined the microbiological safety of sous-vide processed foods are:

- Intensity of heat treatment and duration
- Temperature achieved by rate of cooling
- Management of chilled storage of product (temperature and time duration)

Generally sous-vide foods are not safeguarded by low pH or low water activity, and typically do not contain preservatives (GARCÍA-LINARES et al., 2004). Therefore, the microbiological safety of these foods depends on the mild heat treatment consolidated with effective refrigeration storage (Stringer and Metris, 2018).

1.4.4 Sensory Analysis

In order to assess the sensory attributes of each different sous-vide cooked salmon preparation, sensory analysis was carried out in St. Angela’s College in Sligo. Sensory science is rapidly developing new methodologies of profiling and sensory mapping which differs from the conventional profiling and also in the statistical analysis of the results. Multiple Factor Analysis (MFA) was the projective mapping or napping technique we used in this study to collect data regarding the sensory attributes of the sous-vide prepared salmon. Untrained panellists were used to locate the cooked salmon.
in different dimensions, relative to the similarities and differences. MFA analyses several variables which differ in nature and number from other samples (Albert et al., 2011).
1.4.5 AIM OF STUDY

The aim of the present study was to evaluate the effects of sous vide cooking process on the bio-functionality, lipid profile and sensory properties of salmon in several cooking preparations based on different temperatures and brining applied. In order to achieve such a goal, the present study aimed to:

- Extract bioactive lipids from salmon fillets that were cooked by the sous-vide technique in different temperatures and brining cooking conditions, and appropriately separate/fractionate and further analyse these lipids.
- Evaluate the anti-thrombotic bioactivities of the extracted bioactive lipids of sous vide cooked salmon fillets against human platelet aggregation induced by inflammatory and thrombotic mediators such as PAF and thrombin, but also by well-established platelet agonists such as collagen and ADP
- Determine by LC-MS analysis the fatty acid composition of the bioactive TPL extracts from the sous vide cooked salmon fillets
- Perform a Sensory analysis to determine consumer perception of the varying sous vide cooked salmon fillets preparations
Chapter 2:

Materials and methods

2.1 Chemicals and Reagents

All plastic and glass consumables, reagents, and solvents were of analytical grade and were purchased from Fischer Scientific Ltd. (Dublin, Ireland). Evacuated sodium citrate S-monovettes and 20G safety needles for blood sampling were purchased from Sarstedt Ltd (Wexford, Ireland). The platelet aggregation bioassay was carried out on a Chronolog-490 two-channel turbidimetric platelet aggregometer (Havertown, PA, USA), coupled to the accompanying AGGRO/LINK software package. All platelet aggregation consumables were purchased from Labmedics LLP (Abingdon on Thames, UK). Standard PAF, thrombin, and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich (Wicklow, Ireland), while collagen and ADP were from CHRONOLOG (Havertown, PA, USA). All platelet aggregation consumables were purchased from Labmedics LLP (Abingdon on Thames, UK). Standard PAF, thrombin, egg phospholipid extract, and BSA were purchased from Sigma Aldrich (Wicklow, Ireland). Centrifugations were carried out on an Eppendorf 5702R centrifuge (Eppendorf Ltd., Stevenage, UK). Spectrophotometric analysis was carried out on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). All salmon samples were placed in vacuum pouches and a Multivac Vacuum machine (Sepp Haggenmuller GmBh, Germany) was used. The salmon samples were cooked in a Grant JB Nova water bath (Grant Instruments, Cambridge, UK).

2.2 Sous-vide process

All salmon (Irish organic farmed salmon (*Salmo salar*) samples (fillets & heads) were provided by Marine Harvest (Co. Donegal, Ireland). All fish were reared in a natural environment ensuring that the fish possessed good body shape and sufficient muscle tone. The salmon samples provided were from the same provider (Marine Harvest) in specific conditions that are not altered throughout year.

More specifically, Irish organic farmed salmon was produced by Marine Harvest at their farming facility in Co.Donegal, Ireland, with a diet containing only organic approved natural ingredients from sustainable sources with fish meal and oil derived from the
trimmings of fish caught for human consumption. All salmon fillets tested were harvested from thus farmed salmon of the same batch and LOT number. It should be noted that Marine Harvest have more than one cage to farm salmon simultaneously. Therefore, from the same cage of farmed salmon (1\textsuperscript{st} batch), I was provided with raw salmon fillets to be used for first in line experiments. From the second cage of farmed salmon (2\textsuperscript{nd} batch), I was provided again with salmon fillets to be used for the 2\textsuperscript{nd} line of experiments. Therefore, these second line experiments were conducted in salmon samples of the same batch of fish but from a different cage of farming than the first in line experiments. Similar procedure was carried out for being provided of all salmon samples tested (n=22 in total) that were farmed at the same time (same LOT number). All the above were carried out several times within the same LOT number to ensure experimental reproducibility/repeatability, and thus the observed differences in bioactivities are related to the differences in experimental procedures (i.e. due to the various sous vide cooking temperatures and conditions) rather than the farming conditions and seasonal variability of the salmon.

Salmon fillets were prepared in approximately 50g portions. A 10\% sodium chloride to water solution was created. Various sous vide cooking temperatures (52°C, 65°C and 80°C) were used and for each different temperature a sample was either brined or un-brined. After the sous-vide cooking process, the cooked salmon fillet was homogenised into organic solvents in accordance with the Bligh & Dyer method of lipid extraction (Bligh and Dyer, 1959)

![Diagram of sous-vide cooking process](image)

**Figure 2.1:** The sous-vide cooking process using brined/un-brined salmon fillets and various cooking temperatures. Salmon fillets were portioned into approximately 50g portions. Samples were either brined or un-brined. Samples were then placed into sous-vide pouches and placed in vacuum chamber. Samples were placed in water bath set at various temperatures (52°C, 65°C or 80°C) and cooked for 15 minutes.
Figure 2.2. Flow Chart for the extraction, fractionation, analysis and evaluation of the bioactivities of lipid extracts derived from several preparations of sous vide cooked salmon fillets
2.3 Extraction and separation of bioactive polar lipid compounds from cooked salmon

Three different sous vide cooking temperatures were used (52°C, 65°C & 80°C) and samples were either brined (10% sodium chloride in water) or un-brined. Several (n=22) 50-60g of each sous vide cooked salmon samples was homogenised mechanically using a Waring blender (Fisher Scientific Ltd) and its total lipids (TL) were extracted into chloroform according to the method of (Bligh and Dyer, 1959) and flash vaporised under N₂ stream, weighed, re-dissolved in 2mL of CHCl₃/MeOH 1:1 v/v, and stored at -20°C. For each sample, approximately 1/10 of the TL was stored in sealed vials at -20°C, whilst the remainder of TL was further separated into total neutral lipids (TNL) fraction and the total polar lipids (TPL) fraction using the counter-current distribution method developed by Galanos and Kapoulas, 1962. The TPL and TNL recovered from this method for each sous vide salmon preparation were weighed and stored for further analysis under a nitrogen stream at -20°C.

Figure 2.3. Diagram demonstrating the counter-current distribution method.

The TL was dissolved in 30ml of 87% pre-equilibrated ethanol solution and added to the first separation funnel containing 90ml of pre-equilibrated petroleum ether. After each addition to the separatory funnel, the funnel was shaken well to ensure the maximum
interaction of lipids with the solvents. Once phase separation occurs, the lower phase was transferred to the second separatory funnel, shaken well and the lower phase containing the TPL was extracted to a round-bottom flask. This process was repeated 8 times until all the TL had been washed through leaving the TNL in both separatory funnels dissolved in petroleum ether (P.E) which was extracted to a separate round-bottom flask and the TPL in other round bottomed flask

2.4 Platelet aggregometry bioassay

Blood collection from several healthy donors (n=12) and preparation of human platelet-rich plasma (hPRP) was conducted as previously described (Tsoupras et al., 2019d). The Ethics Committee of the University of Limerick approved the protocol, which was performed in accordance with the Declaration of Helsinki. Healthy donors were fully aware that their blood samples were used in our study and written consent was provided. Briefly, the blood samples were collected from each donor by a specialised phlebotomist using sodium citrate anticoagulant (0.106 mol/L in a 1:10 ratio of citrate to blood; Sarstedt Ltd., Wexford, Ireland) and centrifuged at 194×g for 18 minutes at 24°C with no brake applied. The supernatant hPRP was then transferred to polypropylene tubes at room temperature for the aggregation bioassays, whereas platelet-poor plasma (PPP) was obtained by further centrifuging the remainder of the blood specimens at 1465×g for 20 minutes at 24 °C with no brake applied. hPRP was adjusted to 500,000 platelets /μL if required by addition of the respective volume of PPP according to the absorbance of the hPRP measured using a spectrophotometer at 530 nm (Shimadzu UV-1800, Kyoto, Japan). Aliquots of standard PAF stock solution in chloroform/methanol (1:1 v/v) were evaporated under a stream of nitrogen and redissolved in BSA (2.5 mg BSA/mL saline) to obtain PAF solutions with final concentrations in the aggregometer cuvette ranging from 0.26 nM to 0.26 µM. The examined salmon PL samples were also dissolved in BSA (2.5 mg BSA/mL saline). Standard stock solutions of active thrombin, collagen, and ADP dissolved in saline were further diluted in saline prior testing. Then, 250 µL of PRP was added to an aggregometer cuvette at 37°C with stirring at 1000 rpm. The PRP was calibrated using the PPP as a blank. The maximum-reversible platelet aggregation curve induced by PAF, thrombin, collagen or ADP was determined as 100% aggregation, which was also used as a baseline (0% inhibition) in the absence of any lipid sample, by adding the appropriate amounts of each platelet agonist in the aggregometer cuvette, in order to reach specific final concentrations: for PAF approximately 0.1–1 nM, for thrombin
approximately 0.01–0.4 U/mL, for collagen approximately 1–5 µg/mL and for ADP at approximately 2–10 µM. The PAF, thrombin, collagen, and ADP-induced aggregation of hPRP was calculated first at 0% inhibition of baseline in a cuvette (100% aggregation) in the absence of any lipid sample, whereas after the pre-incubation of hPRP with several amounts (µg) of the lipid samples in a different cuvette for 2 min, the same amount of PAF, thrombin, collagen, or ADP that induced maximum-reversible platelet aggregation was added and the reduced aggregation was calculated. Thus, a linear curve at the 20%–80% range of the percentage of inhibition against PAF, thrombin, collagen, and ADP-induced aggregation of hPRP was deduced for each sample. From this curve, the concentration (µg) of the lipid sample that led to 50% of agonist-induced aggregation of hPRP was calculated as the 50% inhibitory concentration value also known as the IC50 value (half-maximal inhibitory concentration) for each sample. The resulting IC50 values were expressed as a mean value of the mass of lipid (µg) in the aggregometer cuvette ± standard deviation (SD). All experiments were performed several times (n≥10), using a different donors’ blood samples for each replicate to ensure reproducibility.

**Figure 2.4.** Flow diagram of the light transmission aggregometry technique for the evaluation and the monitoring of platelet defects and the effects of platelet aggregation agonists and antiplatelet compounds. Reproduced with modifications after permission from Dr Tsoupras (Tsoupras et al 2019d)
2.5 LC-MS analysis

The salmon TL and TPL fractions were analysed by LC-MS in Limerick Institute of Technology. Each of these lipids were separated into two vials and dried under nitrogen stream. The first vial was subjected to saponification with the addition of 1.5mL of saponification reagent, [2.5 M KOH: methanol (1:4, v/v)], then vortexed. The vials were then incubated at 72°C for 15 minutes before the addition of 225µL of formic acid. 1725µL of chloroform and 375µL of Milli-Q water were added and vortexed. A biphasic separation was observed. The chloroform layer containing the free fatty acids was then transferred to amber vials and evaporated to dryness before storing at -20°C until LC-MS analysis took place.

