Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Studies of metabolic changes in sugar kelp (*Saccharina latissima*) after different processing and post-mortem changes in Atlantic salmon (*Salmo salar*) upon addition of sugar kelp during storage by NMR spectroscopy

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Supervisor: Alexander Dikiy and Elena Shumilina
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Preface

I would like to thank SINTEF research institute and Prof. Grazina Juodeikiene (University of Kaunas) for our collaborations and their contributions to the research I have done in regard to this master's thesis.

To my supervisors Prof. Alexander Dikiy and Elena Shumilina, I am thankful for all the work and effort they have spent on me. During this last semester you have given me a taste of the life as a researcher. It was the interest Alex showed in me as a potential master student and his passion for NMR studies at our first meeting that made me decide to join your research lab and work with NMR metabolomics. I am very grateful for the close collaboration I have had with Elena during my work in the lab and on the thesis. I have always been welcome in her office, where she always has sat aside time for answering all my questions and given me valuable guidance with my thesis and laboratory work. Even during some weekends! For the future I will try to follow Alex' advice to not worry and stress less about everything all the time.

To my family, I am grateful for the support you have showed me during my 5 years of Biotechnology studies at NTNU Gløshaugen. Also, many thanks to my sister for taking the time to give some appreciated last thoughts and opinions on this thesis.

Lastly, I would like to thank Anna and Martin for lending me their apartment during the end of my master period; It made finishing the thesis easy and convenient for me.

Even Moen Kirkholt
Trondheim, 12th of July 2019

Abstract

Combating hunger and ensuring food security are topics included in the 17 sustainable development goals by the United Nations, which addresses many global issues. The possibility of cultivating marine organisms for human food consumption can be one of the solutions to combat the future need of increased food production. The European market requires more detailed information about nutritional value and safety of consuming seaweeds as food. Sugar kelp (*Saccharina latissima*) is one of the brown seaweeds that are cultivated in Norway. Its nutritional value upon different treatment was investigated in this thesis using a metabolomics approach. Nuclear magnetic resonance (NMR) spectroscopy coupled with multivariate analysis were the techniques used to assess the metabolic changes.

The thesis is comprised of three studies: i) Assessment of post-harvest changes of the polar metabolome of sugar kelp upon storage at different temperature and treatment; ii) Monitoring changes within the sugar kelp metabolome during fermentation by lactic acid bacteria (LAB) and iii) Analysis of the metabolic changes during storage of Atlantic salmon muscle (*Salmo salar*) that were caused by the addition of sugar kelp.

1. Whole and cut sugar kelp were stored at 4 °C and room temperature to model real conditions of seaweed post-harvest storage on board ships. The autolysis and fermentation processes within sugar kelp tissue were detected and assessed. This was done by comparing the concentration of different amino and organic acids (valine, isoleucine, tyrosine, phenylalanine, formate and pyruvate) between the samples. Conclusions were done based on data obtained from the analysis of three replicas (a total of 35 samples). The results demonstrated that storage of cut seaweed at room temperature caused significant deviations of the seaweed metabolome compared to the other treatments.

2. Three sugar kelp samples were fermented by using different LAB strains. The concentrations of lactic acid in fermented samples were at least 14.3 times higher compared to non-fermented sugar kelp. The concentration of essential amino acids valine and phenylalanine were increased in all fermented sugar kelp. The concentrations of umami taste related compounds glutamic and succinic acid were increased by fermentation with two of the LAB strains. One LAB strain caused increased formation of glycerol and acetone. It was shown that sugar kelp can be used as a substrate for LAB fermentation. The nutritional value and taste of the final product depends on the LAB strain used for fermentation.

3. Muscle tissue of Atlantic salmon was wrapped in wet sugar kelp and stored at 4 °C for 3 weeks. Polar and non-polar metabolites were determined and quantified in 65 salmon samples after storage, as well as in sugar kelp tissue and dripped liquid. Mutual diffusion of sugar kelp and salmon metabolites caused significant decrease of the formation of trimethylamine (TMA) and biogenic amines cadaverine and putrescine, and it caused an increase of aspartate in sugar kelp-treated salmon samples. Studies of the changes of the non-polar metabolic profile (lipids and

carotenoids) showed significant changes in the carotenoid content of the sugar kelp-treated samples during storage compared to the reference samples.

The studies demonstrated that different processing of seaweeds causes significant changes in the metabolic content. Therefore, it is necessary to set and strengthen the standards of seaweed handling during shipping and storage on board ships. This will ensure delivery of seaweed with predictable quality to the land industry. Sugar kelp was found to be a valuable substrate for LAB fermentation. Fermentation might enhance flavor of seaweeds depending on the LAB strain being used. Wrapping fish fillets into seaweed with kelp liquid changes the metabolic composition of fish. The final product will have different nutritional characteristic and shelf life compared to non-treated fish.

Table of contents

1 Introduction

1.1 Motivation and background of thesis

Sustainability and utilization of marine resources are topics that have been put on the agenda globally, as an attempt to combat future challenges. The United Nations have proposed sustainable development goals that addresses many of these future issues (1), where one of the goals is related to hunger and food security. The UN claims 815 million people are undernourished globally (year 2019) and also that an additional 2 billion people will be undernourished by the year of 2050 (2). The need of more food and sustainable food production is evident. To be viewed as sustainable, the food produced today has to satisfy the current need of food. Also, it must not compromise future generations from also utilizing this type of food production.

The oceans cover approximately 70 % of the Earth's surface and contain a massive amount of marine resources that can be utilized by humans. The possibility of cultivating marine organisms for human food consumption, known as *mariculture*, can be one of the solutions to combat the future need of increased food production. Ocean farms, which are facilities at sea where marine organisms such as fish and seaweed can be cultivated, are thought to be more sustainable than land-based agriculture. Cultivation of seaweeds at ocean farms is suggested to be a more sustainable option than land-based agriculture due to fresh water, chemical fertilizers and land area not being required in open ocean seaweed cultivation (3).

Saccharina latissima, formerly known as *Laminaria saccharina*, is a brown seaweed commonly known as sugar kelp. It is described to be a cold-water species that grows along coasts in the Northern Hemisphere (4). This include northern European countries such as Norway. Contrary to Asian countries, where the tradition of using seaweed in foods is strong, seaweed is not represented in traditional Norwegian cuisine. Although consumption of the Japanese dish *sushi* has become quite popular in Norway over the last years, where seaweed for instance is used to make *Maki Sushi.* Seaweed as a main dish or food product in the regular Norwegian grocery store is not common. The potential of incorporating seaweed, such as sugar kelp, into mainstream food consumption culture can lead to more sustainable food production and a reduction in the strain on land-based agriculture. Information from researching post-harvest changes in sugar kelp and how it influences other food systems can be beneficial to the food industry. It can provide knowledge on how to process and incorporate sugar kelp with other food systems in effective and favorable ways for both seaweed cultivator and consumer.

The aim of this thesis was to characterize metabolic changes of the seaweed *S. latissima* upon different treatment and storage temperature after harvest. The influence of adding wet sugar kelp to premium and standard quality Atlantic salmon (*Salmo salar*) was also studied, based on changes in nutritional value and shelf life. Lactic acid bacteria (LAB) fermentation of *S. latissima* was performed to assess the nutritional value and taste properties of fermented sugar kelp, which was based on its metabolic composition for food consumption purposes. Metabolic characterization of *S. latissima* and Atlantic salmon was performed by NMR spectroscopy.

1.2 Metabolism

Metabolism describes a set of chemical reactions occurring in cells that are necessary for growth and normal function of living organisms. It can be divided into anabolism and catabolism (5). During anabolism new cell components are produced by use of energy and reducing power, while during catabolism energy and reducing power are produced from breaking down organic molecules (5). The following subsections will describe metabolic pathways that are relevant for seaweeds.

1.2.1 Photosynthesis

Converting energy from sunlight to chemical energy stored in carbohydrate molecules is described to be the source of mainly all metabolic energy in a biological system (6). This process is known as *photosynthesis*, and it can be simplified to equation I:

$$6\,CO_2 + 6\,H_2O + sunlight \rightarrow C_6H_{12}O_6 + 6\,O_2 \qquad \text{(I)}$$

The process of photosynthesis involves the light and dark reactions. In the light reactions, photosynthetic pigments absorb photons from sunlight and drive the synthesis of ATP and NADPH together with the oxidation of water (H_2O) to oxygen (O_2). Adenosine-5'-triphosphate (ATP) is an energy-rich molecule used by cells to fuel energy-demanding processes, while nicotinamide adenine dinucleotide phosphate (NADPH) can be described as a coenzyme in cells used for donating electrons and hydrogens to biochemical reactions (6). Produced ATP and NADPH are used in the dark reactions, which do not require sunlight, to drive the synthesis of carbohydrates (such as glucose, $C_6H_{12}O_6$) from carbon dioxide (CO_2) and water. Photosynthetic organisms include plants and seaweeds.

1.2.2 Breakdown of glucose

Breakdown of glucose, which are molecules of stored chemical energy, is a form of metabolism used by multicellular organism such as humans and animals to fuel cells with energy that is required for living organisms. It involves consuming oxygen to oxidize glucose to carbon dioxide and water (the opposite reaction of photosynthesis, equation II) in order to generate ATP that can be used as cellular fuel (6). This process is known as *respiration*, which can occur under *aerobic* and *anaerobic* conditions.

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O \qquad \text{(II)}$$

A simplified overall reaction scheme for respiration is shown in figure 1.1. Aerobic respiration occurs in cells when oxygen is available to them. It can be described to involve

glycolysis, oxidative decarboxylation of pyruvate, citric acid cycle and oxidative phosphorylation. Glucose is converted to pyruvate during glycolysis, and pyruvate is converted to acetyl-CoA after oxidative decarboxylation. Acetyl-CoA is used in the citric acid cycle to generate electron carrier molecules (among other), such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), which in turn are used in the electron transport chain to drive the generation of ATP by oxidative phosphorylation. The ATP yield from glucose breakdown can be between 36-38 ATPs per molecule of glucose (6).

Under anaerobic conditions, where cells are in the absence of oxygen, breakdown of glucose yields a much lower amount of ATP (6). Aerobic respiration is therefore always preferred when oxygen is available to cells. Under anaerobic conditions, pyruvate produced from glycolysis can be converted to ethanol or lactic acid (6).

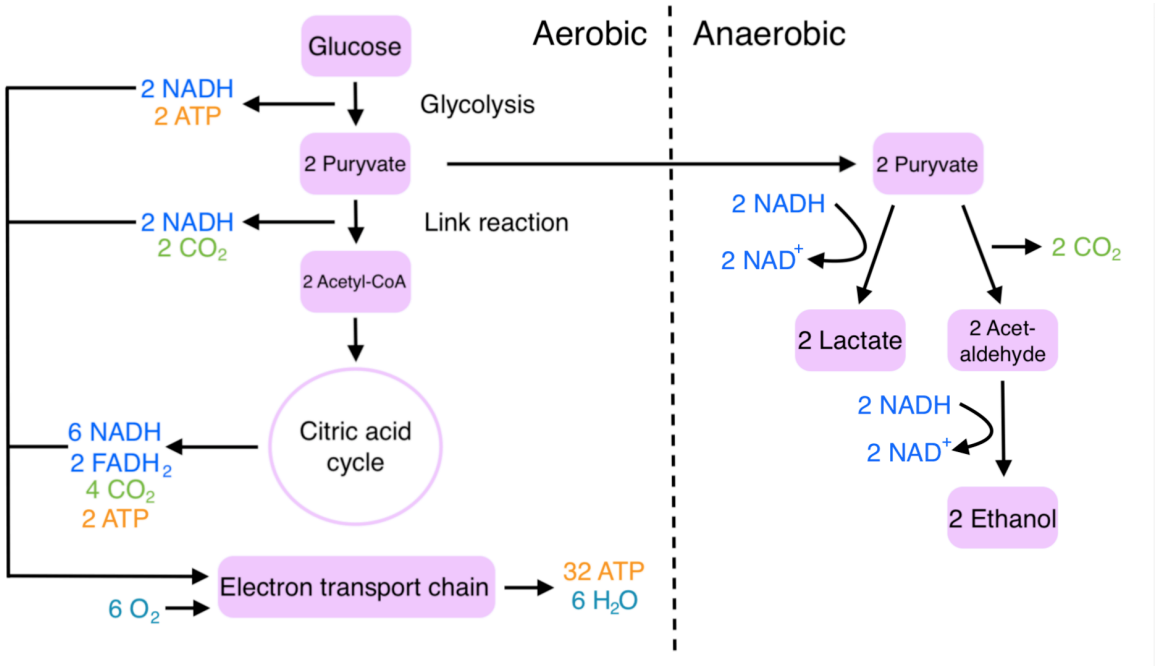

Figure 1.1. Simplified overview of some key intermediate metabolites and reactions involved during respiration of glucose under aerobic and anaerobic conditions (6). The figure does not show complete net reactions involved in respiration of glucose.

1.2.3 Fermentation

Fermentation can be described as a form of metabolism often used by microorganisms, such as *Bacteria*, to produce ATP to fuel cellular activity. Müller describes it in general as a reaction where a substrate is oxidized, the produced intermediate compound is reduced and excreted and that most generated ATP is a result of substrate level phosphorylation (7). Glucose can be used as a substrate for fermentation, and the excreted end-product is often a desired value product for food and industrial purposes. Fermentation products are for instance used to make dairy products such as yoghurt and cheese, and fermentation of sugars can produce ethanol and lactic acid as end-products (figure 1.1, anaerobic section).

Some major fermentation pathways of sugars have been described by Müller (7), which are summarized in figure 1.2. The genus of *Escherichia* bacteria can perform mixed acid fermentation, producing fermentation products such as ethanol, lactate, acetate, succinate and formate. The acid form of some of the mentioned compounds are lactic acid, acetic acid, succinic acid and formic acid; the two different forms will be used interchangeably during this thesis. The genus of *Enterobacter* is shown to perform 2,3-butanediol fermentation producing fermentation products such as ethanol, 2,3-butanediol, lactate and formate. Figure 1.3 shows a simplified overview of mixed acid and 2,3-butanediol fermentation pathways (8). Acetoin is an intermediate metabolite of this pathway. It is described as a compound with yoghurt odor and creamy butter taste, which is used as an additive in foods to enhance flavor (9). The microorganism *Bacillus subtilis* has been described to perform mixed acid-butanediol fermentation (10). Formic acid is a compound used to preserve livestock feed, such as silage. Described properties of formic acid include arrest of degradation processes and retainment of nutritive value of feed (11).

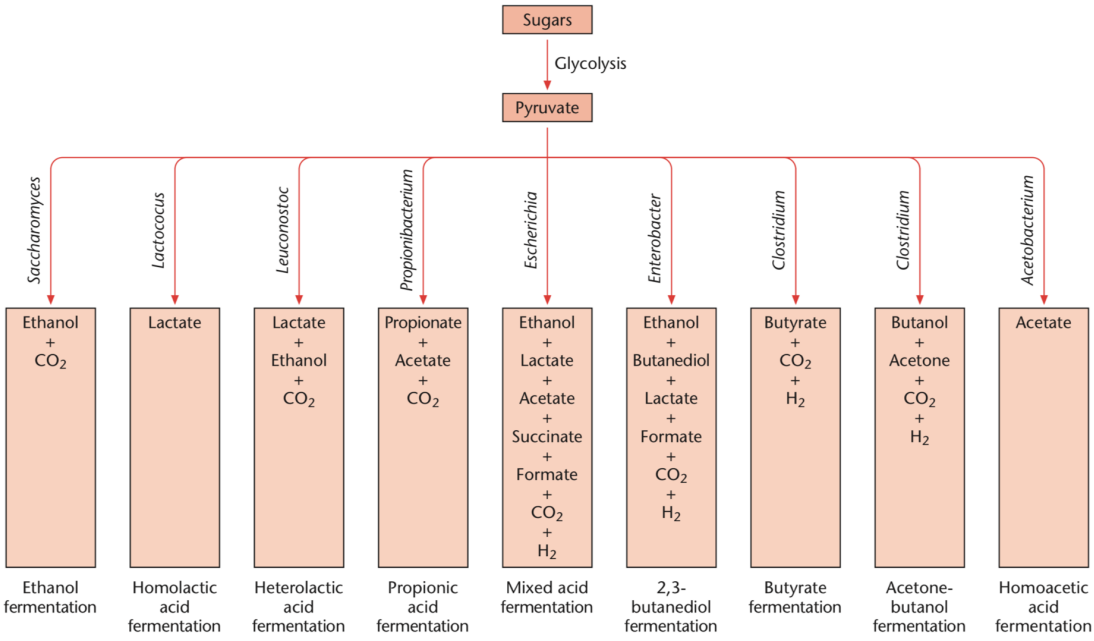

Figure 1.2. Overview of some major fermentation pathways for sugars and their respective end-products. Genera of microorganisms known to perform the different types of fermentations are listed. Figure by Müller (7).

