Master's thesis

NTNU
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Department of Biotechnology and Food Science

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Shelf life of Atlantic salmon – effect of modified atmosphere packaging and curcumin as a natural antioxidant

Master's thesis in Biotechnology
Supervisor: Turid Rustad
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Kunnskap for en bedre verden

Preface

This master thesis is a 60 credits project work done to fulfill a 2- year international master's program in Biotechnology, MSBIOTECH, at the faculty of natural sciences of the esteemed Norwegian University of Science and Technology, NTNU. The research was conducted at the Department of Biotechnology and Food Science and the duration was from fall 2021 to Spring 2022. I want to thank my supervisor and mentor, Professor Turid Rustad for her guidance, and patience, and for providing me an opportunity to dive in deeper and explore the field of food science as per my interests. She has been a continuous source of inspiration, motivation, and step-by-step guidance.
I am also very thankful to Siri Stavrum, Tarjei Haugbro, and Martin Haider for the help and guidance with the laboratory work, and equipment hence making the atmosphere in the laboratory welcoming.

To my parents. The reason for what I become today. Thanks for your great support and continuous care. To my siblings. I am grateful to you all. You have been my inspiration. To my grandparents, family, and my soul mate.

The fear of the LORD is the beginning of wisdom, and the knowledge of the Holy One is insight.

Sadoon Ali Zahid

Trondheim, May 15th, 2022

Summary

Seafood offers healthier alternatives to meat, which is one of the reasons why consumer demand for fresh and quality fish products had increased. Atlantic salmon is a good source of omega-3 fatty acids, proteins, vitamins, and minerals. The increased demand is facing challenges due to biochemical and microbial spoilage in the fish. Since fish is highly perishable food there is a need for new and improved preservation methods. The application of modified atmospheric packaging (MAP) and antioxidants in combination have been shown to be promising in preventing microbial spoilage and delaying lipid oxidation, thus prolonging the shelf life of salmon. The aim of this thesis was to study the combined effects of vacuum or MAP packaging with the application of curcumin antioxidant to prolong the shelf life of Atlantic salmon.

Curcumin antioxidant solutions were extracted and evaluated for antioxidant activity using DPPH and ABTS assays. Atlantic salmon was used to conduct two storage experiments, each lasting 15 days. In the first storage experiment, vacuum, and vacuum in combination with curcumin solutions while in the second experiment, MAP (CO_2:N_2 60:40) and MAP in combination with curcumin solution were stored at 0°C until further sampling was done on days 3, 8, 13 and 15. The drip loss, color, and microbial growth were the parameters used to analyze the quality changes. The total lipid content was determined, and the primary and secondary oxidation products were measured using PV and TBARS.

The drip loss increased during storage, and vacuum treated samples showed higher drip losses than MAP treated samples. Both vacuum and MAP treatment and the use of curcumin failed to prevent microbial spoilage. The microbial growth reached the recommended consumption after 8 and 13 days of storage. As the curcumin solutions possessed significant antioxidant activity, the PV in MAP with curcumin-treated samples was found to be lower than vacuum treated samples with the storage days. TBARS values increased in all samples with the storage days indicating the formation of secondary oxidation products. Both PV and TBARS values suggested that despite the lipid oxidation, the lipids were not rancid.

In conclusion, the study showed that MAP is advantageous compared to vacuum in retaining the quality of salmon. The use of antioxidants combined with MAP also affected the parameters to some extent. The study also suggested that curcumin solutions have the potential to cause a delay in lipid oxidation.

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Abbreviations

Acronym	Full form
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
PUFA	Polyunsaturated fatty acid
RH	Unsaturated fatty acid
$R^{\bullet}$	Lipid alkyl radical
$ROO^{\bullet}$	Lipid peroxyl radical
ROOH	Lipid hydroperoxide
MAP	Modified atmosphere packaging
AH	Antioxidant
$A^{\bullet}$	Antioxidant radical
$RO^{\bullet}$	Alkoxyl radical
ROH	Hydroxyl derivate
EO	Essential oil
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
PV	Peroxide value
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
SSO	Specific spoilage organism
MAP	Modified atmospheric packaging
LAB	Lactic acid bacteria
TMA	Trimethylamine
CFU	Colony forming units
SDOM	Standard deviation of the mean

1 Introduction

The following master's thesis is the fulfilment of 60 credits for the program international master's in biotechnology at NTNU. The aim of this thesis is to analyze the quality and shelf life of Atlantic salmon treated with natural antioxidant curcumin combined with vacuum and modified atmosphere packaging.

1.1 Background and motivation

Rapid population growth increased demands for food, and changes in dietary habits made it tremendously difficult to ensure universal access to healthy food without creating negative environmental, economic, or social impacts (1). It is estimated that 470 million metric tons of animal protein will be required annually by 2050 to feed the rapidly growing Earth's human population that was 2.5 billion people in the year 1950. It is projected that the population will peak at 9.73 billion people in 2064. It is unclear what proportion of the supply will come from land, the sea and lab-grown supply and how those proportions will change with the evolution of future technologies. Food produced from the ocean will certainly play a crucial role in the global food security because it is healthy and involves less CO_2 emission than animal meat. Thus, seafood has a low carbon footprint, most important of all is that the large proportion of land and water utilization for agriculture for animal meat production could be minimized (2). Identifying and consuming heathier food is crucial for global efforts to fight malnutrition, obesity, and associated illnesses. Seafood including fisheries offer healthier alternatives for meat and dairy products in a world in which malnutrition and undernutrition remain the primary health and well-being threats for many people because large population around the globe is not getting enough food (3).

Fish consumption possesses numerous health benefits to humans. The omega-3 fatty acids in fish, serve to lower cholesterol levels, prevent the risk of stroke, heart disease, and preterm delivery, and enhance cognitive development (4). FAO emphasize the important contribution that sustainable aquaculture makes to food security, nutrition, poverty alleviation, and human development(5).

A number of human interventions have contributed to the declines and collapses of fish populations, including overexploitation of fisheries, habitat degradation, and invasive species(6) failures of reproduction (7) fluctuation in temperatures and climate change (8). Global aquaculture practices have proven that sustainable aquaculture incorporates environmental, economic, and social parameters on a larger scale. However, sustainability in aquaculture requires not only optimizing benefits but also minimizing negative impacts on natural and social environments (9). The humans are now consuming more fish produced by aquaculture, that are produced sustainably to minimize the impact on environment caused by fish farming and wild fisheries (10).

Human nutrition nowadays relies heavily on fish and seafood products but in the absence of preservation, these products spoil rapidly (11). The old fishing tribes used simple and efficient ways to preserve fish that included techniques like drying, salting, smoke-treatment and freeze drying (12). Nowadays people demand more fresh fish to eat, in that case the traditional methods could not be used to prolong the shelf life. Foods containing high levels of unsaturated fat especially PUFAs in fish, are at great risk of deterioration due

to a very complex and important process, the lipid oxidation (11). The continuously increasing consumer demand for fresh products with prolonged shelf life causes a surge in energy cost used for freezing and frozen storage. The conventional techniques involving cold storage have been in practice to preserve the shelf life of fish, but the disadvantage is very high energy consumption (13). In this regard, efforts are being made by the fish processing industry to produce fish with improved shelf life and increased marketing potential meanwhile reducing energy cost. The use of alternatives such as chemical decontamination, ultra-high pressure and modified atmospheric packaging (MAP) could also contribute to improving the shelf life (14). The use of natural antioxidants in combination with vacuum and MAP packaging will be discussed further in section 1.6 and 1.11.

1.2 Atlantic Salmon (*Salmo salar*)

The Atlantic salmon is born in fresh waters, spend the first years of their lives there and then move to oceans. After maturation, the fish returns to the fresh water to spawn (15). The Norwegian salmon aquaculture circulates around sea and river, and it has a significant role in financial statistics of the country. In the year 2019 the first hand value of salmon in Norway was 68 billion NOK (16). Despite playing a significant role for social and economic gains in Norway, the salmon industry is prone to many developmental hurdles. Norway is taking advantage of its long coastline, enriched freshwaters, and sea in salmon farming. This is a growing industry in Norway, the country intend to very high revenue from the salmon business (17).

1.3 Fish muscle and nutritional value

Fish meat is mainly composed of water, protein, and lipids accounting for 98% of the total mass, the rest of the meat contains carbohydrates, vitamins, and minerals that have a crucial role in biochemical functioning of the fish. In percentage, fish meat has 66-81% water, 16-21% protein, 0.2-5%, fat, 1.2-1.5% vitamins and 0-0.5% carbohydrates. The lipid and ash content differ considering the size of the fish, in which season fish was harvested, the habitat that fish was grown and also depends on feeding of the fish (18). The lipid/fat content found in Norwegian salmon fillets varies from 11-19 % (19).

Fish muscles are divided into red, white and mosaic skeletal muscles that are varying functionally. The 30 % red muscles in salmon are rich in lipids, these muscles help fish in aerobic activities like swimming. Red muscles also contain trimethylamine oxide. The high lipid content makes them prone to lipid oxidation whereas trimethylamine oxide can undergo enzymatic or non-enzymatic degradation generating dimethylamine and formaldehyde (20, 21). The 70% white muscles are involved in anerobic activity because of fewer mitochondria and less ability to convert glycogen into CO_2 and water, instead glycogen is broken down into lactic acid. Mosaic muscles are found in salmon in a great number as they are formed by cooccurrence of red and white muscles in the same areas of fish muscle. The shelf life of fish is highly dependent biochemical and chemical processes taking place in the muscle. During storage, the lipid oxidation and lipid-protein interaction may cause protein aggregation resulting in quality change of the salmon (22).

The dry weight of salmon tissue has 65-75% protein, has a well-balanced amino acid composition and is a good source of the essential amino acids lysine and methionine in the human diet (23). The omega 3-fatty acids are the polyunsaturated fatty acids involved in

lowering of the serum cholesterol, thus helpful in prevention of heart diseases (24). The long chain omega-3 fatty acids of salmon, contain eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) that are beneficial to health (25, 26). The WHO recommended daily EPA and DHA intake is 200-500 mg, therefore regular salmon consumption can meet this need (27). A study showed that Norwegian farmed salmon contains 1-1.6 gram of EPA and DHA per 100 gram of fish meat (28). Another benefit of salmon consumption is that it provides the vitamins and minerals. Salmon is an excellent source of vitamin A and D and trace elements like phosphorus, magnesium, selenium and iodine (29). A study showed that salmon also contains other vitamins and also provides minerals such as Ca, Na, K, Mg, P, Mn, Fe, Cu, Zn and Se (30).

1.4 Fish Lipids

Lipids is an important constituent of salmon, there is difference in lipid distribution depending upon the muscle types and parts (31). Salmon tissues contain both neutral and polar lipids. Triacyl-glycerol and esters are categorized as neutral lipids while polar lipids include cholesterol, phosphatidyl choline, phosphatidyl ethanolamine and in low amounts cardiolipin, phosphatidyl inositol, phosphatidyl serine and spingomyelin (32). As described earlier sea food especially salmon is rich in long chain polyunsaturated fatty acids, eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3) (33). The structures of PUFAs eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3) are shown in Figure 1.2 (34).

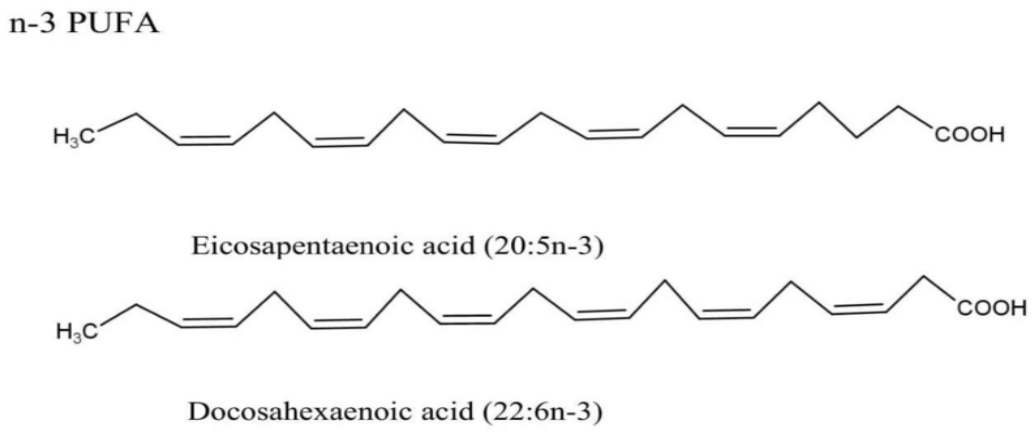


Figure 1.2 Structure of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (34)

The cis double bonds separated by methylene groups in PUFAs, make them prone to lipid oxidation. The hydrogen atoms bound to the methylene bridges (-CH_2-) that are easily removed and therefore act as reactive hydrogen atoms. After reacting with oxygen, the reactive hydrogen atoms result in the formation of hydroperoxyl radicals that facilitates lipid oxidation (35).

1.5 Lipid Oxidation

The free radicals of the fatty acids undergo complex reactions with oxygen causing oxidative degradation or lipid oxidation also termed as rancidity. The free radicals and reactive aldehydes can interact with proteins and vitamins to alter their chemical functioning. The lipid oxidation can be affected by structure and types of fatty acids, and storage environment depending on temperature, light, oxygen availability and water activity (36). The PUFA of salmon are oxidized by a free radical-induced lipid peroxidation (LPO) when subjected to an oxidative stress (37).

The process can be enzymatic or non-enzymatic. The non-enzymatic process is sub categorized into two types. The first type is autoxidation that is facilitated by free radicals and second type is photooxidation that is initiated by UV light or singlet oxygen. On the other hand, the enzymatic process involves cellular enzymes such as cyclooxygenases, lipoxygenases, and cytochrome P450 that catalyze the enzymatic oxidation of PUFAs (38).

The lipid oxidation process involves three steps that are initiation, propagation, and termination. During initiation, the oxygen results in the formation of alkyl radical groups ($R^{\bullet}$). In addition to oxygen, light or metal ions can also catalyze the formation of alkyl radical groups. The propagation step involves the rapid reaction of alkyl radical ($R^{\bullet}$) with oxygen to generate lipid peroxyl radical ($ROO^{\bullet}$), this radical takes a hydrogen from another lipid molecule to generate a new lipid radical ($R^{\bullet}$) that continues the chain reaction and also results in the generation of lipid hydroperoxide (ROOH). During termination, non- reactive species are formed as a result of reaction between two radicals. The reaction can occur when two peroxyl or two alkyl radicals react or one of these two react with each other. The reaction depends on the oxygen availability (39). The steps in lipid oxidation are shown in Figure 1.2 (40).