Before LC-MS analysis took place, all the dried lipids were re-constituted in 500 µL of methanol: dichloromethane (2:1 v/v), centrifuged at 13,000 rpm for 6 minutes prior to filtering through 3 kDa ultra-centrifuge filters (Amicon Ultra 3k). Polar lipid and free fatty acid profiles were obtained in a LC (Agilent 1260 series) equipped with a Q-TOF mass spectrometer (Agilent 6520) and the source type was ESI. The column used for separations was an Agilent C18 Poroshell 120 column (2.7 µm, 3.0 x 150mm). The composition of the mobile phase (A) was 2 mM ammonium acetate in water and 2 mM ammonium acetate in 95% acetonitrile for mobile phase (B). Chromatographic separation was performed by gradient elution starting with 60% B for 1 minute, then increasing to 90% B over 2.5 minutes. Then, 90% B was held for 1.5 minutes and increased afterwards to 100% over 5 minutes. Subsequently, 100% B was held for 4 minutes, reducing afterwards to 60% B over 0.5 minutes and held for 1 minute until the next run. The mobile phase flow rate was 0.3 mL/minute until 5 minutes elapsed, increasing up to 0.6 mL/minute after 10 minutes and held at this flow rate until the end of the run. The injection volume was 10 µL. The mass spectrometer was operated in negative ionisation mode, scanning the lipids from m/z 50-1100. Drying gas flow rate, temperature, and nebuliser pressure were at 5 L min⁻¹, 325°C, and 30 psi, respectively. Fragmentor and skimmer voltages were kept at 175V and 65V, respectively, and the capillary voltage was 3500 V. In the negative ion mode, the monitoring reference masses used were 1033.988 and 112.9855, respectively. Assignment of free fatty acids and phospholipids species is based upon a combination of survey, daughter, precursor, and neutral loss scans. The identity of phospholipid species were verified using the LIPID MAPS: Nature Lipidomics Gateway (www.lipidmaps.org), by using the lowest delta values combined with the results obtained from the LC-MS analysis of FFA.
2.6 Sensory Analysis

Descriptive sensory profiling is a method commonly used to identify the key sensory drivers in product development and reformulation in order to access consumer acceptance and the marketing of such products. Fast descriptive sensory profiling techniques such as Napping and CATA are frequently used in the food industry to assess the sensory aspects of products. Napping is a method where the food products are projected onto a two-dimensional space which is based on how similar the sensory aspects of each food product are in relation to each other. “Global” napping (including all sensory aspects) or “partial” napping (focusing on specific sensory aspects e.g. texture, smell or taste) are the most frequently used napping methods used in sensory analysis presently. Napping provides a rapid snapshot of a group of samples using all data from the assessors to create a consensus image. Once the panel provide the 2D data, the results are further analysed to create multiple dimensions similar to Principal Component Analysis (PCA) plots, which are usually viewed two dimensions at a time. The results generated from napping can then be correlated to PCA plots generated from descriptive analysis. These two methods give similar results, as assessed by a multivariate correlation coefficient called the RV coefficient. The Check-all-that-apply (CATA) method in sensory analysis focuses on describing a product using appropriate selected descriptive words from a given list (Dehlholm et al., 2012). It evaluates sensory and non-sensory perception of products and has been shown to be in agreement with traditional panel-developed sensory profiles. The methodologies used to capture consumer perceptions of products are relatively less time consuming and generally easier to conduct than using the traditional descriptive analysis approach which uses a trained sensory panel. CATA participants are given a list of terms and asked to endorse those that characterise the sample (Bennett and Hayes, 2012). These questions are often used in market research to reduce the response burden of participants (Raginski et al., 1994) as participants do not have to generate their own descriptors for each sample. CATA and napping were suitable sensory analysis techniques used in this thesis due to

- Only one sensory analysis session was required
- Less expensive to run than other descriptive analysis approaches
- Doesn’t require expert sensory assessors
- Assessors evaluate samples based on what is important to them
• Data from analysis can be acquired quickly using computers with sensory software
• Gives an overall snapshot of samples

Six salmon samples and a replicate (control Brined 80°C) were all tested by 30 assessors using napping followed by check-all-that-apply. Tests were conducted in designated individual booths under artificial daylight. 30 assessors (age 18-60, 19 female) with experience in sensory evaluation but no previous experience in napping evaluated the products. The assessors were asked to provide information in relation to consumption of salmon (40% would consume salmon weekly, 27% monthly and the rest occasionally) and the majority of them would consume it baked (42%) or smoked (31%). This study does not rely on subjective measurement of the salmon samples but objective measurements of the attributes. Therefore, the frequency of consumption does not affect the final sensory results. In other words, the assessors were not asked to express preference or liking which would be affected by the frequency of consumption.

For the napping test they were given instructions in the beginning of the test

“Please taste all the samples, in the order presented to you. Place the samples by dragging the code across into the rectangle according to the strategy that two samples placed closer to each other are more alike than two samples placed further apart. The criteria for how to separate the samples just has to make sense to you. There is no right or wrong answer.”

Furthermore, a scientist was present during the testing in order to answer the assessors’ questions. The seven salmon samples (six samples and the replicate) were simultaneously presented in a random order to each assessor. Random order was used in order to counter-act first order and carry-over effects, despite the fact that assessors were allowed to re-taste the samples (Reinbach et al., 2014).

The assessors were requested to taste the samples and to lay them out on in a rectangular space on the screen in such a way that two salmon samples were very near if they seemed similar and that two salmon samples were distant from one another if they seemed different. For each salmon product, both X co-ordinate and Y co-ordinate were collected through Fizz Biosciences software. Assessors were also asked to give reasons for creating their groups. The frequencies of the terms used for the group creation were calculated.
The next stage of testing included a check-all-that-apply (CATA) test were the participants were asked to check a number of terms for each of the samples they were presented. The 17 attribute terms used for the CATA session (Appearance (A)-pink/salmon, discoloured, fatty/oily; Odour (O)- strong fishy, mild fishy; Texture (Tx)-soft, firm, dry, moist, flaky; Taste (T)- sweet, salty, umami and Aftertaste (At) – strong fishy, mouth drying, oily and sweet) were developed during a vocabulary session with untrained participants, where all six samples (without the replicate) were presented monadically to the panellists and they were asked to write down as many terms as possible in relation to the appearance (A), texture (Tx), taste (T), odour (O) and aftertaste (AT) of the samples.

2.7 Statistical Analysis
All experiments were completed in triplicate with the obtained results expressed as a mean value ± standard deviation (SD). These values were obtained using one-way analysis of variance (ANOVA) and the Kruskal-Wallis non-parametric test to determine significant statistical differences between analyses if present for all data except for the analyses of the LC-MS data where one-way analysis of variance (ANOVA) and the Tukey’s honest significant difference (HSD) multiple comparison post-hoc test was used (SPSS Inc., Chicago, 215 IL, USA).

The analysis of data from sensory evaluation included analysis of the napping data and data from check-all-that-apply. For the data generated from napping, Multiple Factor Analysis as described by other researchers (Le Dien and Pagès, 2003) was used to generate the bi-plots of the products using XL Stat software. Factomine-R software was used to generate the factor map/score with confidence ellipses as described by Christian Dehlholm 2014 (in Novel techniques in Sensory Characterisation and Consumer Profiling by P. Varela and G. Ares (2014). Although CATA results are usually considered exploratory or descriptive (Fleming et al., 2013) for this study CATA data were analysed using a Cochran’s Q test in order to find differences between samples. This was conducted using XLStat and the symmetric plots were generated. Pairwise comparisons between products were performed using the McNemar approach.
Chapter 3: 
Results

3.1 Yield of extraction and separation of bioactive lipids from cooked salmon

Total lipids (TL) of all salmon samples (n=22) were extracted and separated into total polar lipids (TPL) and total neutral lipids (TNL). The recovered amounts of TL, TPL and TNL (expressed as g of lipids/100g of fish tissue) are given in Table 3.1. The majority of the lipids that were extracted were of neutral lipid nature which is agreement with the literature (Tsoupras et al., 2018a). The neutral lipid fraction of raw salmon fillets generally comprises of approximately 60-84% of the TL, whilst the total polar fraction contributes to approximately 16-40% of the TL (Tsoupras et al., 2018a).

The TL was extracted in triplicate (n=3) from six separate samples of the salmon using several sous-vide cooking procedures. The TL of these samples was further separated to TPL and TNL. These results are displayed on Table 3.1 expressed as g of lipids per 100g of sample, with the TPL and TNL also being displayed as a percentage of the TL.

The content for total lipids (TLs), neutral lipids (NLs) and PLs is presented in Table 3.1 and is expressed as g per 100 g of fish tissue for all samples. Salmon is generally categorised as oily fish species, thus their lipid content is usually higher than other fish species. In this present study also, all salmon samples had high lipid content as reflected by their TLs, which is in accordance with the literature (Tsoupras et al., 2018a). Interestingly, the cooking of all sous vide salmon preparations yielded similar values for TL when compared to that of TL from raw salmon as reported by (Tsoupras et al., 2018a). Moreover, all samples exhibited higher levels of TNL when compared to the raw salmon samples

The 52°C SVSB contained significantly less amounts of TPL when compared to all other salmon preparations. The effect of brining exhibited no differences in the yield of extraction in TL, TNL and TPL.

Tsoupras et al., 2018a reported that the majority of lipids extracted from raw salmon fillets were neutral lipids (NLs) since NLs contribute to approximately 60-84% of the TL of raw salmon, thereby the TPL fraction of the raw salmon fillets comprises of
approximately 16-40% of the TL. Notably, the % TNL of all sous-vide cooked salmon samples exhibited higher % of TNL. Interestingly, all of the sous-vide cooked salmon samples displayed lower % TPL.

The Bligh & Dyer method of extraction is one of the most well-established methods for high yield of extraction of lipids in combination with the high stability of key bioactive lipid substances extracted, since it does not need heat treatment. In contrast, other well-established techniques (such as the Soxhlet method), even though they may well exhibit higher yields of extraction, because they are applying higher temperatures, there is an increased possibility of modifications in both structures and relative bioactivities of these key substances. Within this study, we chose to use the Bligh & Dyer method of extraction and the subsequent Galanos & Kapoulas counter current distribution for extracting the bioactive polar lipids of salmon, since they have been tested effectively in several marine sources including salmon, with high yield of bioactive polar lipids (Tsoupras et al., 2018a, Panayiotou et al., 2000, Nasopoulou et al., 2014). By applying this experimental approach, almost all of the PL fraction was recovered from salmon in accordance with previous reported results in salmon and other fish species.
Table 3.1. The Total lipid (TL), total neutral lipid (TNL) and total polar lipid (TPL) expressed as g/100g of sample with TNL and TPL also being expressed as a percentage of TL (range values, n=3)

<table>
<thead>
<tr>
<th></th>
<th>TL (g/100g)</th>
<th>TNL (g/100g)</th>
<th>TNL (%) TL</th>
<th>TPL (g/100g)</th>
<th>TPL (%) TL</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSF</td>
<td>3.61-7.41</td>
<td>2.58-6.74</td>
<td>60-84</td>
<td>0.50-1.22</td>
<td>16-40</td>
</tr>
<tr>
<td>52°CCSVSNB</td>
<td>4.81-8.73</td>
<td>4.21-8.01</td>
<td>85.59-90.47</td>
<td>0.49-0.83</td>
<td>9.54-14.42</td>
</tr>
<tr>
<td>52°CCSVSB</td>
<td>5.87-8.21</td>
<td>5.09-7.35</td>
<td>86.82-92.86</td>
<td>0.74-0.92</td>
<td>7.14-13.18</td>
</tr>
<tr>
<td>65°CCSVSNB</td>
<td>7.27-7.61</td>
<td>6.54-6.84</td>
<td>89.24-90.74</td>
<td>0.69-0.79</td>
<td>9.25-10.75</td>
</tr>
<tr>
<td>65°CCSVSB</td>
<td>7.47-8.19</td>
<td>6.76-7.36</td>
<td>89.89-90.41</td>
<td>0.72-0.82</td>
<td>9.59-10.11</td>
</tr>
<tr>
<td>80°CCSVSNB</td>
<td>7.25-8.37</td>
<td>6.30-7.32</td>
<td>86.70-87.68</td>
<td>0.96-1.04</td>
<td>12.32-13.30</td>
</tr>
<tr>
<td>80°CCSVSB</td>
<td>7.25-8.37</td>
<td>7.25-7.87</td>
<td>87.51-90.03</td>
<td>0.88-1.04</td>
<td>9.97-12.49</td>
</tr>
</tbody>
</table>

Abbreviations: RSF = raw salmon fillets; 52CSVSNB = 52°C sous-vide cooked salmon no brine; 52CSVSB = 52°C sous-vide cooked salmon brined; 65CSVSNB = 65°C sous-vide cooked salmon no brine; 65CSVSB = 65°C sous-vide cooked salmon brined; 80CSVSNB = 80°C sous-vide cooked salmon no brine; 80CSVSB = 80°C sous-vide cooked salmon brined

3.2 The effect of several sous-vide cooking preparations on the bio-functionality of salmon lipids against platelet aggregation

The effect of several sous-vide cooking preparations on the bio-functionality of salmon lipids against platelet aggregation induced by inflammatory and thrombotic mediators such as PAF and thrombin, but also by well-established platelet agonists, such as collagen and ADP, is presented in Appendix II, in relation to raw salmon fillets (Tsoupras et al., 2019c) and unpublished data communicated from Dr. Alexandros. Tsoupras 2019.

The in vitro antithrombotic activities of TPLs from sous-vide processed salmon using different cooking procedures was evaluated for the first time by their inhibitory effects against human platelet aggregation induced by PAF, thrombin, collagen, and ADP. These effects were expressed by their IC50 values (half-maximal inhibitory
concentrations) against each platelet agonist. The absolute results for these IC50 values are shown in Appendix II. It should be noted that the lower the IC50 value the stronger the inhibition against the platelet agonist tested.

The IC50 values obtained against platelet aggregation, induced by all these platelet agonists, showed that all of the TPL extracts exhibited strong antithrombotic effects. More specifically, apart from the TPLs derived from the 80°C CSVSNB and the 80°C CSVSB sous vide salmon preparations, TPLs from the other sous vide salmon preparations strongly inhibited the PAF pathway of platelet aggregation. These anti-PAF effects of the TPLs from 52°C CSVSNB, 52°C CSVSB, 65°C CSVSNB and 65°C CSVSB sous vide salmon preparations were found within the same range whilst remaining similar to that previously reported anti-PAF effects of the TPL extracts of raw SFS as reported by (Tsoupras et al., 2019c) (p > 0.05 in all these comparisons) and other raw marine sources (Nasopoulou et al., 2007, Panayiotou et al., 2000). On the other hand, the TPLs derived from sous vide salmon preparations using higher temperatures, namely the 80°C CSVSNB and 80°C CSVSB preparations, exhibited approximately the same anti-PAF effects, which however were significantly lower than the anti-PAF effects of all the other sous vide salmon preparations (p<0.05 in all comparisons).

Moreover, the IC50 values of the anti-PAF effects of the TPLs derived from the 80°C CSVSNB and 80°C CSVSB preparations were approximately one order of magnitude lower than those of the previously reported raw salmon fillets (Tsoupras et al., 2019c) (p<0.05 in all comparisons).