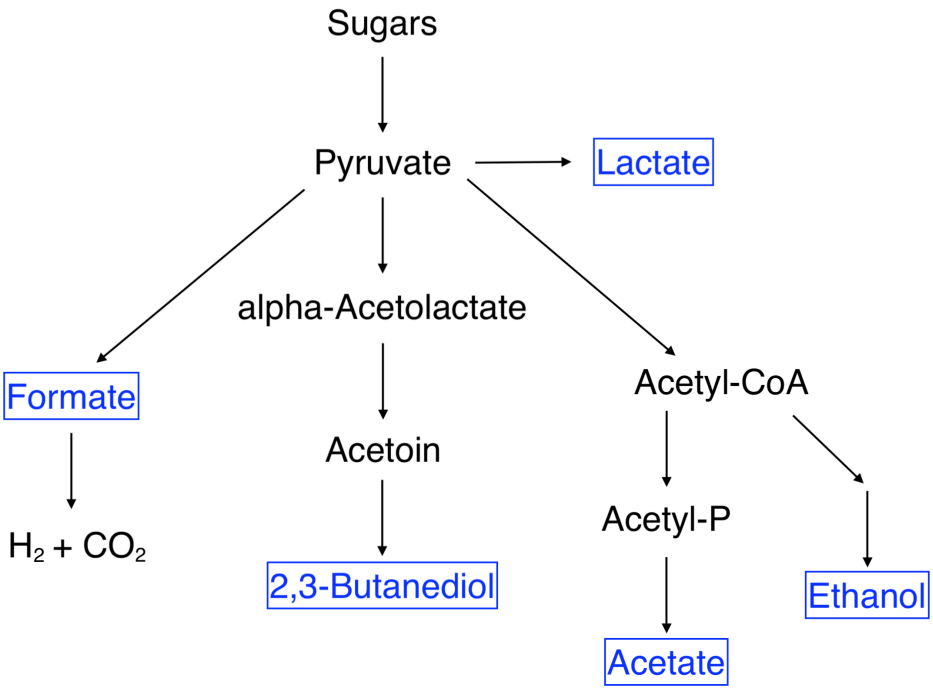

Figure 1.3. Simplified overview of mixed acid and 2,3-butanediol fermentation pathways with sugars as substrate. The genera of *Escherichia* and *Enterobacter* are microorganisms known to perform these types of fermentation. Selected fermentation products are marked in blue. Figure modified from Boumba (8).

1.3 Properties of *Saccharina latissima* and brown seaweeds

The main components of *S. latissima* are reported to be mannitol, alginate, laminarin, cellulose, proteins and salts (12). The pigment responsible for the appearance of brown seaweed is reported to be fucoxanthin (13). Moen described in his thesis some structural properties of brown seaweeds (figure 1.4): The cell walls of brown seaweed contain compounds such as alginate, fucans, cellulose and proteins, while the bulk of the alginate is located in the intercellular matrix (14). Alginate are molecules built up of mannuronic and guluronic acid, and alginate provides seaweed with mechanical structure by binding divalent metal ions (14). Polysaccharides, diterpens and phlorotannins are components in brown seaweed that are thought to give bioactive properties such as anti-oxidation and properties that are anti-viral and anti-diabetic (13).

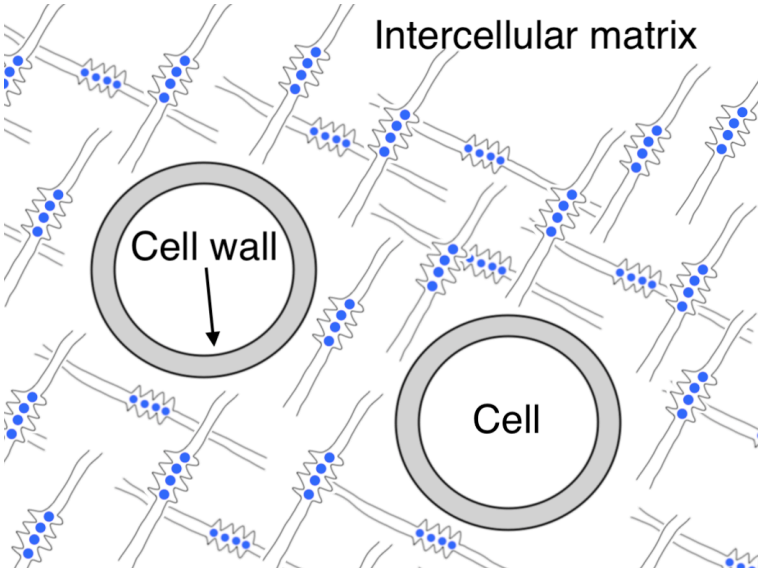

Figure 1.4. Simplified overview of a cross-section of brown seaweed tissue. The cells are located within a three-dimensional alginate network (intercellular matrix). Blue dots represent calcium ions bridging polyguluronate blocks. Figure modified from Moen (14).

1.3.1 Lactic acid bacteria fermentation of *Saccharina latissima*

Seaweeds such as sugar kelp can be used as substrate for fermentation. Lactic acid fermentation of vegetables has been described as a favorable way for both processing and preservation of the food product, together with yielding new flavors for the fermented product (15). Also, lactic acid fermentation of food products are described to promote resistance against production of toxins and microbial spoilage (15). The effects of lactic acid bacteria (LAB) fermentation on sugar kelp has been studied by Bruhn *et al*. LAB fermentation of sugar kelp by *Lactobacillus plantarum* was shown to reduce the amount of trace metals (heavy metals) such as cadmium and mercury (16). The mentioned metals are among others reported to have high degrees of toxicity and are proposed human carcinogens (17). The sensory properties of sugar kelp after LAB fermentation were described to have improved visual and odor properties compared to fresh sugar kelp (16).

1.4 Metabolic post-harvest changes of seaweeds

Two known processes of metabolic change to occur in organisms after harvest or death can be categorized as autolytic and bacteriological. Chesters *et al.* described decomposition of seaweeds to include autolysis of the seaweed protoplast and growth of microorganisms (18). The protoplast can be defined as the cell content within the cell wall. Most seaweeds consist of eukaryotic cells, and a major degradation pathway described for proteins in eukaryotic cells is lysosomal proteolysis (19).

When organisms die, the deterioration of their cells can be due to activity of their own enzymes. In general, the rate of enzyme catalyzed reactions increase with higher temperatures until the enzymes become thermally inactivated (20). Lysosomes are

organelles within the eukaryotic cell (figure 1.5) that contain digestive enzymes, such as proteases (19). Proteases are enzymes that promotes hydrolysis of peptide bonds, which connect amino acids in peptides and proteins. Normally the lysosomes are membrane-enclosed in living cells and thereby uncontrolled degradation of the cell content outside of the lysosomes is prevented (19). However, upon cell death and/or processing such as cutting of seaweed, the cells or lysosomes can be disrupted, and digestive enzymes can start to leak out to the rest of the cell content or onto surrounding cell tissue. Degradation of cells or cell content by peptidases can lead to an increase in free amino acids, as proteins and peptides are broken down to its main building blocks.

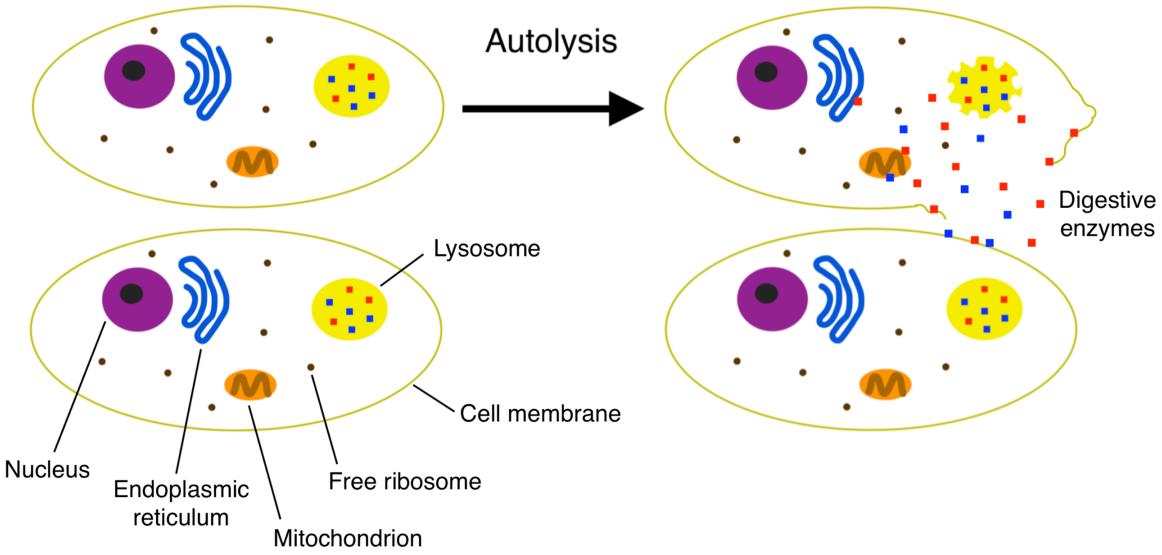

Figure 1.5. A simplified overview of a eukaryotic cell and some of its organelles. During autolysis, the cell is destroyed as a result of the activity of its own enzymes.

When organisms such as animals and plants die, their immune system stops functioning and the remaining organic matter can be decomposed by microorganisms. It was described by Moen that microorganisms have to hydrolyze complex organic matter into short polymer chains for assimilation, and that extracellular enzymes can be produced by the microorganisms for this purpose (14). When organic matter has been broken down into amino acids, sugars and fatty acids, they can be utilized by microorganisms for aerobic and anaerobic respiration. Sugar kelp is organic matter that contains compounds such as carbohydrates, and these can be decomposed by microorganisms after harvest. Østgaard *et al.* showed that mannitol and laminarin were effectively consumed for methane fermentation by microorganisms under anaerobic digestion of *Laminaria saccharina* (21). Alginate was shown to be fermented more slowly (21).

1.5 Shelf life of salmon

The shelf life of a food product can be defined as the time period the product can be stored and is suitable for human consumption. Increasing the shelf life of food products by combating processes that are responsible for their deterioration is favorable for food industry; it is more sustainable as it can reduce food waste. Salmon is often given a commercial shelf life duration of 14 days (2 weeks) after slaughter.

1.5.1 Post-mortem biochemical processes in fish

Two well-known post-mortem biochemical processes that influence the eating quality of fish during storage are autolysis and bacterial activity (22), which are shown in figure 1.6. Post-mortem changes due to autolysis in cod occurs app. the first 6 days after slaughter, followed by changes due to bacterial activity from app. day 7 after slaughter (22). After slaughter the fish's cells will start to deteriorate due to the activity of its own enzymes. Huss describes that production of ATP in post-mortem salmon muscle will have to switch to anaerobic respiration as oxygen-rich blood is no longer circulated through the fish body (22). This results in lactic acid and pyruvic acid as the main end-products, and a reduction in the pH of the muscle tissue. Anaerobic respiration does not yield sufficient amount of ATP for normal levels, so the salmon muscle will enter rigor mortis state. Resolution of the rigor mortis state, leading to softening of the muscle tissue, is thought to be related to the activity of naturally occurring muscle enzymes (22). These will activate softening by destroying components necessary for proper function of the rigor mortis complex. Another autolytic process that is coincidental with resolution of rigor mortis is the ATP degradation (22), which is described further in section 1.5.2.1.

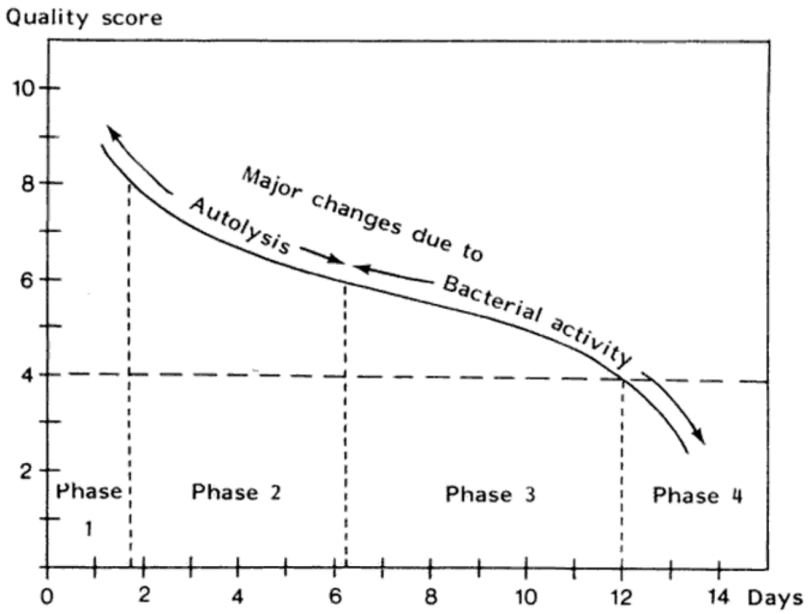

Figure 1.6. Plot of quality score of cod as a function of time after slaughter. Major post-mortem changes are results of autolysis and bacterial activity. Figure by Huss (22).

Microorganisms are reported to be present on all outer surfaces and in the intestines of caught fish (22). Huss describes that bacteria can proliferate in the fish flesh after death of the fish, because the immune system is no longer functioning. Huss further suggests spoilage of fish flesh is a result of bacterial enzymes diffusing into the flesh from the outside, and that nutrients diffuse to the outside of the fish where most microbial growth takes place (22). During storage of fish, growth of spoilage bacteria results in production of odors and flavors that decrease the quality of the fish and reduces the shelf life (22).

1.5.2 Biochemical evaluation of fish quality

Tolerance levels of compounds that indicate fish spoilage has been established to ensure food safety. They can be used to determine fish quality, which can be evaluated from quantitative standards. Quantitative NMR analysis can therefore be used to assess fish spoilage based on the amounts of spoilage indicators present in the muscle tissue. The following subsections will describe some of the indicators that can be used for biochemical evaluation of fish quality.