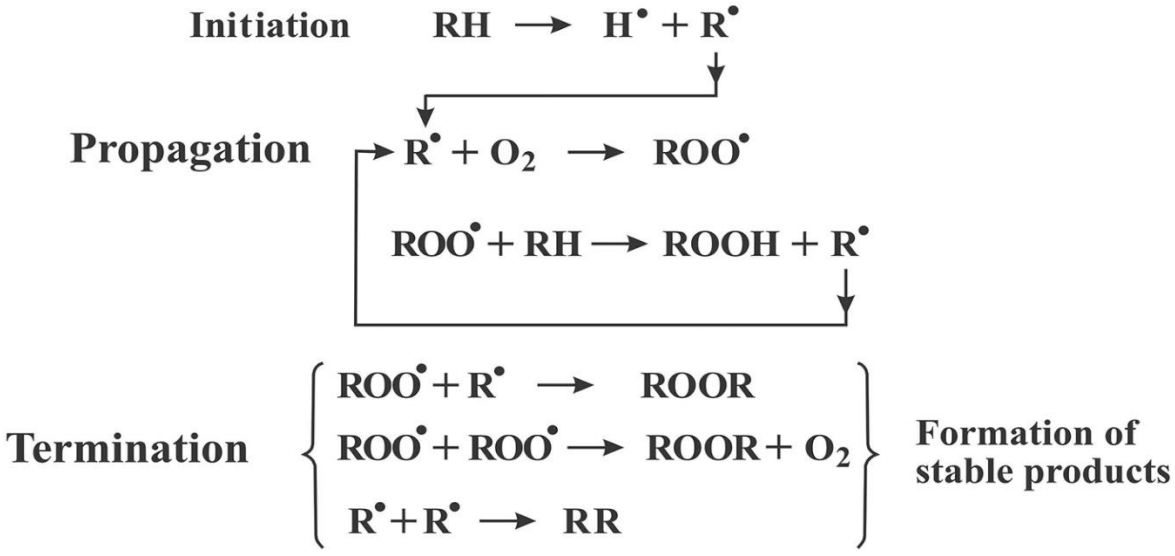


Figure 1.2 Initiation, propagation, and termination steps of Lipid oxidation process (40)

The lipid oxidation yields both primary and secondary oxidation products. The primary oxidation products are peroxides and hydroperoxides (41). These primary oxidation

products react further to form secondary products like aldehydes, ketones, alcohols or tertiary products dimers and oligomers. The process of oxidation is highly affected by the oxygen, light, enzymes, metal ions, so the process is categorized based on these factors or the mechanism (39).The aim of this work was to combine the use of antioxidant curcumin, chilled storage of modified atmospheric packed and vacuum packed salmon to prevent the lipid oxidation in the fish. The external factors like oxygen and temperature are controlled, by replacing oxygen with N_2 and CO_2 or vacuum packaging and storing at chilled conditions. The application of antioxidants is elaborated further in the chapter below.

1.6 Antioxidants

Antioxidants are stable chemical compounds that are capable of neutralizing a free radical by donating an electron or hydrogen, thus preventing the oxidation reactions (42). Depending on their mode of action the antioxidants can be classified into primary and secondary antioxidants. Primary antioxidants are characterized by their chain-breaking ability, and this is done by donating an electron to the free radical, thus terminating the chain-reaction. Secondary antioxidants exhibit the mechanism to remove the reactive species initiators by quenching the chain-initiating catalyst or removing the prooxidant (43). The free radical scavenging ability of antioxidants protect against cellular damage. Most of the antioxidant molecules have low molecular weight. The antioxidants react with free radicals, terminate the chain reaction, and protect the important molecules like lipids from the damage. The catalytic effect of metal ions in lipid peroxidation is decreased by lowering the metal concentration by metal chelating agents, that act as secondary antioxidants. By limiting their redox potential the oxidized metal ions are stabilized by secondary antioxidants (44). The antioxidants are known to prevent lipid oxidation as a result the shelf life of food containing lipids is improved. The endogenous antioxidants in the human body include glutathione, ubiquinol and uric acid (45).

Phenolic antioxidants are examples of primary antioxidants. The working principle of primary antioxidant is to neutralize free radicals either by hydrogen atom transfer (HAT) mechanism or by a single electron transfer (SET) mechanism. During HAT, the antioxidant AH, with a hydrogen atom interacts with free radicals to neutralize them and generate an antioxidant free radical ($A^{\bullet}$) and a non-radical substrate (RH, ROH or ROOH). The whole mechanism is shown in Figure 1.3. On the other hand, secondary antioxidants such as ethylenediaminetetraacetic acid (EDTA) and citric acid neutralize the prooxidant catalyst like Fe and Cu metal ions. β-carotene reacts and neutralize singlet oxygen and such antioxidants are exhausted after reacting with one free radical (46).

Figure 1.3 Hydrogen atom transfer mechanism of primary antioxidants (46)

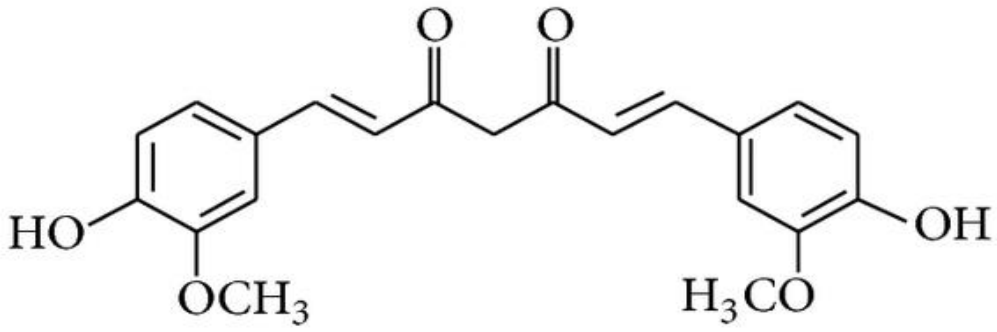
Antioxidants come from a variety of natural sources and can also be produced synthetically. Plant products like herbs, spices, seeds, fruits, and vegetables are key natural source for antioxidants. The plant antioxidants are economically valuable, have enhanced biological value and can be produced from food by-products so that plant species are less exploited. These factors make the use of natural antioxidants advantageous over synthetic antioxidants because studies have shown that the latter can pose adverse effects in human (47). The food industry has been using synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tert-butyl hydroquinone (TBHQ), 2-naphthol (2NL), 4-phenylphenol (OPP) and 2,4-dicholorphenoxyacetic acid (2.4-DA) (48). Studies have shown health issues such as skin allergies, GI tract problems and rarely cancer due to long-term consumption of synthetic antioxidants. High doses of synthetic antioxidants can result in DNA damage (49). The previous studies conducted on animals have shown that BHA and BHT affects liver and resulted in carcinogenic effects (50, 51). Due to these reasons natural antioxidants are used frequently, are replaced and chosen over the use of synthetic antioxidants (52). Consumers perception towards natural antioxidants is also increasing and studies have shown consumers show more interest towards natural antioxidants when it comes to the use of synthetic compounds in food for color and preservation (53-55).

The natural antioxidants from plant sources are categorized into phenolic compounds, vitamins, and carotenoids (56). Normal food items such as fruits, vegetables, herbs, spices, and edible mushrooms can be good sources of natural antioxidants. Some medicinal plants also have health benefits as antioxidant source (57). The polyphenols of fruits and vegetables have potential to prevent lipid oxidation (58). The herbs and spices like oregano, thyme, dittany, rosemary, and lavender contain essential oils as potent source of antioxidants but the strong flavor of these species make its use limited (52, 59). Tea extracts in this regards is beneficial as these contains catechins, tannins and flavonoids and have no strong flavor (60). Curcumin a major component of the Asian spice turmeric, has been known to possess antioxidant and anti-inflammatory properties. Further details about curcumin are explained in next section (61).

1.7 Curcumin

Curcumin is derived from the rhizome of *Curcuma longa* (turmeric) and several other *Curcuma* species. It is a natural polyphenol known as diferuloylmethane and has chemical formula of 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (62). Curcumin, $C_{21}H_{20}O_6$ having a molecular weight of 368.4, is a beta-diketone. It is also known as diferuloylmethane (63). Figure 1.4 Shows the structure of curcumin (64). *Curcuma longa* is found to be used traditionally, has significance across Asia for its medicinal properties as an excellent antioxidant and anti-inflammatory, antimutagenic, antimicrobial, anti-cancerous nature(65-67).

The cellular targeting of multiple signaling molecules showed potential health benefits of curcumin mentioned in previous literature (68). The antioxidant effects of curcumin also make it beneficial therapeutically. US FDA has approved curcumin and curcuminoids by categorizing them as "Generally recognized as safe" to use (62). The clinical trials have shown that the daily doses of 4000-8000 mg are in safe and tolerable range (69). The inhibition of the formation of reactive oxygen species (ROS) makes curcumin an excellent antioxidant and the cyclooxygenase (COX) inhibition gives its anti-inflammatory attribute.



Structure of curcumin

Figure 1.4 Structure of Curcumin (64)

1.8 Determination of Antioxidant activity

The suppression of oxidation of lipids and proteins by controlling the oxidative chain reaction is known as antioxidant activity (70). The phenolic compounds exhibit antioxidant property involving four basic mechanisms of action. Firstly, hydroxyl groups of phenolic antioxidants are excellent hydrogen donors, secondly, they react with ROS, thirdly they can interact with reactive nitrogen species (RNS) and lastly, they terminate the cycle of formation of new radical groups. As already mentioned the antioxidant activity for phenolic antioxidants is the single electron transfer (SET) mechanism, proton transfer (HAT) and ability to chelate metal ions (71).

The radical scavenging antioxidant capability has been determined by both direct and indirect methods, but indirect methods are advantageous in terms of ease of use. DPPH and ABTS are two decolorizing assays of indirect methods used to determine the radical

scavenging activity of antioxidants. The free radical scavenging activity is determined by DPPH assay. This method takes a short time and DPPH is therefore a widespread method. The ability of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) to react with species that donate hydrogen atoms, is quite stable and the absorption spectrum can be obtained at 515nm. The radical scavenging activity of the DPPH by an antioxidant, results in a reduction reaction that leads to the decolorization of DPPH methanol solution. Hence, the reducing capability of the antioxidant towards DPPH radical is measured using this assay (72).

Another assay that determines the radical scavenging activity of the antioxidants is the ABTS assay. In this assay, the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) generated in the aqueous phase is scavenged by the antioxidants, using propyl gallate as a standard. For this purpose, the ABTS salt is allowed to react with a strong oxidizing agent and this way ABTS radical is formed. The hydrogen atom donation by antioxidants causes a reduction of the color of the blue green ABTS free radical and is determined as change in absorption at 734 nm (73).

1.9 Determination of oxidation status

As mentioned previously, lipid oxidation results in primary and secondary oxidation products. The peroxides are generated as primary oxidation products of the lipid oxidation, these hydroperoxides are quite stable and are used to assess the lipid oxidation status in food specimen. The secondary oxidation products such as aldehydes, ketones, epoxides, hydroxy compounds, oligomers, and polymers formed can also be used to determine the oxidation status. The characteristics of the secondary oxidation products often show variations in terms of volatility, polarity and molecular weights (74).

For the determination of oxidation status determination of PV and TBARS are widely practiced in labs. The concentration of primary oxidation products such as hydroperoxides is measured by determining the peroxide value (PV) using iodometric titration, while other methods are also used. To measure the secondary oxidation products, the TBARS value is used that measures the aldehydes. The determination of PV works on the basic principle of taking iodine from potassium iodide by the hydroperoxides. The reaction is shown in equation 1.

$$ROOH + KI \rightarrow ROH + KOH + I_2 \quad (1)$$

The concentration of ROOH from primary oxidation products can be determined by measuring the iodine generated. This is possible by titrating with sodium thiosulfate.

The reaction is shown in equation 2

$$I_2 + 2Na_2S_2O_3 \rightarrow 2NaI + Na_2S_4O_6 \quad (2)$$

The peroxides concentration is calculated by the sodium thiosulphate ($Na_2S_2O_3$) consumed and PV is represented as milli-equivalents (m_{eq}) peroxide per 1 kg lipid of the food specimen. If the PV of a lipid is lower than 10-20 m_{eq}/kg that means the rancidity is low (75).

The secondary oxidation products can be measured by another assay, the thiobarbituric acid reactive substance (TBARS) assay. The unsaturated fatty acid oxidation products are measured because of their reaction with the thiobarbituric acid (TBA) that generates a pink complex TBARS that has absorbance at 532-535 nm in spectrophotometer. (76).

1.10 Microbial spoilage in fish

The microorganisms like *Listeria monocytogens*, *Clostridium botulinum*, *Salmonella spp*, *Vibrio cholera*, *Vibrio parahaemolyticus* are the foodborne pathogens growing in unprocessed and processed fish (77). The bacterial growth and metabolism affects the pH, causes toxicity, gives bad odors, results in slime formation, changes flavor and also affects the color of the food (78). The most commonly bacteria found among natural microflora include *Pseudomonas*, *S.putrefaciens* and *Photobacterium (79)*. The pathogens *Vibrio parahaemolyticus, Staphylococcus aureus,* and *Aeromonas* spp. could be introduced to the fish as a result of capture, processing or during preparation at home or in restaurant (80-82). The chilled-stored fish is reported to have aerobic spoilage bacteria *Pseudomonas* and *Shewanella* species. In contrary the CO_2 resistant species *Photobacterium phosphoreum* was found in modified atmospheric packed fish (83). Studies have also reported that aerobic ice stored fish contains *Pseudomonas spp.* and *S.putrefaciens*. It is also reported that, when stored at 25°C the most common bacteria in fish microflora are mesophilic *Vibrionaceae* (84). Jorgensen et al. (1988)(84) reported *S.putrefaciens* grew on ice stored cod and the bacterial count affect the shelf life of the fish showing an inverse relationship. In a finding by Hussain et al. (1976)(85) vacuum packed fish on ice storage showed growth of Gram-positive bacteria. The spoilage in processed salmon is linked to the *Pseudomonas spp.*, *Shewanella spp.* and *Photobacterium spp.* that grows naturally on live salmon. Studies show that *Photobacterium phosphoreum* spoils the fish by producing trimethylamine while *Shewanella spp*. produces both TMA and volatile sulfides that cause deterioration (86). In a study conducted on fresh cold-smoked vacuum-packed salmon the natural microflora contained *Brochothrix thermosphacta*, *Yersinia ruckeri*, *Photobacterium* and *Carnobacterium spp.*, whereas *Lactobacillus* and *Photobacterium spp*. were found when this fish was stored for 19 days at 7°C (87).

The vacuum packaging (VP) or modified atmosphere packaging (MAP) is used to enhance the shelf life of fish by limiting the growth of bacteria thus preventing spoilage. The VP involves the removal of air and MAP replaces the air by a combination of CO_2 and N_2 gases (88). Studies showed MAP is more effective than vacuum packaging in inhibiting the bacterial flora in cold stored fish. The MAP treatment involves CO_2 that prevents the increase in total bacterial count and therefore species like *P.phosphoreum* are inhibited (89).

1.11 Vacuum and MAP packaging

The removal of air to limit oxygen and then using hermetic seal to package is known as vacuum packaging. To reduce the economic loss of fish due to spoilage, cold storage is supplemented by vacuum packaging to extend the shelf life by delaying spoilage thus ensuring quality and safety. Vacuum packaging protects against rancidity, causes a reduction in aerobic microorganisms, prevents damage during handling and serves as oxygen-water vapor barrier. The technique also minimizes the shrink loss, protects color and limits microflora to grow (90). The limitations of this technique are adequate control of

the systems and chances of strict anaerobic bacteria to grow even after cold storage of vacuum treated specimen.