The TPLs from the 52°C CSVSNB, 52°C CSVSB and 65°C CSVSB exhibited the strongest inhibitory effects against thrombin-induced platelet aggregation in comparison to the TPLs extracted from all the other sous vide preparations in higher temperatures. These anti-thrombin effects were also found similar to previously reported anti-thrombin effects of the TPL extracts of raw SFS as reported by Tsoupras et al. 2019 (p > 0.05 in all these comparisons). On the other hand the TPLs derived from sous vide salmon preparations using higher temperatures, namely the 80°C CSVSNB and 80°C CSVSB preparations, exhibited approximately the same anti-thrombin effects, which however were significantly lower than the anti-thrombin effects of all the other sous vide salmon preparations (p<0.05 in all comparisons) and from those of the previously reported raw salmon fillets (Tsoupras et al., 2018a) (p<0.05 in all comparisons).
Figure 3.1 Antithrombotic activities of TPL extracts from various salmon samples (raw and various sous-vide preparations) against platelet-activating factor (PAF)-induced aggregation of human platelets. Results are expressed as IC50 (half-maximal inhibitory concentration) values that reflect the inhibitory strength of each PL extract against PAF-induced platelet aggregation and is expressed as mean values of μg of PLs in the aggregometer cuvette that causes 50% of inhibition of PAF-induced aggregation of platelets in human platelet-rich plasma (hPRP) ± SD. It should be noted that the lower the IC50 value, the stronger the inhibition against the platelet agonist tested. * Indicates statistically significant differences of the less bioactive extract (p < 0.05). Abbreviations: TPL: Total Polar Lipids Raw SF = raw salmon fillets; 52CSVSNB = 52°C sous-vide cooked salmon no brine; 52CSVSB = 52°C sous-vide cooked salmon brined; 65CSVSNB = 65°C sous-vide cooked salmon no brine; 65CSVSB = 65°C sous-vide cooked salmon brined; 80CSVSNB = 80°C sous-vide cooked salmon no brine; 80CSVSB = 80°C sous-vide cooked salmon brined SD: standard deviation. Interestingly, the above mentioned anti-thrombin effects of the TPLs from all sous-vide preparations were significantly lower than their relative anti-PAF effects, suggesting a higher specificity of these TPL against the PAF-pathway, rather than the thrombin pathway, in a similar pattern with the previously reported anti-PAF and anti-thrombin
activities of the TPL from raw salmon fillets, using the same extraction method and counter-current distribution method (Tsoupras et al., 2018a).

**Figure 3.2.** Antithrombotic activities of TPL extracts from various salmon samples (raw and various sous-vide preparations) against thrombin-induced aggregation of human platelets. Results are expressed as IC50 (half-maximal inhibitory concentration) values that reflect the inhibitory strength of each PL extract against PAF-induced platelet aggregation and is expressed as mean values of μg of PLs in the aggregometer cuvette that causes 50% of inhibition of thrombin-induced aggregation of platelets in human platelet-rich plasma (hPRP) ± SD. It should be noted that the lower the IC50 value, the stronger the inhibition against the platelet agonist tested. # Indicates statistically significant differences of the less bioactive extract (p < 0.05). Abbreviations: TPL: Total Polar Lipids  RSF = raw salmon fillets; 52CSVSNB = 52°C sous-vide cooked salmon no brine; 52CSVSB = 52°C sous-vide cooked salmon brined; 65CSVSNB = 65°C sous-vide cooked salmon no brine; 65CSVSB = 65°C sous-vide cooked salmon brined; 80CSVSNB = 80°C sous-vide cooked salmon no brine; 80CSVSB = 80°C sous-vide cooked salmon brined SD: standard deviation.
Figure 3.3 Antithrombotic activities of TPL extracts from various salmon samples (raw and various sous-vide preparations) against ADP-induced aggregation of human platelets. Results are expressed as IC50 (half-maximal inhibitory concentration) values that reflect the inhibitory strength of each PL extract against ADP-induced platelet aggregation and is expressed as mean values of μg of PLs in the aggregometer cuvette that causes 50% of inhibition of PAF-induced aggregation of platelets in human platelet-rich plasma (hPRP) ± SD. It should be noted that the lower the IC50 value, the stronger the inhibition against the platelet agonist tested. # Indicates statistically significant differences of the less bioactive extract (p < 0.05). Abbreviations: TPL: Total Polar Lipids RSF = raw salmon fillets; 52CSVSNB = 52°C sous-vide cooked salmon no brine; 52CSVSB = 52°C sous-vide cooked salmon brined; 65CSVSNB = 65°C sous-vide cooked salmon no brine; 65CSVSB = 65°C sous-vide cooked salmon brined; 80CSVSNB = 80°C sous-vide cooked salmon no brine; 80CSVSB = 80°C sous-vide cooked salmon brined SD: standard deviation.

Furthermore, the TPLs from sous vide preparations, the ones treated in low temperatures (52°C-65°C), exhibited strong inhibitory effects against ADP-induced platelet aggregation, whilst their strong anti-ADP effects were similar with the anti-ADP effects of TPLs from raw SF (Unpublished data communicated from Dr
Alexandros Tsoupras). Instead, the 80°C CSVSNB and 80°C CSVSB samples exhibited significantly lower anti-ADP effects from all the other sous vide preparations or even from raw SF (p<0.05 in all these comparisons).

Figure 3.4. Antithrombotic activities of TPL extracts from various salmon samples (raw and various sous-vide preparations) against collagen-induced aggregation of human platelets. Results are expressed as IC50 (half-maximal inhibitory concentration) values that reflect the inhibitory strength of each PL extract against collagen-induced platelet aggregation and is expressed as mean values of μg of PLs in the aggregometer cuvette that causes 50% of inhibition of PAF-induced aggregation of platelets in human platelet-rich plasma (hPRP) ± SD. It should be noted that the lower the IC50 value, the stronger the inhibition against the platelet agonist tested. # Indicates statistically significant differences of the less bioactive extract (p < 0.05). Abbreviations: TPL: Total Polar Lipids  RSF = raw salmon fillets; 52CSVSNB = 52°C sous-vide cooked salmon no brine; 52CSVSB = 52°C sous-vide cooked salmon brined; 65CSVSNB = 65°C sous-vide cooked salmon no brine; 65CSVSB = 65°C sous-vide cooked salmon brined; 80CSVSNB = 80°C sous-vide cooked salmon no brine; 80CSVSB = 80°C sous-vide cooked salmon brined SD: standard deviation.
Nevertheless, the anti-ADP effects of TPL form all sous vide preparations were stronger than their anti-thrombin and lower than their anti-PAF effects (p<0.05 in all these comparisons), respectively, apart from the TPL of both the 80°C CSVSNB and 80°C CSVSB samples that had similar anti-ADP and anti-PAF effects (p>0.05 in all these comparisons).

Differently than the effects of the TPL from sous vide preparations against all the other platelet agonists, the anti-collagen effects of the TPL from all sous vide preparations exhibited different pattern. More specifically, the TPLs from both 65°C CSVSNB and 65°C CSVSB samples had the strongest inhibitory effects against collagen-induced platelet aggregation than all samples tested. The anti-collagen effect of these two sous vide preparations were significantly stronger not only when compared with the anti-collagen effects of the TPL from 80°C CSVSNB and 80°C CSVSB, but also when compared with the anti-collagen effects of the TPL from 52°C sous vide salmon preparations and that of the raw salmon fillets (see Appendix II) (Unpublished data communicated from Dr Alexandros Tsoupras) (p<0.05 in all these comparisons).

Interestingly, the anti-collagen effects of TPL from both 65°C CSVSNB and 65°C CSVSB samples were stronger from their anti-PAF, anti-ADP and anti-thrombin effects in these conditions applied. This suggests that both 65°C CSVSNB and 65°C CSVSB seem to contain TPL moieties with higher specificity against the collagen pathway than all the other samples.

The in vitro antithrombotic activities of TPLs from Raw SH, 80°C CSVSHNB and 80°C CSVSHB were evaluated by their inhibitory effects against human platelet aggregation induced by well renowned platelet agonists namely PAF, thrombin, collagen and ADP. These effects were expressed using IC$_{50}$ values against each individual platelet agonist.

The IC$_{50}$ values obtained against platelet aggregation, induced by PAF, collagen and ADP, showed that all PL extracts possessed strong antithrombotic effects.

For PAF, collagen ADP no difference was observed between cooked and raw (p>0.05 in all comparisons. Whereas, there was a decrease in the anti-thrombin effect after cooking at 80°C.
Figure 3.5. Antithrombotic activities of TPL extracts from salmon heads against PAF/thrombin/ADP/collagen-induced aggregation of human platelets. Results are expressed as IC50 (half-maximal inhibitory concentration) values that reflect the inhibitory strength of each TPL extract against collagen-induced platelet aggregation and is expressed as mean values of μg of TPL in the aggregometer cuvette that causes 50% of inhibition of collagen-induced aggregation of platelets in hPRP ± SD. It should be noted that the lower the IC50 value, the stronger the inhibition against the platelet agonist tested. * and # indicate statistically significant differences (p < 0.05) for the most and least bioactive extracts, respectively. Raw SH: raw salmon head; hPRP: human platelet-rich plasma; 80CSVSHNB = 80°C sous-vide cooked salmon head no brine; 80CSVSHB = 80°C sous-vide cooked salmon head brined

3.2 The effect of several sous-vide cooking procedures on the fatty acid content and structures of bioactive salmon lipids.

In order to evaluate the fatty acid composition of salmon TPL from all sous vide preparations, AS WELL AS TO GET INSIGHTS FOR THE ABOVE RESULTS TPL were saponified and analysed by LC-MS analysis. Representative chromatograms of
such an analysis for the saponified salmon TPL are shown in Figure 3.6. In addition, the changes of the obtained fatty acid composition of salmon PLs from all sous vide preparations can be seen in Figure 3.7 (The absolute values for FA-content can be observed in APPENDIX III). It is clear that these PL samples were rich in PUFA and especially in ω3 PUFA with a low ratio of n-6/n-3. More specifically, in PLs from 52°C CSVSB, the SFA were the most abundant fatty acid class followed by the PUFA and monounsaturated fatty acids (MUFA) ( ). PLs from 52°C CSVSB contain high amounts of ω3 PUFA, with the most abundant n-3 fatty acids being DHA (22:6ω3) and EPA (20:5ω3). Similar amounts of n-6 fatty acids were also present with the most abundant being linoleic acid (LA; 18:2ω6) followed by less amounts of DPA and arachidonic acid (ARA; 20:4ω6). Furthermore, the most abundant of the MUFA was found to be oleic acid (18:1 c9), followed by lower but considerable amounts of gadoleic acid (20:1 c9).

In the SFA the most abundant was the palmitic (16:0), followed by lower but considerable amounts of stearic acid (18:0). The overall n-3 fatty acid content of the PLs from 52°C CSVSB was similar to that of their n-6 fatty acids and thus the ratio of n-6/n-3 in PLs from 52°C CSVSB was approximately 0.95. Similarly, in the 52°C CSVSNB PLs the SFA were the most abundant fatty acid class followed by PUFA and MUFA (Appendix II). PLs from 52°C CSVSNB contain high amounts of n-3 PUFA, with the most abundant n-3 fatty acids being EPA (20:5ω3), while considerable but less amounts of DHA (22:6ω3) were also present. Considerable amounts of ω6 fatty acids were also present with the most abundant being linoleic acid (LA; 18:2ω6) followed by less amounts of arachidonic acid (ARA; 20:4ω6). Moreover, the most abundant of the MUFAs was found to be oleic acid (18:1 c9) and followed by gadoleic acid (20:1 c9). Palmitic acid (16:0) was deemed to be the most prevalent SFA followed by stearic acid (18:0). The overall ω3 fatty acid content of 52°C CSVSNB was similar to that of n-6 fatty acids and thus the ratio of n-6/n-3 was found to be approximately 1.03 (Appendix II).

Similarly, in the PLs 65°C CSVSB the SFA were the most abundant fatty acid class followed by the PUFA and less amounts of the MUFA (Appendix II). PLs from 65°C CSVSB contained high amounts of n-3 PUFA, while the most abundant n-3 fatty acid present being DHA (22:6ω3) followed by EPA (20:5ω3), while considerably less quantities of α-linolenic acid (18:3ω3) were also present.
Figure 3.6. Representative chromatograms of the LC-Ms analysis of the saponified salmon TPL from all sous vide preparations. A1 represents salmon cooked using the sous vide process at 52°C brined, A2 represents salmon cooked using the sous vide process at 52°C un-brined. B1 represents salmon cooked using the sous vide process at 65°C brined, B2 represents salmon cooked using the sous vide process at 65°C un-brined, C1 represents salmon cooked using the sous vide process at 80°C brined, C2 represents salmon cooked using the sous vide process at 80°C un-brined.
Figure 3.7. Depicts the FFA composition of the sous vide salmon preparations, using different cooking temperatures and conditions, fractions acquired by LC-MS analysis after saponification of these samples. 52CSVSNB = 52°C sous-vide cooked salmon no brine; 52CSVSB = 52°C sous-vide cooked salmon brined; 65CSVSNB = 65°C sous-vide cooked salmon no brine; 65CSVSB = 65°C sous-vide cooked salmon brined; 80CSVSNB = 80°C sous-vide cooked salmon no brine; 80CSVSB = 80°C sous-vide cooked salmon brined.
Considerable amounts of n-6 fatty acids were also present with the most prevalent being linoleic acid (LA; 18:2ω6) followed by considerably less amounts of arachidonic acid (ARA; 20:4ω6). Notably, similar to the PLs of 52°CVSBNB, the overall n-3 fatty acid content of the PL from 65°CVSBSB was similar to that of the n-6 fatty acids and therefore the ratio of n-6/n-3 was found to be approximately 1.04. In addition, the most abundant of the MUFA in PL from 65°CVSBSB was deemed to be oleic acid (18:1 c9) followed by gadoleic acid (20:1c9) and in the SFA the palmitic acid (16:0) and the stearic acid (18:0).