1.5.2.1 ATP degradation and freshness considerations

Degradation of ATP-products are some of the autolytical changes occurring in fish after death (22). Post-mortem ATP degradation products in fish muscle include adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine-5'-monophosphate (IMP), inosine, hypoxanthine, xanthine and uric acid (22). Saito *et al.* developed a formula (equation III) for fish freshness based on the ratio of ATP degradation products present in fish muscle during its storage (23). The *K* index gives a relative freshness estimation, where a high *K* value corresponds to low freshness level. Of the mentioned ATP degradation products, hypoxanthine is reported to give bitter off-flavor of spoiled fish (24), and IMP is thought to produce fresh fish flavor present in top quality seafood (22). Inosine has been reported to give bitter taste and was proposed to contribute bad taste to foods (25). A *K*-value rejection level is specific for each species of fish. For Atlantic salmon it has been set to 70-80 % (26).

$$K\ \% = \frac{[Ino] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [Ino] + [Hx]} \cdot 100 \qquad \text{(III)}$$

$$K_1\ \% = \frac{[Ino] + [Hx]}{[IMP] + [Ino] + [Hx]} \cdot 100 \qquad \text{(IV)}$$

$$H\ \% = \frac{[Hx]}{[IMP] + [Ino] + [Hx]} \cdot 100 \qquad \text{(V)}$$

Indices that do not require quantification of ATP, ADP and AMP have also been proposed. As ATP is quickly degraded to IMP after slaughter in most species of fish, other indices than *K*-value may be more informative for evaluating freshness of fish several days after slaughter. K_1 and *H* values (27, 28) can be calculated from equation IV and V, respectively. Good correlation between salmon quality and amount of fish spoilage indicators such as trimethylamine (TMA) and biogenic amines has been reported by use of *H*-value (29). Based on TMA concentration levels, an *H* value above 75 % has been suggested to correspond to grade II quality (30).

1.5.2.2 Formation of trimethylamine

Trimethylamine (TMA) is a volatile amine described to be associated with the unpleasant odor of spoiled seafood (22). TMA is produced from bacterial reduction of the compound trimethylamine oxide (TMAO), which is found naturally in the tissue of marine organisms (22, 31). Figure 1.7 shows an overview of TMAO metabolism and intermediate products. A suggested maximum rejection level for TMA concentration in fish is 29.5 mg/100 g (30). TMA concentrations in the range of 0-4.2 mg/100 g fish has been set for prime quality (grade I), while TMA concentrations in the range of 4.2-29.5 mg/100 g fish has been set for acceptable quality (grade II) (30).

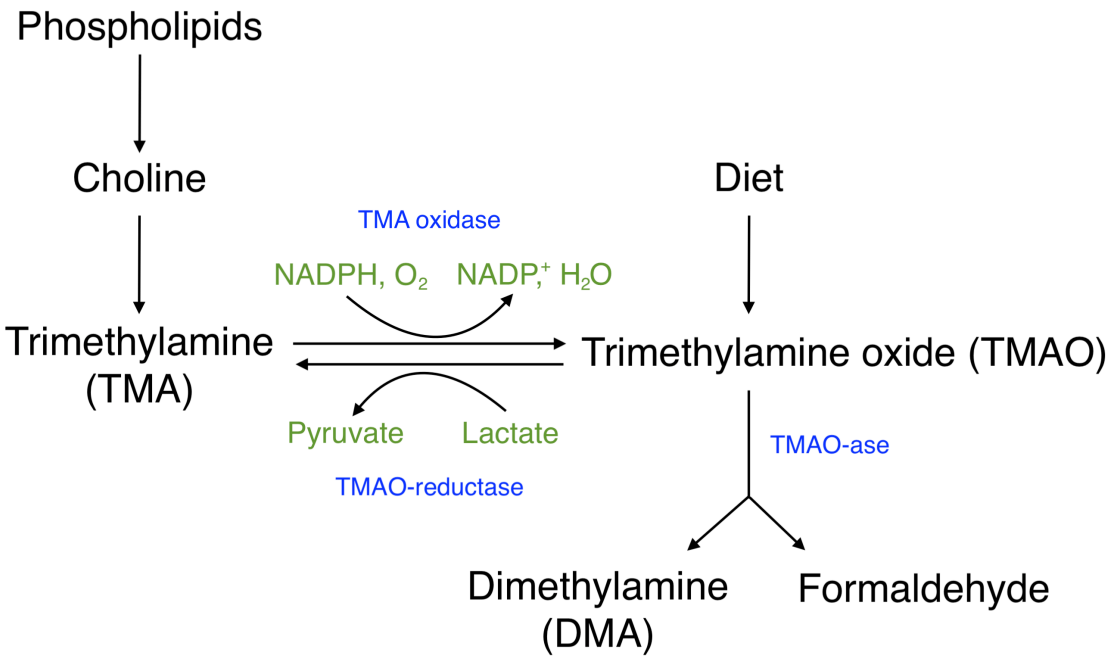

Figure 1.7. Metabolism of trimethylamine oxide (TMAO). Enzymes and metabolites involved in the metabolism are marked in blue and green, respectively. Figure modified from van Waarde (31).

1.5.2.3 Biogenic amines

Spoilage bacteria can produce biogenic amines by decarboxylation of amino acids (22). Cadaverine, histamine, tyramine and putrescine are biogenic amines that are produced by decarboxylation of their respective amino acid precursors: lysine, histidine, tyrosine and ornithine. Rejection levels for biogenic amines have been set for food products as they can be toxic for human consumption at too high concentrations, and quantification of biogenic amines can therefore be used for evaluating fish quality. Suggested rejection levels for biogenic amines in foods from literature are summarized in table 1.1.

Table 1.1. Summary of selected biogenic amines, their amino acid precursors and suggested rejection levels from literature for their concentration in foods (32-34).

Amino acid precursor	Biogenic amine	Rejection level of biogenic amine (mg/100 g)
lysine	cadaverine	51
histidine	histamine	10-20
ornithine	putrescine	17
tyrosine	tyramine	95

1.5.3 Sensory evaluation of salmon quality

Appearance of salmon muscle is a sensory property that is considered as an important quality parameter. Pink-red color of flesh is characteristic for salmonid fish species (35). Redder salmon flesh is perceived by consumers to be of higher quality, have better taste and flavor and be of higher quality (35). The color is a result of astaxanthin, which is a lipid-soluble carotenoid pigment (35, 36). The red color results from conjugated double bonds at the center of the astaxanthin molecule (figure 1.8), and these types of conjugated double bonds have antioxidant properties (37). Astaxanthin is described to be sensitive to oxidation, heat and light because of unsaturated molecule structure (38).

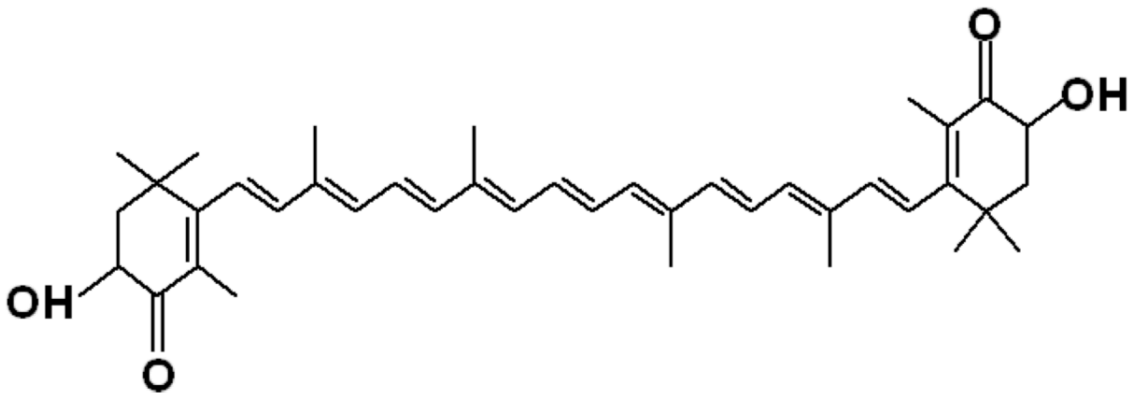

Figure 1.8. Chemical structure of astaxanthin. Figure by Ambati *et al.* (37)

1.5.4 Processing and quality of fish

Different processing methods of fish after harvest has been shown to yield fish products of different quality. Sokolová described in her thesis processing methods of fish that can yield fish products of high and normal quality (39). Traditional processing of fish is normally done post-rigor, and resolution of rigor-mortis state in fish after slaughter is described to normally occur 3-5 days after slaughter. Filleting of fish post-rigor therefore produces standard quality fish or fish products. Processing methods of fish after slaughter that yield fish products of premium quality are described to be performed pre-rigor. Rigor mortis state normally occurs hours after slaughter, and it is reported to occur in Atlantic salmon approximately 8 hours after slaughter (40). This means pre-rigor processing must be performed within 8 hours to produce premium quality fish products of Atlantic salmon. A study by Rosnes *et al.* showed increased quality for pre-rigor Atlantic salmon fillets based on microbial counts, odor and gaping evaluations (41).

1.6 Nutritional value and flavor of food products

Organisms such as humans, animals and plants have a biological requirement for amino acids. They are required for synthesis of proteins and other compounds containing nitrogen that are essential for normal growth and function (42). Amino acids that mammals do not synthesize themselves, which must be obtained through diet, are described as *essential amino acids*. Table 1.2 lists 9 reported essential amino acids for mammals. Foods containing essential amino acids (within recommended dietary concentrations) will have increased nutritional value.

Table 1.2. Summary of 9 essential amino acids for mammals (42).

Amino acid	Symbol
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
Threonine	Thr
Tryptophan	Trp
Valine	Val

Amino acids are also known to influence taste properties of food products. Pure L- and D-forms of amino acids were shown by Solms to exhibit different taste properties (43). Taste properties of selected amino acids are summarized in table 1.3. L-Glutamic acid (glutamate in anion form) has been reported to have flavor-enhancing properties in mixture with other compounds (43). L-glutamate salts, together with 5'-ribonucleotides, are responsible for the *umami* taste (44). Umami flavor, which can be described as savory, is present in food systems such as stocks and bouillon. Succinic acid is a compound that has been described as

an umami related substance (45). Glycerol is a sugar alcohol described to have slightly sweet taste (46). It was further described by Solms that the resulting flavor of a complex food product is the sum of all taste interactions, which is a result that often exceeds the taste properties of pure compounds by themselves (43).

Table 1.3. Taste properties of amino acids. Amino acids exhibiting bitter and sweet taste were compared qualitatively with caffeine and sucrose solutions, respectively. Table modified from Solms (43).

	Taste	
Amino acid	L-form	D-form
Alanine	sweet, 0.54 % sucrose	flat
Aspartic acid (aspartate)	flat	flat
Isoleucine	flat	flat
Leucine	bitter, 0.011 % caffeine	sweet, 1.30 % sucrose
Glutamic acid (glutamate)	unique	flat
Glycine	sweet, 0.45 % sucrose	
Methionine	sulfurous, meaty	sulfurous, meaty
Phenylalanine	bitter, 0.069 % caffeine	sweet, 2.20 % sucrose
Tyrosine	bitter, 0.017 % caffeine	sweet, 1.65 % sucrose

1.7 Metabolomic approach for analyzing food systems

Metabolomics is defined by Tomassini *et al*. as the total amount of metabolites in a biological system (47). The term *metabolite* can be understood as a small organic molecule with a molecular weight less than 1800-1500 Dalton (48), which is an intermediate or end product of metabolism (49). Analyzing food systems based on their metabolome gives information about its biochemical composition, which can be used to determine what metabolites are responsible for properties such as taste, color, aroma and nutritional value of food systems (47). It is stated by Kosmides *et al.* that the metabolome of an organism at a given time will represent the state of the organism at that specific time, and that it can be viewed as a *metabolic fingerprint* (50).

1.7.1 High-resolution nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is an analytical tool that can be used to analyze food metabolomes. The basics of NMR experiments are explained by Friebolin to involve irradiating nuclei, such as protons (^{1}H) and carbon-13 (^{13}C), in a sample with a superimposed field B_0 to induce transitions between different energy levels (51). Transitions occur when the resonance condition (equation VI) is met, meaning that the frequency of the electromagnetic radiation v_1 used to irradiate a nuclide is equal to the Larmor frequency v_L (51). The magnetogyric ratio y represents the detection sensitivity of a nuclide in a NMR experiment.

$$v_L = v_1 = \left|\frac{y}{2\pi}\right| B_0 \qquad \text{(VI)}$$

An NMR spectrum can be produced from the detectable signals resulting from excited nuclei in a sample run for an NMR experiment. Bothwell and Griffin describes that excited nuclei will over time relax to their lower energy ground state, producing emission of photons (electromagnetic radiation) (52). Free induction decay (FID) is described to be produced by summarizing the photons from a population of relaxing nuclei, and it will represent the decay of the intensity of emitted electromagnetic radiation over time (52). Fourier transformation is performed on the FID to convert the spectrum from time to frequency domain (52). This is observed as a "peak" in the spectrum (figure 1.9). Other processing of acquired NMR spectra includes phase and baseline correction.

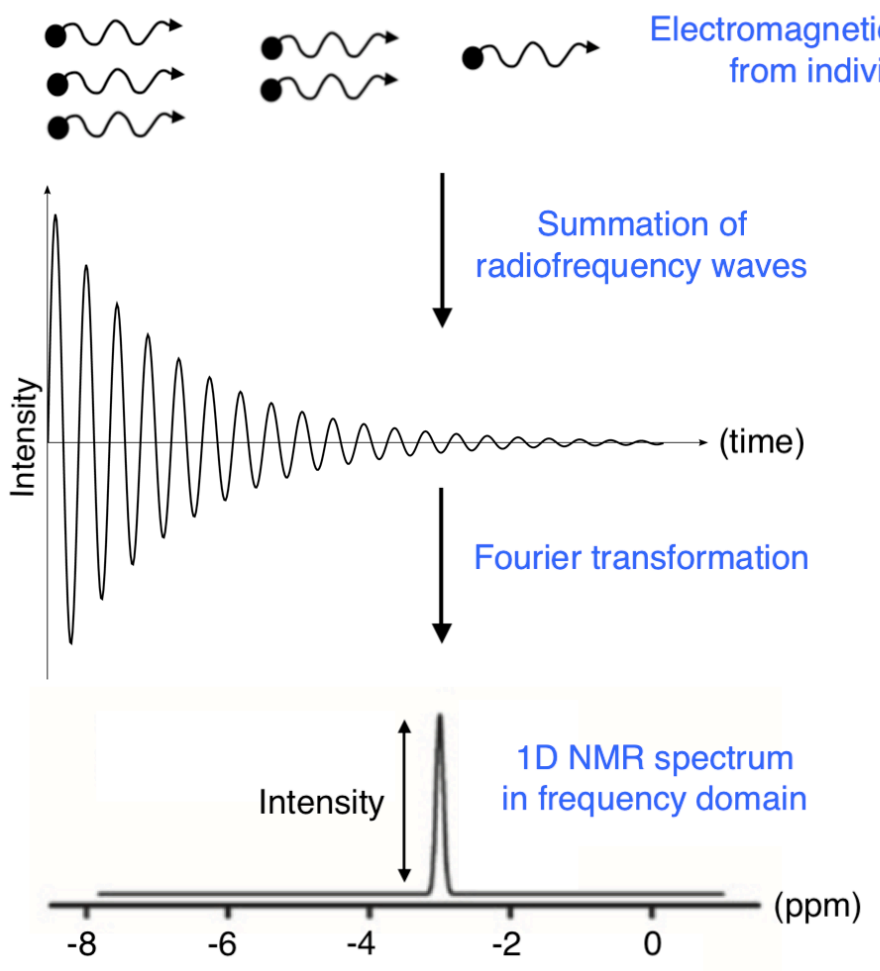

Figure 1.9. Simplified overview showing how emission of electromagnetic radiation originating from excited nuclei can produce detectable free induction decay (FID). Fourier transformation is used to convert the time domain in FID to the frequency domain which is used in NMR spectra. Figure modified from Bothwell and Griffin (52).