Stammen (1990) explained MAP as the use of different gas mixture to replace the air for packaging. During the process, the ratio of gases are kept constant during filling and until the final seal was intact (91). The shelf life of fish packed by MAP as compared to atmospheric packaging shown 50-400% enhanced shelf life (92). The MAP prevents growth of majority of spoilage microflora of fresh fish and gained a lot of attention in the market (88). O_2, N_2 and CO_2 were used as gases in MAP of seafood products, but the use of oxygen was limited due to involvement in lipid oxidation. N_2 instead of O_2 was used to prevent microbial growth and avoid rancidity. The mixture of CO_2: N_2 was found to have low solubility in water and fat so is widely used. The effectiveness of MAP is dependent on the use of best quality of packaging material (93).

A study by Coyne (1933) showed that spoilage from *Pseudomonas spp*. could be prevented by using only 5% of CO_2 (94). A study reported that additional to the bacterial growth inhibition MAP is also effective to prevent the oxidation reactions, but this varies with species, their fat content, use of gas mixture, initial bacterial count and temperature for storage (95). A study conducted by Stammen showed that CO_2 used in MAP gets dissolved in the product. CO_2 dissolved in tissues and form H_2CO_3 reducing the pH. When the product is opened the slow release of gas from the product exerts CO_2 residual effect that gives preservative effect to the MAP packed specimen (91). Research shows that storage temperature plays a very important role in MAP, so if not stored at right temperature MAP is not effective. This could be limitation of MAP that it requires strict control of storage temperature. Studies show that when 25% CO_2 was used, growth of a large proportion of bacteria was inhibited on fish and when CO_2 was used in higher concentrations, no bacteria were reported for 4 days (93).

1.12 Aim of the thesis

The previous efforts in prolonging the shelf life of Atlantic salmon were either studying the effect of antioxidants or MAP alone. The combinatorial effects of both these techniques were less studied. Moreover, the combination of both these techniques to analyze the shelf life and salmon's quality parameters such as color, texture, and drip loss, were also having limited previous work. The aim of this thesis was to study the combined effect of vacuum or MAP packaging with the application of curcumin as antioxidant to prolong the shelf life of Atlantic salmon.

2 Materials and Materials

2.1 Equipment

Equipment	Details
Tray sealing machine	Webomatic TL250
Vacuum chamber machine	Webomatic Supermax
Headspace gas Analyzer	PBI Dansensor CheckMate 9900
Cold room	
Digital scale	.01 g accuracy range
Digital analytical balance	.0001 g accuracy range
Stomacher bag	Graded with dimensions 180 mm X 300 mm X 70 µm
Stomacher homogenizer	
Vortex mixer	MS2 Minishaker IKA
Filter Petri plates	Compact Dry TC
Incubator	Termaks
Chromameter	Konica Minolta CR400
Homogeniser	Ultra Turrax T28
Centrifuge	Multifuge X1R, room temperature
Centrifuge	Sorvall RC-5B Plus
Spectrophotometer	Ultrospec 2000
Drying oven	Termaks
Freezer	
Evaporating unit with N_2-gas	Pierce Reacti-Vap Reacti-Therm
Heating module	
Automatic titrator	TM235 TitroLine 7000 Coupled with a platinum electrode Pt 62/61

2.2 Chemicals

Chemicals	Details
Antioxidant solutions	Curcumin extracts
Distilled water	
Ethanol (CH_2H_5OH)	
Methanol (CH_3OH)	
DPPH ($C_{18}H_{12}N_5O_6$) solution	1,1-diphenyl-2-picrylhydrazyl 0.15 mM DPPH in 96% ethanol
Propyl gallate (PG, $C_{10}H_{12}O_5$) stock solution	10 mM PG in 80% methanol
ABTS ($C_{18}H_{18}N_4O_6S_4$)	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid 7 mM
Potassium persulfate ($K_2S_2O_8$)	140 mM
Sodium carbonate (Na_2CO_3)	20%
Carbon dioxide (CO_2), nitrogen gas (N_2)	60:40, for MA packaging
Saline peptone water	0.1% peptone, 0.85% NaCl
Sodium chloride (KCl) solution	0.15 M
Chloroform (CH_4Cl)	
Nitrogen gas (N_2)	
Acetic acid (CH_3COOH)	Glacial
Potassium iodide (KI) solution	4.63 M
Sodium thiosulphate ($Na_2S_2O_3$)	0.001 M
TBA (2-thiobarbituric acid) stock solution	0.04 M
Sodium sulphite (Na_2SO_3)	0.3 M
TCA (trichloroacetic acid) solution	0.28 M
TEP (1,1,3,3-tetraethoxypropane) solution	0.01 M
BHT (butylated hydroxytoluene) solution	3% BHT in ethanol
Sodium hydroxide (NaOH)	0.1 M

All the chemicals used were of analytical grade.

2.3 Preparation of Antioxidant extract

The curcumin antioxidant extraction was done in September 2021. The commercial turmeric powder by product name TRS was bought from Melon Midtbyen in Trondheim Sentrum. The issue that limits the capability to extract curcumin is its poor solubility in water. Although solvent extraction solves this problem but the use of ethanol and methanol in the extraction process are not suitable to use extracts on food items. This study involves the ultrasonication extraction in water bath (96). 1 gram of raw turmeric powder (macro curcumin) was partially dissolved by mixing in 50 ml of doubly distilled water in Falcon tubes. The tubes were kept in a water bath set at 60°C for 25 minutes. The tubes were then put in small beakers with water to put in a sonication unit. The sonication frequency of 45 kiloHertz was utilized with a sonication time of 30 minutes. After sonication, the tubes were centrifuged (25°C 3000 g for 1 hour). The supernatant was collected in new tubes, covered in aluminum foil to prevent photooxidation, and stored at 4°C to measure the antioxidant activity.

15ml of the extract was mixed with 100 ml of doubly distilled water to dilute and then filtered using 0.20µm non-pyrogenic sterile syringe filters to make it sterile. 2 ml of this diluted extract was applied on each sample of fish fillet of 80±15g on the day of packaging using sterilized pipette tips.

2.4 Determination of antioxidant activity

The antioxidant activity of diluted curcumin extracts was determined by DPPH and ABTS assays assessing the free radical scavenging.

2.4.1 DPPH

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined by the method given by Nenadis et al. (2007) (97) and Thiansilakul et al. (2007) (98). Using 96% ethanol as a reference, absorbance was measured at 517 nm, and antioxidant activity was determined as propyl gallate (PG) equivalents.

2.4.2 ABTS

The 2,2'-Azino-Bis-3-Ethylbenzothiazolin e-6-Sulphonic Acid (ABTS) assay as given by Re et al. (1999) (99), Nenadis et al. (2004) (100) and Nenadis et al. (2007)(97), was employed to analyze the radical scavenging activity of the antioxidant extracts. Distilled water was used as reference and absorbance was measured at 734 nm to determine radical scavenging activity as PG equivalents.

2.5 The Salmon packaging and storage experiments

The packaging and storage experiments were done in three successive rounds. Rounds 1 and 2 involved Vacuum packaging while round 3 involved MAP and vacuum packaging. The first storage experiment was used mainly as a training, both on sampling and to see if the chosen storage time and amount of added antioxidant was suitable. The trimmed *Salmo salar* (Atlantic Salmon) fillets, were bought from Domstein Sjomat AS, with a production and processing date of 12.10.2021 and arrival in Kalvskinnet NTNU's lab on 13.10.2021 for the first round of packaging. The fillets were trimmed at the tail and belly to keep the fat content and sample size as uniform as possible. Figure 2.1 illustrates the trimming and sample sizing, the outer fillet marked grey was removed (101). The large fillets were cut

into 30 small fillet samples (N=30), and that day was set as day 0 of packaging and storage experiments.

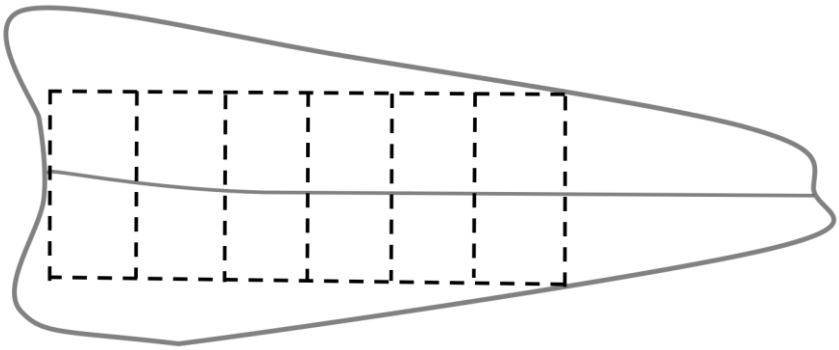


Figure 2.1: Schematic representation of the sample trimming and sizing (dotted line) of the Salmon fillet. The outer area was trimmed off and removed

For the first experiment, the samples were divided into two groups, one control group (CON) and one group treated with curcumin (CUR). The CUR samples were treated with 2 ml curcumin antioxidant applied using a micropipette. All samples were marked with days to perform storage experiments gradually for example CON3.1, CON 3.2, CUR3.1, CUR3.2, and CUR3.3 indicating all the samples to be observed on day 3 while CON6.1, CON 6.2, CUR6.1, CUR6.2, and CUR6.3 to be observed on day 6 and so on for day 8, day 10, day 13 and day 15. All samples were vacuum-packed using a Webomatic Supermax vacuum chamber machine. The samples were then put in ice packed storage box, transported to NTNU Gløshaugen, and finally stored in the refrigerator at 0°C until sampling. The sampling was done on days 3, 6, 8, 10, 13, and 15.

The second round of vacuum packaging was performed on 12.11.2021 on fish that arrived on 11.11.2021. The fillets were cut and trimmed into 16 smaller fillet samples (N=16) in the same way as described for vacuum packaging round 1. After applying 2ml of curcumin antioxidant, the sample containers were covered with aluminum foil and let sit in the fridge for 30 minutes so that the fillets could absorb the curcumin antioxidant. This improvement was done after observation in the first round of experiment. The samples were then packed and marked as mentioned above. The two groups, one control (CON) and one group treated with 2 ml curcumin extract (CUR) were marked as sampling days as mentioned above for example CON3.1, CON3.2, CUR3.1, CUR3.2 to be observed on day 3 and so on for day 8, 13 and 15.

The third and last round of packaging involved MAP packaging with controls that were vacuum packaged. The fish arrived on 25.01.2021 and packaging was done on 26.01.2021. The fillets were trimmed and cut into 20 samples (N=20). The samples were divided into three groups CON, CUR, and CON V.P representing MAP control, MAP with 2 ml curcumin extract, and controls that were vacuum packaged respectively. These vacuum packaged controls were analyzed on day 3 and day 15 only. The representation and marking were the same as in other rounds for example CON3.1, CON3.2, CUR3.1, CUR3.2, CON V.P 3.1, CON V.P 3.2 for all samples observed on day 3, and so on for days 8, 13 and day 15. The modified atmospheric packaging (MAP) was done using the plastic tray sealing machine

Webomatic TL250, for which the gas composition was maintained at approximately 60:40 of CO_2: N_2. The plastic sealed trays (box) had dimensions of 18.5x13.0x4.5 cm and a volume of 660 ml. The gas composition for MAP packaging was analyzed using a headspace gas analyzer, PBI Dansensor CheckMate 9900, showing an average gas composition of CO_2: N_2 60.34:39.60 during packaging. The fish processing, packaging, storage, and experimental setup for determining parameters are shown in a flow chart in Figure 2.2.

2.6 Experimentation to analyze parameters

On each day of the storage experiment, drip loss, microbial growth by total colony count on plates, color, and total lipid content were measured. The microbial growth was determined by plating on compact dry TC plates and observing the colony counts after 48 hours of growth. The lipids were extracted each day, chloroform phase was collected in Falcon tubes, marked, and stored at -20°C freezers for further observations comprising PV and TBARS.

2.7 Drip loss

The drip loss was analyzed on each of the sampling days. For that, a digital scale up to an accuracy of 0.01 g was employed. The fillet samples along with the packaging bag/sealed MAP tray were weighed. After that, the bag/sealed MAP tray was opened and the sample for microbial growth was cut out, to ensure sterility for microbiology. The excess drip on the fillet sample and opened bag/MAP tray were dried using a paper towel, then the weight of the bag and fillet was determined separately again. The mass of drip was calculated by taking the difference of package before and after removal of free water which also includes the sample cut out for microbiology. The mass of the initial product is the initial weight of the fish sample before drying. The percentage drip loss was determined by the equation 3.

Percentage Drip loss (%) = (Mass of drip/Mass of initial Product) X 100 (3)

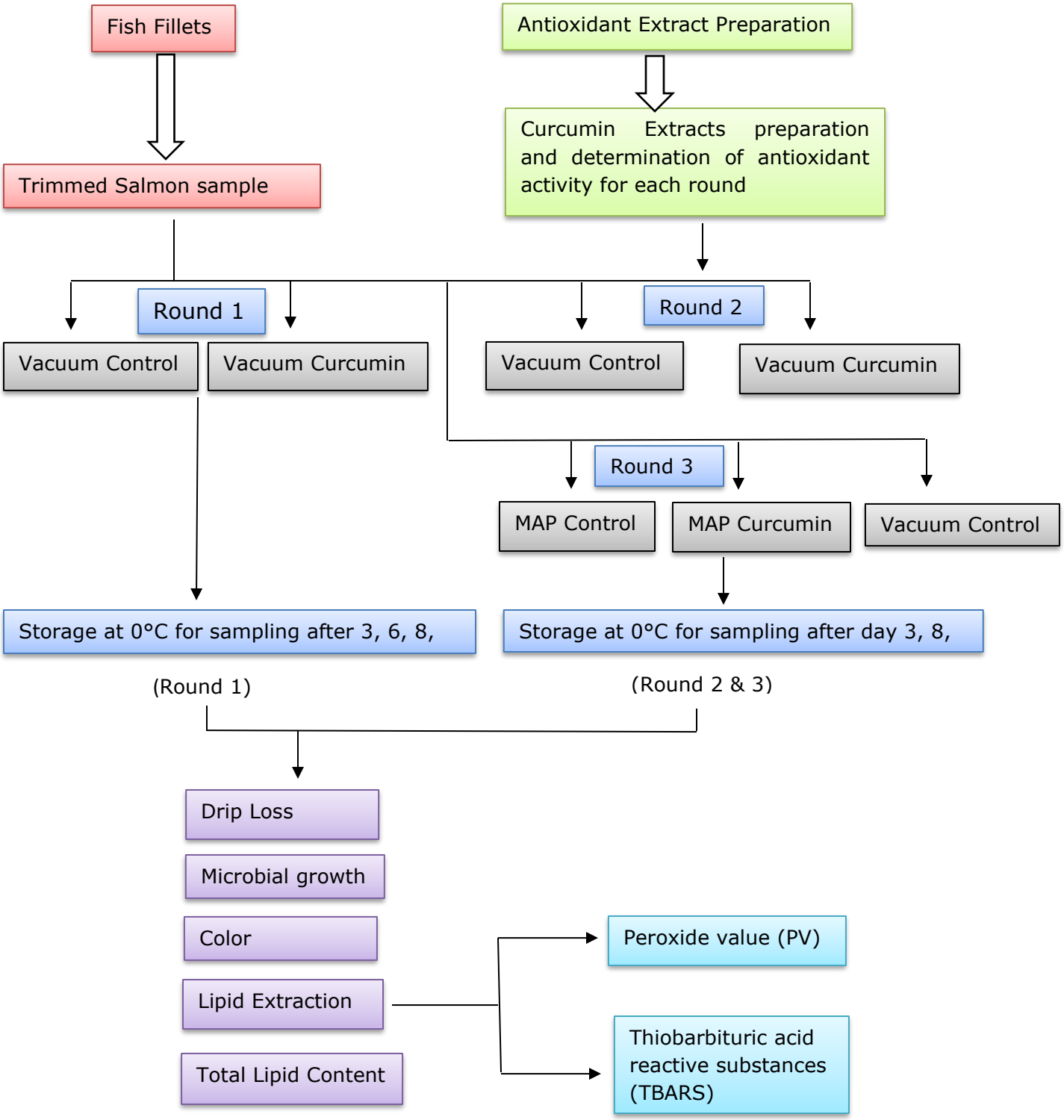


Figure 2.2 Flowchart of the experimental setup, showing three rounds of packaging, followed by storage and determination of drip loss, microbial growth, color, lipid extraction, and total lipid content on each day of sampling. The lipid extracts were stored for later analysis of PV and TBARS, after the completion of experimental days.