Similar to that of the previous samples, SFA was the most abundant fatty acid class followed by PUFA and MUFA. The majority of the PUFA contained in the PLs from 65°CVSBNB was found to be n-6 fatty acids, with the most abundant being linoleic acid (LA; 18:2ω6) and arachidonic acid (ARA; 20:4ω6). The most abundant n-3 fatty acids being DHA (22:6ω3) followed by considerably lower amounts of EPA (20:5ω3) (Appendix II). Notably, due to the higher overall n-6 fatty acid content in comparison to the n-3 fatty acids, the ratio of n-6/n-3 was found to be approximately 1.63 which is highest of the n-6/n-3 ratios in this study. In addition, the most abundant of the MUFA in PL from 65°CVSBNB was found to be oleic acid (18:1c9) and gadoleic acid (20:1c9) and the SFA the palmitic acid (16:0) and the stearic acid (18:0).

Following a similar trend, the PLs from 80°CVSBSB exhibited SFA as the most abundant fatty acid class followed by PUFA and MUFA (Appendix II). The majority of the PUFA contained in PLs from 80°CVSBSB were found to be n-6 PUFAs with the most abundant n-6 fatty acid being linoleic acid ((LA; 18:2ω6) and di-homo linoleic (20:2ω6). The most abundant n-3 fatty acids being DHA (22:6ω3) and EPA (20:5ω3). The overall the overall n-3 fatty acid content of the PL from 80°CVSBSB were similar to that of the n-6 fatty acids and thus the ratio of n-6/n-3 was found to be approximately 1.07. PLs from 80°CVSBSB contained relatively high amounts of SFA with the most abundant being palmitic acid (16:0) followed by less, but considerable, amounts of stearic acid (18:0) and MUFA, with the most abundant of the MUFA being oleic acid (18:1 c9) and gadoleic acid (20:1c9).

Finally, the SFA were the most abundant fatty acid class in the PL from 80°CVSBNB, followed by the PUFA and to a lesser extent the MUFA (Appendix II). The PUFA contained in the PL from 80°CVSBNB were mostly found to be n-6 PUFA with the majority being linoleic acid (LA; 18:2ω6). The most abundant n-3 fatty acids being DHA (22:6ω3) followed by lower amounts of EPA (20:5ω3). The n-6/n-3 ratio was
found to be approximately 1.19. PLs of 80°C CSVSNB exhibited relatively high amounts of SFA with palmitic acid (16:0) followed by stearic acid (18:0) but also considerable amounts of MUFA with the most abundant being oleic acid (18:1c9) and gadoleic acid (20:1c9).

### 3.4 Sensory Analysis

Data collected from napping were analysed using multiple factor analysis. As seen from the scree plot (supplementary data) the first two factors can explain the 57.10% of variance. As seen in Figure 3.8, factor 1 separates the sous vide samples at 52°C from the rest of the samples. Factor 3.9 separates the brined samples from the un-brined ones. The judges were asked to write down the attributes of the products which led to their groupings and it was found that 25 out of the 30 judges created their groups based on the texture of the products. Whereas only eleven times taste was mentioned and only one judge mentioned saltiness in specific. Seven times appearance was mentioned as a contributing factor for creating groups. Groupings generated from napping can be further explained in CATA data are taken into consideration.

![Figure 3.8: Principal coordinate analysis (PCA) plots from multiple factor analysis on the napping data](image)

Figure 3.8: Principal coordinate analysis (PCA) plots from multiple factor analysis on the napping data
The Figure 3.9 represents the symmetric plot of the products and the attributes as resulted from the principal coordinate analysis (PCA) of the CATA data. The first two dimensions explain 83.99% of the variation and the scree plot indicates the two first dimensions are sufficient to interpret the relationships between the attributes and the products. In this figure, it is observed that the two sous-vide samples at 52°C were perceived more similar and were characterised by attributes such as Fatty/Oily Appearance, Strong Fishy Odour, Moist Texture. Whereas the control samples and the samples sous-vide at 65°C were closer in the PCA plot and they were characterised as Flaky, Firm. This was expected since these samples were cooked at temperatures above 60°C.

Figure 3.9: Symmetric plot of products as revealed from the principle coordinate analysis on the CATA data. Abbreviations: At: Aftertaste; Tx: Texture; O: Odour; A: Aroma; T: Taste; Sousvide 52: salmon cooked sous-vide @ 52°C no brine; Sousvide B 52: salmon cooked sous-vide @ 52°C brined; Sousvide 65: salmon cooked sous-vide @ 65°C no brine; Sousvide B 65: salmon cooked @ sous-vide @ 65°C brined; Control 80:
salmon cooked sous-vide @ 80°C no brine; Control B80: salmon cooked sous-vide @ 80°C brined.

**Table 3.4**: Proportion of 1s across assessors for each combination of products and attributes. Multiple pairwise comparisons of products applying the McNemar test on the data from CATA. Abbreviations: A:Aroma; O:Odour; Tx:Texture; T:Taste; At:Aftertaste. 52CSVSNB = 52°C sous-vide cooked salmon no brine; 52CSVSB = 52°C sous-vide cooked salmon brined; 65CSVSNB = 65°C sous-vide cooked salmon no brine; 65CSVSB = 65°C sous-vide cooked salmon brined; 80CSVSNB = 80°C sous-vide cooked salmon no brine; 80CSVSB = 80°C sous-vide cooked salmon brined. *values across the rows sharing the same letter do not differ significantly.

<table>
<thead>
<tr>
<th>Attributes</th>
<th>p-values</th>
<th>52CSVSNB</th>
<th>52CSVSB</th>
<th>65CSVSNB</th>
<th>65CSVSB</th>
<th>80CSVSNB</th>
<th>80CSVSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-Pink/Salmon</td>
<td>0.991</td>
<td>0.600 (a)</td>
<td>0.567 (a)</td>
<td>0.533 (a)</td>
<td>0.533 (a)</td>
<td>0.567 (a)</td>
<td>0.600 (a)</td>
</tr>
<tr>
<td>A-Discoloured</td>
<td>0.290</td>
<td>0.200 (a)</td>
<td>0.100 (a)</td>
<td>0.200 (a)</td>
<td>0.267 (a)</td>
<td>0.100 (a)</td>
<td>0.233 (a)</td>
</tr>
<tr>
<td>A-Fatty/Oily</td>
<td>0.000</td>
<td>0.633 (b)</td>
<td>0.500 (b)</td>
<td>0.200 (ab)</td>
<td>0.200 (ab)</td>
<td>0.067 (a)</td>
<td>0.233 (ab)</td>
</tr>
<tr>
<td>O-Strong Fishy</td>
<td>0.342</td>
<td>0.300 (a)</td>
<td>0.233 (a)</td>
<td>0.167 (a)</td>
<td>0.100 (a)</td>
<td>0.100 (a)</td>
<td>0.167 (a)</td>
</tr>
<tr>
<td>O-Mild Fishy</td>
<td>0.791</td>
<td>0.400 (a)</td>
<td>0.467 (a)</td>
<td>0.567 (a)</td>
<td>0.500 (a)</td>
<td>0.533 (a)</td>
<td>0.167 (a)</td>
</tr>
<tr>
<td>Tx-Soft</td>
<td>0.000</td>
<td>0.567 (ab)</td>
<td>0.667 (b)</td>
<td>0.433 (a)</td>
<td>0.367 (ab)</td>
<td>0.200 (ab)</td>
<td>0.267 (ab)</td>
</tr>
<tr>
<td>Tx-Firm</td>
<td>0.000</td>
<td>0.133 (ab)</td>
<td>0.033 (a)</td>
<td>0.533 (bc)</td>
<td>0.267 (ab)</td>
<td>0.600 (c)</td>
<td>0.567 (c)</td>
</tr>
<tr>
<td>Tx-Dry</td>
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<td>0.033 (a)</td>
<td>0.067 (ab)</td>
<td>0.200 (ab)</td>
<td>0.167 (ab)</td>
<td>0.500 (b)</td>
<td>0.233 (ab)</td>
</tr>
<tr>
<td>Tx-Moist</td>
<td>0.001</td>
<td>0.767 (b)</td>
<td>0.667 (ab)</td>
<td>0.367 (ab)</td>
<td>0.533 (ab)</td>
<td>0.333 (a)</td>
<td>0.400 (ab)</td>
</tr>
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<td>Tx-Flaky</td>
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<td>0.100 (a)</td>
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<td>0.300 (a)</td>
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</tr>
<tr>
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<td>0.067 (a)</td>
<td>0.133 (a)</td>
<td>0.167 (a)</td>
<td>0.033 (a)</td>
</tr>
<tr>
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<td>0.267 (a)</td>
<td>0.267 (a)</td>
<td>0.133 (a)</td>
<td>0.167 (a)</td>
</tr>
<tr>
<td>T-Umami</td>
<td>0.714</td>
<td>0.200 (a)</td>
<td>0.200 (a)</td>
<td>0.100 (a)</td>
<td>0.200 (a)</td>
<td>0.200 (a)</td>
<td>0.233 (a)</td>
</tr>
<tr>
<td>At-Strong Fishy</td>
<td>0.107</td>
<td>0.367 (a)</td>
<td>0.300 (a)</td>
<td>0.200 (a)</td>
<td>0.167 (a)</td>
<td>0.233 (a)</td>
<td>0.067 (a)</td>
</tr>
<tr>
<td>At-Mouth Drying</td>
<td>0.000</td>
<td>0 (a)</td>
<td>0.067 (ab)</td>
<td>0.367 (ab)</td>
<td>0.133 (ab)</td>
<td>0.433 (b)</td>
<td>0.233 (ab)</td>
</tr>
<tr>
<td>At-Oily</td>
<td>0.000</td>
<td>0.533 (b)</td>
<td>0.267 (ab)</td>
<td>0.100 (a)</td>
<td>0.233 (ab)</td>
<td>0.067 (a)</td>
<td>0.233 (ab)</td>
</tr>
<tr>
<td>At-Sweet</td>
<td>0.321</td>
<td>0.200 (a)</td>
<td>0.100 (a)</td>
<td>0.100 (a)</td>
<td>0.200 (a)</td>
<td>0.133 (a)</td>
<td>0.133 (a)</td>
</tr>
</tbody>
</table>

In Table 3.4 shows the proportions of 1s across assessors for each combination of products and attributes. A high proportion means the attribute is frequently ticked by the judges for the product under investigation. For a given attribute, the Cochran’s Q test allowed to test for a given product whether the judges feel the attribute or not. For the cases of significant p-values multiple pairwise comparison where conducted by applying the McNemar test using XLstat 2018. Products with the same letter do not differ significantly. As seen on the table the main differences across samples are observed in
the attributes related to texture, such as firm, soft, dry and moist. There were also differences observed in the appearance attribute fatty/oily and in the aftereffect attributes mouth-drying and oily.

A contingency table with the sum of attribute tables across the assessors is used to construct a correspondence analysis (supplementary data). The independence between the rows (attributes) and the columns (products) is tested using chi-square test and it is found that there is dependence between the products and the attributes (p<0.0001).
Chapter 4:
Discussion

4.1 Introduction to discussion

Inflammatory and thrombotic events are implicated in all stages of atherosclerosis and CVD (Tsoupras et al., 2018b). Activation and aggregation of platelets are crucial parts of vascular physiology and pathophysiology, and thus in cardiovascular diseases (Tsoupras et al., 2018b, Stokes and Granger, 2012). Such processes can be induced by several platelet agonists, with the inflammatory and thrombotic mediators PAF and thrombin being the most potent ones, but also to a lesser extent by collagen and ADP (Tsoupras et al., 2018b, Demopoulos et al., 2003, Li et al., 2010, Tsopanoglou and Maragoudakis, 2009, Stokes and Granger, 2012, Tsoupras et al., 2019c).

Healthy dietary patterns have exhibited beneficial outcomes against atherosclerosis and CVD (Tierney et al., 2019). Several bioactive compounds from foods and fish of such diets, including salmon, have exhibited strong antithrombotic effects against platelet aggregation induced by PAF and thrombin (Tsoupras et al., 2018b, Lordan et al., 2017, Tsoupras et al., 2019a, Tsoupras et al., 2018a, Nasopoulou et al., 2011, Kalogeropoulos et al., 2008, Antonopoulou et al., 2005). Salmon is characterised as an oily fish that several nutritional guidelines have proposed to be included in healthy diets (Lara et al., 2007). Recently, bioactive lipids of salmon have been found to possess strong antithrombotic effects against not only PAF and thrombin but also against collagen and ADP pathways (Tsoupras et al., 2019a, Tsoupras et al., 2019c, Tsoupras et al., 2019d).

Epidemiologic studies have shown that high dietary consumption of salmon and other oily fish, such as herring and trout, is associated with reduced rates of myocardial infarction, atherosclerosis, and other ischemic pathologies (Appendix 1). However, it is important to state that salmon is mainly consumed cooked. The majority of the studies related to the effects of cooking to salmon are related mainly to the effects on its fatty acid composition, while reduction of its health benefits are mostly correlated with any changes in n-3 PUFA content in cooked salmon (Tierney et al., 2019, Goel et al., 2018, Ferraro et al., 2013).

To the best of our knowledge, there aren’t any studies reporting the effects of cooking, and especially of mild cooking techniques such as sous vide on the bioactive salmon PLs. This is the first study examining the effects of mild sous vide cooking procedures
on the bio-functionality and fatty acid content of salmon PLs in different conditions applied. More specifically, in this study we applied different temperatures 52°C, 65°C and 80°C) and brining conditions in order to evaluate the optimum conditions of sous vide that has the minimum effects on the functionality and fatty acid content of salmon PLs.