The energy of the emitted electromagnetic radiation during NMR spectroscopy is described to be expressed as the *chemical shift* (δ) (52). This is an exact value that can be connected to the identity of a compound. A relative scale is used in in NMR spectra, where the frequency difference (Δv) between the resonance signals of a sample and a reference compound (internal standard) is determined and used to calculate the chemical shift (equation VII) (51). Reference compounds used as internal standards in NMR spectroscopy includes trimethylsilylpropanoic acid (TSP) for hydrophilic solvents and tetramethylsilane (TMS) for hydrophobic (organic) solvents. The δ-values are in general a very small number; they are given in parts per million (ppm).

$$\delta_{sample} = \frac{\Delta v\ [Hz]}{v_{reference}\ [MHz]} \qquad \text{(VII)}$$

To ensure separation of chemical shifts in NMR spectra, as interpreting distinct resonance signals are crucial for compound determination, *shimming* is a way to optimize separation. Good signal separation is described to depend on a homogenous magnetic field B_0 and a homogenous chemical environment in the sample (52). When the sample is placed in the magnetic field of the magnet during NMR spectroscopy, it disturbs the field. Re-homogenization of the magnet field, known as shimming, is therefore necessary to perform before spectral acquisition. This ensures spectra with good resonance signal separation.

The acquired spectrum from 1D ^{1}H NMR spectroscopy will contain nuclei resonance signals, which are positioned in the spectrum at different chemical shifts based on the chemical environment and the chemical groups the nuclide is bound to. As food systems most often contain a mixture of compounds, the resulting NMR spectrum of a food system will constitute of many resonance signals originating from magnetically equivalent nuclei from different compounds. Due to a phenomenon known as *spin-spin coupling*, splitting patterns of resonance signals can be observed as doublet, triplet and multiplet signals (52). This is a result of nuclei behaving as tiny magnets and influencing each other's energy within a molecule (52). As most compounds consist of several chemical groups, containing for instance hydrogen, a single compound within a food system can also give rise to several resonance signals within the same spectrum. Each distinct compound will therefore produce a distinct pattern of resonance signals in an NMR spectrum, which can be used for determining metabolites (47). An NMR spectrum of a food system will therefore most often give rise to multiple patterns of resonance signals originating from different metabolites, and these patterns and signals can be used to determine the identity of a metabolite. This can be done by comparing the obtained spectral data to reference data, which are available in online databases such as *The Human Metabolome Database* (53) and *Biological Magnetic Resonance Data Bank* (54).

1.7.2 Quantification of metabolites

As described by Tomassini *et al.*, the intensity or area of resonance signals in NMR spectra are proportional to the concentration of the chemical group they originate from, which ultimately means the intensity of a resonance signal is proportional to the concentration of the compound it originates from (47). Determined metabolites from NMR spectra can therefore also be quantified, by use of NMR processing software such as *TopSpin* by Bruker. Shumilina *et al.* described how the concentration of a metabolite in an NMR sample tube C_M can be calculated (29), which is shown in equation VIII. It involves comparing the integral (area) of a resonance signal originating from the metabolite (P_M) to the integral of the reference substance TSP (P_R). This value is multiplied by the ratio of the proton number in TSP and metabolite (n_R/n_M) and the known concentration of TSP (C_R).

$$C_M = (P_M/P_R) \cdot (n_R/n_M) \cdot (C_R) \quad \text{(VIII)}$$

Spectrum accumulation is necessary as the intensity of an individual FID produces small signals compared to random electronic noise. Summation of FIDs from several rounds of nuclei excitation averages out contribution from noise and builds up the contribution of the signal (51). Increasing the spectral acquisition parameter *number of scans* (NS) for an NMR experiment will therefore increase the signal-to-noise (S/N) ratio, which is proportional to the square root of the number of scans (NS) (51):

$$S/N \sim \sqrt{NS} \quad \text{(IX)}$$

Signal-to-noise ratios of resonance signals affect the reliability of signals in an NMR spectrum to be used for determination and quantification of metabolites. It was assumed by Maniara *et al.* that only signals with an S/N ratio greater than 3 can be considered as detectable (55). For using resonance signals to quantify metabolites, the S/N ratio ought to be greater than 10 for adequate reliability (55). Receiver gain (RG) is another acquisition parameter in NMR spectroscopy that is chosen to maximize the SN ratio (56). In regard to sample requirement for NMR spectroscopy, it was proposed that minimum 1 mg of sample (concentration 5 mM) is sufficient for acquisition of ^{13}C spectra, and minimum 15 µg of sample (concentration 0.05 mM) is often enough for acquisition of ^{1}H spectra (57).

1.7.3 Targeted and untargeted analysis of NMR spectra

NMR spectra of food samples can be acquired by using NMR active nuclei such as ^{1}H and ^{13}C. It will give information about all of the metabolites that were present in the food sample at the time of sampling, based on the nucleus selected to produce the NMR spectrum. As described by Tomassini *et al.*, the task of transforming NMR spectra into a data matrix that can be used for quantification of metabolites or metabolic profiling can follow different

strategies (47). The two described strategies can be labelled as *targeted* and *untargeted* (the latter is referred to as *pattern recognition* by Tomassini *et al.*). Targeted analysis is described as determining resonance signals in spectra that originate from metabolites of interest, measuring their peak intensities and making a data set containing only non-redundant information. In an untargeted analysis, all the information from the spectra are included in the data set, making it redundant. Analysis of variance (ANOVA) and multivariate data analysis can be performed to reduce the number of insignificant variables and dimensions of large data sets so that metabolic relationships between metabolites can be discovered (47).

1.7.4 Statistics

Processing redundant data produced from acquired NMR spectra is necessary in order to obtain meaningful data on metabolomics. Most often the metabolites of interest are unknown prior to an experiment. Methods of statistical analysis can be applied to determine metabolites that are significant for the metabolic composition of a food system. As mentioned in section 1.8.3, ANOVA and multivariate data analysis can be performed. This compares the mean differences between the groups and analyze if there is an interaction between the independent variables on the dependent variable (58). Statistically significant interactions or statistically significant independent variables can be estimated from computed *p*-values and *F*-values from the ANOVA. For a chosen significance level of 5 %, a result with a *p*-value less than 0.05 can be considered statistically significant. The *p*-value describes the probability of obtaining a result at least as extreme as the one being observed, given that the result is insignificant – the null hypothesis (59). The null hypothesis can be rejected when the *p*-value of a result is less than 0.05, and the result can be considered significant. Results from an ANOVA of a dataset with *p*-values greater than 0.05 can therefore be considered statistically insignificant and potentially redundant information. *F*-values describe the variance between groups compared to the variance within the groups (60). Null-hypothesis are rejected at larger *F*-values, and it can be used to further distinguish the statistical significance of results with *p*-values close to 0 (60).

A commonly used method for multivariate data analysis is *principal component analysis* (PCA). This is an algorithm that reduces data with many dimensions to a small number of dimensions, which explains as much of the variation in a data set as possible (50). Principal components (PCs) are produced, where PC-1 and PC-2 will contain the biggest portion of variation from the data set. The PCs can be plotted in a map to give a visual representation of the results of the PCA analysis. The data points produced in the PCA map will often cluster; each cluster of data points can be understood as representing a specific metabolic fingerprint. Kosmides *et al.* stated that metabolic fingerprints can be used to find biomarkers, which are explained to be metabolites that significantly differ between classes (50). For understanding how addition of seaweed affects the salmon metabolome during storage, or how different treatments and storage conditions affect seaweed during storage, PCA analysis of obtained spectral data can be used to find biomarkers, which in turn will reveal what metabolites are influenced.

2 Materials and methods

All mentions of sugar kelp and seaweed during the thesis refers to *Saccharina latissima*.

2.1 Storage experiment of sugar kelp

Storage experiment and washing/extraction of sugar kelp were carried out by SINTEF research institute as described in subsections 2.1.1 , 2.1.2 and 2.1.4.

2.1.1 Sample harvest

Sugar kelp was harvested from a cultivation site at Frøya, Norway on the 13th of June 2018. The sugar kelp was harvested at 16.00, allocated to plastic boxes with lids and transported to the lab within 3 hours of harvest.

2.1.2 Experimental design

The metabolite content of sugar kelp as a function of storage time, after being exposed to different treatments and storage conditions, was studied by analyzing algal water extracts by 1D ^{1}H NMR spectroscopy. Treatments included cut and whole algal tissue (figure 2.1). Cut seaweed was prepared by cutting it with scissors to approximately 1-2 x 1-2 cm pieces. Storage conditions included cold room (4 °C) and room temperature. Samples for cold storage were put into the cold room at 21.35. Algal water extracts originating from cold room stored samples were sampled at time points 2, 5 and 12 days of storage (C-4°C and W-4°C). Algal water extracts originating from room temperature stored samples were sampled at time points 2, 5 and 8 days of storage (C-RT and W-RT). Figure 2.2 gives an overview of the experimental design.

Figure 2.1. Photos of whole (left) and cut (right) sugar kelp in plastic boxes during storage at room temperature. Photos by SINTEF.

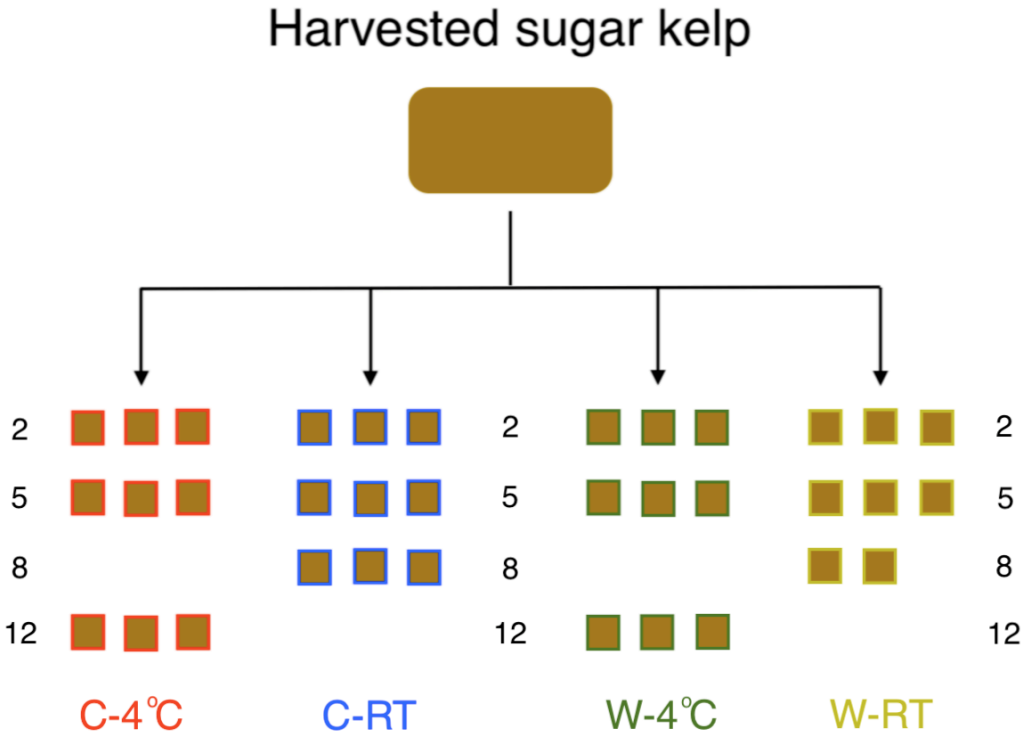

Figure 2.2. Overview of treatment and storage conditions for 3 parallels of harvested sugar kelp. Treated sugar kelp was stored from 2-12 days before sampling and water extraction. Abbreviations: C-4°C = cut sugar kelp stored at 4°C; C-RT = cut sugar kelp stored at room temperature; W-4°C = whole sugar kelp stored at 4°C; W-RT = whole sugar kelp stored at room temperature.

2.1.3 Chemicals

Deuterium oxide (D_2O, 99.9 %) from Cambridge Isotope Laboratories Inc. (Andover, MA, USA); 3-(Trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP, 98 atom % D) from Armar Chemicals (Dottingen, Switzerland); Trichloroacetic acid (TCA) from Sigma-Aldrich (St. Louis, MO, USA).

2.1.4 Extraction procedure

Polar metabolites were extracted from the sugar kelp samples by weighing out 5 g of sample and adding 10 mL of distilled water. The samples were shaken for some seconds, followed by centrifugation at 6200 g for 10 min. The supernatants were collected and frozen at -20 °C in 50 mL centrifuge tubes.

2.1.5 Sample preparation for NMR spectroscopy

Sugar kelp extracts from 50 mL-centrifuge were thawed, and pH of the extracts were measured with an Orion Star A111 pH meter from Thermo Scientific. Samples of parallels 2 and 3 with pH values higher than 8 were adjusted to pH 7 by use of TCA (7.5 %). 1 mL of sample was transferred to a 1,5 mL centrifuge tubes. The samples were centrifugated at 20 000 g for 5 minutes at 20 °C. 540 µL of supernatant was transferred to new 1,5 mL-Eppendorf tubes and 60 µL of TSP (1 mM TSP in 20 mM Sodium Phosphate buffer pH 7 in D_2O) was added. The mixture was vortexed for some seconds. 530 µL of the mixed solution was transferred to standard 5 mm-NMR tubes.

2.1.6 NMR data acquisition

1D ^1H NMR spectra of algal water extracts were acquired at 300 K with a Bruker Avance 600-MHz spectrometer equipped with 5-mm z-gradient TXI (H/C/N) cryoprobe at the NMR center of the Faculty of Natural Sciences and Technology at the Norwegian University of Science and Technology (NTNU) in Trondheim. The NMR spectra were acquired with the Bruker pulse sequences *noesygppr1d* and the following acquisition parameters: NS = 48; SW = 20 ppm; RG = 50.8; O1 = 2820 Hz; D1 = 4 seconds.

2.2 Lactic acid bacteria fermentation of sugar kelp

2.2.1 Pretreatment of sugar kelp from Seaweed Energy Solutions

Vacuum packed and frozen sugar kelp was obtained from Seaweed Energy Solutions AS (SES). The packaging date was set to the 27th of April 2017, and the sugar kelp was stored at -40 °C.

The seaweed seedlings were produced in SES' hatchery in Trondheim and further brought to SES' sea farm at Frøya for cultivation and harvesting. Seedlings were deployed in January-February and harvested during the time period of April-May to reduce biofouling and achieve good food grade quality. The macroalgae was stored in flow-through seawater between harvesting and packaging, where the buffering period was normally less than 3 days. The sugar kelp was rinsed in sterilized seawater before packaging and further frozen at -20 °C on the same day as packaging.

2.2.2 Sample preparation

Lactic acid bacteria (LAB) strains used in the experiment were from the LAB collection of Kaunas University of Technology, Lithuania. The LAB fermentation was carried out at the mentioned university as described below.

Lactic acid bacteria (LAB) strains *Lactobacillus paracasei* LUHS244, *Pediococcus acidilactici* KTU05-7 (Pa7) and *Pediococcus pentosaceus* KTU05-9 (Pp9) were grown in MRS broth (de Man, Rogosa and Sharpe) at 35 °C for 48 hours before they were used for fermentation. 3 x 10 g samples of sugar kelp (pretreatment described in section 2.2.1) were mixed with 2 mL of respective pure LAB suspensions (containing app. 10^9 CFU/mL) and incubated at 35 °C for 48 hours. After fermentation, the fermented samples were stored at -18 °C.

2.2.3 Experimental design

Fermentation of *Saccharina latissima* by LAB strains *Lactobacillus paracasei* LUHS244, *Pediococcus acidilactici* KTU05-7 and *Pediococcus pentosaceus* KTU05-9 was studied by analyzing the polar metabolite content of the fermented sugar kelp, and a non-fermented sample for reference, by NMR spectroscopy. The resulting LAB fermented sugar kelp was evaluated as a potential food product based on nutritional value and taste properties of its polar metabolite composition.

2.2.4 Chemicals

Liquid nitrogen; Deuterium oxide (D_2O, 99.9 %) from Cambridge Isotope Laboratories Inc. (Andover, MA, USA); 3-(Trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP, 98 atom % D) from Armar Chemicals (Dottingen, Switzerland). Sodium hydroxide from Sigma-Aldrich was used to adjust pH in samples.