2.8 Microbial growth during storage

The NKML method 184 'Aerobic count and specific spoilage organisms in fish and fish products' as colony forming units (CFU) was utilized to observe and analyze the microbial activity in fish during storage (102). An approximately 10 g sample was cut, weighed using a digital balance of 0.01 g and put into a stomacher bag of dimensions 180 mm X 300 mm X 70 µm. 100 ml of sterile saline peptone water containing 0.1 % w/v peptone, and 0.85 % w/v NaCl was added to the stomacher bag and put in a stomacher homogenizer that was run at 230 rpm for 30 seconds. The purpose of using saline peptone water was to sustain the microbial growth during incubation. The homogenized mixture was considered as dilution 10^{-1}. Further series dilution of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were done in 15 ml falcon tubes, using 9 ml of sterile saline peptone water and 1 ml of 10^{-1} homogenized mixture from the stomacher bag using sterilized micropipette tips. The method of serial dilution was followed as suggested by Duun and Rustad (2007)(103). 1 ml of each series of diluted solutions were plated out on Compact Dry TC filter Petri plates, marked, and incubated at 22°C for 48 hours. The grown colonies appearing red on plates were counted manually and microbial activity was determined as CFU per gram of sample. The overgrown plates were denoted as TMTC (too many to count), while undergrown as TFTC (too few to count) and as no growth where no colonies were shown.

2.9 Changes in Color

The Konica Minolta Chromameter CR400 was used to determine changes in the color of the fillet during storage. The chromameter readings as L*, a*, and b* denoted the lightness, red-green range, and yellow-blue range respectively in the fish fillets. Before starting the measurement, the chromameter was calibrated to initials on a clear white calibration plate. The chromameter was directed on fish fillets where there were fewer white fat lines so that the fat lines did not cause fluctuations in the color. For each sample, three parallel readings were done and an average of L*, a*, and b* was reported as the color changes. The technique used in determining the parameter of color was learned from a lab engineer.

2.10 Lipid Extraction

The extraction of lipids from sample fillets was done by performing the method described by Bligh and Dyer (1959)(104). The purpose of extracting lipids was to determine the oxidation activity by measuring the degree of oxidation, employing the methods of peroxide value (PV) determination, and also determining the thiobarbituric acid reactive substances (TBARS) in lipid samples.

The chloroform and methanol were placed on chilled ice to keep them cool. An accurate 10±0.9 g of fish sample was cut and put in a chloroform resistant homogenizer tube with caps. 16 ml distilled water, 20 ml chloroform, and 40 ml methanol was added to the tubes and the mixture was homogenized for 2 minutes, using an Ultra Torrax homogenizer T28. Then, 20 chloroform was added and homogenized for 40 seconds. After that 20 ml of distilled water was added and homogenized for 40 seconds. After that, the tubes were centrifuged (0°C 9000 g for 10 minutes). The pinkish chloroform phase containing lipids was transferred to 50 ml falcon tubes. 2 ml of chloroform phase was used instantly for determination of total lipid content while the rest was stored at -20°C for later analysis of PV and TBARS.

2.10.1 Lipid content determination

2 ml of freshly extracted lipid in the chloroform phase was transferred in pre-weighed Kimax tubes. The chloroform was evaporated by placing the Kimax tubes were under an N_2 gas stream in a Pierce Reacti-Vap evaporating unit and a Reacti-Therm heating module set at 60°C for 7-10 minutes. The tubes were cooled in a desiccator for 1 hour and weighed again. The percentage lipid content per gram of fish sample was calculated using the equation 4.

$$\text{Lipid content (\%)} = \frac{\text{Lipid in tube X total chloroform used during the extraction}}{\text{Amount of sample weighed in (g) (Chloroform used in Kimex tube)}} \quad (4)$$

2.10.2 Peroxide Value (PV)

The peroxide value was determined by iodometric titration as described in AOCS official methods Cd 8b-90 to analyze the primary oxidation products, in the chloroform samples containing lipids(105). TitroLine 7000 assembled with a platinum electrode, was an automatic titrator instrument used to perform the titration. A saturated solution of potassium iodide was used, which was prepared by adding 10 g of KI to 13 g of distilled water. A mixture of 18 ml glacial acetic acid, 12 ml chloroform (solvent mixture), and 0.5 ml KI solution in a titration beaker were run for getting the reading for the blank sample. After 1 minute of stirred mixing, 30 ml distilled water was added, and titration was run. The volume of titrant poured by the titrator was used as a reference later while running titration on samples. For determining PV in samples, 12 ml chloroform phase containing lipids, 18 ml solvent mixture (acetic acid and chloroform), and 0.5 ml of KI were stirred mixed for 1-minute, later titration was started after adding 30 ml of distilled water. Some samples gave large, deviating values during titration, which was corrected by repetition. The PV of samples as m_{eq} peroxide/kg lipids was evaluated by using the equation 5.

$$PV = \frac{(V-B) \times T \times M}{(w)} \quad (5)$$

Where V is the volume (ml) of titrant consumed during titration of the sample, B is the volume (ml) of titrant consumed during titration of blank, T is the molarity of the titer (0.001), M is the molarity conversion factor and w is the weight (g) of the lipid sample.

2.10.3 TBARS

The thiobarbituric acid reactive substance (TBARS) was determined as described by Ke and Woyewoda as a micro method (1979) and used to assess the secondary oxidation products in the lipid samples (106). The lipids were weighed in Kimex tubes, 5 ml TBA working solution was added and mixed by vortex for 15-20 sec. The Kimex tubes were incubated in a boiling water bath set at 90°C for 45 minutes and then cooled by placed in cold water. 2.5 ml of TCA solution was added to each tube and mixed by inverting. The tubes were then centrifuged using Multifuge X1R, (25°C 3000 g for 7 minutes). A pink water phase was separated from the chloroform phase. The top layer was collected in spectrophotometer cuvettes to measure the absorption at 538 nm using Ultrospec 2000 spectrophotometer. The standard curve for the blank samples was obtained using the same procedure as above but instead of lipids, 1,1,3,3-tetraethaoxypropan (TEP) was added to Kimax tubes. Two parallels with 1, 3, 5, 10, 15, and 20 nanomoles of 0.0001 M TEP were prepared. Doubly

distilled water was used as a reference before taking absorption readings. The TBARS value was calculated by equation 6 and expressed as μmol TBARS per gram lipid.

$$\text{TBARS} = \frac{(A-b)}{a \times w \times 1000} \quad (6)$$

Where A is the absorbance of the sample, a and b represents the slope and intercept of the standard curve, w denotes the weight of lipids in samples and 1000 is the conversion factor from nanomoles to μmol.

2.11 Data Handling

The compilation and analysis of the large set of data were organized by Microsoft Excel in Windows 10. The ToolPak data analysis tool was utilized for the interpretation of data into correlations and significance for the correlation coefficient. Finally, the results were presented in the manner of the average standard deviation of the mean (SDOM).

3 Results and Discussion

This chapter gives a presentation of the results as well as a detailed discussion of the experiments. This section also includes the limitations during this study. The original experimental data and the relevant examples for calculation are outlined under Appendix A section.

3.1 Antioxidant Activity

The DPPH and ABTS were used to determine the antioxidant activity, Table 3.1 shows the propyl gallate (PG) equivalents results for antioxidant activity. The experimental data, standard curves and calculations are listed under Appendix A.1.

Table 3.1 DPPH and ABTS assays showing the antioxidant activity of curcumin extracts as propyl gallate (PG) equivalents including SDOM.

Antioxidant Assay	**Curcumin**
DPPH	23.42 ± 0.08
ABTS	68.03 ± 0.15

As shown in the table, the antioxidant activity of curcumin solution showed higher antioxidant activity in ABTS assay with PG equivalents. The antioxidant ability of curcumin to prevent the formation of reactive oxygen species (ROS) and to act as secondary antioxidant is mentioned earlier in the introduction section.

The effectiveness of antioxidants in terms of the antioxidant activity and polyphenol content is highly dependent on the choice of extraction process (107). The solvent extraction uses both alcohols and aqueous mediums, while in the current study aqueous extraction of curcumin was performed. The antioxidant activity of curcumin is variable with curcumin level in different species, that also depend upon the habitat climate, environment, seedling process, harvest age, altitude, cultivation method, and nutrients in soil (108). The antioxidant concentration is also an important determinant factor because at wrong concentration the antioxidant can act as a prooxidant (109). Based on the structural, chemical, and experimental properties, the attribute of an extract to act as an antioxidant or prooxidant must be carefully studied before application. Therefore, the natural antioxidants possessing significant antioxidant activity should not be conclusive, instead its ability to delay lipid oxidation in foods is more important parameter to consider. The antioxidant activity helping in delaying the lipid oxidation is dependent on constituents of herb or spice used, structure of antioxidant, their phenolic content and also on properties like structure, concentration used and factors like light and temperature (110).

The antioxidant activity for curcumin calculated in the current study could be different from curcumin-based extracts used in previous studies and work. The curcumin extracts in this study exhibit the antioxidant properties determined by DPPH and ABTS assay. The practical antioxidant activity of curcumin is further discussed in later sections with reference to results from microbial growth, PV and TBARS.

3.2 Drip loss

The calculation of drip loss and raw data is provided in Appendix A.2. The volume of curcumin extract was used as 2ml of the extract was used on the samples. It is assumed that the drip loss in samples treated with curcumin extracts is affected due to weight of samples. The issue of the excess drip in curcumin (CUR) samples is addressed by incubating the treated samples covered in aluminum foil in 4°C refrigerator for 30 minutes. The lipophilic property of curcumin makes it able to penetrate the cell membrane (111). It is assumed that after 30 minutes maximum of extract in treated samples is absorbed leaving no drip behind. There could be a possible different strategy for the experiments to measure the drip loss. In that strategy, the antioxidant extracts could be applied to some extra samples and after incubation for 30 minutes the remaining extract if not absorbed could have been wiped to get an accurate estimate of extract contributing to drip, but that is the limitation of the current study that could be improved in further work. Figure 3.1 illustrates the percentage drip loss as average for two parallel samples i.e., vacuum and MAP packed, each of these groups had two parallels for each sample.

Figures 3.1 shows that there is a significant increase of drip loss with the progress of storage days in both vacuum and MAP packed samples. The drip loss was found to increase with the passage of days in cold storage in previous studies. In a study, the freeze stored fillets showed significantly higher drip loss as compared to unfrozen and showed highest drip loss on day 15 of cold storage (112). Another study indicates that drip losses between 1-2% could not be considered as high drip loss (113).

The value of drip losses in vacuum packaging was found to be higher than in MAP packaging in general. In vacuum samples, even for day 3 of storage, very high value of drip loss was recorded. The categorization of drip loss in current study into low, medium, and high can be done on the basis of values of drip loss obtained. The lowest drip loss values were in range of 1.2-2.9 % on average and were exhibited in MAP samples in day 3 to day 8 of storage. The medium drip loss values ranged between 3-5% and were recorded by both group of samples for different storage days, while the highest drip loss values were between 6-8.6% and were recorded for vacuum packed samples. These values highly deviate from drip losses in previous studies, so can be regarded as high drip loss values. A previous master thesis reported the drip losses during vacuum and MAP in salmon showing 0.5-1.6% as low, 1-3% as medium and 2.6-4.8% as high values of drip losses (114). The drip loss values for vacuum samples were higher than 4% from day 3 to day 15. The effect of mechanical force during vacuum packaging could be related to elevated values right from day 3 of storage. Dunn and Rustad (2008)(115) reported that during super-chilling drip loss was not found to be a major problem. The highest drip loss found during that study was 1.6 % in salmon stored at – 1.4°C that was not regarded as high. It was also found that drip loss increased in partially frozen stored fish. As mentioned previously 1-2% could not be regarded as high drip loss values.

It has been obvious from previous studies that drip losses are increased and show greater values in vacuum packaging than in MAP (116). Specifically, Atlantic salmon have been reported to show higher drip loss values when packed under vacuum than in MAP (89).

However, there could be deviation from normal trend as one study showed salmon with MAP showed higher drip losses than vacuum (117). This deviation could possibly because of different storage temperatures (0 or 4°C), storage days, MAP gas composition, and different vacuum chamber machines.

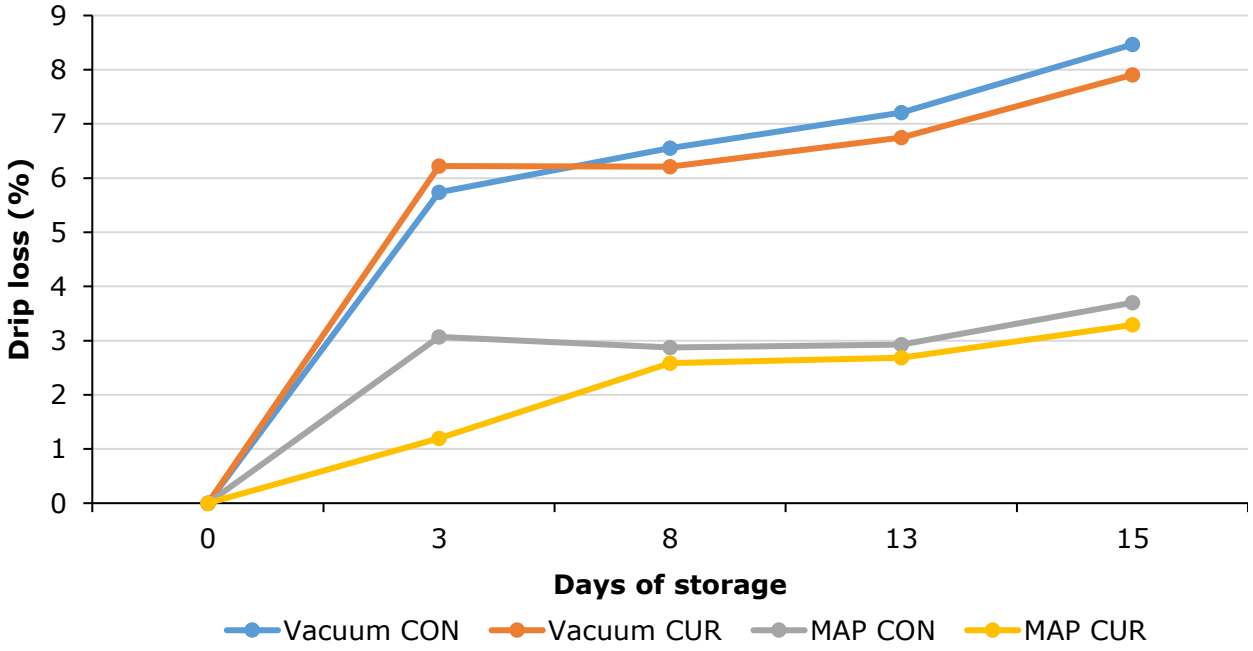


Figure 3.1: Drip loss (%) of sample weight for average of two parallels for two groups of packaging experiments (Vacuum and MAP), Vacuum CON (blue), vacuum CUR (orange), MAP CON (grey) and MAP CUR (yellow) during storage days 3, 8, 13, and 15.