4.2. Effects of different sous vide preparations on the yield of extraction and separation of bioactive salmon PLs

The TL, TNL and TPL were extracted from the Irish organic farmed salmon (Salmo salar) subsequent to the various different sous-vide cooking preparations and used for analysis. Previous research has demonstrated the antithrombotic bioactivities that the TPL of raw Irish organic farmed salmon possess against PAF, thrombin collagen and ADP (Tsoupras et al., 2019c, Tsoupras et al., 2018a, Tsoupras et al., 2019a) and unpublished data communicated from Dr. Alexandros Tsoupras. Several other lipid extraction methods exist, for example (Folch et al., 1957). However, for this study we used a well-established extraction technique combined with the appropriate counter-current distribution technique to extract the highest efficacy of TPL from the TL extract of natural source, namely the Bligh & Dyer method (Bligh and Dyer, 1959). By applying such methodology in several fish species, including salmon, it is now well established that within the TPL fraction of such separations contains highly bioactive marine polar lipids. For this reason in the present study, we also applied similar methodologies with the one used in raw salmon (Tsoupras et al., 2018a) in order to gain maximum efficacy in obtaining the bioactive TPL from cooked salmon samples.

Salmon, which is classified as an oily fish species, is reported to contain high levels of TL as opposed to other fish species (Zhang et al., 2012b). Furthermore, the majority of extracted lipids belong to the more neutral classes of lipids, which combined are classified as TNL. As a result, of the TNL fraction contributing to approximately 60-84% of the raw salmon TL. Subsequently raw salmon TL contain lower amounts of the more polar classes of lipids, which combined are classified as TPL; the TPL fraction consist approx.16-40% of the salmon TL (Tsoupras et al., 2018).

Previous studies performed by Tsoupras et al., 2018 investigated the antithrombotic activities of polar lipids extracted from raw Irish organic farmed salmon (Salmo salar) and lower levels of TL, TNL and TPL were extracted upon comparison to all cooked
salmon samples in this study. Larsen et al., 2010 hypothesized that the lipids were extracted more effectively from cooked fish samples rather than raw fish due to the bound lipids being released as free lipids during the cooking process therefore, leading to an increased lipid extraction. The increase in lipid extraction from cooked salmon upon comparison to raw salmon could be as a result of mechanical factors during the homogenisation step of the Bligh & Dyer method (1959). The softer tissue of cooked fish is more efficiently homogenised enabling more lipids to be extracted. Various different lipid extractions have been used throughout the literature on fish therefore, it remains difficult to compare lipid extraction quantities on the same fish species (Al-Saghir et al., 2004, Candela et al., 1998, Ågren and Hänninen, 1993).

Therefore, it may be possible that in our samples, it seems that a higher release of NL during the cooking process lead to an increase in lipid extraction in TNL and subsequently in TL, whereas the TPL was similar. The % TL was lower in the cooked salmon samples when compared to raw, the fact that the absolute values of TPL (g/100g) did not change between the raw salmon and the sous-vide cooked salmon. It was reported that an increase in TL from cooked fish samples was due to the loss and changes in other lipid materials in the fish muscles (Mai et al., 2006). It was also suggested that TL in cooked fish samples was due to the polymerisation and/or oxidation of the triglycerides (Bakar et al., 2008).

4.3 The effect of several sous-vide cooking preparations on the bio-functionality of salmon PLs against platelet aggregation

The consumption of fish and fish oils has improved platelet function, human thrombosis, and haemostasis in several disorders (Knapp, 1997, Mori, 2018, Axelrod et al., 1994, Mori et al., 1997, Goel et al., 2018). These effects have primarily been attributed to the EPA and DHA content of fish through mechanisms related to the eicosanoid pathways (Goel et al., 2018). However, several recent reviews and meta-analyses have indicated that there is insufficient evidence for any benefits from n-3 PUFA supplements, in the form of purified fatty acids or in the form of esters, on reducing the risk for CVD (either on primary or in secondary prevention) and on lowering the risk of all-cause mortality, cardiac death, sudden death, myocardial infarction, stroke, or cancer (Enns et al., 2014, Walz et al., 2016, Manson et al., 2019, Rizos et al., 2012, Kwak et al., 2012, Chowdhury et al., 2012, Lordan et al., 2017)
Remarkably, several of these studies have also proposed that the beneficial effect of fish intake on cerebrovascular risk is likely to be mediated through the interplay of a wide range of nutrients abundant in fish (Chowdhury et al., 2012, White and McHowat, 2007, Lordan et al., 2017). Within this concept, promising outcomes have been attributed to polar lipids (PLs) of marine origin (Lordan et al., 2017) especially those bearing n-3 PUFA within their structure that were found also in salmon (Lordan et al., 2017, Antonopoulou et al., 2005).

PLs from raw salmon have been recently found to possess strong anti-inflammatory, antithrombotic, and cardio-protective activities against platelet activation and aggregation induced by inflammatory and thrombotic mediators such as PAF and thrombin, but also against well-established platelet agonists such as collagen and ADP (Tsoupras et al., 2019a, Tsoupras et al., 2019c, Tsoupras et al., 2019d) and unpublished data communicated by Dr Alexandros Tsoupras.

It is well established that platelet activation and aggregation is a well-defined risk factor for CVD (Tsoupras et al., 2018b). Moreover, European Food Safety Authority (EFSA) has declared as one of the health claims related to CVD for a food or a food product (functional food, food supplement, nutraceutical, etc) its ability to reduce platelet aggregation. Therefore, in this study we studied the effects of cooking on the capability of salmon bioactive compounds to inhibit platelet aggregation. More specifically, within this study, we examined the effects of mild cooking procedures, such as sous vide, in several conditions of temperatures and the effect of brining on the bio-functionality of salmon TPL against platelet aggregation induced by the platelet agonists, PAF, thrombin, collagen and ADP, in human platelet preparations (EFSA 2011).

The effects of cooking on fish PL bioactivities against platelet aggregation has been previously studied in cod (Panayiotou et al., 2000) and sardines (Nasopoulou et al., 2013. Moreover, in these studies they have used washed platelet preparations, and examined solely, PAF. The novelty of this study is the effects of cooking on the bio-functionality of TPL obtained from salmon, a native Irish oily fish, against platelet aggregation of human platelet rich plasma (hPRP), induced not only by PAF, but also by thrombin, collagen and ADP.

In this study we performed milder cooking preparations according to the sous vide technique which differs from to the cooking techniques used in cod and sardine cooking.
studies (frying and grilled), in order to evaluate if in these milder conditions have better preservation of the bioactivities of salmon TPL.

The bioactivities of TPL from cooked salmon against platelet aggregation were tested in human platelet preparations of hPRP according to (Tsoupras et al., 2019d) and not in washed platelets as it was investigated in the cases of cod and sardine. The use of hPRP provide the benefit to investigate the bioactive salmon TPL in more physiological media, and its binding is not altered by plasma proteins and other plasma constituents, which is closer to the in vivo conditions.

Furthermore, in contrast to the previous studies in cod and sardine, in the present study we examined the effects of sous vide cooking on the bio-functionality of salmon TPL against platelet aggregation induced not only by PAF, but also by other well established thrombotic and platelet activation agonists such as thrombin, collagen and ADP, in human platelet preparations. This was done so, in order to obtain an overall evaluation of the effects of cooking on the anti-thrombotic activities of salmon TPL with respect to most well renowned pathways involved in platelet aggregation that are also recognised by EFSA. Such holistic approaches facilitate the overall evaluation of the effects of sous vide on the bio-functionality of salmon TPL against all platelet aggregation related pathways.

It should be mentioned that in the present study, samples were of Irish organic farmed salmon origin were used in order to compare the anti-thrombotic effects of sous vide cooked salmon from the salmon of the same lot number, the same supplier and of the same quality to previous studies performed by (Tsoupras et al., 2018a, Tsoupras et al., 2019a).

Salmon fillets were cooked by the sous vide technique in several cooking temperatures (52°C, 65°C and 80°C), while for each different temperature a sample was either brined or un-brined by a 10% sodium chloride to water solution. From the literature, the optimum sous-vide cooking temperature for salmon, in order to maximise taste and texture qualities was 52°C (Brookmire et al., 2013). The 80°C temperature was used as this is a standard temperature for the pasteurisation of food (González-Fandos et al., 2005). The median temperature of 65°C was used in order to evaluate the effects of the intermediate temperature of sous-vide cooking of salmon samples on its anti-thrombotic properties. Brining is a common technique used by chefs worldwide to improve the texture of cooked fish to prevent it from flaking upon cooking, hence that is why we
used the brining process (Jittinandana et al., 2002). It is thus important to study on an individual basis the effect of different temperatures and brining conditions on the composition of lipids and any possible change on the biological activity of these lipids.

The in vitro antithrombotic properties of the TPL of all these samples against human platelet aggregation the action of PAF/Collagen/Thrombin/ADP-induced in human PRP (hPRP) with the results displayed in Figures 3.2-3.5. All the results are expressed as IC50 values, which are described as the mass of lipid measured in micrograms (µg) that is necessary to induce half the maximal-reversible aggregation caused by PAF/Collagen/Thrombin/ADP-induced platelet aggregation (Tsoupras et al., 2019d). In general, the half maximal inhibitory concentration (IC50) is a measure of the potency of a substance in inhibiting a specific biological or biochemical function. IC50 is a quantitative measure that indicates how much of a particular inhibitory substance (e.g. drug/bioactive compound, etc.) is required to inhibit, in vitro, a given biological process or biological component by 50%, and thus it is the most effective and well-established value for comparing the potency of two or more bioactive inhibitors/compounds. These are some of the reasons for using in our study IC50 values for comparisons of the inhibitory strength of each lipid sample from all the sous vide salmon preparations against PAF, thrombin, ADP and Collagen induced platelet aggregation.

In the present study, it was found for the first time that mild sous vide preparations from 52°C until 65°C did not affect the bio-functionality of salmon TPLs with strong antithrombotic bioactivities against well-established platelet-agonists, namely PAF, thrombin, collagen, and ADP. The strong anti-platelet effects of the TPL from these sous vide preparations were also found to be comparable to the relevant effects of the raw SFs and to other fish species (Tsoupras et al., 2019a, Tsoupras et al., 2018a, Nasopoulou et al., 2007, Nasopoulou et al., 2013, Panayiotou et al., 2000)

More specifically, PLs from brined and un-brined sous vide salmon preparations at 52°C and 65°C were found to strongly inhibit the PAF pathway of human platelet aggregation, an effect that was found to be similar with the PLs from the raw SFs (Tsoupras et al., 2018a, Tsoupras et al., 2019a) but also with other edible fish species (Lordan et al., 2017, Nasopoulou et al., 2013, Panayiotou et al., 2000).

However, by applying higher temperatures, such as those used for pasteurisation (80°C) the anti-PAF effects of cooked salmon were decreased, in both brined and un-brined sous vide salmon preparations, compared to all the other sous vide salmon preparations.
and when compared to raw SF. When the temperature increases in the sous-vide cooking process, a considerable reduction in the potency of anti-PAF effects was observed. These results are in accordance with the previous reported results in cooking preparations (grilling) in another oily fish, namely sardines, in which a decrease in the anti-PAF activities of sardine TPL was also observed in high temperature of grilling in comparison to raw sardines (Nasopoulou et al., 2013).

PAF is a potent inflammatory mediator implicated in the development and progression of atherosclerosis, CVDs and other inflammation-related chronic disorders. (Tsoupras et al., 2018a). The preservation of the bio-functionality of TPL when applying sous vide producing cooked salmon as a functional food, even if cooked, with strong anti-PAF activities and thus potent anti-thrombotic and cardio-protective properties.

In raw SF the anti-PAF effects of salmon TPL were stronger than their antithrombin (Tsoupras et al., 2018a). These results have proposed that the bioactive TPL from SF possess higher specificity against the PAF-pathway, rather than the thrombin pathway (Tsoupras et al., 2018a). After sous vide cooking to 52°C-65°C the antithrombin effects of TPL from cooked SF were similar to the anti-thrombin effects of raw SF TPL. Therefore, the anti-thrombin effects of TPL from sous vide preparations in both 52°C and 65°C were again less potent than their anti-PAF activities, suggesting that the higher specificity of salmon TPL against PAF in comparison to their anti-thrombin effects was preserved.

However, by applying higher temperatures, such as those used for pasteurisation (80°C) the anti-thrombin effects of cooked salmon were again decreased, in both brined and un-brined sous vide salmon preparations, compared to all the other sous vide salmon preparations and when compared to raw SF.

This is the first study examining the effect of general cooking in bio-functionality of bioactive PLs from a food source in relation to their anti-thrombin effects but also against collagen and ADP induced platelet aggregation. Platelets are endowed with a repertoire of surface receptors that enable them to adhere, activate and aggregate upon vascular injury by several mediators and their related pathways, such as PAF (Tsoupras et al., 2018c). Apart from PAF, platelet adhesion is governed by the interaction between vascular collagen and its related receptors on platelet membranes and the engagement of other soluble agonists, predominantly ADP, Thromboxane A2 (TXA2) and thrombin, with their related Gq-protein coupled receptors (Vilahur et al., 2018). Therefore, an
understanding of the platelet signalling mechanism involved in thrombus formation by all these agonists and their related pathways is necessary to identify the overall antithrombotic properties of food related antiplatelet agents, such as the bioactive salmon TPL.