2.2.5 Extraction procedure

Frozen, fermented sugar kelp samples were freeze dried with liquid nitrogen and roughly grinded to small chunks with a kitchen mortar. 4 samples of 2.8 g frozen sugar kelp (3 different fermented samples and 1 reference) were quickly weighed out in weighing boats, chopped vigorously with a carbon steel surgical blade and transferred to 50 mL centrifuge tubes. 15 mL of deionized water was added to the samples, followed by nutation of the samples for 3 hours using a VWR® Nutation Mixer. The samples were centrifugated for 10 min at 20 °C and 6800 g. The resulting supernatants were decanted into new 50 mL centrifuge tubes by using glass funnels with filter paper (grade 589[1] black ribbon, ashless). The pH of the water extracts from the seaweed samples was measured by using a Sentron SI series pH meter (SI 400 7400-010), followed by adjustment of the samples' pH to 7 by use of sodium hydroxide (9 M).

2.2.6 Sample preparation for NMR spectroscopy

Same procedure as described in section 2.1.5.

2.2.7 NMR data acquisition

Same procedure as described in section 2.1.6.

2.3 Storage experiment of premium quality Atlantic salmon

2.3.1 Sample preparation

3 packages of SALMA® belly loin fillets (*Salmo salar*, sashimi-grade quality) with slaughter date the 18th of October 2018 was purchased at a local grocery store on the 23rd of October 2018. Day 5 after slaughter was the reference point for the salmon fillets initial condition before the experiment was conducted.

3 pieces of salmon á ~ 5 g was cut out from each respective salmon filet and processed immediately to be used as reference point for the salmon filets initial metabolite content. 14 pieces á ~ 5 g was cut from one salmon filet. 7 salmon pieces were packed in zip lock

plastic bags and stored at 4 °C (reference pieces), while the other 7 salmon pieces were covered in sugar kelp from SES (pretreatment described in section 2.2.1 placed on a weighing boat and packed in zip lock plastic bags and stored at 4 °C. The samples were marked so that 1 pair of salmon pieces (reference and covered in sugar kelp) was assigned one out of seven different storage durations. 3 parallels of samples were made by repeating the procedure for the 2 remaining salmon filets, resulting in a total of 42 pieces of salmon for storage (figure 2.3).

Figure 2.3. Overview of premium quality salmon (PQS) and sugar kelp samples prepared for storage. After storage, polar metabolites were extracted by trichloroacetic acid (TCA) and analyzed with 1D ^{1}H NMR spectroscopy.

2.3.2 Experimental design

The effect of sugar kelp on the metabolite content of SALMA® (*Salmo salar*) was studied by analyzing sugar kelp-treated and reference salmon muscle samples stored at 4 °C from day 5-21 after slaughter as a function of storage time. Samples were processed for metabolite extraction at day 5, 7, 9, 11, 14, 17, 19 and 21 after slaughter (figure 2.4). NMR spectroscopy was performed for determination and quantification of polar metabolites.

Figure 2.4. A flowchart giving a representation of the experimental design of the premium quality salmon (PQS) storage experiment. Pink squares represent reference samples. Green squares represent seaweed-covered salmon samples. The metabolic composition of all samples was analyzed by NMR spectroscopy after different storage durations (from day 5-21 after slaughter) by TCA extraction of polar metabolites.

2.3.3 Chemicals

Deuterium oxide (D_2O, 99.9 %) from Cambridge Isotope Laboratories Inc. (Andover, MA, USA); Trichloroacetic acid (TCA) from Sigma-Aldrich (St. Louis, MO, USA); 3-(Trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP, 98 atom % D) from Armar Chemicals (Dottingen, Switzerland); Sodium hydroxide from Sigma-Aldrich was used to adjust pH in samples.

2.3.4 Trichloroacetic acid extraction procedure

After removing sugar kelp from the covered salmon pieces and collecting remaining algal tissue and liquid in the weighing boat and zip lock plastic bag, each pair of salmon pieces from the 3 parallels were individually cut into small pieces by using a carbon steel surgical blade. The cut salmon pieces were transferred to 50 mL centrifuge tubes. 18 cooled iron balls were added to each centrifuge tube, and the samples were homogenized at frequency 30 s^{-1} for 5 min using a Retsch Mixer Mill MM 400. For samples stored until day 14-21 days after slaughter the amount of balls for sugar kelp-treated samples was modified to 18 small and 6 medium sized balls to improve homogenization. 30 mL of cooled TCA (7.5 %) was added to the samples for extraction of polar metabolites. The samples were homogenized further at frequency 20 s^{-1} for 4 min. The samples were centrifugated for 10 min at 8 °C and 6800 g. The resulting supernatant of the samples was decanted and filtrated into a new 50 mL centrifuge tube by using filter paper (grade 589[1] black ribbon, ashless) and glass funnels.

The filtered-out extracts were adjusted to pH 7 by using sodium hydroxide (9 M). The volume of the samples after pH-adjustment was noted.

2.3.5 Sample preparation for NMR spectroscopy

540 µL of sample extract was allocated to 1,5 mL Eppendorf tubes and mixed with 60 µL of TSP (1 mM TSP in 20 mM Sodium Phosphate buffer pH 7 in D_2O) by vortex. The sample was centrifugated for 5 min at 20 °C and 20 000 g. 530 µL of resulting supernatant was transferred to a standard 5 mm-NMR tube.

2.3.6 NMR data acquisition

1D ^{1}H NMR spectra of TCA extracted PQS, sugar kelp tissue and liquid were acquired at 300 K with a Bruker Avance 600-MHz spectrometer equipped with 5-mm z-gradient TXI (H/C/N) cryoprobe at the NMR center of the Faculty of Natural Sciences and Technology at the Norwegian University of Science and Technology (NTNU) in Trondheim. The NMR spectra of TCA extracted PQS were acquired with the Bruker pulse sequences *noesygppr1d* and the following acquisition parameters: NS = 48; SW = 20 ppm; RG = 144; O1 = 2820 Hz; D1 = 4 seconds.

2.4 Storage experiment of standard quality Atlantic salmon

2.4.1 Sample preparation

1.9 kg of salmon filet (*Salmo salar*) was purchased from Ravnkloa, Trondheim on the 18th of March 2019. Slaughter date was 11th of March 2019, and the salmon was described to be farmed in Norway and captured in the northeastern Atlantic Ocean. Day 7 after slaughter was the reference point for the salmon fillet's initial metabolic content before the experiment was conducted.

The upper middle part of the salmon fillet (back loin) was cut out and used to make samples for TCA and acetone extraction. 20 pieces of salmon muscle were cut out from the fillet in total. 2 pieces á ~ 5 g was processed immediately to be used as reference point for the salmon fillets initial metabolite content (TCA and acetone extraction). Of the remaining 18 pieces á ~ 5 g that were cut out, 9 of these pieces were covered in sugar kelp, as described earlier. For the TCA extraction, 6 pairs of reference and seaweed-covered samples were prepared for storage. For the acetone extraction, 3 pairs of reference and seaweed-covered samples were prepared for storage. Figure 2.5 shows an overview of the prepared samples for the storage experiment.

Figure 2.5. Overview photo of standard quality salmon (SQS) and sugar kelp samples prepared for storage. After storage, polar and non-polar metabolites were extracted by trichloroacetic acid and acetone, respectively, and analyzed by 1D ^{1}H NMR spectroscopy.

2.4.2 Experimental design

The effect of addition of sugar kelp on upon storage of standard quality Atlantic salmon (*Salmo salar)* was studied by analyzing the samples stored at 4 °C as a function of storage time. The samples were processed for polar metabolite extraction at day 7, 9, 11, 14, 17, 19 and 21 after slaughter, and non-polar metabolite extraction at day 7, 11, 17 and 21 after slaughter (figure 2.6). The samples were stored at 4 °C from day 7-21 after slaughter. NMR spectroscopy was used for determination and quantification of metabolites.

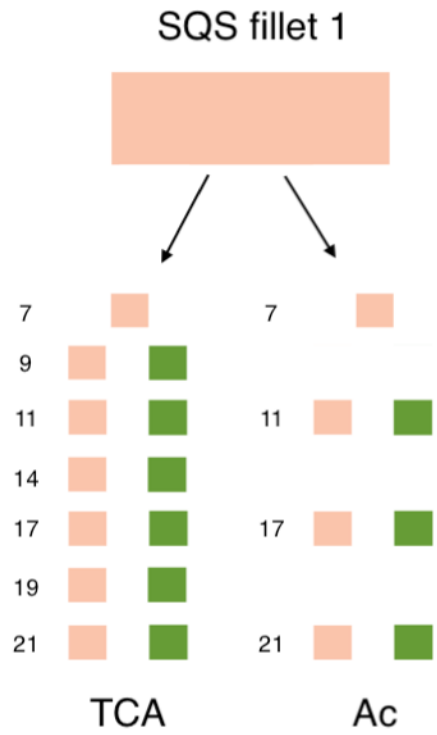

Figure 2.6. A flowchart giving a representation of the experimental design of the standard quality salmon (SQS) storage experiment. Pink squares represent reference samples. Green squares represent seaweed-covered salmon samples. The metabolic composition of selected samples was analyzed by NMR spectroscopy at different storage time (from day 7-21 after slaughter) by trichloroacetic acid (TCA) and acetone extraction of polar and non-polar metabolites, respectively.

2.4.3 Chemicals

Liquid nitrogen; Deuterium oxide (D_2O, 99.9 %) from Cambridge Isotope Laboratories Inc. (Andover, MA, USA); Trichloroacetic acid (TCA) from Sigma-Aldrich (St. Louis, MO, USA); 3-(Trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP, 98 atom % D) from Armar Chemicals (Dottingen, Switzerland); Acetone from VWR International S.A.S (Fontenay-sous-Bois, France); Acetone-d6 (99+ atom % D) from Acros Organics (New Jersey, USA); Sodium hydroxide from Sigma-Aldrich was used to adjust pH in samples.

2.4.4 Trichloroacetic acid extraction procedure

Same procedure as described in section 2.3.4 . For samples stored until day 17-21 after slaughter, the amount of balls was modified to 18 small and 6 medium sized balls to improve homogenization for both reference and sugar kelp-treated samples.

2.4.5 Acetone extraction procedure

After removing sugar kelp from the covered salmon pieces and collecting remaining algal tissue and liquid in the weighing boat and zip lock plastic bag, each pair of salmon pieces from the 3 parallels were individually cut into small pieces by using a carbon steel surgical blade. The cut salmon pieces were transferred to 50 mL centrifuge tubes. 18 cooled iron balls were added to each centrifuge tube, and the samples were homogenized at frequency 30 s^{-1} for 5 min using a Retsch Mixer Mill MM 400. 15 mL of cooled acetone was added to the samples for extraction of metabolites. The samples were homogenized further at frequency 20 s^{-1} for 4 min. The samples were centrifugated for 10 min at 8 °C and 6800 g. The resulting volume of supernatant of one sample was decanted to two 15 mL centrifuge tubes, dividing the volume approximately equal. The 15 mL centrifuge tubes containing supernatant were subjected to 2 hours of centrifugation at 35 °C and 0.2 vacuum pressure to remove the acetone solvent, and the apparatus used was a SpeedVac Vacuum Concentrator from Thermo Fischer Scientific. The remaining contents in the 15 mL centrifuge tubes were frozen with liquid nitrogen followed by 24 hours of freeze drying at optimal conditions -61 °C and 0.028 mbar pressure. This was done using an Alpha 1-4 LO plus laboratory freeze-dryer from CHRIST.

2.4.6 Sample preparation for NMR spectroscopy

2.4.6.1 Trichloroacetic acid extracts of salmon muscle tissue

Same procedure as described in section 2.3.5.

2.4.6.2 Acetone extracts of salmon muscle tissue

300 µL of acetone-d6 was added to one of the two 15 mL centrifuge tubes (tube 1) containing freeze-dried content. The content was mechanically mixed with the acetone-d6 using a glass stirrer. The liquid content was decanted from tube 1 to tube 2. Another 300 µL of acetone-d6 was added to tube 1. The content of tube 1 and 2 was mechanically mixed using a glass stirrer. The content of tube 1 and 2 were mixed for some seconds using a vortex mixer and further centrifugated for 3 min at 18 °C and 4800 rpm. The resulting supernatant of tube 2 was transferred to a 1,5 mL Eppendorf tube, while the resulting supernatant of tube 1 was transferred to tube 2. The content of tube 2 was mixed for some seconds using a vortex mixer, followed by another round of centrifugation for 3 min at 18 °C and 4800 rpm. The resulting supernatant of tube 2 was transferred to the same 1,5 mL Eppendorf tube mentioned earlier, combining the volumes of acetone-d6 that initially was divided between tubes 1 and 2. The content of the 1,5 mL Eppendorf tube was centrifugated for 5 min at 20 °C and 20 000 g. The resulting supernatant was transferred to a standard 3 mm NMR tube.

2.4.7 NMR data acquisition

1D ^{1}H NMR spectra of the TCA and acetone extracted SQS and sugar kelp tissue were obtained by using a Bruker Avance 600 MHz spectrometer equipped with a 5 mm z-gradient TXI (H/C/N) cryoprobe at the NMR center of the Faculty of Natural Sciences and Technology at the Norwegian University of Science and Technology (NTNU) in Trondheim. The NMR spectra originating from SQS TCA extraction were acquired with the Bruker pulse sequences *noesygppr1d* and the following acquisition parameters: NS = 48; SW = 20 ppm; RG = 144; O1 = 2820 Hz; D1 = 4 seconds. The NMR spectra originating from SQS acetone extraction were acquired with the Bruker pulse sequences *zg30* and the following acquisition parameters: NS = 512; SW = 20 ppm; RG = 2.8; O1 = 2820 Hz; D1 = 4 seconds.

2.5 Sugar kelp tissue and liquid from storage of Atlantic salmon

1D ^{1}H NMR spectra of sugar kelp tissue and liquid from the PQS and SQS storage experiments, and sugar kelp liquid from the original package, were acquired. NMR spectra of sugar kelp tissue were acquired by the same procedures as described for reference sugar kelp in sections 2.2.5 NMR spectra of sugar kelp liquid were acquired by the same procedure as described in sections 2.1.5 Collected sugar kelp liquid with volume below 540 µL was diluted to 540 µL with de-ionized water.

2.6 Processing and analysis of NMR spectra

NMR spectra were processed with *TopSpin* 3.6.1 (Bruker, Germany). Metabolite assignment was performed by using own laboratory database and by comparing NMR resonance signals and data observed in the spectra to published data of metabolite reference standards (*Biological Magnetic Resonance Data Bank* and *Human Metabolome Database*). Quantification of metabolites was performed by using equation VIII described in section 1.7.2 ^{1}H NMR spectra of polar and non-polar extracts were calibrated to resonances of TSP and TMS, respectively at 0 ppm. All spectra of non-polar metabolites (acetone extraction) were phase and baseline corrected.

2.6.1 Analysis of variance and principal component analysis

Principal component analysis (PCA) was performed to do an untargeted analysis of the metabolic composition of PQS, SQS and sugar kelp during storage. All spectra originating from TCA salmon muscle extraction were normalized to average mass of extracted tissue and volume of extractions. The acquired salmon and sugar kelp spectra were divided into smaller regions called buckets, so that regions ideally containing only 1 resonance signal from different spectra could be compared and significant changes between spectra could be identified within specific regions. *Amix-Viewer* (Bruker, version 4.0) was used to manually create variable sized buckets in the region of 0 – 10 ppm of the salmon and sugar kelp spectra, producing a pattern. By subjecting this pattern to all acquired spectra, a bucket

table containing integral values of all regions from all spectra was produced. Known resonance signal regions of the compound anserine was excluded from the pattern. *SPSS Statistics* (IBM, version 25) was used to make a reduced bucket table, in order to obtain a robust and stable PCA model. This was done by performing ANOVA, followed by removing buckets that were insignificant ($p > 0.05$). The following initial and reduced number of buckets within the tables were produced: storage of PQS, contained 96 and reduced to 45 buckets; storage of SQS, contained 131 and reduced to 22 buckets; storage of sugar kelp, contained 106 and reduced to 71 buckets. The bucket tables were imported to *The Unscrambler X* (CAMO Software AS, version 10.5) where the PCA was performed.