The drip loss affects the product quality of fish and meat; thus, it is a highly deciding concern for consumer attention. There are several reasons to keep drip loss low, higher drip loss provides a good medium for bacterial growth. The loss of water also affects the juiciness and tenderness of the meat product, may also influence the texture, so there is need to prevent drip loss (118). The drip loss also leads to percentage weight loss in fish so it has an economic importance to reduce the drip loss (119). Additionally Wani et al. (2014)(120) reported that the drip loss results in pool of spoilage to allow pathogenic microbes to grow, this can reduce the shelf life of fish. The microbial spoilage is explained in next section.

In conclusion for drip loss, the results of this study indicate that the overall drip loss values are higher than what had been reported in previous studies, for both treatment groups. However, the similar trend of increasing drip loss values from previous studies is observed with increased days of storage and samples treated with vacuum showed higher drip loss than MAP. Moreover, the results show that the use of antioxidant may have no effect on drip loss.

3.3 Microbial spoilage

The microbial growth during storage is described graphically in Figure 3.2 as log CFU/gram sample of dilution 10^{-3} for average of two parallels of control (CON) and curcumin (CUR) each for two treatment groups i.e., vacuum and MAP. The plate counts were used to calculate the CFU, and the data is presented in Appendix A.3. The use of vacuum or MAP showed no effect on microbial growth. Both groups showed significant growth, so it is assumable that the shelf life was not improved by choosing one packaging method over the other. There was a significant increase in the microbial growth with the passage of storage days, the total count increased ranging from 6.25 to 6.64. The plating after serial dilution 10^{-3} varied between 15-73 colonies on day 3 to 175-560 colonies on day 15 in both treatment groups.

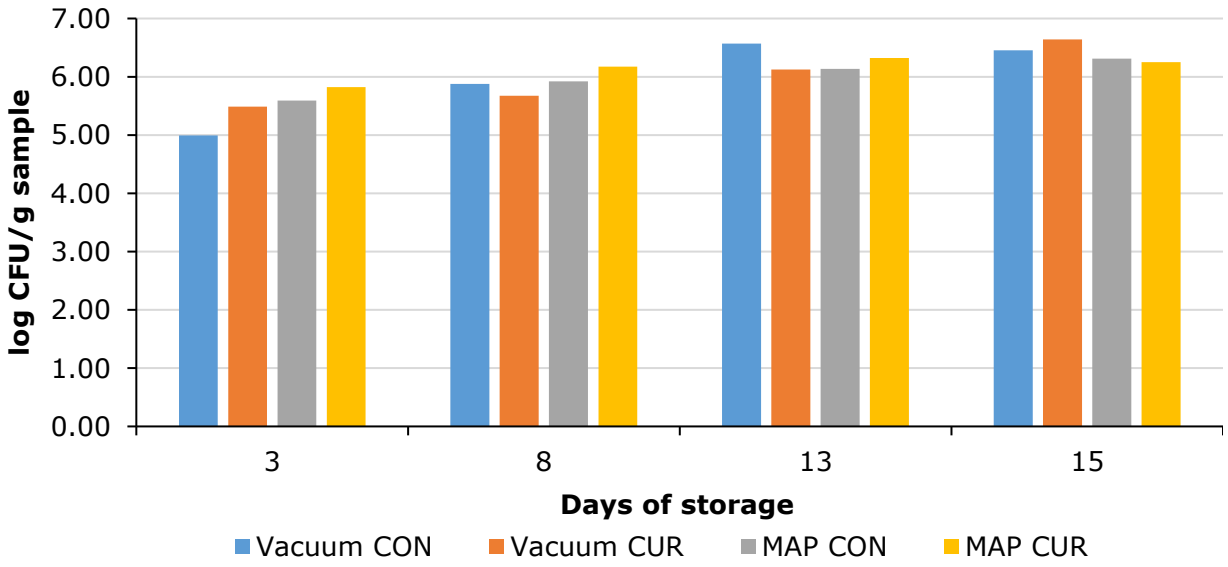


Figure 3.2: Log CFU per gram sample counted during 3-15 days of storage for average of two parallel samples for vacuum and MAP packaging methods, Vacuum CON (blue), vacuum CUR (orange), MAP CON (grey) and MAP CUR (yellow).

In chilled and frozen fish, the acceptable microbial criteria for aerobic plate count (APC) at 25°C and *E. coli* set as criteria by International Commission on Microbiology Specifications for Foods (ICMSF), is log 6 CFU/g. The value is considered safe for consumption by humans (121). The microbial growth results for current study ranged from 5-6.64 log CFU/g during storage days for all samples that indicates that the microbial growth reached the recommended consumption limit for the fish. On day 13 log CFU value for some sample groups was above log 6 CFU/g. In a previous thesis conducted in similar manner as the current study, log 6 CFU/g exceeded after day 6 and an average of 8.43 log CFU/g was found after 15 days of storage (114). That study also showed no significant differences between packaging methods. Hansen et al. (2009)(89) reported that vacuum packaging was less efficient than MAP in prolonging the shelf of salmon. Their study indicated that microbial growth surpassed the log 6 CFU/g on day 15 and 18 in vacuum packed while in MAP the log 6 CFU/g was not reached on day 15. In another finding by Van Haute, Sam, et

al. (2017)(122) it was clear that the microbial shelf life of MAP salmon is longer than vacuum packed salmon. However, the current study indicates no significant difference between vacuum packaging and MAP which is not in agreement of the previous research. Previous studies reported that CO_2 level higher than 25% ensure that extended shelf life (93). The previous literature also showed that with the passage of storage days CO_2 levels drop significantly (89). However, during the current study the CO_2 levels were not measured. As described previously from the literature that the aerobic species *Shewanella* and *Pseudomonas* show limited growth in MAP, but *Photobacterium phosphoreum* show resistance to CO_2 and grow during MAP treatment (89).

The spoilage process cannot be sufficiently justified by mere bacterial count, rather it depends on the specific spoilage organisms (SSO) and their resultant metabolites. The SSOs are responsible for producing spoilage metabolites such as ammonia, amines, organic acid, and sulfur compounds as result of amino acids degradation (123). Different SSOs are involved in production of different spoilage metabolites. The identification of specific spoilage organisms could have been done by plating the colonies on species dependent agar, but the timespan for this study was too limited to perform this kind of approach. In addition, the work on metabolites was also not carried out during this study.

Despite not using any techniques for identification of SSOs by growing them on species dependent agar plates, the previous studies and research provide evidence to hypothesize about the possible microflora. As explained in the previous chapter, the most common bacteria included in natural microflora of salmon are aerobic *Pseudomonas*, anaerobic *S. putrefaciens* and gram negative *Photobacterium*. The use of MAP or vacuum packaging causes growth prevention of aerobic microflora but some CO_2 resistant bacteria like *Photobacterium phosphoreum* can grow even in absence of oxygen.

As described in previous sections, the literature showed that chilled stored salmon treated with MAP had *P. phosphoreum* as dominant spoilage bacteria (83). As mentioned in previous chapter, the psychrotrophic aerobic Gram-negative Pseudomonas *spp.* and *S. putrefaciens* grows under strict aerobic conditions. Studies show that salmon treated with MAP showed dominantly *P. phosphoreum*, lactic acid bacteria (LAB) like *Carnobacterium maltaromaticum*. Some other bacteria found in MAP treated salmon were *Serratia proteamaculns*, *C. divergens*, *Serratia* spp, *Lactobacillus fuchuensis*, and *Y. intermedia. P. phosphoreum*, *Carnobacterium maltaromaticum* were also reported to be found in vacuum treated salmon along with *Serratia spp* (124).

The antimicrobial effect of curcumin treatment on MAP and vacuum samples might have prevented some bacteria from the microflora but it is not obvious from the study as no identification tests were conducted. Curcumin possess antibacterial property due to polyphenolic content (125). The curcumin solutions used in this study show significant antioxidant activity as determined in section 3.1 but the inhibition of specific bacteria from microflora is not determined. In conclusion, the microbial activity was found to be in close range to 6 log CFU/g sample that was considered safe to consume in agreement with the previous studies, there was no difference in CFU of MAP from vacuum treatment or the use of curcumin extract showed no change in microbial activity in this study.

3.4 Color

The pink-red color of muscles that gives special feature to the Atlantic salmon, comes from pigment deposition in the muscles. This color is due to pink-red pigment astaxanthin that is a carotenoid. Synthetic astaxanthin is added to the feed of fish, that imparts the color in muscles. Adding more astaxanthin makes the fish meat redder, however it is expensive and make up a large proportion of the feed cost in salmon farming. The characteristic reddish color for salmon or degree of redness is highly involved in attracting consumer's attention. The freshness and flavor of salmon was judged more on redness so that determines the consumer's willingness to pay (WTP) (126). Astaxanthin binds to the proteins, but color is highly dependent on the lipid content. High lipid content causes dilution of astaxanthin in fillets (127).

The color analysis data is given in Appendix A.4. There have been no significant changes in lightness (L*-values) for vacuum packed samples but MAP samples showed a significant increase in lightness. This is in agreement with the previous studies, Sigurgisladottir et al. (127) found a moderate increase in L* values during cold storage, whereas Hansen et al. (89) reported the increasing L* value during MAP storage, that was indicative of pigment oxidation during increased length of storage. There were no significant changes in redness (a*-values) during storage regardless of packaging method. However, the yellowness (b*-values) or yellow-blue range showed a different trend. The b*-values in samples treated with curcumin (Vacuum CUR and MAP CUR) showed much variance from those of control samples (Vacuum CON and MAP CON). The graphical representation shown in Figure 3.3 shows average b*-values for two parallels of control and curcumin treated samples in vacuum packed and MAP. The yellowness (b*-values) for control samples were ranging between 10-12 but for curcumin treated samples the b*-values ranged from 17-21, in two storage groups. The effect of color changes in salmon treated with curcumin was not reported in previous studies, but it is obvious that curcumin imparts yellow color due to its strong yellowish color in the antioxidant solution. Moreover, it has been shown that as a natural food colorant curcumin gives yellow color to the food it is used on. In a study, yellowness of curcumin reported to have potential use as smart packaging film in seafood (128). The b*-values for vacuum samples treated with curcumin showed a slight increased readings than MAP samples treated with curcumin. The overall yellowness was found to show a small decrease over the time of storage.

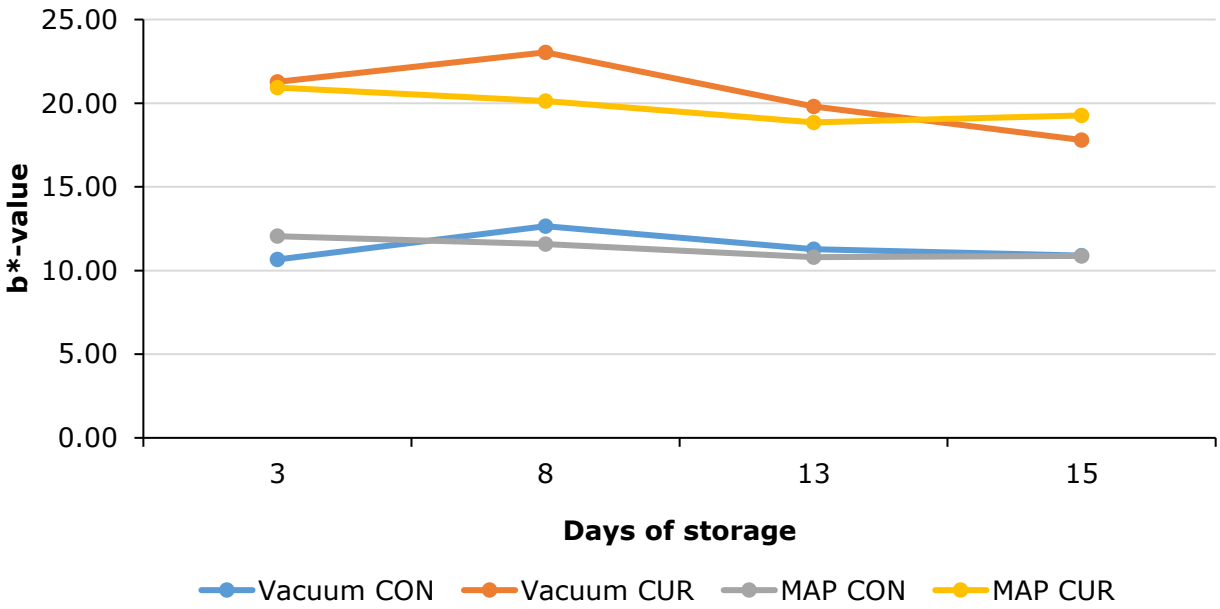


Figure 3.3: Average b*-values plotted against days of storage (3-15) for two parallels of Vacuum CON (blue), Vacuum CUR (orange), MAP CON (grey) and MAP CUR (yellow).

There were smaller but insignificant variations in L*, a*, and b* values within samples, that was possibly due to the use of different fillets for sampling that could have come from different fish. As mentioned earlier in this section, high lipid content affects the color as it dilutes astaxanthin in fillet. The texture of salmon is also related to color, as studies reported that salmon fillets with high L*-values are softer than those with low values (129). However, the texture analysis was not performed in the current study. In conclusion, the lightness (L*-values) increased in MAP samples during storage days, the redness (a*-values) showed no significant changes, and the yellowness (b*-values) showed high increased values in samples treated with curcumin. The yellowness is related to the natural yellow color of curcumin antioxidant solution that imparted yellow color to the samples.

3.5 Total lipid content

The example for calculation of total lipid content (%) and raw data for total lipid content (%) are given in Appendix A.5 as Table A.5 and Table A.5.1. The average total lipid content (%) for two parallels of control and curcumin samples in vacuum treated and MAP are illustrated in Figure 3.4.