Concerning the anti-ADP effects of salmon TPL, by applying mild temperatures in the sous vide preparations (52°C and 65°C) either brined or un-brined, the strong inhibitory effects of salmon TPL against ADP-induced platelet aggregation were preserved in similar potency with those of the raw SF (Unpublished data communicated by Dr Alexandros Tsoupras). Similar to the changes observed on the anti-PAF activities of the salmon TPL after sous vide cooking, it was also observed that the anti-ADP effects of salmon TPL were significantly reduced when higher temperatures were applied (80°C brined and un-brined) during sous vide preparations. PAF-pathway of platelet aggregation is related to the intracellular changes within platelets after the binding of PAF on its specific for PAF G-coupled protein receptor, namely PAF-receptor. Intracellular signalling triggered by such an initial PAF-related signal is that of the eicosanoids and of ADP, which concludes in release and secretion of ADP from platelet granules to enhance the initial signal (related to PAF) by a further activation of platelets by the ADP-pathway too, resulting in platelet aggregation and thrombus formation (Tsoupras et al., 2019b). Thus, PAF, eicosanoids and ADP pathways are inter-related, and the anti-PAF effects of food food-derived bioactive TPL including salmon TPL seem to be related with their anti-ADP effects. The aforementioned explain the observed similarities between the changes found in the anti-PAF and anti-ADP activities of salmon TPL after cooking.

Another interesting pathway for inducing platelet aggregation that recently has received plenty of attention as a target for drug and food supplement development, is that related to collagen (Vilahur et al., 2018). One of the events that triggers platelet tethering to the site of vascular injury occurs upon subendothelial collagen exposure, mostly fibrillar types I and III, to the circulation. Collagen mediates platelet adhesion by interacting with a range of unique surface platelet receptors. Binding of Collagen on these receptors activates by specific intracellular signalling the aggregation of platelets by specific pathways. The ability of salmon TPL to inhibit the collagen pathway is of great importance for the endothelium platelets interaction during atherosclerosis and atherothrombosis. Notably, bioactive TPL from raw salmon have recently exhibited
strong anti-collagen effects (Unpublished data communicated by Dr Alexandros Tsoupras).

Within the present study, it was also found that mild cooking sous vide preparations in 52°C preserved these strong anti-collagen effects of salmon TPL. Interestingly, and differently than what happened to all the effects of salmon TPL against all the other platelet agonists, the TPL from 65 brined and un-brined sous vide salmon preparations exhibited highly potent anti-collagen effect which was significantly stronger than the anti-collagen effects of TPL from either the 52°C or the raw SF.

Similarly, the anti-collagen effects of the TPL from salmon sous vide preparations were preserved even when higher temperatures were applied (80°C) either in brined or in un-brined salmon preparations.

Differently than SF, salmon PLs from sous vide preparations of SH at 80°C exhibited similar antithrombotic properties against platelet aggregation induced by PAF, ADP and collagen with those of salmon PLs from raw SH (Tsoupras et al., 2019c). A reduction was observed only in the anti-thrombin properties of SH-PLs from both brined and un-brined sous vide preparations at 80°C, in comparison with those of the raw SH in human platelets (Tsoupras et al 2019). SH were chosen to be also tested in this study, since they are the main by-product of fish industries of salmon products, and thus its valorisation is crucial for sustainable development (Tsoupras et al., 2019c). In addition, SH are consumed in several places globally (Ferraro et al., 2010). Higher temperatures (80°C) were applied in order to overcome the different and more dense structure of these salmon samples. Notably, the higher preservation of the antithrombotic properties of salmon PLs from cooked SH in comparison to that of the cooked SF seems to be related with this structural and textural difference of SH to SF samples, since the overall tissue structure of the SH seem to encase the bioactive PLs within. However, more studies are required to fully evaluate the differences observed in the antithrombotic properties of salmon PLs from sous vide SH and SF preparations.

The variance on the changes observed in the anti-platelet effects of the bioactive salmon TPL against not only PAF, but also against all the other platelet agonists studied (thrombin, collagen and ADP), during sous vide processing in different temperatures and brining conditions, seem to be related to alterations of the fatty acid composition in these salmon TPL. For example, changes in the content of DPA of the bioactive salmon TPL in different temperatures may be related to the differences in their anti-collagen
activities, since DPA is related to inhibition of the collagen pathway (Murugappa and Kunapuli, 2006). DPA content was increased in the TPL from sous vide salmon preparations in 65°C, compared to those in other temperatures, which may explain the stronger anti-collagen effects of the TPL derived from sous vide salmon in this temperature, rather than those in raw salmon fillets or in lower (52°C) and higher (80°C) temperatures. In addition, changes observed in both EPA and DHA content when observed when higher temperatures were applied may also be related to the reduction of the anti-PAF, anti-thrombin and anti-ADP effects of salmon TPL.

An overall evaluation of the effects of sous vide cooking on the changes of the fatty acid composition of bioactive salmon TPL in relation to their observed anti-platelet effects against all platelet agonists tested (PAF, thrombin, collagen and ADP) is further discussed in the following section.

4.4 The effect of several sous-vide cooking preparations on the fatty acid content in relation to the bio-functionality of salmon PLs

Previous elucidation of the structure activity relationship suggested that specific phospholipid moieties bearing ω3 PUFA, such as EPA and DHA, in their sn-2 position of their glycerol backbone, either belonging to alkyl-acyl-PLs or acyl-acyl-PLs, seem to be present in most bioactive subclasses of the SFs, providing a rational for their strong antithrombotic effects (Tsoupras et al., 2018a, Tsoupras et al., 2019a). Furthermore, it has been previously reported that cooked salmon also contains phospholipids bearing considerable amounts of n-3 PUFA

Within our study, by applying LC-MS analysis according to Tsoupras et al (Tsoupras et al 2019a), we also found that sous vide preparations of salmon in several different temperatures and/or brined/un-brined contain high amounts of PLs bearing PUFA within their structure, the majority of which were n-3 PUFA and especially DHA and EPA, similar to the raw salmon (Tsoupras et al., 2018a). These results were shown in Figure 3.6 and the absolute values appear in the APPENDIX III. Considerable amounts of n-6 fatty acids were also present with the most abundant being linoleic acid followed by less amounts of arachidonic acid. On the other hand, all samples exhibited high levels of saturated fatty acids, specifically, palmitic acid being the most abundant, followed by stearic acid in all samples. Furthermore, the most prominent MUFA was found to be oleic acid followed by gadoleic acid. All these results are in accordance
with previous reports in raw (Tsoupras et al., 2018a) and cooked salmon (See Table 1.1 and Table 1.4).

More specifically, in both brined and un-brined salmon sous vide preparations in 52°C (brined and un-brined) the fatty acid composition of salmon we reported that SFA and MUFA increased, whereas PUFA were decreased because n-6 were decreased but n-3 remained similar to raw SF. Therefore, it is possible that the double bonds of n-6 PUFA broken from cooking into the relative SFA and MUFA. It seems that n-6 are more susceptible to cooking procedures rather than the n-3. Instead, the n-3 PUFA remained unaffected by these mild procedures. Overall, n6/n3 was found to be approximately at the range of the value 1/1, which is much lower than that of Westernised diets that is within the range of 5/1–20/1 (Simopoulos, 2002). It has been reported that high values of this ratio are correlated with a higher risk in cardiovascular disease and other chronic diseases (Simopoulos, 2002). Taking this into account, the observed favourable n-6/n-3 ratio in the bioactive TPL from 52°C further supports the preservation of the cardioprotective properties for PLs from 52°C sous vide salmon since low values for this ratio seem to provide several beneficial health outcomes in CVD and other chronic disorders (Simopoulos, 2016). Interestingly, this ratio was also preserved in values within the range of 1/1 even when higher temperatures were applied. In these conditions of sous vide processing in 65°C and 80°C, there was a decrease observed in both n-6 and n-3 which may explain why the n-6/n-3 ratio remained relatively unaffected.

Interestingly, when higher sous-vide cooking temperatures were applied, a reduction was observed in the EPA content of salmon TPL in both 65°C and 80°C in un-brined sous vide preparations, while a similar reduction was also observed in their DHA content only in the 80°C un-brined samples. These changes are related to the reduction of the anti-platelet effects of salmon TPL against all platelet agonists tested (PAF, thrombin, collagen and ADP) when temperatures of 80°C was applied, since the most bioactive TPL in salmon have been previously proposed to be the ones bearing such n-3 PUFA within their structures (Tsoupras et al., 2018a, Tsoupras et al., 2019a). It is proposed that the breakdown of these n-3 PUFA on the salmon TPL as a result of the higher temperature was applied, are related to the reduction of the bioactivities of the most bioactive salmon TPL against platelet aggregation. These results are in agreement with previous results on sardine cookery (Nasopoulou et al., 2013).

Even though there was such a reduction observed in both the EPA and DHA content and therefore in the anti-platelet activities of salmon TPL in high cooking temperatures
against PAF, thrombin and ADP, still the bioactivities of salmon TPL against these platelet agonists in these high temperatures were not diminished and were preserved at considerable levels, while their anti-collagen effects remained relatively unaffected. These outcomes further suggest that salmon is still a functional food against platelet aggregation with low n-6/n-3 ratio with cardioprotective properties, even when cooked in such high temperatures, lower than 100°C (80°C). Further studies are needed in order to evaluate the effects of applying higher temperatures with appropriate cooking techniques on the overall anti-platelet effects of salmon TPL.

In contrast to the un-brined samples, in brined samples when the same higher temperatures were applied, there was not a significant decrease of both EPA and DHA content in the bioactive salmon TPL. This finding in all brined salmon samples before and after sous vide preparations differ from previously reported reduction of EPA and DHA when brining another oily fish such as sardines (Nasopoulou et al., 2013). Our results suggest that brining in salmon may not affect the n-3 content of salmon even when cooked between the temperatures of 52°C to 80°C by mild sous vide cooking process.

Furthermore, a decline in the anti-platelet effects of salmon TPL was observed against PAF, thrombin and ADP. Therefore, such a reduction on the anti-platelet effects of brined samples against these platelet agonists seem to be related to the effects of higher temperatures in other type of bioactive PLs. It should also be stressed that the bioactive marine TPL extracts contain several bioactive PL subclasses either of phospholipid or glycolipid moieties or of both, with several fatty acid content. The bioactivities of these PL are usually related with the degree of unsaturation of their FA content and the positions of their double bonds. But this is not the case for all bioactive PLs. For example, there are bioactive alkyl-acyl PLs with strong anti-PAF activities, without being highly unsaturated (Tsoupras et al., 2018b). In addition, in sea bass, specific peculiar glycolipids exhibited the most profound anti-platelet effects against PAF (Nasopoulou et al., 2014, Tsoupras et al., 2011,2012). Therefore, further studies are required with implementing overall structural analysis of the PL involved, for further evaluation of the changes in the total structures of the bioactive salmon TPL by such sous vide preparations and their structure activity relationships.

Nevertheless, by applying mild cooking sous vide processing the preservation of the potent antithrombotic properties of the bioactive salmon TPL against inflammatory and thrombotic mediators such as PAF and thrombin, but also against well-established
platelet agonists such as collagen and ADP, along with the preservation of their n-3 content and n-6/n-3 ratios, highlight both the bio-functionality of cooked salmon and the efficacy of the sous vide technique.

This is of great significance, since salmon is consumed mostly cooked. Therefore, if in cooked salmon such bioactive compounds are preserved, during consumption of cooked salmon a considerable proportion of such bioactive food-derived PL remain intact during digestion (Lordan et al., 2017, Burri et al., 2012) After their absorption into the blood stream through the intestinal tract, the polar head of bioactive PL amphiphilic moieties facilitate their superior incorporation into blood lipoproteins and their more effective transfer to several tissues and blood cells membranes, including platelets, when compared with other neutral forms of ω3 PUFA, such as FFA, esters or TAG (Burri et al., 2012, Lordan et al., 2017, Tsoupras et al., 2019a). Thus, the amphiphilic properties of such bioactive PL facilitates their superior bioavailability of their ω3 PUFA content, which indicates that they are more effective and influential on platelet functionality in comparison with the aforementioned neutral forms of n-3 PUFA (Tsoupras et al., 2018a, Tsoupras et al., 2019a).

When in the membranes of blood cells, including platelet membranes, such bioactive salmon PLs, can beneficially inhibit platelet-activation, either by their direct binding and effects on platelet membrane receptors related to platelet-aggregation, or by their indirect effects on the lipid-related microenvironment of such receptors, and/or by an overall indirect effect on platelet aggregation by affecting the functionality and polarisation of platelet membranes (Tsoupras et al., 2018b, Mori, 2017, Li et al., 2010, Tierney et al., 2019, Lordan et al., 2017, Tsoupras et al., 2018a, Tsoupras et al., 2019a) Since recent findings have exhibited that platelet-activation induced by pathways associated with PAF, thrombin, collagen, and ADP, seems to be inter-connected and interrelated in several inflammation and thrombosis related manifestations (Tierney et al., 2019, Tsoupras et al., 2018a, Tsoupras et al., 2019a, Stokes and Granger, 2012, Mastenbroek et al., 2015, Vilahur et al., 2018, Keating and Schneider, 2009, Melnikova et al., 2008, Zimmerman et al., 1985), the overall beneficial antiplatelet effects of SPL against these platelet agonists strongly suggest that they possess cardioprotective properties, and well preserved when salmon is cooked in mild cooking conditions such as those of the sous vide technique.
4.4 Sensory Analysis

The sous-vide cooking process allows for food stuffs of high nutritional quality and high sensory attributes to be preserved with a longer shelf life than other cooking-cooling processes. Therefore, in this study, we performed sensory analysis on all sous vide salmon preparations in order to detect the changes in smell, taste, colour, juiciness as well as the appearance of off-flavours and off-odours during the sous vide cooking process (Diaz et al., 2009).

In Table 3.8, it can be observed that the two sous-vide samples at 52°C were perceived more similar and were characterised by attributes such as Fatty/Oily Appearance, Strong Fishy Odour, Moist Texture. Whereas, the control samples (80°C) and the samples sous-vide at 65°C were closer in the PCA plot and they were characterised as Flaky, Firm. This was expected since these samples were cooked at temperatures above 60°C.