3 Results and discussion

3.1 Assignment of metabolite resonances in 1D ^{1}H NMR spectra

In order to detect and quantify metabolic changes occurring in sugar kelp and salmon muscle during different processing and storage, a resonance assignment of 1D ^{1}H NMR spectra of the described experiments (see section 2) was carried out. NMR assignment was performed by interpreting acquired spectra, using own metabolite database of our laboratory and published data for reference standards from *Biological Magnetic Resonance Data Bank* and *Human Metabolome Database*. Assigned metabolites are summarized in table 3.1, and they are representative for the resonance signals used for quantification in all results presented in the following sections.

Table 3.1. Assignment of metabolite resonance signals in 1D ^{1}H NMR spectra. The resonance signal(s) in the second column were used for metabolite quantification. Multiplicity: d = doublet; dd = doublet of doublets; t = triplet; q = quadruplet; quin = quintet; m = multiplet.

Compound	Chemical shift of resonance signal (ppm)	Signal multiplicity	Assignment	Other resonance signals (ppm)
β-Alanine	2.56	t	CH_2	3.18
2,3-butanediol	1.15	d	two CH_3	3.62/3.72
Acetic acid	1.92	s	CH_3	-
Acetoin	1.37	d	CH_3	2.21/4.42
Acetone	2.24	s	two CH_3	-
Alanine	1.48	d	CH_3	3.78
Anserine	2.67	m	CH_2	3.18/7.13/8.33
Aspartic acid	2.82	dd	CH_2	2.66/3.86
Betaine	3.89	s	CH_2	3.26
Cadaverine	1.72	m	two CH_2	1.46/3.01
Creatine/phosphocreatine	3.04	s	CH_3	3.90
Ethanol	1.19	t	CH_3	3.66
Formic acid	8.46	s	CH	-
Fumaric acid	6.52	s	two CH	-
Glutamic acid	2.36	m	CH_2	2.06/2.12/3.75
Glycerol	3.57	m	two CH	3.64/3.78
Glycine	3.57	s	CH_2	-
Glycogen	5.38	broad signal	four CH	-
Histamine	3.30	t	CH_2	3.03/7.14/7.99
Histidine	7.11	s	CH	7.95
Hypoxanthine	8.20	s	CH	8.22
Inosine-5'-monophosphate (IMP)	8.58	s	CH	8.20
Inosine	8.34	s	CH	6.09/8.23
Isoleucine	1.01	d	CH_3	0.93/1.46/1.97
Lactic acid	1.33	d	CH_3	4.11
	4.11	q	CH	1.33
Leucine	0.96	t	two CH_3	1.70/3.73
Lysine	1.73	m	CH_2	1.47/1.90/3.02/3.76
Maltose	5.40	d	CH	3.42/3.58/3.70
Mannitol	3.81	d	CH	3.69/3.77/3.88
	3.88	dd	CH	3.69/3.77/3.81
Methionine	2.14	m	CH_2 and CH_3	2.19/2.64/3.95
Niacinamide	8.72	dd	CH	7.60/8.25/8.93
Nicotinic acid	8.95	d	CH	7.52/8.25/8.60
Ornithine	3.02	t	CH_2	1.73/1.93/3.78
Phenylalanine	7.43	m	two CH	7.31/7.36
Putrescine	1.77	m	two CH_2	3.09
Pyruvic acid	2.38	s	CH_3	-
Succinic acid	2.41	s	two CH_2	-
Taurine	3.43	t	CH_2	3.25
Threonine	1.32	d	CH_3	3.59/4.27
Trimethylamine (TMA)	2.90	s	CH_3	-
Trimethylamine oxide (TMAO)	3.27	s	CH_3	-
Tyramine	7.22	d	two CH	6.91
Tyrosine	7.19	d	two CH	6.90
Uracil	5.81	d	CH	7.53
Valine	1.05	d	CH_3	0.98/2.27/3.61

3.2 Processing of sugar kelp

Metabolic changes in processed sugar kelp was quantified by NMR spectroscopy. The results of the metabolite quantification are available in appendix A, which summarizes all metabolite concentrations being referred to in the following subsections.

3.2.1 Principal component analysis of sugar kelp during storage

A PCA of the obtained spectroscopic data from the storage experiment of sugar kelp was performed to assess if the distribution of the sugar kelp samples was dependent on their treatment and storage condition. Samples clustering in the PCA scores plot would indicate similarities in metabolic composition of the samples. Figure 3.1 shows the resulting PCA1/PCA2 scores (A) and correlation loadings (B) of the sugar kelp storage experiment. The C-RT samples stored for 5 and 8 days were distinctively separated from the other samples. It suggests the storage of cut sugar kelp at room temperature causes major differences in seaweed metabolic composition compared to the other treatments.

The correlation loadings (figure 3.1, B) shows the significant buckets/variables that cause samples clustering in the scores plot. The grouping of the variables on the left side of the correlation loadings plot suggests that related metabolites occur at higher concentrations in the C-RT samples compared to the other treated sugar kelp samples. Table 3.2 shows a list of assigned compounds that significantly differed in the C-RT sugar kelp samples compared to the other treated samples.

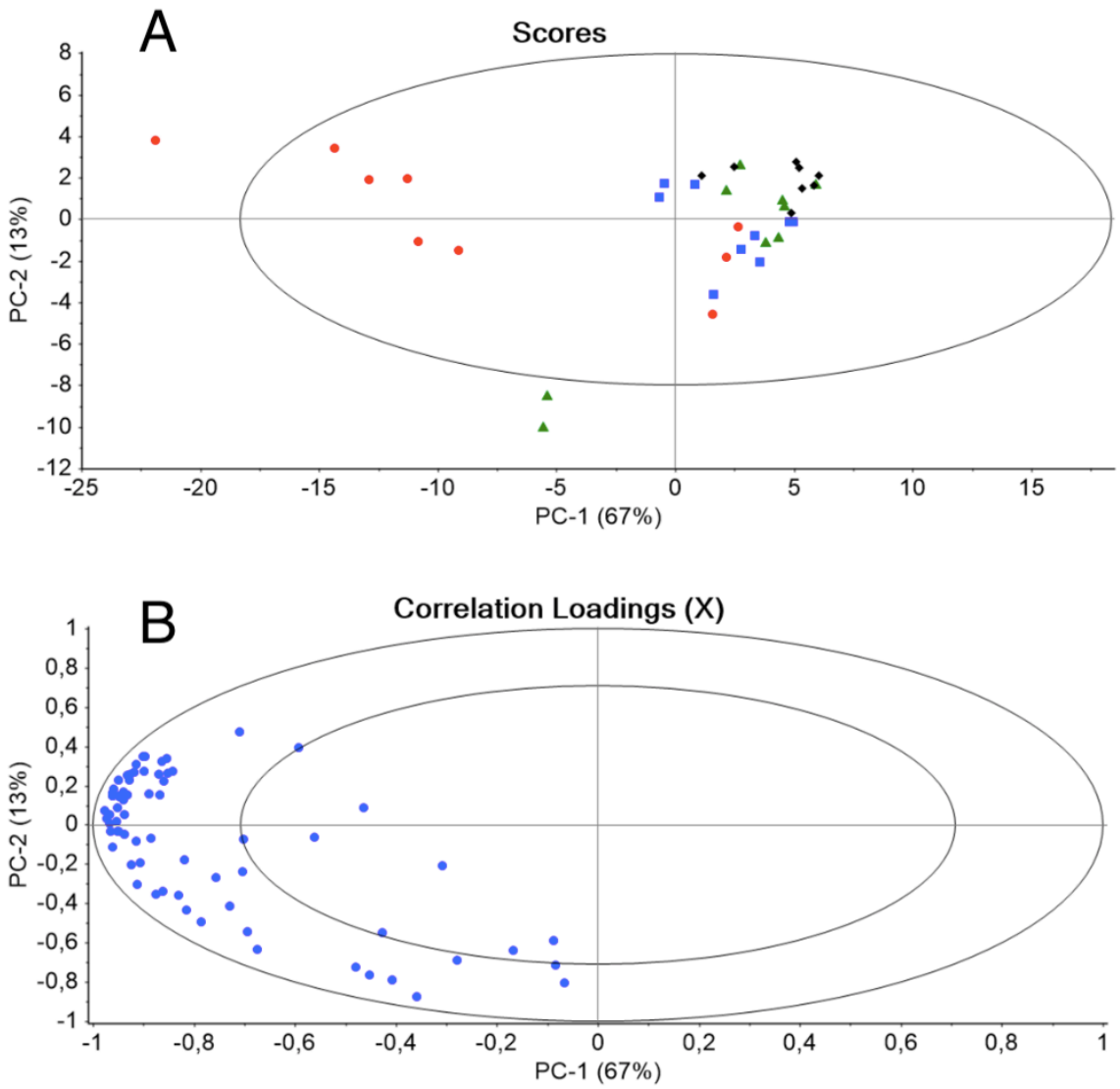

Figure 3.1. Scores plot (A) and correlation loadings (B) of mean-centered PCA performed using reduced bucket table ($p < 0.05$) of ^{1}H NMR spectra of sugar kelp samples exposed to different treatments and storage condition. A: Red circles - cut sugar kelp stored at room temperature for 2-8 days; green triangles - whole sugar kelp stored at 4 °C for 2-12 days; blue squares - cut sugar kelp stored at 4 °C for 2-12 days; black diamonds - whole sugar kelp stored at room temperature for 2-8 days.

Table 3.2. Summary of significant buckets ($p < 0.05$), relative signal assignments, significance levels and *F* values from ANOVA for cut, room temperature-stored (C-RT) sugar kelp at 5-8 days of storage.

Chemical shift of bucket (ppm)	Compound assignment	Sig. level	*F* value
Cluster of C-RT sugar kelp			
1.05	Valine	0.000	13.710
1.02	Isoleucine	0.000	11.270
0.97	Leucine	0.000	9.033
1.15	2,3-butanediol	0.005	5.218
1.33	Lactic acid	0.000	12.854
1.48	Alanine	0.003	5.669
1.92	Acetic acid	0.031	3.363
2.35	Glutamic acid	0.017	3.980
2.38	Pyruvic acid	0.000	14.954
7.19	Tyrosine	0.000	9.451
7.39	Phenylalanine	0.000	8.706
8.46	Formic acid	0.016	4.026
8.71	Niacinamide	0.007	4.915

Parallels 1 and 2 of the W-4C samples stored for 2 days were also observed to be distinctively separated from the other samples (figure 3.1, A). During analysis of the mentioned samples' respective spectra, the chemical shifts in the amino acid region of valine, isoleucine and leucine was observed shifted. The separated clustering of these two samples can result from resonance signals shifting upon differences in pH. However, shifting of resonances was not observed in other samples with pH values deviating from 7 (table 3.3). Another cause of resonance shifting can be change in temperature of samples. The concentration of mannitol was low in samples with high pH compared to those with pH values closer to 7 (figure 3.2). These samples included W_4C_12_3; W_RT_T05 parallels 2 and 3; W_RT_T05 parallels 1 and 2. It is suggested that mannitol in these samples was used as substrate for producing basic fermentation products by microorganisms, that increased the pH of the samples.

Table 3.3. Measured pH values of sugar kelp extracts exposed to different treatments and storage temperatures. Abbreviations: C_4C = cut sugar kelp stored at 4 °C; C_RT = cut sugar kelp stored at room temperature; W_4C = whole sugar kelp stored at 4 °C; W_RT = whole sugar kelp stored at room temperature; T02-T12 - storage time in days.

	pH		
Sample name	Parallel 1	Parallel 2	Parallel 3
C_4C_T02	6.69	6.11	6.39
C_4C_T05	6.76	7.11	7.05
C_4C_T12	5.82	6.36	6.42
C_RT_T02	6.31	6.59	6.15
C_RT_T05	6.54	5.59	6.31
C_RT_T08	5.67	6.06	5.68
W_4C_T02	6.20	6.14	6.54
W_4C_T05	6.49	5.87	6.54
W_4C_T12	6.27	7.02	8.08
W_RT_T02	6.14	6.77	6.90
W_RT_T05	6.87	9.09	8.27
W_RT_T08	8.51	9.07	-

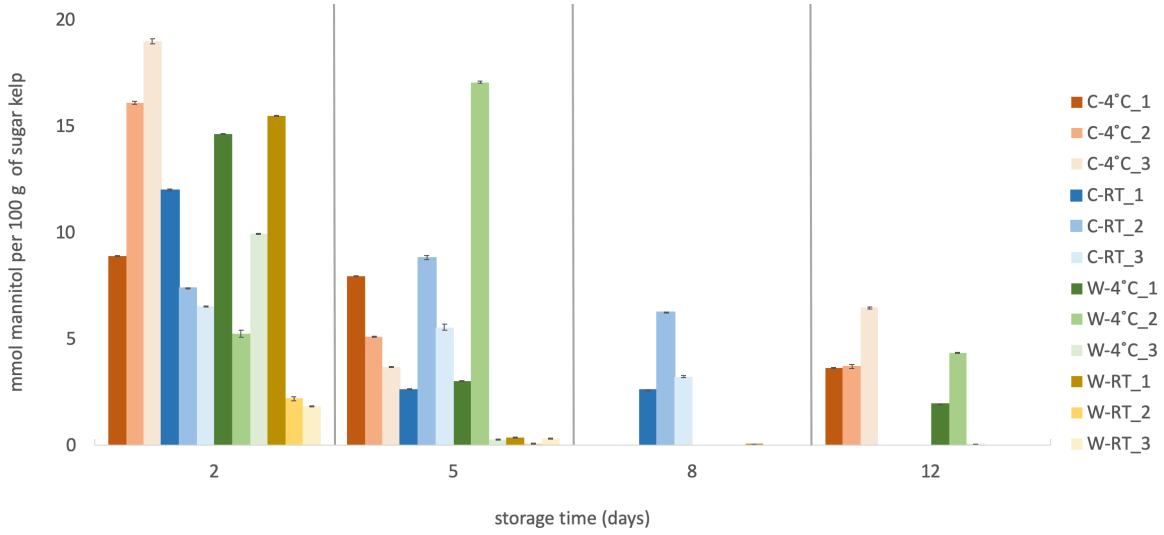

Figure 3.2. Concentrations of mannitol in treated sugar kelp samples as a function of storage time. Abbreviations: C-4 °C_1 = parallel 1, cut sugar kelp stored at 4 °C; C-RT_1 = parallel 1, cut sugar kelp stored at room temperature; W-4 °C_1 = parallel 1, whole sugar kelp stored at 4 °C; W-RT_1 = parallel 1, whole sugar kelp stored at room temperature. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

3.2.2 Significant metabolites of cut, room temperature treated sugar kelp

The concentrations of amino acids valine, isoleucine, tyrosine and phenylalanine generally increased over time during storage of C-RT sugar kelp (blue-gradient bars, figures 3.3-3.6). An increase in the concentration of free amino acids can suggest breakdown of peptides and proteins. Enzymes are in general more active at higher temperatures and cutting of the sugar kelp is suggested to have promoted autolysis by release of cellular content and digestive enzymes. Increased concentrations of essential amino acids valine, isoleucine and phenylalanine could increase nutritional value of the sugar kelp (42). Increased concentrations of phenylalanine and tyrosine could influence taste. For both mentioned amino acids, L-form gives bitter taste and D-form gives sweet taste in pure solutions (43).