The lipid content varied from 8.9 % to 18.9 % in both vacuum treated and MAP samples. There was a non-uniform difference in lipid content observed in sample for storage days and between packaging methods. The high variation in lipid content was possibly due to the use of pieces of fillets from different fish for sampling. Previous studies showed that salmon fat deposition is highly related to amount and composition of fish feed. Salmon fillet has difference in fat distribution, with tail having lowest while belly flap and dorsal region having highest fat content (130). So, it can be assumed that the use of different fillets during sampling resulted in non-uniform random lipid content for all samples. The water-content for the samples was not determined in this study.

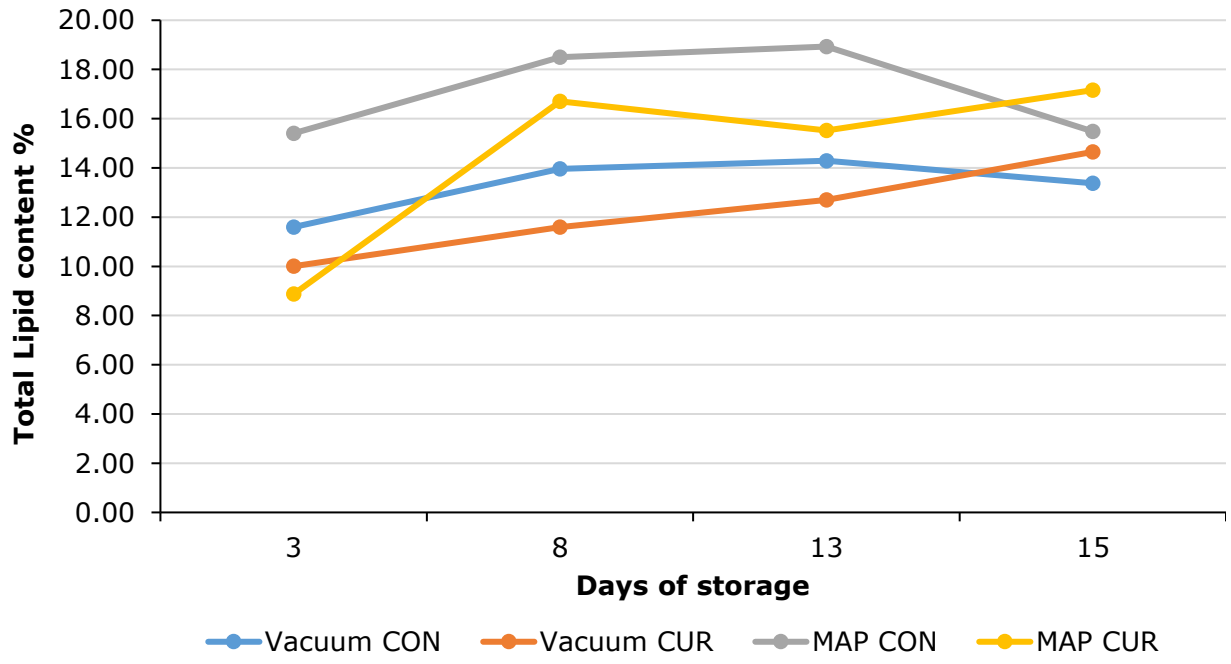


Figure 3.4: Average total lipid content (%) of wet weight plotted against days of storage (3-15) for two parallels of Vacuum CON (blue), Vacuum CUR (orange), MAP CON (grey) and MAP CUR (yellow).

In summary, the total lipid content (%) showed great variations within storage days, packaging methods for both control and curcumin treated samples.

3.6 Primary and secondary oxidation products

The primary and secondary oxidation products were measured by PV and TBARS assay. The raw data for these two procedures is given in Appendix A.6.

3.6.1 PV

The raw data for the calculated PV and example calculation are given in Appendix A.6 and below Table A.6.1. The data for lipid content (%) in 12 ml chloroform extract, was used from Table A.5.2. The PV results are illustrated graphically in Figure 3.5. The PV ranged from 0.14 to 2.07 m_{eq} peroxide / kg lipids for the samples. The increased storage time increased the PV for control samples in vacuum packaging while it decreased for curcumin treated samples in vacuum packaging. The PV in MAP control samples was observed to be increasing from day 3 to day 8 and then decreasing in day 13 and 15. However, MAP curcumin samples showed a decrease in PV with storage days. Overall, the PV decreased during the storage except control sample in vacuum packaging. The PV for MAP showed a significant decrease over storage days. This indicates that samples with MAP packaging and MAP in combination with curcumin, have slowed down the lipid oxidation in these samples. There is a clear difference in PV between MAP and vacuum samples, even vacuum sample treated with curcumin showed a decreasing PV over time. The use of curcumin antioxidant and its effect on lowering PV could be attributed to the antioxidant activity of curcumin solutions. However, the results from PV are not decisive as the secondary oxidation products are measured by TBARS that is discussed in the next section.

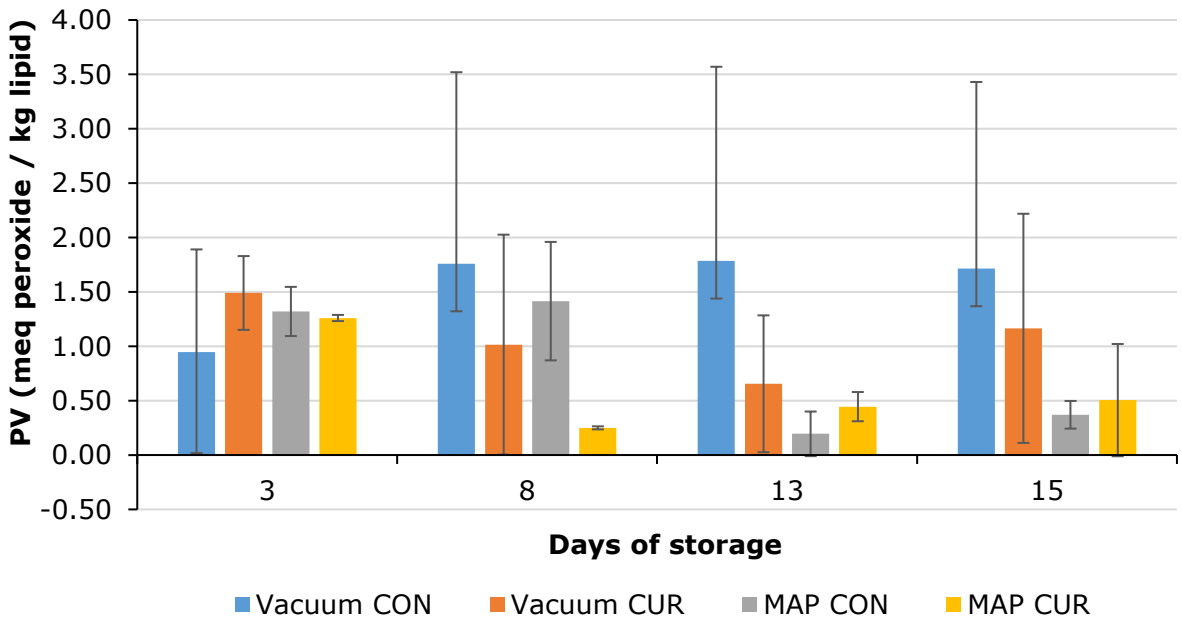


Figure 3.5: Average peroxide values (m_{eq} peroxide / kg lipids) plotted against days of storage for two parallels of Vacuum CON (blue), Vacuum CUR (orange), MAP CON (grey) and MAP CUR (yellow). The error bars represent the SDOM.

The association of producers of omega-3 LCPUFA products is Global Organization for EPA and DHA Omega-3s (GOED), that recommends products with omega-3-LCPUFA having a peroxide value less than 5 meq peroxide/kg lipid (PV<5meq O_2 /kg). This recommendation is according to GOED voluntary monograph that suggests that PV should be below 5 in fish oils intended for human consumption (131). As mentioned above the range of PV for this study ranged between 0.14-2.07 m_{eq} peroxide / kg lipids, that is far less than 5 meq/kg. On day 3 the PV was between 0.95-1.5, then from day 8 the PV started decreasing except in the vacuum control samples that kept on increasing until day 15. Studies have shown that PV could be decisive in sensory evaluation of fish oil where the oxidation status of eicosapentaenoic acid and docosahexaenoic acid could help in determining the sensory quality. As measuring PV does not involve the smelling or tasting of the samples , PV could not be decisive in sensory evaluation (132). However, the sensory analysis was not done in the current study.

Studies explained that the primary oxidation products i.e., hydroperoxides levels can be measured quantitatively by measuring the PV (132). The lower PV in this study shows that the primary oxidation products were present in low amount. Other possibility is that the decomposition of hydroperoxides into secondary oxidation products that can determined by measuring TBARS (133). The determination of TBARS by assessing the secondary oxidation products is explained in next section.

Studies have shown that PV has some limitations, there could be possible oxidation during extraction of lipids or performing the PV titration. Moreover, the method has lack of sensitivity and colored lipid soluble compounds can hinder the PV measurement to give wrong results. The rancidity in oil is measured by measuring both primary and secondary

oxidation products or perform sensory analysis. Therefore, only PV cannot be decisive in measuring rancidity (134). Previous studies also mentioned the nature of oil to indicate off-flavor quality, for example olive oil could be called rancid at 20 meq/kg PV whereas fish oil shows rancidity around 1 meq/kg PV (135).

The PV for some samples were deviating by showing very high values. The problem was solved by repeating the procedure for the second time. As the method required 12 ml of chloroform extract for titration, there could be problem of running out of samples. This occurred for a few samples when performing the repetition. The practical limitation in this study was about 10 g of fish sample was used to extract the lipids, that yielded 27-33 ml of chloroform extract. This volume was used to determine total lipid content (2 ml), PV (12 ml), and TBARS (2 ml).

In summary, the PV increased with storage time in vacuum control samples and decreased in other samples. The decreased PV with storage days were observed in samples treated with curcumin, and MAP samples. The secondary oxidation products are explained further in the next section.

3.6.2 TBARS

TBA (thiobarbituric acid) was known to mainly measure malondialdehyde (MDA) and is expressed as mg MDA/kg, but studies show that TBA reacts with numerous other compounds, so it is referred as TBARS (Thiobarbituric acid reactive substances) (134). In this study, considering all products of secondary oxidation, the TBARS was expressed in µmol TBARS/g lipids. Figure 3.6 illustrates the obtained values in TBARS for samples during storage days.

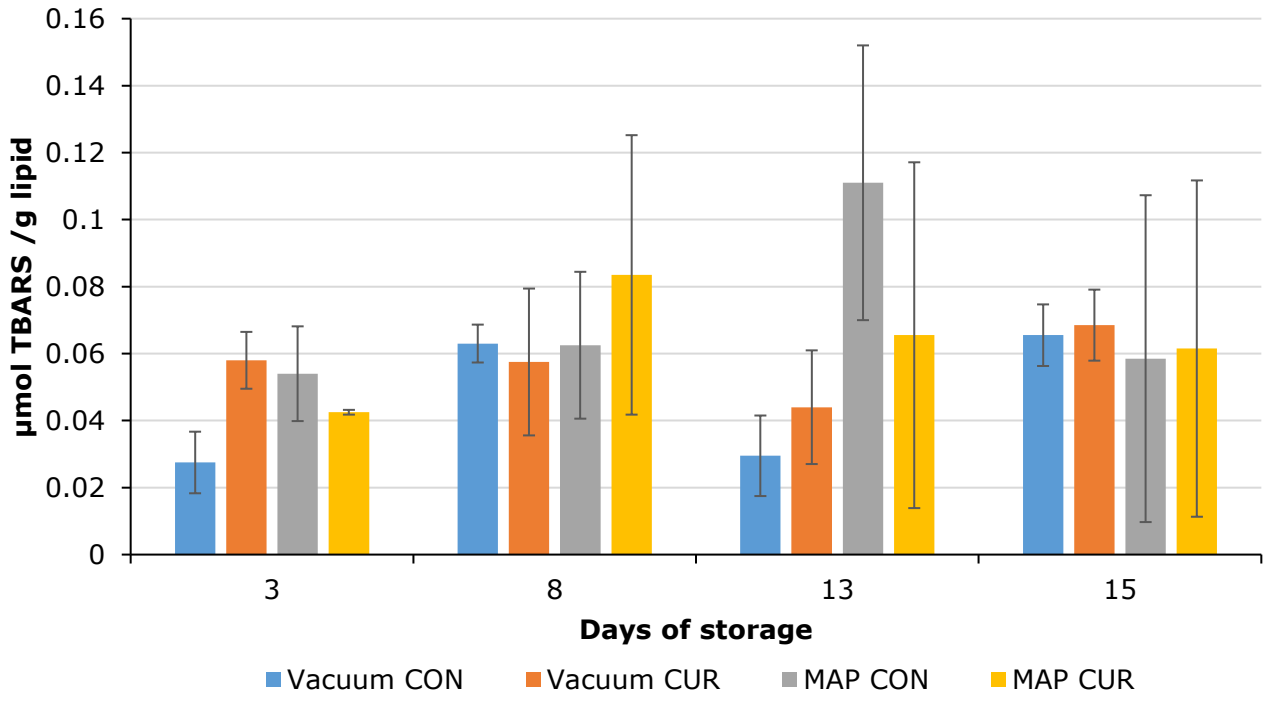


Figure 3.6: Average TBARS values (µmol TBARS/g lipids) plotted against days of storage for two parallels of Vacuum CON (blue), Vacuum CUR (orange), MAP CON (grey) and MAP CUR (yellow). The error bars represent the SDOM.

Overall, the TBARS values appeared to be increasing with the storage days in all samples. From day 3 to day 6 the TBARS values for all samples increased, then on day 13 there was a decrease in values in all samples except MAP control that showed increased value. On day 15 the values were increased except MAP control that showed as decrease as compared to value on day 13. There was a complexity in the oxidation process when comparing TBARS values to those of PV. The decreased PV with storage days indicated the possible decomposition of primary oxidation products into secondary products. This reason could be relevant to explain that with passing days of storage, the TBARS values increased, so it might be assumed that the secondary oxidation occurred. Moreover, the curcumin treatment to the samples showed no change in the samples by affecting the TBARS values.

A previous study from a master thesis showed lower detection limit of 0.7 µmol TBARS/g lipids in fish oils (136). Studies also showed that the degree of rancidity in fish could be determined by TBARS method (134). It has been reported that in chilled stored fish the accepted TBARS value is 5 mg MDA/kg lipids, with a maximum limit of 8 mg MDA/kg lipids determining the consumption limit for fish (137). As in this study, µmol TBARS/g lipids was used to express TBARS, the converted limit of is 5-8 mg MDA/kg lipids would be in range 0.069 to 0.111 µmol TBARS/g lipids. The TBARS values, as shown in figure has only one sample exceeding this mentioned limit, so theoretically it could be indicated that the rancidity in this study has not been reached for a large number of samples and that the fish is good for consumption. A master thesis (114) similar to this study reported TBARS values close to or above 0.069 to 0.111 µmol TBARS/g lipids. Another study by Ke et al. (138) describes that if TBARS value in fish is 0.58 mg MDA/kg lipids it could not be considered

rancid, whereas the values between 0.58-1.51 mg MDA/kg lipid could be considered slightly rancid and values of 1.51 mg MDA/kg lipid is referred to be rancid. According to this study, the samples in the current study were not in rancid condition.