Cooking fish and meat results in improved taste, softer texture, increased safety, and improved nutrient digestibility (KADAM and PRABHASANKAR, 2012). The muscle of the fish separates into flakes at temperatures around 46-49°C (Ofstad et al., 1995). While collagen which is present in connective tissues, shrinks and solubilises at around 60°C and above 65°C is transformed into gelatine. Ofstad et al., 1993 tested salmon and cod fillets cooked between the temperatures of 50-70°C and reported that the maximum water loss from salmon flesh was at 50°C. After this point at temperatures above 60°C, various structural changes in the conjunctive tissue of the fish take place which can explain the changes in structure. These changes were detected by the judges. Moreover, a more recent study by Kato et al., (2016) showed that the texture of fish cooked under sous-vide at temperatures higher than 65°C was significantly different from the raw fish. The changes in texture were measured using a texture analyser and no sensory data were collected for these samples (Kato et al., 2016).

Interestingly there were not significant differences due to taste as it would be expected between the brined and un-brined samples. This was the case for both the CATA data and the napping data as seen in Figure 3.7 and Figure 3.8.

Previous research (Alexi et al., 2018) has shown that CATA results obtained from semi-trained panellists on CATA terms can be comparable to results from descriptive profiling. However, in this case although the panellists had conducted other sensory tests were not trained in the attributes used for CATA therefore they cannot be compared with the semi-trained panellists from the study by Alexi et al (2018). On the other hand, the findings
from CATA are comparable to the findings from napping as seen in Figure 3.8. Indeed, as seen in Figure 3.7 samples cooked at 52°C were perceived different from the rest of the samples. The fact that CATA and napping findings were in agreement to corroborated by the findings of Reinbach et al., (2014) who got comparable information from CATA and napping on beer samples.
Chapter 5:

Conclusions

- Sous vide salmon preparations in mild conditions (52°C-65°C) of both brined and un-brined salmon resulted in preservation of the antithrombotic properties of bioactive salmon PLs against human platelet aggregation induced by the inflammatory and thrombotic mediators’ platelet-activating factor (PAF) and thrombin, but also by the well-established platelet agonists adenosine diphosphate (ADP) and collagen.

- However, higher temperatures such as those applied for pasteurization (80°C) resulted in reduction of these antithrombotic properties, which was accompanied by a reduction in the EPA and DHA content of salmon PLs, especially in the un-brined sous vide salmon preparations at 80°C.

- Nevertheless, fatty acid compositional analysis of all sous vide salmon preparations, showed that even though there were variances observed on the FA content in different temperatures, especially for the un-brined salmon samples, the overall n-6/n-3 ratio of their PUFA content was also preserved in low values within the beneficial range of the 1/1 value for CVD.

- Sous vide salmon preparations in all conditions did not affect the taste, whereas in 65°C and 80°C slight textural differences were observed.

- Emerging cooking techniques, such as sous vide seem to be the optimal procedures for preserving the bioactivities and fatty acid composition of salmon, without affecting its taste and texture

- Further studies are needed in order to fully evaluate the benefits and drawbacks of sous vide in fish cookery and functional food processing.
# APPENDIX I: Studies on the benefits of fish-consumption

<table>
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<tr>
<th>Author</th>
<th>Study Design</th>
<th>Study Focus</th>
<th>Outcome</th>
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<td>(Rundblad et al., 2018)</td>
<td>8-week randomized parallel study</td>
<td>Comparison of krill oil (KO) supplementation and fish consumption with similar amounts of n-3 FA on the cardiovascular risk markers</td>
<td>Fasting serum TAG did not change. TL (P=0.007), PL (P=0.015), cholesterol (P=0.009), cholesteryl ester (P=0.022), all increased in KO supplementation. Blood glucose decreased (P=0.0024) in KO group. Vitamin D increased in fish group (P=0.024). Plasma levels of marine n-3 increased in KO and fish group compared to control (P≤0.0003)</td>
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<tr>
<td>(Raatz et al., 2013)</td>
<td>Randomized 3 period crossover-designed trial (4-week treatment, 4-8 week washout)</td>
<td>Compare the effects of twice per week consumed farmed Atlantic salmon at doses 90, 180 and 270g and its effects on plasma phospholipids FA proportions and various CVD risk biomarkers</td>
<td>No changes were observed in any CVD risk biomarkers in either group. Salmon intake changed Plasma Phospholipid n-3 and n-6 FA proportions. Arachidonic acid decreased (P=0.0002) with salmon dose independent. No change in LA in all treatments. Total n-3 reduced (P&lt;0.0001). EPA increased in a dose-dependent manner (P&lt;0.0001). DHA increased in all treatments (P&lt;0.0001). Total n-3 increased dose dependent manner (P&lt;0.0001) and EPA+DHA</td>
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<tr>
<td>Study</td>
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<td>Intervention Details</td>
<td>Findings</td>
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<tr>
<td>(Elvevoll et al., 2006)</td>
<td>Dietary intervention (n=71)</td>
<td>To assess the impact of the source of n-3 FA on their incorporation in serum, on blood lipid composition and on cellular activation. 5 groups involved in trial. 3 groups given 400g fish per week for 8 weeks (smoked salmon, salmon &amp; cod). 4&lt;sup&gt;th&lt;/sup&gt; group given 15ml/day cod liver oil and 5&lt;sup&gt;th&lt;/sup&gt; group as control with no supplementation</td>
<td>EPA and DHA in cooked salmon increased (129% in EPA and 45% in DHA) compared to CLO (106% EPA and 25% DHA). No significant changes were observed in CVD risk biomarkers</td>
</tr>
<tr>
<td>(Lara et al., 2007)</td>
<td>Dietary Intervention (n=41) 4-week period followed by a 4-week washout period</td>
<td>To test the hypothesis that oil-rich fish consumption improved CHD risk factors. Non-obese healthy subjects consumed 125g/day of salmon over 4-week period followed by 4-week washout period. Subjects were instructed to maintain dietary and physical activity patterns during trial</td>
<td>Compared to no-fish, salmon consumption significantly reduced systolic, diastolic and mean arterial BP by 4%, TG by 15% and LDL-C by 7%. HDL-C increased significantly by 5% (P&lt;0.05). Plasma adiponectin showed a trend toward improvement (8.39µmmol/L with salmon and 7.52µmmol/L with no-fish; P=0.086). No significant changes were found either in plasma, leptin, glucose or insulin after salmon consumption</td>
</tr>
<tr>
<td>(Lindqvist et al., 2007)</td>
<td>Randomized crossover trial</td>
<td>The effect of a 4-week herring diet compared to a reference diet on biomarkers for CVD in obese patients. Subjects were randomly assigned to 4-week diet of 150g baked herring/day for 5 days/week or reference diet (pork and chicken fillets) and switched diets after 2-week washout.</td>
<td>P-HDL was significantly higher in herring diet (1.22 vs 1.13 mmol/L P=0.036). Small but no significant decreased in TAG, no effect on other biomarkers. TEAC and FRAP, but not ORAC-values indicated that plasma antioxidants may have been reduced. CRP levels were lower in herring diet.</td>
</tr>
<tr>
<td>(Din et al., 2008)</td>
<td>Dietary Intervention</td>
<td>Assessed the effect of dietary intervention with oily fish on platelet-monocyte aggregation in healthy subjects. 14 subjects had their diet supplemented with 500g of oily fish per week for 4 weeks. The control group of 14 subjects had no dietary supplementation.</td>
<td>Oily fish group had an increase in n-3 FAs in plasma phospholipids (14.2 ± 3.4% vs 5.8 ± 1.3%, P&gt;0.001. In contrast to control group, platelet-monocyte aggregation was reduced by 35% with oily fish consumption (16.0 ± 9% to 24.8 ± 10.9%, P&lt;0.01) and returned to basal levels 4 weeks after discontinuation of supplement. Inverse correlations were observed between platelet-monocyte aggregation and plasma n-3 FA concentrations (R=0.421, P=0.006). No changes in plasma markers of platelet aggregation.</td>
</tr>
<tr>
<td>(Giacco et al., 2007)</td>
<td>Randomized clinical trial</td>
<td>The effect of high intake of lean or fatty fish on glucose tolerance, leucocyte membrane FA composition and leucocyte function in overweight adults. 68 healthy</td>
<td>High intake of fatty fish, not lean fish, significantly improved glucose regulation 120mins postprandially (P=0.012) but did not affect fasting glucose concentrations.</td>
</tr>
</tbody>
</table>
overweight/obese participants consumed 750g/week of either lean or fatty fish and control group avoided fish for an 8-week period. Energy, macronutrient and physical activity was not altered during the study.

A small increase in fasting to 120mins postprandial insulin C-peptide concentration was observed after fatty fish intake (P=0.012). Lean fish consumption increased DHA in leucocyte membranes (P=0.010) and fatty fish increased n-3 PUFA content (P=0.00016) and reduced n-6 PUFA content (P=0.00057) in leucocyte membrane.

(Aadland et al., 2015) Randomized controlled trial with a crossover design

The aim of this study was to elucidate the potentials of 2 main dietary protein sources (lean seafood and non-seafood) to modulate fasting and postprandial lipids in healthy subjects. After 3 week run in periods and separated by a 5 week washout period, 20 healthy subjects consumed 2 balanced diets that varied in main protein sources (60% of total dietary proteins from lean seafood or non-seafood sources for 4 weeks

Compared to the non-seafood intervention, the lean-seafood intervention group reduced fasting (relative differences by diets: 0.31mmol/L: P=0.03) and postprandial (P=0.01) serum triacylglycerol concentrations. The lower serum triacylglycerol in chylomicrons and VLDLs (P=0.004), reduced fasting VLDL particle size (P=0.04) and a reduced postprandial concentration of medium sized VLDL particles (P=0.02). The lean seafood intervention prevented the elevated ratio of total cholesterol to HDL cholesterol in the fasted serum (P=0.03) and postprandial serum (P=0.01) observed after non-seafood diet.
### Dietary Intervention

The effect of fatty and lean fish intake on the lipoprotein subclasses was investigated. 33 patients with CHD aged 61.0 ± 5.8 yrs were assessed. Subjects were randomly assigned to a fatty fish diet (n=11), a lean fish diet (n=12) or a control diet (n=10) for 8 weeks. Fish diets included at least 4 fish meals/week. Control group consumed lean beef, pork and chicken. NMR was used to determine lipoprotein subclasses and their lipid components.

Concentrations of n-3 FAs and DHA increased in fatty fish group. Concentrations of cholesterol, cholesterol esters and total lipids in very large HDLs increased in fatty fish group (P=0.005, P=0.002 and P=0.007 respectively). Mean size of HDL particles in fatty fish group increased (9.8 ± 0.3 Nm at baseline and 9.9 ± 0.4nm at baseline and 9.9 ± 0.4nm at the end of study; overall difference P=0.004). The fish diets did not affect VLDL or LDL size.

### Parallel-arm randomized intervention study

126 Chinese women with hypertriacylglycerolaemia, aged 35-70 years, were assigned to 4 groups to consume 80g fillets of oily fish (salmon, herring or pompano) or a mix of commonly eaten meats (pork/chicken/beef/lean fish) for 5 days/week over an 8 week period.

The oily fish subgroup exhibited significant increases in intake of n-3 LC PUFA while decreasing dietary n-6:n-3 PUFA ratio. Compared to the control group, significant increases of DHA, EPA + DHA and total n-3 PUFA in plasma choline phosphoglyceride was observed in oily fish group. Plasma TAG only reduced in herring and salmon groups. Upon baseline comparisons, the 3 oily fish diets significantly decreased serum concentrations of TAG, apoCII and apoCIII, but only salmon and herring diets significantly lowered TNF-α and raised adiponectin levels in...
<table>
<thead>
<tr>
<th>Study (Year)</th>
<th>Intervention Type</th>
<th>Description</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Harris et al., 2007)</td>
<td>Dietary Intervention</td>
<td>The comparison of the rate and extent of enrichment of blood cell membranes and plasma phospholipids with n-3 FAs from either fish or fish oil capsules. Healthy premenopausal female volunteers were randomly assigned a daily average of 485mg EPA and DHA either from 2 servings of oily fish (salmon or albacore tuna) per week or from 1-2 capsules/week.</td>
<td>After wk 16, EPA and DHA in RBCs increased from 4.0 ± 0.6% of total FAs to 6.2 ± 1.4% whereas, it rose from 4.3 ± 1% to 6.2 ± 1.4% in the capsule group (P&lt;0.0001) for both. Similar results were observed in plasma phospholipids. EPA and DHA stabilized in the latter after 4 weeks but continued to rise to week 16 in RBCs. EPA in RBCs significantly increased (P=0.01) more rapidly in the fish group than in the capsule group during initial 4 weeks, but rates did not significantly differ between groups after that. Total FA variances were less in RBCs than in plasma phospholipids (P=0.04).</td>
</tr>
<tr>
<td>(Moore et al., 2006)</td>
<td>Randomized dietary Intervention</td>
<td>The effects on CVD risk markers of reducing dietary n-6:n-3 PUFA by changes in linoleic acid:α-linolenic acid and/or increasing LC n-3 PUFA. This study also tested whether decreases in LA:LNA modulate effects of LC n-3 PUFA. 142 subjects over a 24wk period were</td>
<td>Modest dietary manipulations of n-6 and n-3 PUFAs results in significant group X time interactions for serum triacylglycerols (TAGs; P=0.05) At 24 weeks, the control and 2 oily fish groups showed lower TAG than the white fish/sunflower group (P=0.05). Reductions in TAG,</td>
</tr>
</tbody>
</table>
randomly assigned to a control group or 1 of 4 interventions. Intervention groups received 2 portions of oily fish (0.7g EPA+DHA) per week, and replaced habitual household fats with ones high in sunflower (high LA:LNA) or rapeseed (low LA:LNA) oil associated with increased oily fish intakes, were maximized when combined with lower dietary LA:LNA. There was no significant changes in several other CVD risk markers.