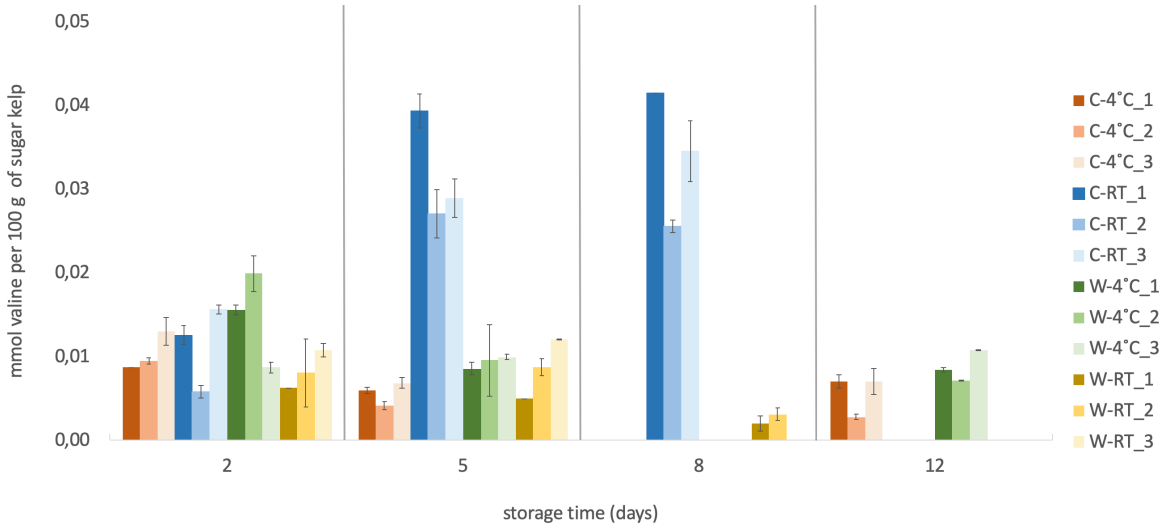

Figure 3.3. Concentrations of valine in treated sugar kelp samples as a function of storage time. Abbreviations: C-4 °C_1 = parallel 1, cut sugar kelp stored at 4 °C; C-RT_1 = parallel 1, cut sugar kelp stored at room temperature; W-4 °C_1 = parallel 1, whole sugar kelp stored at 4 °C; W-RT_1 = parallel 1, whole sugar kelp stored at room temperature. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

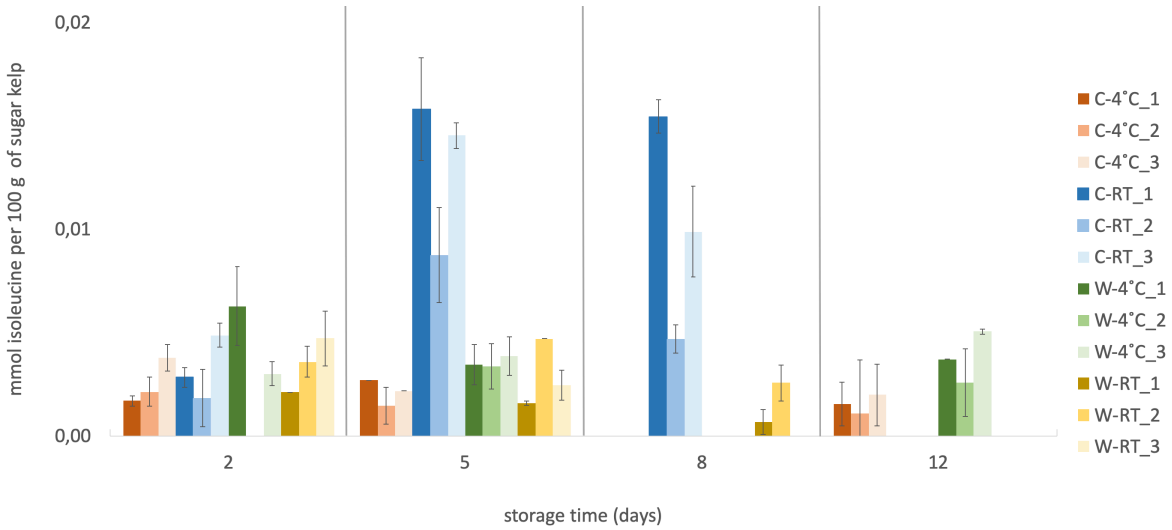

Figure 3.4. Concentrations of isoleucine in treated sugar kelp samples as a function of storage time. Abbreviations: C-4 °C_1 = parallel 1, cut sugar kelp stored at 4 °C; C-RT_1 = parallel 1, cut sugar kelp stored at room temperature; W-4 °C_1 = parallel 1, whole sugar kelp stored at 4 °C; W-RT_1 = parallel 1, whole sugar kelp stored at room temperature. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

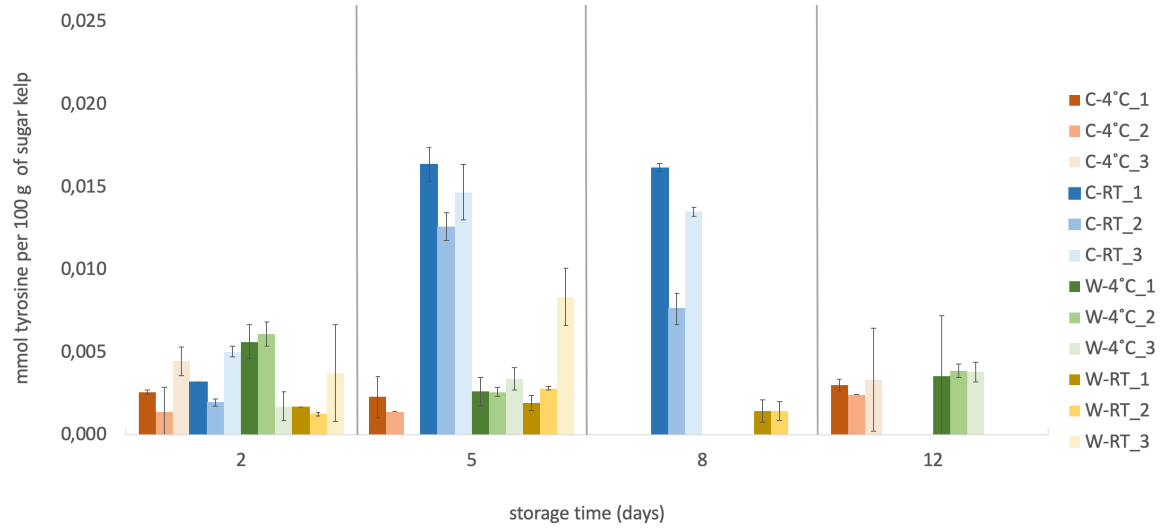

Figure 3.5. Concentrations of tyrosine in treated sugar kelp samples as a function of storage time. Abbreviations: C-4 °C_1 = parallel 1, cut sugar kelp stored at 4 °C; C-RT_1 = parallel 1, cut sugar kelp stored at room temperature; W-4 °C_1 = parallel 1, whole sugar kelp stored at 4 °C; W-RT_1 = parallel 1, whole sugar kelp stored at room temperature. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

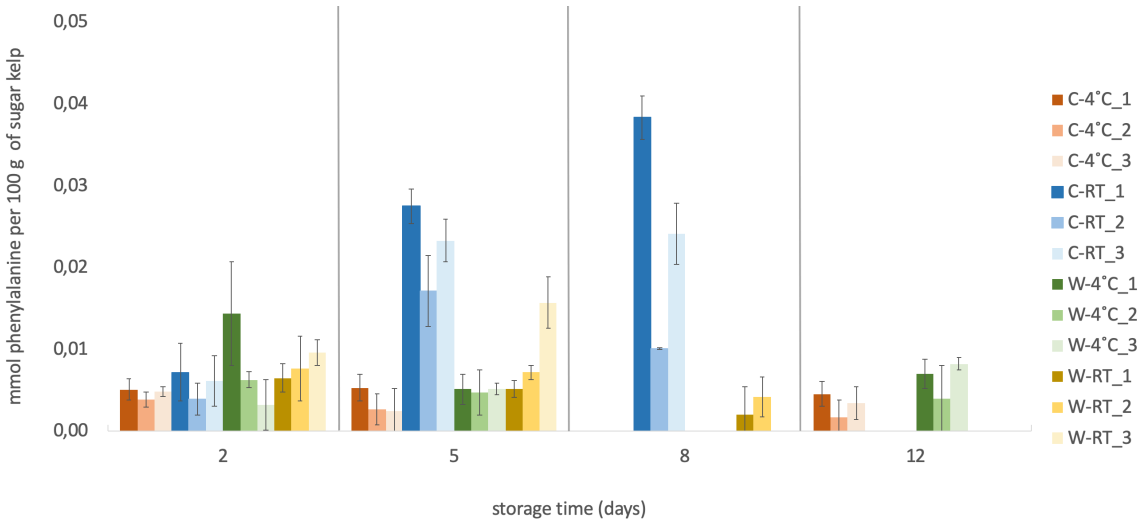

Figure 3.6. Concentrations of phenylalanine in treated sugar kelp samples as a function of storage time. Abbreviations: C-4 °C_1 = parallel 1, cut sugar kelp stored at 4 °C; C-RT_1 = parallel 1, cut sugar kelp stored at room temperature; W-4 °C_1 = parallel 1, whole sugar kelp stored at 4 °C; W-RT_1 = parallel 1, whole sugar kelp stored at room temperature. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

The concentration of formic, pyruvic and acetic acid generally increased over time during storage of C-RT sugar kelp (blue-gradient bars, figures 3.7-3.9). An increase of organic acids in a sample can reduce the pH value and result in a sour product. Formic acid is among others an end-product of mixed acid and 2,3-butanediol fermentation, which is performed by the genera *Escherichia* and *Enterobacter* (7). An increase of formic acid may suggest presence of bacteria of the mentioned genera. Acetic acid is also a potential end-product of fermentation of sugars; increased concentration of acetic acid over time can suggest sugar fermentation by bacteria of the genera *Acetobacterium* and *Escherichia* (7). Also, acetic acid has an unpleasant odor, which at too high concentration can give a food product undesired odor.

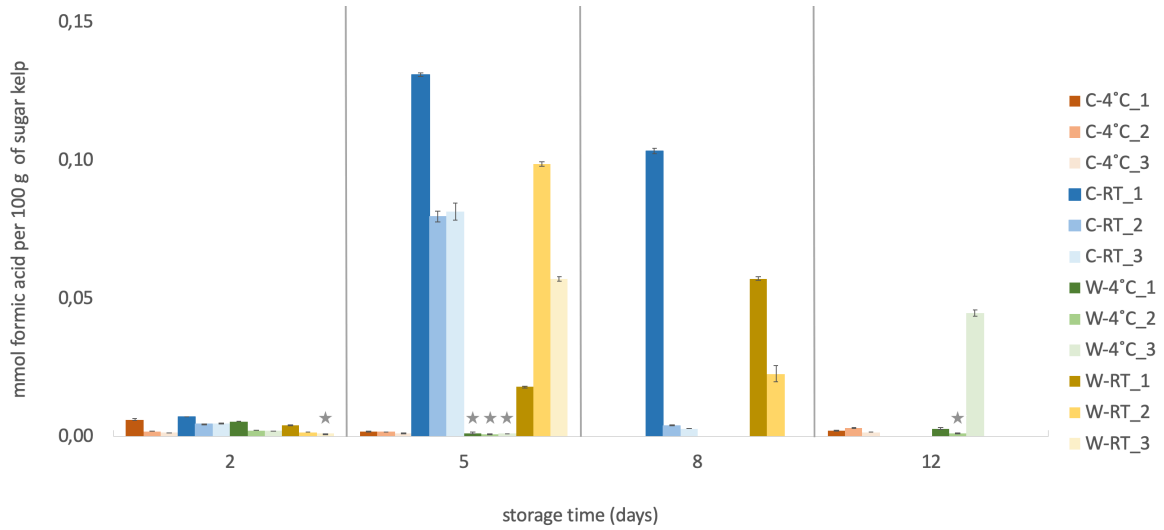

Figure 3.7. Concentrations of formic acid in treated sugar kelp samples as a function of storage time. Gray stars located above bars indicate S/N ratio < 10. Abbreviations: C-4 °C_1 = parallel 1, cut sugar kelp stored at 4 °C; C-RT_1 = parallel 1, cut sugar kelp stored at room temperature; W-4 °C_1 = parallel 1, whole sugar kelp stored at 4 °C; W-RT_1 = parallel 1, whole sugar kelp stored at room temperature. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

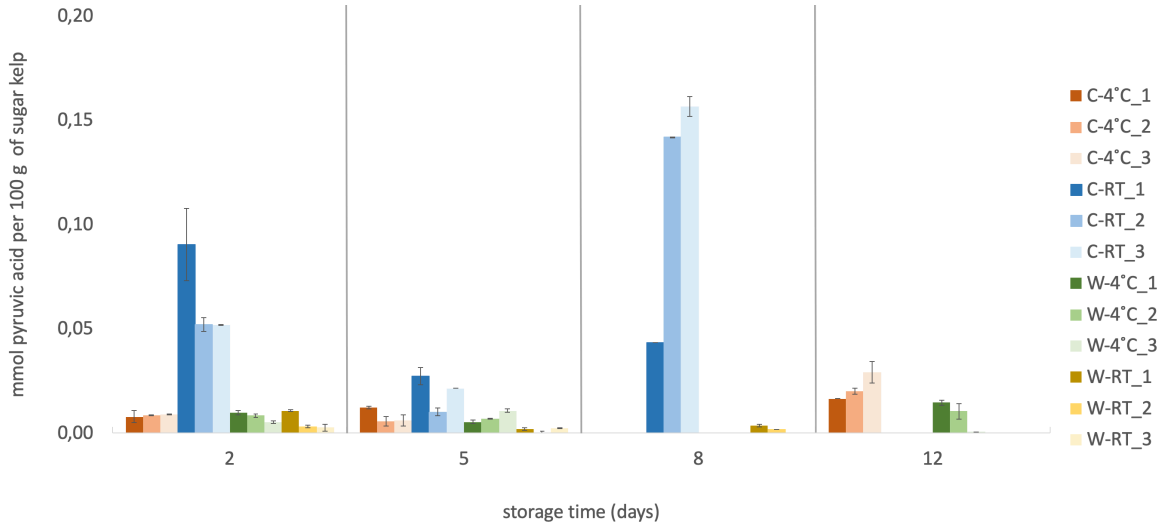

Figure 3.8. Concentrations of pyruvic acid in treated sugar kelp samples as a function of storage time. Abbreviations: C-4 °C_1 = parallel 1, cut sugar kelp stored at 4 °C; C-RT_1 = parallel 1, cut sugar kelp stored at room temperature; W-4 °C_1 = parallel 1, whole sugar kelp stored at 4 °C; W-RT_1 = parallel 1, whole sugar kelp stored at room temperature. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

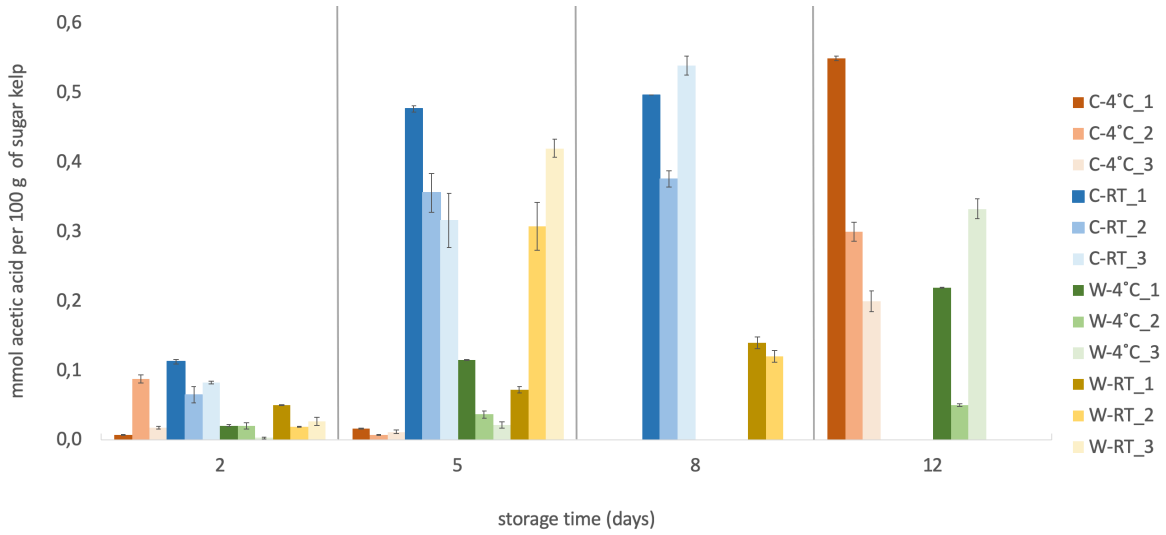

Figure 3.9. Concentrations of acetic acid in treated sugar kelp samples as a function of storage time. Abbreviations: C-4 °C_1 = parallel 1, cut sugar kelp stored at 4 °C; C-RT_1 = parallel 1, cut sugar kelp stored at room temperature; W-4 °C_1 = parallel 1, whole sugar kelp stored at 4 °C; W-RT_1 = parallel 1, whole sugar kelp stored at room temperature. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

The concentration of 2,3-butanediol was also observed to generally increase over time during storage of C-RT sugar kelp (blue-gradient bars, figure 3.10). As 2,3-butanediol is an end-product of 2,3-butanediol fermentation of sugars, together with formic acid among others, the increase of this compound during storage of sugar kelp may suggest the presence of *Enterobacter* bacteria in the samples (7).