In summary, the TBARS values generally showed increased values with the storage day, whereas PV were decreasing, so possibly secondary oxidation products were formed. The TBARS values were low, showing that the lipids were not rancid. The use of antioxidant showed no effect on TBARS values.

3.7 Limitations of study and future work

A low number of parallels caused fewer data to interpret, this is considered the main limitation of the study. There were variations in color and lipid content due to use of pieces of fillets from different fish. These variations could be minimized by using fillets of same fish to keep parameters uniform. The recommendation for future work is to have a greater number of samples in each group, that would provide more reliable results. Moreover, for measuring the oxidation products more parallels per sample would give more accurate results. The several parallels in PV and more parallels in TBARS would avoid the need for repetitions. In this regard, it is recommended to extract more lipid samples per fish, if there is a need for repetition in case of deviating results from the titrator. Although there are solvent extraction methods available for curcumin extraction, in this study, curcumin was extracted using ultrasonication in water bath. It is recommended, to find more aqueous-based extraction methods for curcumin instead of using ethanol or methanol in solvent extraction. During this work, samples were incubated in refrigerator for 30 minutes after treating with curcumin. It is also recommended that when treating samples with curcumin by pipetting or spraying, the samples must be incubated for 1 hour before packaging, so that maximum extract is absorbed.

Time was a key factor for limitations in the current study. There were immense and hectic days of laboratory work during this study despite not determining some parameters like water content, ash content, protein content. One must be careful when one has to work with too many parallels and also more parameters at the same time. If the future work is intended to perform these parameters, a well-developed strategic planning is recommended.

It is recommended for future work that sensory analysis is also performed. It will give reliable results about texture, color, taste, and smell that would define parameters like microbial spoilage, PV, pH, color, and rancidity. Another important recommendation is to plate for spoilage specific organisms (SSO) that would be helpful in differentiation of microbes involved in spoilage during storage. Moreover, determining the metabolites produced by microflora would give more insights on microbial spoilage. It is also recommended that small details like measuring the CO_2 levels would provide reliability when analyzing MAP samples.

4 Conclusion

The results for this study indicated that drip loss increased during storage, vacuum treated samples showed higher drip loss than MAP and the values were significantly higher than drip loss calculated in previous studies. Neither packaging method nor the use of antioxidant curcumin was helpful in prolonging the shelf life by preventing microbial spoilage. The microbial growth reached the recommended consumption limit after 8 and 13 days of storage. The color analysis showed that curcumin treatment imparts yellow color to the samples. There was a variation in lipid content probably because of using fillets from different fish for sampling. The results from antioxidant activity determination suggested that the extracted curcumin solutions possess significant antioxidant activity to cause a delay in the lipid oxidation. The fact that curcumin delays lipid oxidation could not be confirmed during the study after measuring primary and secondary oxidation products. The PV in MAP with curcumin-treated samples was found to be lower than vacuum treated samples with the storage days. TBARS values increased with storage days in all samples indicating the formation of secondary oxidation products. Although, the TBARS values confirmed that the lipid oxidation occurred but the PV and TBARS values showed that lipids were not rancid. Moreover, the PV and TBARS analysis indicated low but not high effect of curcumin in delaying lipid oxidation.

In summary, MAP was found be beneficial packaging method to prolong the shelf life of Atlantic salmon. There were some advantageous effects of using MAP in combination with curcumin when considering the lipid oxidation process, so this technique could lead to future prospects. More research is needed for the use of curcumin as an antioxidant source.

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Appendix A; Experimental Data and calculation examples

A.1 Antioxidant activity

Figure A.1 and A.2 showing the standard curve for the antioxidant assays DPPH and ABTS respectively.

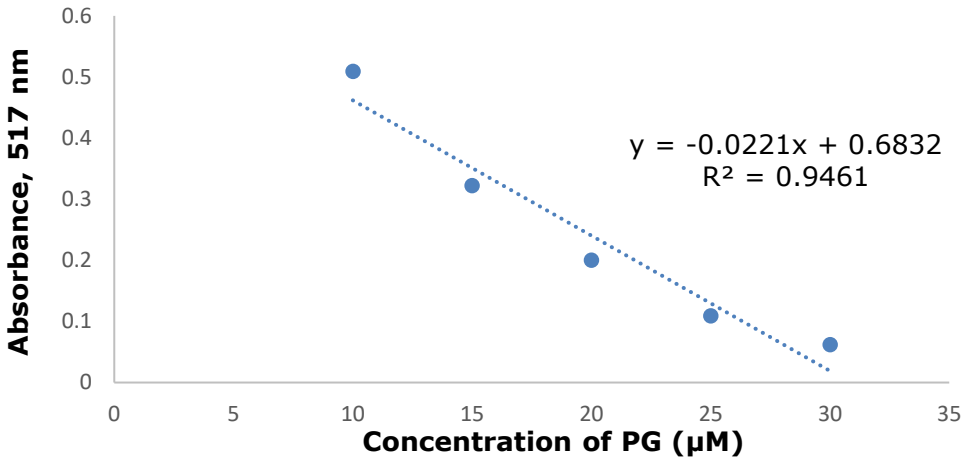


Figure A.1 showing the standard curve for antioxidant activity assay, DPPH by plotting the absorbance at 517nm against the concentration of PG (µM) while using 80% methanol as blank. A linear approximation generates the equation y = -0.0221x + 0.6832 and a coefficient of determination R^2 = 0.9461.

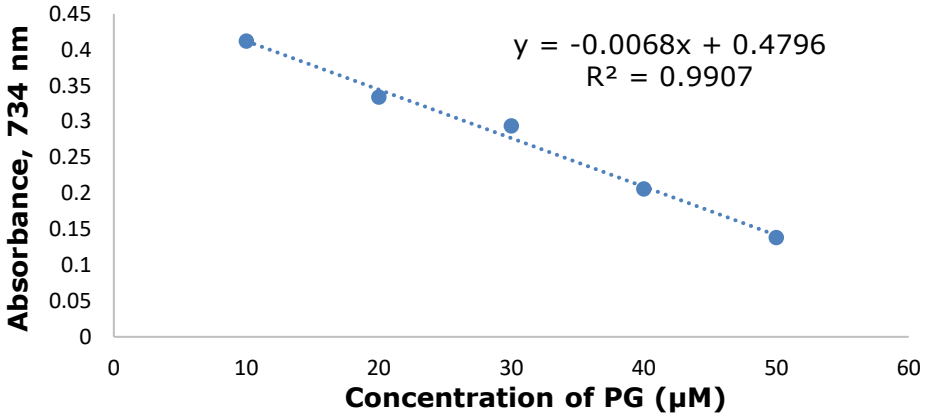


Figure 2 showing the standard curve for antioxidant activity assay, ABTS by plotting the absorbance at 734nm against the concentration of PG (µM) while using 80% methanol as blank. A linear approximation generates the equation y = -0.0068x + 0.4796 and a coefficient of determination R^2 = 0.9907.

The absorbance values (A) were obtained by taking the average of three parallels. The standard curve is shown to yield different values for slope (a) and intercept (b) to calculate

the PG equivalents by using equation 6. The absorbance values for three parallels for the samples are given in Table A.1.

Table A.1. The antioxidant capability of the curcumin sample solutions measured in DPPH and ABTS assays.

	Absorbance	DPPH	ABTS
Curcumin	1	0.168	0.018
	2	0.164	0.017
	3	0.165	0.016
	Average	0.166	0.017

The PG equivalence of curcumin antioxidant in DPPH assay was calculated by equation 6 as an example as follows.

$$\text{PG equivalence (DPPH), curcumin} = \frac{0.166 - 0.6832}{-0.0221} = 23.40$$

A.2 Drip loss

The example for calculation of percentage drip loss is shown in Table A.2 for raw data on experiment day 8, using Equation 3.

Percentage Drip loss (%) = (Mass of drip/Mass of initial Product) X 100 (3)

Mass of drip (g) = 104.19 – 102.18 = 2.01

Drip loss (%) = 2.01 X 100/ 99.20 = 2.03

Table A.2 Day 8 experimental data for calculation of drip loss for vacuum CON, vacuum CUR, MAP CON and MAP CUR. The values from initial sample to mass of drip are given in grams (g) while drip loss is given in percentage.

	Vacuum CON	Vacuum CUR	MAP CON	MAP CUR
Initial sample	99.20	103.60	74.10	63.80
Fish before drying	104.19	108.52	83.76	74.29
Microbiology sample (MB)	11.68	10.74	11.27	13.48
Fish after drying	90.50	91.50	64.48	58.61
Dried fish+MB	102.18	102.24	75.75	72.09
Mass of drip	2.01	6.28	8.01	2.2
Percentage Drip loss (%)	2.03	6.06	9.60	2.96

Table A.2.1 shows the drip loss for all the experimental days (3, 8, 13 and 15) for two groups of experiments i.e., Vacuum packaging and MAP packaging. Both these groups

contained two parallel samples of control (CON) and curcumin (CUR). The average for two parallels, that was presented in results is shown in Table A.2.2.

Table A.2.1 showing the percentage drip loss (%) of samples for two groups vacuum and MAP packaging, in each group two parallels for control CON and curcumin CUR are represented. The percentage drip loss for these samples is expressed for all experimental days

	% Drip Loss			
Sample	**Day 3**	**Day 8**	**Day 13**	**Day 15**
Vacuum CON 1	5.45	6.50	7.65	8.30
Vacuum CON 2	6.02	6.61	6.77	8.63
Vacuum CUR 1	6.77	6.06	6.34	7.61
Vacuum CUR 2	5.68	6.36	7.16	8.20
MAP CON 1	3.22	3.60	3.68	3.38
MAP CON 2	4.92	2.15	2.17	4.02
MAP CUR 1	0.53	2.96	2.45	1.70
MAP CUR 2	1.86	2.21	2.92	4.88

Table A.2.2 showing the average percentage drip loss for two parallels in each group of vacuum and MAP packaging

		% Drip Loss average		
Sample	**Day 3**	**Day 8**	**Day 13**	**Day 15**
Vacuum CON	5.74	6.56	7.21	8.47
Vacuum CUR	6.23	6.21	6.75	7.91
MAP CON	3.07	2.88	2.93	3.70
MAP CUR	1.20	2.59	2.69	3.29

A.3 Microbial growth

The counted colony forming unit (CFU) are shown in table A.3

Table A.3 showing the microbial growth counted in the form of CFU for the two groups of packaging samples on the experiment days 3, 5, 8 and 15. Two parallels for each sample control (CON) and curcumin (CUR) were plated in both experimental groups MAP and vacuum, also the weight of samples is enlisted. The CFU is expressed for both 10^{-3} and 10^{-5} dilution.

Day	**Sample**	**Weight of sample**	**Dilution**	
			10^{-3}	**10^{-5}**
Day 3	Vacuum CON 1	14.39	15	0
	Vacuum CON 2	13.79	13	0
	Vacuum CUR 1	10.89	33	0
	Vacuum CUR 2	8.87	28	0

	MAP CON 1	12.49	44	0
	MAP CON 2	11.48	37	0
	MAP CUR 1	8.61	70	0
	MAP CUR 2	9.92	73	1
Day 8	Vacuum CON 1	11.68	71	1
	Vacuum CON 2	11.00	105	1
	Vacuum CUR 1	10.74	42	1
	Vacuum CUR 2	10.11	62	1
	MAP CON 1	11.27	170	0
	MAP CON 2	10.48	61	0
	MAP CUR 1	13.48	97	0
	MAP CUR 2	10.74	260	1
Day 13	Vacuum CON 1	10.29	28	6
	Vacuum CON 2	12.29	48	3
	Vacuum CUR 1	9.29	260	6
	Vacuum CUR 2	10.91	90	1
	MAP CON 1	12.12	277	1
	MAP CON 2	9.54	86	1
	MAP CUR 1	10.56	250	2
	MAP CUR 2	13.23	223	4
Day 15	Vacuum CON 1	10.19	56	10
	Vacuum CON 2	13.31	220	9
	Vacuum CUR 1	14.87	550	7
	Vacuum CUR 2	8.52	390	5
	MAP CON 1	11.00	212	3
	MAP CON 2	13.29	202	4
	MAP CUR 1	9.20	175	3
	MAP CUR 2	9.39	218	9

The CFU was used to calculate the log CFU/g using the following equation and values of medium volume (100 ml), dilution factor (d), volume plated (1ml) and weight of sample used.

log CFU = log (CFU X 100 ml / d X 1 ml X w)

A.4 Color

The chromameter readings for color are enlisted in table A.4 for the sample on each day of experimentation.

Table A.4 showing the experimental average color measurements of lightness L*, red-green range a*, and yellow-blue range for two groups of samples. Vacuum and MAP showing two parallels for control (CON) and curcumin samples.

Day	Sample	L*	a*	b*
Day 3	Vacuum CON 1	47.79	11.41	9.92
	Vacuum CON 2	41.32	13.38	11.40
	Vacuum CUR 1	41.82	9.99	22.02
	Vacuum CUR 2	44.39	8.08	20.55
	MAP CON 1	43.54	12.54	11.54
	MAP CON 2	41.86	13.18	12.56
	MAP CUR 1	47.77	8.27	22.27
	MAP CUR 2	46.36	8.71	19.60
Day 8	Vacuum CON 1	44.60	13.99	13.4
	Vacuum CON 2	45.70	11.92	11.90
	Vacuum CUR 1	41.59	9.48	23.87
	Vacuum CUR 2	45.46	8.99	22.21
	MAP CON 1	42.47	12.63	11.43
	MAP CON 2	44.10	12.80	11.74
	MAP CUR 1	46.18	8.22	19.82
	MAP CUR 2	46.50	7.84	20.45
Day 13	Vacuum CON 1	43.00	13.07	11.49
	Vacuum CON 2	43.95	12.49	11.06
	Vacuum CUR 1	40.87	9.66	20.09
	Vacuum CUR 2	39.73	9.15	19.50
	MAP CON 1	44.54	12.18	10.65
	MAP CON 2	46.11	12.14	10.95
	MAP CUR 1	49.65	9.32	19.00
	MAP CUR 2	47.35	9.27	18.71
Day 15	Vacuum CON 1	40.65	12.92	9.99
	Vacuum CON 2	42.21	12.90	11.81
	Vacuum CUR 1	39.83	11.46	17.73
	Vacuum CUR 2	39.06	10.22	18.17
	MAP CON 1	42.28	12.81	11.02
	MAP CON 2	43.31	13.57	10.73
	MAP CUR 1	49.52	9.34	19.03
	MAP CUR 2	48.12	9.46	19.52

A.5 Total Lipid content

The total lipid content calculation is shown from an example calculation from table A.5, in which two parallel vacuum control samples on day 8 of the experiment were used as an example.