(Zampelas et al., 2005) Cross-sectional study To investigate the association between fish consumption and levels of inflammatory markers among adults with no prior evidence of CVD. 1,514 men and 1,523 women enrolled. CRP, IL-6, TNF-α, serum amyloid A and WBC analysed. Dietary habits evaluated. 88% of men, 91% of women reported fish consumption at least once a month. Compared to non-fish consumers, those that consumed >300g of fish/week reported an average 33% lower CRP, 33% lower IL-6, 21% lower TNF-α, 28% lower SAA and 4% lower WBCs (all P<0.05). Significant results were observed when lower quantities (150-300g) of fish consumed.

(Raatz et al., 2016) Cross-over design investigated the effect of dose-dependent intake of farmed Atlantic salmon on lipoprotein particle (P) size and concentration. They hypothesized that low density lipoprotein (LDL)-P and high density lipoprotein (HDL)-P size and concentration would increase with salmon intake in a dose-dependent manner. Overweight, Intake of salmon reduced plasma and serum triglyceride (TG) concentrations and increased plasma HDL-C concentrations. The concentrations of large very low-density lipoproteins (VLDL)-P and chylomicron (CM)-P were reduced. Large LDL-P concentrations were increased in a dose-dependent manner. The mean size of VLDL-P was
adult participants (n=19) were enrolled to a cross-over designed clinical trial evaluating intake of farmed Atlantic salmon. In random order, participants were assigned to 90, 180 or 270g of salmon twice weekly for 4-week dietary treatments. Following a 4-8 week washout period participants crossed over to another dose of fish intake until all treatments were completed. Plasma lipid concentrations were determined and serum lipoprotein concentrations and particle size were determined by nuclear magnetic resonance.

reduced and that of LDL was increased. Total TG was reduced as was the TG content of VLDL-P and CM-P. twice weekly intake of farmed Atlantic salmon portions influences the lipoprotein particle size and concentration in a manner associated with CVD risk reduction.
APPENDIX II

The IC$_{50}$ numerical values

Table 5.1. The *in vitro* antithrombotic properties of the TPL of all sous vide salmon preparations samples against platelet aggregation induced by PAF, thrombin, collagen and ADP, expressed in numerical mean IC$_{50}$ values in micrograms (µg) of salmon TPL ± SD (N=3-7).

<table>
<thead>
<tr>
<th></th>
<th>IC-50 values of salmon TPL against PAF</th>
<th>IC-50 values of salmon TPL against Thrombin</th>
<th>IC-50 values of salmon TPL against collagen</th>
<th>IC-50 values of salmon TPL against ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSF</td>
<td>45.0 ± 22.0</td>
<td>382.0 ± 39.0</td>
<td>122.0 ± 24.0</td>
<td>135.0 ± 33.0</td>
</tr>
<tr>
<td>52°CCSVSNB</td>
<td>66.9 ± 46.5</td>
<td>292.0 ± 55.7</td>
<td>91.8 ± 25.1</td>
<td>152.1 ± 21.9</td>
</tr>
<tr>
<td>52°CCSVSB</td>
<td>115.2 ± 62.5</td>
<td>300.0 ± 122.0</td>
<td>74.5 ± 14.4</td>
<td>132.6 ± 16.7</td>
</tr>
<tr>
<td>65°CCSVSNB</td>
<td>119.4 ± 23.1</td>
<td>325.6 ± 121.2</td>
<td>59.1 ± 5.1</td>
<td>149.6 ± 15.2</td>
</tr>
<tr>
<td>65°CCSVSB</td>
<td>78.6 ± 5.3</td>
<td>519 ± 326.4</td>
<td>53.1 ± 2.5</td>
<td>143.5 ± 17.9</td>
</tr>
<tr>
<td>80°CCSVSNB</td>
<td>268.7 ± 49.7</td>
<td>728.6 ± 181.8</td>
<td>145.6 ± 64.8</td>
<td>255.2 ± 109.2</td>
</tr>
<tr>
<td>80°CCSVSB</td>
<td>222.7±110.2</td>
<td>706.3±191.8</td>
<td>113.3±53.0</td>
<td>232.2 ± 49.6</td>
</tr>
</tbody>
</table>

Abbreviations: RSF = raw salmon fillets; 52CSVSNB = 52°C sous-vide cooked salmon no brine; 52CSVSB = 52°C sous-vide cooked salmon brined; 65CSVSNB = 65°C sous-vide cooked salmon no brine; 65CSVSB = 65°C sous-vide cooked salmon brined; 80CSVSNB = 80°C sous-vide cooked salmon no brine; 80CSVSB = 80°C sous-vide cooked salmon brined
APPENDIX III

Table 5.2 Fatty acid composition of the saponified Total Polar Lipids (TPL) from various cooking preparations of salmon fillets. Results are presented as percentage of the total fatty acid content (mean values, n=3)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>C</th>
<th>52 TPL B</th>
<th>52 TPL NB</th>
<th>65 TPL B</th>
<th>65 TPL NB</th>
<th>PSVS TPL B</th>
<th>PSVS TPL NB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caprylic</td>
<td>8:0</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Pelargonic</td>
<td>9:0</td>
<td>0.04</td>
<td>0.06</td>
<td>0.04</td>
<td>0.04</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>Capric</td>
<td>10:0</td>
<td>0.02</td>
<td>0.03</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Undecylic</td>
<td>11:0</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lauric</td>
<td>12:0</td>
<td>0.02</td>
<td>0.04</td>
<td>0.01</td>
<td>0.00</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Myristic</td>
<td>14:0</td>
<td>1.11</td>
<td>1.73</td>
<td>1.07</td>
<td>1.30</td>
<td>1.25</td>
<td>1.24</td>
</tr>
<tr>
<td>Pentadecylic</td>
<td>15:0</td>
<td>0.00</td>
<td>0.06</td>
<td>0.02</td>
<td>0.01</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Palmitic</td>
<td>16:0</td>
<td>21.94</td>
<td>20.43</td>
<td>24.19</td>
<td>22.15</td>
<td>21.34</td>
<td>19.32</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>16:1 ω7 c9</td>
<td>0.47</td>
<td>1.22</td>
<td>0.69</td>
<td>0.81</td>
<td>0.75</td>
<td>0.83</td>
</tr>
<tr>
<td>Margaric</td>
<td>17:0</td>
<td>0.42</td>
<td>0.34</td>
<td>0.42</td>
<td>0.69</td>
<td>0.62</td>
<td>0.29</td>
</tr>
<tr>
<td>Stearic</td>
<td>18:0</td>
<td>13.10</td>
<td>12.95</td>
<td>13.83</td>
<td>12.15</td>
<td>14.10</td>
<td>12.35</td>
</tr>
<tr>
<td>Oleic</td>
<td>18:1 ω9 c9</td>
<td>21.14</td>
<td>20.56</td>
<td>17.82</td>
<td>19.75</td>
<td>18.34</td>
<td>33.90</td>
</tr>
<tr>
<td>Fatty Acid</td>
<td>Mol. Wt</td>
<td>8.08</td>
<td>9.04</td>
<td>8.49</td>
<td>8.47</td>
<td>9.26</td>
<td>6.83</td>
</tr>
<tr>
<td>----------------------------------</td>
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<td>-------</td>
</tr>
<tr>
<td>Linoleic</td>
<td>18:2 ω6 c9,c12</td>
<td>8.08</td>
<td>9.04</td>
<td>8.49</td>
<td>8.47</td>
<td>9.26</td>
<td>6.83</td>
</tr>
<tr>
<td>Linolenic (α)</td>
<td>18:3 ω3 c9,c12,c15</td>
<td>2.36</td>
<td>2.22</td>
<td>1.46</td>
<td>1.71</td>
<td>1.79</td>
<td>2.28</td>
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<tr>
<td>Stearidonic</td>
<td>18:4 ω3 c6,c9,c12,c15</td>
<td>0.89</td>
<td>1.92</td>
<td>1.75</td>
<td>1.98</td>
<td>1.53</td>
<td>1.00</td>
</tr>
<tr>
<td>Nonadecylic</td>
<td>19:0</td>
<td>0.03</td>
<td>0.03</td>
<td>0.17</td>
<td>0.08</td>
<td>0.06</td>
<td>0.03</td>
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<tr>
<td>Arachidic</td>
<td>20:0</td>
<td>0.13</td>
<td>0.15</td>
<td>0</td>
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<tr>
<td>Gadoleic</td>
<td>20:1 ω11 c9</td>
<td>7.60</td>
<td>8.95</td>
<td>9.03</td>
<td>10.71</td>
<td>9.49</td>
<td>8.02</td>
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<tr>
<td>DihomoLinoleic</td>
<td>20:2 ω6 c11,c14</td>
<td>3.71</td>
<td>3.68</td>
<td>1.34</td>
<td>2.14</td>
<td>2.05</td>
<td>2.81</td>
</tr>
<tr>
<td>Dihomolinolenic</td>
<td>20:3 ω6 c8,c11,c14</td>
<td>0.21</td>
<td>0.24</td>
<td>0.19</td>
<td>0.39</td>
<td>0.26</td>
<td>0.36</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>20:4 ω6 c5,c8,c11,c14</td>
<td>1.78</td>
<td>1.99</td>
<td>2.35</td>
<td>2.81</td>
<td>2.41</td>
<td>0.19</td>
</tr>
<tr>
<td>EPA</td>
<td>20:5 ω3 c5,c8,c11,c14,c17 (EPA)</td>
<td>6.90</td>
<td>6.92</td>
<td>6.18</td>
<td>2.23</td>
<td>5.57</td>
<td>2.97</td>
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<tr>
<td>Heneicosylic</td>
<td>21:0</td>
<td>0.01</td>
<td>0.01</td>
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<tr>
<td>Docosadienoic</td>
<td>22:2 ω6 c13,c16</td>
<td>0.15</td>
<td>0.19</td>
<td>0.11</td>
<td>0.17</td>
<td>0.11</td>
<td>0.21</td>
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<tr>
<td>Eranthic</td>
<td>22:3 ω6 c5, c13, c16</td>
<td>0.03</td>
<td>0.02</td>
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<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
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<tr>
<td>Ardenic</td>
<td>22:4 ω6 c7, c10, c13, c16</td>
<td>0.08</td>
<td>0.07</td>
<td>0.12</td>
<td>0.17</td>
<td>0.11</td>
<td>0.09</td>
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<td>DPA</td>
<td>22:5 ω6 c4, c7, c10, c13, c16</td>
<td>2.53</td>
<td>1.71</td>
<td>4.03</td>
<td>5.74</td>
<td>3.22</td>
<td>2.42</td>
</tr>
<tr>
<td>DHA</td>
<td>22:6 ω3 c4, c7, c10, c13, c16,c19 (DHA)</td>
<td>7.12</td>
<td>5.36</td>
<td>6.57</td>
<td>6.33</td>
<td>7.45</td>
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<td>0</td>
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<td>Lignoceric</td>
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<td>0.09</td>
<td>0.06</td>
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<td>0.07</td>
<td>0.05</td>
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<tr>
<td>Total SFA</td>
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<td>36.92</td>
<td>35.91</td>
<td>39.85</td>
<td>36.51</td>
<td>37.64</td>
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<td></td>
</tr>
<tr>
<td><strong>Total MUFA</strong></td>
<td>21.17</td>
<td>23.12</td>
<td>23.55</td>
<td>23.67</td>
<td>24.34</td>
<td>21.20</td>
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<tr>
<td><strong>Total PUFA</strong></td>
<td>33.84</td>
<td>33.36</td>
<td>32.61</td>
<td>32.17</td>
<td>33.78</td>
<td>23.81</td>
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<tr>
<td><strong>Total ω3 PUFA</strong></td>
<td>17.27</td>
<td>16.42</td>
<td>15.96</td>
<td>12.25</td>
<td>16.34</td>
<td>10.88</td>
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<tr>
<td><strong>Total ω6 PUFA</strong></td>
<td>16.57</td>
<td>16.94</td>
<td>16.65</td>
<td>19.92</td>
<td>17.44</td>
<td>12.93</td>
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<tr>
<td><strong>ω6/ω3</strong></td>
<td>0.96</td>
<td>1.03</td>
<td>1.04</td>
<td>1.63</td>
<td>1.07</td>
<td>1.19</td>
<td></td>
</tr>
</tbody>
</table>


CROPOTOVA, J., MOZURAITYTE, R., BEATE STANDAL, I. & RUSTAD, T. 2019a. Assessment of lipid oxidation in Atlantic mackerel (Scomber scombrus) subjected to different antioxidant and sous-vide cooking treatments by conventional and fluorescence microscopy methods.


KWAK, S. M., MYUNG, S. K., LEE, Y. J. & SEO, H. G. 2012. Efficacy of omega-3 fatty acid supplements (eicosapentaenoic acid and docosahexaenoic acid) in the...


NASOPOULOU, C., NOMIKOS, T., DEMOPOULOS, C. A. & ZABETAKIS, I. 2007. Comparison of antiatherogenic properties of lipids obtained from wild and
cultured sea bass (Dicentrarchus labrax) and gilthead sea bream (Sparus aurata). Food Chemistry, 100, 560-567.


TSOPRAS, A., LORDAN, R. & ZABETAKIS, I. 2018c. Inflammation, not Cholesterol, Is a Cause of Chronic Disease. *Nutrients, 10*.