Table A.5 showing the raw data and total lipid content calculation for two parallel vacuum CON samples on day 8 of the experiment

Total Lipid Content	**Vacuum CON 1**	**Vacuum Control 2**
Fish sample (g)	10.1000	10.1100
Tube + lid (g)	10.5399	10.6354
Tube + lid + Lipids (g)	10.6290	10.6873
Weight of Lipid (g)	0.0891	0.0519
Total Lipid/Fat content (%)	17.64	10.27

The total percentage lipid content (%) of first parallel (Vacuum CON 1) from the above table was calculated by equation 4 as follows, using chloroform used as 40 ml, chloroform phase added in tubes as 2 ml.

Lipid content (%) = 40 ml X 0.0891 X 100 / 2 ml X 10.1000 = 17.64 %

The further calculations for total percentage lipid content of two groups of experiments i.e., vacuum and MAP having two parallel samples of control (CON) and curcumin (CUR) for day 3, 8, 13, and 15 are enlisted in table A.5.1.

Table A.5.1 showing the total percentage lipid content (%) for two parallels each of control (CON) and curcumin (CUR) in two groups i.e., vacuum and MAP determined on experimental day 3, 8, 13, and 15.

Day	Sample	Total lipid content (%)
Day 3	Vacuum CON 1	14.03
	Vacuum CON 2	9.16
	Vacuum CUR 1	7.90
	Vacuum CUR 2	12.11
	MAP CON 1	11.70
	MAP CON 2	19.11
	MAP CUR 1	7.73
	MAP CUR 2	10.01
Day 8	Vacuum CON 1	17.64
	Vacuum CON 2	10.27
	Vacuum CUR 1	10.81
	Vacuum CUR 2	12.38
	MAP CON 1	19.94
	MAP CON 2	17.05
	MAP CUR 1	14.67
	MAP CUR 2	18.74
Day 13	Vacuum CON 1	18.88
	Vacuum CON 2	9.70
	Vacuum CUR 1	14.80
	Vacuum CUR 2	10.60
	MAP CON 1	24.53
	MAP CON 2	13.33
	MAP CUR 1	18.35
	MAP CUR 2	12.70
Day 15	Vacuum CON 1	10.71
	Vacuum CON 2	16.04
	Vacuum CUR 1	18.10
	Vacuum CUR 2	11.20
	MAP CON 1	11.20
	MAP CON 2	19.77
	MAP CUR 1	14.34
	MAP CUR 2	19.98

2 ml of chloroform extract containing lipids were evaporated that gave the weight of lipids (g) per ml of chloroform extract. The values for weight of lipids (g) are provided in Table A.5.2 and these were used in calculation of PV and TBARS.

Table A.5.2 showing the weight of lipids in 2 ml chloroform extract for two parallels each of control (CON) and curcumin (CUR) in two groups i.e., vacuum and MAP determined on experimental day 3, 8, 13, and 15.

Day	Sample	Weight of lipids in 2 ml Chloroform extract(g)	Weight of lipids in 12 ml Chloroform extract(g)
Day 3	Vacuum CON 1	0.0728	0.4368
	Vacuum CON 2	0.0498	0.2988
	Vacuum CUR 1	0.0396	0.2376
	Vacuum CUR 2	0.0612	0.3672
	MAP CON 1	0.0623	0.3738
	MAP CON 2	0.1025	0.6150
	MAP CUR 1	0.0399	0.2394
	MAP CUR 2	0.0538	0.3228
Day 8	Vacuum CON 1	0.0891	0.5346
	Vacuum CON 2	0.0519	0.3114
	Vacuum CUR 1	0.0550	0.3300
	Vacuum CUR 2	0.0674	0.4044
	MAP CON 1	0.1024	0.6144
	MAP CON 2	0.0885	0.5310
	MAP CUR 1	0.0751	0.4506
	MAP CUR 2	0.0953	0.5718
Day 13	Vacuum CON 1	0.0945	0.5670
	Vacuum CON 2	0.0533	0.3198
	Vacuum CUR 1	0.0741	0.4446
	Vacuum CUR 2	0.0556	0.3336
	MAP CON 1	0.1245	0.7470
	MAP CON 2	0.0707	0.4242
	MAP CUR 1	0.0935	0.5610
	MAP CUR 2	0.0668	0.4008
Day 15	Vacuum CON 1	0.0588	0.3528
	Vacuum CON 2	0.0850	0.5100
	Vacuum CUR 1	0.0951	0.5706
	Vacuum CUR 2	0.0577	0.3462
	MAP CON 1	0.0565	0.3390
	MAP CON 2	0.1047	0.6282
	MAP CUR 1	0.0771	0.4626
	MAP CUR 2	0.1052	0.6312

A.6 Primary and Secondary oxidation products

A.6.1 PV

Table A.6.1 enlists the values of volume of titrant consumed V and Blank B, and calculated PV of the samples using equation 6. The above Table A.5.2 was used to take the weight of lipids in 12 ml of chloroform extract.

Table A.6.1 showing the volume of titrant consumed (V) and Blank (B) and peroxide value (PV) for two parallels each of control (CON) and curcumin (CUR) in two groups i.e., vacuum and MAP determined on experimental day 3, 8, 13, and 15.

Day	Sample	Volume of titrant consumed(V)	Blank (B)	Peroxide value (PV)
Day 3	Vacuum CON 1	0.2060	0.0795	0.29
	Vacuum CON 2	0.5578	0.0795	1.60
	Vacuum CUR 1	0.3766	0.0795	1.25
	Vacuum CUR 2	0.7146	0.0795	1.73
	MAP CON 1	0.5939	0.0407	1.48
	MAP CON 2	0.7532	0.0407	1.16
	MAP CUR 1	0.3376	0.0407	1.24
	MAP CUR 2	0.4535	0.0407	1.28
Day 8	Vacuum CON 1	0.8534	0.0795	1.45
	Vacuum CON 2	0.7241	0.0795	2.07
	Vacuum CUR 1	0.6504	0.0795	1.73
	Vacuum CUR 2	0.2004	0.0795	0.30
	MAP CON 1	1.1468	0.0407	1.80
	MAP CON 2	0.5888	0.0407	1.03
	MAP CUR 1	0.1597	0.0407	0.26
	MAP CUR 2	0.1783	0.0407	0.24
Day 13	Vacuum CON 1	0.9499	0.0795	1.54
	Vacuum CON 2	0.7287	0.0795	2.03
	Vacuum CUR 1	0.1748	0.0795	0.21
	Vacuum CUR 2	0.4465	0.0795	1.10
	MAP CON 1	0.0745	0.0407	0.05
	MAP CON 2	0.1861	0.0407	0.34
	MAP CUR 1	0.3439	0.0407	0.54
	MAP CUR 2	0.1801	0.0407	0.35
Day 15	Vacuum CON 1	0.5981	0.0795	1.47
	Vacuum CON 2	1,0791	0.0795	1.96
	Vacuum CUR 1	0.3192	0.0795	0.42
	Vacuum CUR 2	0.7333	0.0795	1.91
	MAP CON 1	0.1966	0.0407	0.46
	MAP CON 2	0.2195	0.0407	0.28
	MAP CUR 1	0.1061	0.0407	0.14
	MAP CUR 2	0.5898	0.0407	0.87

$$PV = \frac{(V-B) \times T \times M}{(w)} \quad (5)$$

PV = (0.2060 – 0.0795) X 1 / 0.4368 = 0.29 m_{eq} peroxide/kg lipids

A.6.2 TBARS

The standard curve obtained during the analysis of two groups of samples i.e., vacuum and MAP is shown in Figure A.6.2

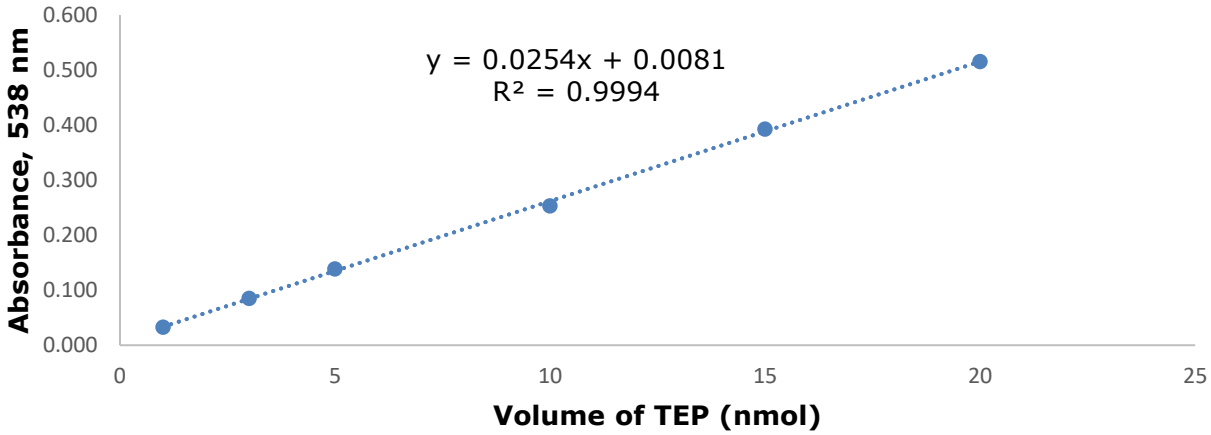
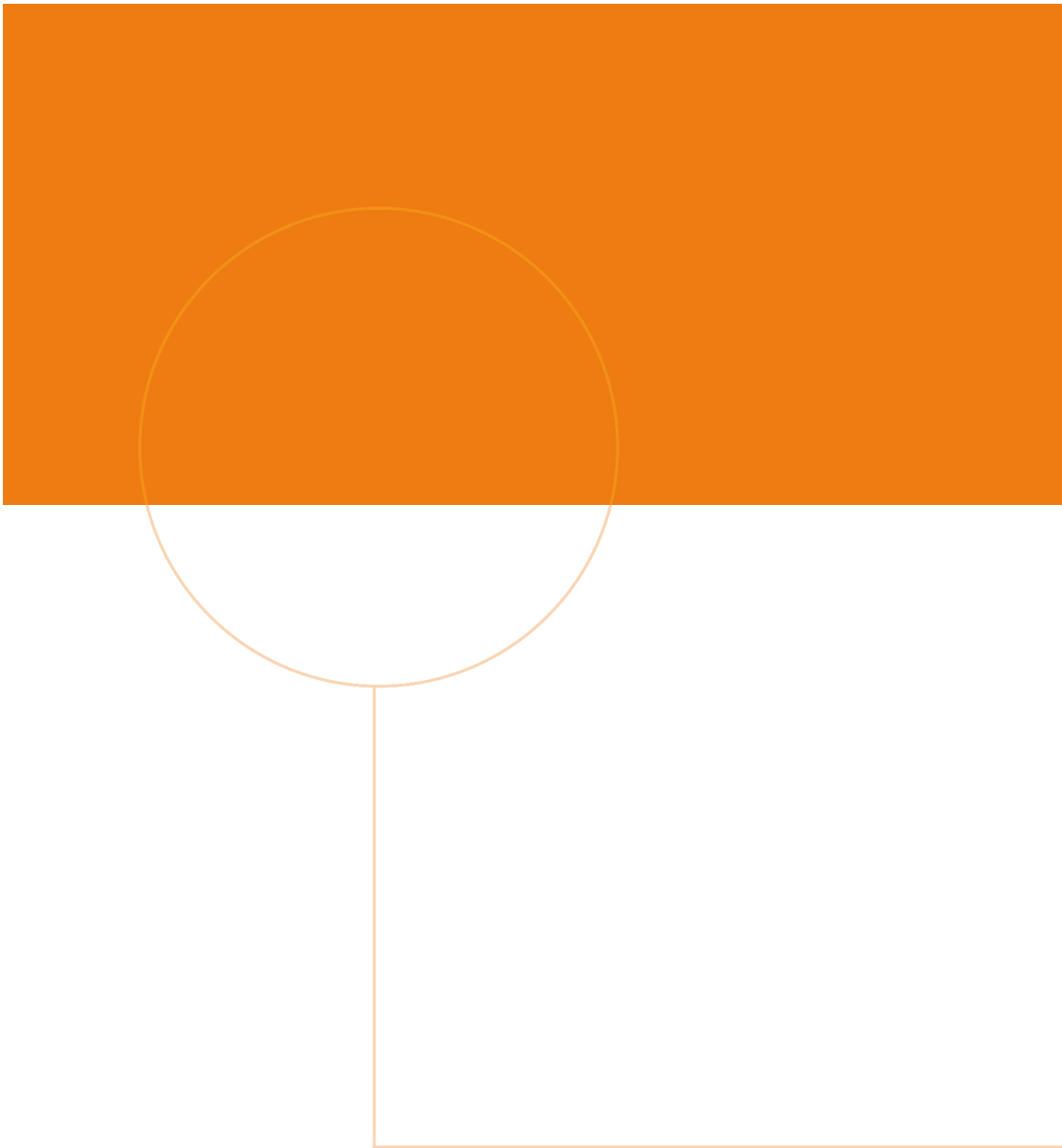


Figure A.6.2: Standard curve used for analysis of TBARS assay for two groups of samples (Vacuum and MAP) obtained by plotting the absorbance at 538 nm against volume of TEP (nmol). The resultant linear approximation gives the equation y = 0.0254x + 0.0081 and a coefficient of determination R^2 = 0.9994.

From the slope (a) and intercept (b) of the standard curve and absorbance (A) readings for samples, TBARS was calculated using equation 6. In addition, the relevant weight of lipids in 2ml chloroform extract was obtained from Table A.5.2. The values for absorbance (A) and calculated TBARS is shown in Table A.6.2.

Table A.6.2 showing the absorbance at 538 nm and calculated TBARS for two parallels each of control (CON) and curcumin (CUR) in two groups i.e., vacuum and MAP determined on experimental day 3, 8, 13, and 15.

Day	Sample	Absorbance at 538 nm	TBARS
Day 3	Vacuum CON 1	0.071	0.034
	Vacuum CON 2	0.035	0.021
	Vacuum CUR 1	0.072	0.064
	Vacuum CUR 2	0.089	0.052
	MAP CON 1	0.077	0.044
	MAP CON 2	0.174	0.064
	MAP CUR 1	0.051	0.042
	MAP CUR 2	0.067	0.043
Day 8	Vacuum CON 1	0.141	0.059
	Vacuum CON 2	0.096	0.067
	Vacuum CUR 1	0.110	0.073
	Vacuum CUR 2	0.080	0.042
	MAP CON 1	0.099	0.078
	MAP CON 2	0.212	0.047
	MAP CUR 1	0.113	0.054
	MAP CUR 2	0.111	0.113
Day 13	Vacuum CON 1	0.281	0.038
	Vacuum CON 2	0.036	0.021
	Vacuum CUR 1	0.114	0.056
	Vacuum CUR 2	0.053	0.032
	MAP CON 1	0.267	0.082
	MAP CON 2	0.259	0.140
	MAP CUR 1	0.250	0.102
	MAP CUR 2	0.057	0.029
Day 15	Vacuum CON 1	0.116	0.072
	Vacuum CON 2	0.135	0.059
	Vacuum CUR 1	0.156	0.061
	Vacuum CUR 2	0.120	0.076
	MAP CON 1	0.142	0.093
	MAP CON 2	0.072	0.024
	MAP CUR 1	0.199	0.097
	MAP CUR 2	0.078	0.026



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