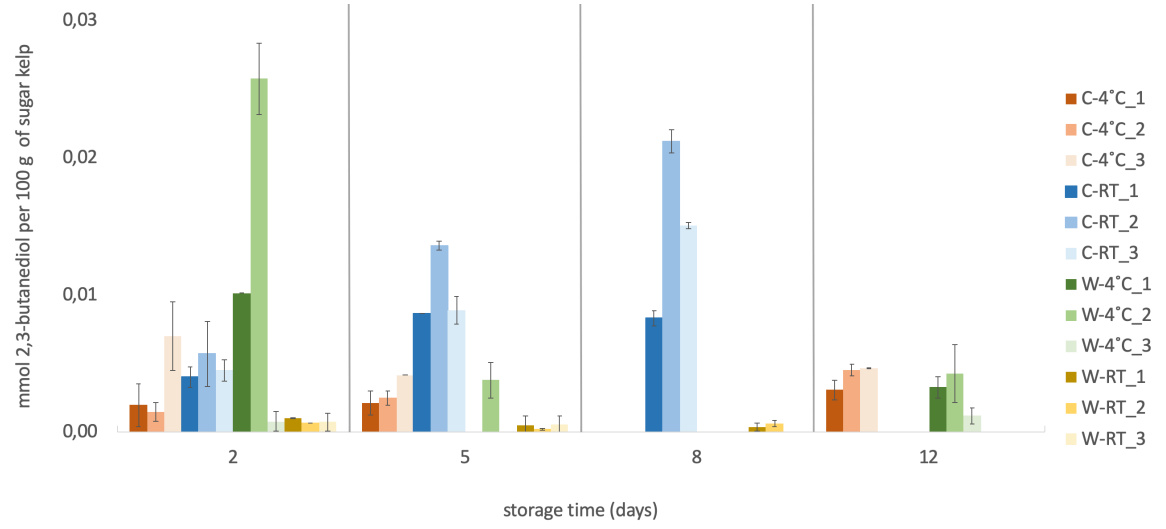

Figure 3.10. Concentrations of 2,3-butanediol in treated sugar kelp samples as a function of storage time. Abbreviations: C-4 °C_1 = parallel 1, cut sugar kelp stored at 4 °C; C-RT_1 = parallel 1, cut sugar kelp stored at room temperature; W-4 °C_1 = parallel 1, whole sugar kelp stored at 4 °C; W-RT_1 = parallel 1, whole sugar kelp stored at room temperature. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

3.3 Lactic acid bacteria fermentation of sugar kelp

In order to assess the taste properties and nutritional value of fermented products of sugar kelp, samples of sugar kelp were fermented with three different LAB strains: *Lactobacillus paracasei* LUHS244, *Pediococcus acidilactici* KTU05-7 (Pa7) and *Pediococcus pentosaceus* KTU05-9 (Pp9). These samples were analyzed by NMR spectroscopy for metabolic characterization. A targeted analysis was performed by quantifying metabolites which intensities were observed to vary either from reference or from the spectra obtained from the other fermented sugar kelp samples. Table 3.4 shows a list of selected metabolites from the fermentation experiment.

The concentration of lactic acid was increased at least 14.3 times in fermented sugar kelp by all three strains, indicating sugar kelp is a good substrate for LAB fermentation by the mentioned strains. The amount of the essential amino acids valine and phenylalanine was observed at higher concentrations in the fermented sugar kelp samples compared to reference. The concentration of phenylalanine was observed at an amount 4.6 times higher in the LUHS244-fermented sugar kelp compared to reference. Fermentation by LUHS244 therefore yielded the highest increase of phenylalanine out of the LAB strains. The amount of acetone was increased in sugar kelp fermented by LUHS244 and Pp9 LAB strains, while the concentration of acetone did not increase significantly after fermentation by Pa7. Acetone is an end-product of acetone-butanol fermentation (7). However, formation of butanol was not observed. This may suggest some other type of sugar fermentation producing acetone as an end-product had occurred. The formation of glycerol was not detected by fermentation of strain Pa7. The LAB strain Pp9 was shown to produce fermented sugar kelp with the highest concentration of glycerol, which can result in increased sweet taste (46). An increase of the concentration of glutamic and succinic acid in sugar kelp could lead to an increase in umami taste (45). The concentration of glutamic acid was 1.2 times higher in Pp9 fermented sugar kelp compared to reference. The concentration of succinic acid was 1.2 times higher in LUHS244 fermented sugar kelp. The concentration of inosine was 2.6 mg/100 g in LUHS244-fermented sugar kelp. As inosine is described to have bitter taste (25), increased concentration of inosine in fermented sugar kelp can be unfavorable for fermented sugar kelp as a food product. The concentration of formic acid was increased 4.8 and 5.4 times in LUHS244 and Pp9 fermented sugar kelp compared to reference, respectively. Increased concentrations of formic acid can arrest degradation processes in the fermented sugar kelp and increase its the shelf life (11).

Table 3.4. Concentrations of metabolites extracted from lactic acid bacteria (LAB) fermented sugar kelp and non-fermented sugar kelp (reference). LAB strains: *Lactobacillus paracasei* LUHS244, *Pediococcus acidilactici* KTU05-7 (Pa7) and *Pediococcus pentosaceus* KTU05-9 (Pp9). Δ1 – calculated concentration difference in times of a given metabolite compared to reference (decrease < 1 < increase). Δ2 – calculated concentration difference of a given metabolite compared to reference.

	Lactic acid bacteria strain						
	LUHS244		Pa7		Pp9		Reference
Compound	Conc. (mg/100 g)	Δ1 (times)	Conc. (mg/100 g)	Δ1 (times)	Conc. (mg/100 g)	Δ1 (times)	Conc. (mg/100 g)
2,3-butanediol	0.15	1.5	0.07	0.8	0.22	2.3	0.10
Acetic acid	33.85	20.9	25.92	16.0	31.84	19.6	1.62
Acetone	2.57	15.1	0.22	1.3	5.33	31.3	0.17
Alanine	52.78	1.0	48.83	0.9	58.19	1.1	52.78
Ethanol	18.45	7.2	15.74	6.1	10.00	3.9	2.57
Formic acid	1.45	4.8	0.89	2.9	1.63	5.4	0.30
Glutamic acid	12.83	0.9	14.08	1.0	16.14	1.2	13.57
Lactic acid	101.58	16.8	100.89	16.7	86.50	14.3	6.06
Mannitol	439.68	0.7	473.96	0.7	511.69	0.8	645.87
Phenylalanine	3.61	4.6	3.09	3.9	2.77	3.5	0.79
Succinic acid	8.67	1.2	7.24	1.0	8.03	1.1	7.01
Tyrosine	1.84	3.3	1.65	3.0	1.61	2.9	0.55
Valine	5.30	3.4	4.84	3.1	5.64	3.6	1.55

	LUHS244		Pa7		Pp9		Reference
Compound	Conc. (mg/100 g)	Δ2 (mg/100 g)	Conc. (mg/100 g)	Δ2 (mg/100 g)	Conc. (mg/100 g)	Δ2 (mg/100 g)	Conc. (mg/100 g)
Glycerol	33.25	33.25	0.00	0.00	150.95	150.95	0.00
Inosine	2.55	-	*	-	*	-	*
Pyruvic acid	1.16	1.16	0.16	0.16	0.32	0.32	0.00
Uracil	0.00	0.00	0.84	0.84	1.46	1.46	0.00

** Signal-to-noise ratio (S/N) < 10*

3.4 Post-mortem changes and shelf life of salmon upon addition of sugar kelp

Metabolic changes in sugar kelp-treated and reference salmon muscle was quantified by NMR spectroscopy. The results of the metabolite quantification are available in appendix B, which summarizes all metabolite concentrations being referred to in the following subsections.

3.4.1 Principal component analysis of premium and standard quality salmon during storage

To assess if the distribution of salmon samples was influenced by addition of sugar kelp, a PCA of the spectroscopic data from premium quality salmon (PQS) and standard quality salmon (SQS) was performed. Figures 3.11 and 3.12 shows the resulting PCA1/PCA2 scores (A) and correlation loadings (B) of the PQS and SQS storage experiments, respectively. A distinctive horizontal separation of clustered reference and sugar kelp-treated samples was

observed in the scores plot A for both PQS and SQS, indicating differences in their metabolic composition during storage. A vertical distribution of both sample clusters was also observed. This distribution is suggested to be dependent on storage time, and it can indicate storage time was having a larger effect on the metabolic composition of reference samples compared to sugar kelp-treated samples. In the SQS scores plot the vertical distribution is prominent in both reference and sugar kelp-treated samples. It is suggested this was a result of the quality difference between the salmon samples. The amount of bacterial spoilage is suggested to have been increased in standard quality salmon, as it was not processed and packed pre-mortis like the premium quality salmon. The addition of sugar kelp to salmon muscle with higher amounts of bacterial spoilage during storage is suggested to be less effective.

The correlation loadings plot (figures 3.11 and 3.12, B) shows a separation of metabolite clustering in storage of both PQS and SQS. The metabolites on the left side were present at significantly higher concentrations in the sugar kelp-treated samples and are characteristic for the metabolic composition of sugar kelp-treated samples. Mannitol, aspartic acid and alanine were the main metabolites for sugar kelp-treated PQS, while mannitol, uracil, acetoin and formic acid were the main determined metabolites for sugar kelp-treated SQS. Increased concentrations of mannitol, aspartic acid and alanine in sugar kelp-treated samples are suggested to be results of diffusion from sugar kelp. Increased concentrations of acetoin and formic acids might be results of fermentation.

On the right side of the correlation loadings plot (figures 3.11 and 3.12, B), metabolites having higher concentration in reference samples are located. The metabolites clustered to the bottom of the right side of the correlation loadings plot of PQS and SQS are metabolites present at higher concentrations compared to the other cluster on the right upper side. These metabolites were forming in reference samples stored until days 17-21 days after slaughter. Some of these include TMA and hypoxanthine, which are compounds known to be indicators of reduced fish quality (22). The signal at 1.71 ppm was assigned to cadaverine, which is a biogenic amine also responsible for the reduction of fish quality. Tables 3.5 and 3.6 summarizes chemical shifts of assigned and unknown metabolites significant for the distribution of PQS and SQS based on metabolic composition.

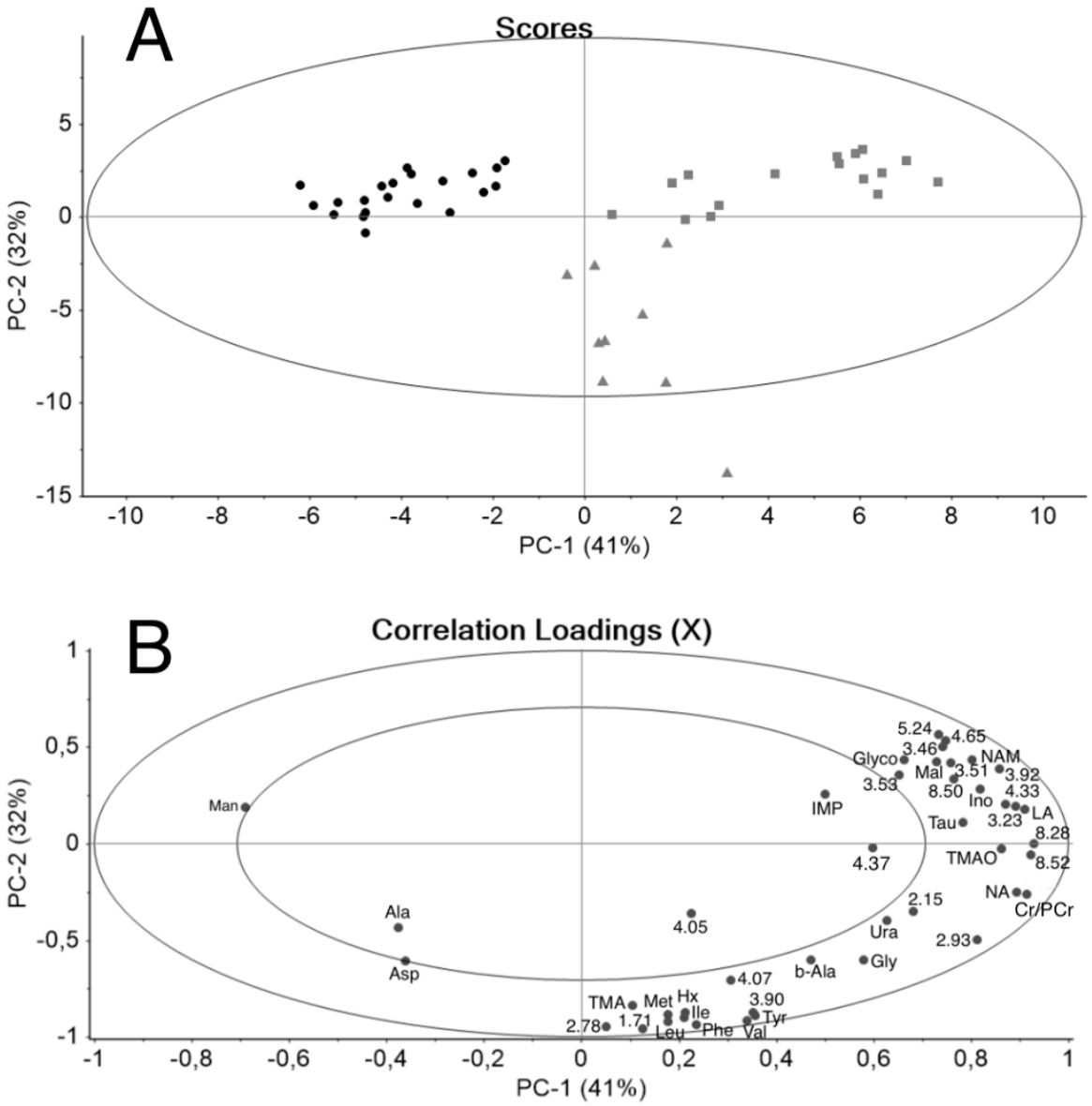

Figure 3.11. Scores plot (A) and correlation loadings (B) of mean-centered PCA performed using reduced bucket table *(p* < 0.05) of ^{1}H NMR spectra of PQS stored from day 5-21 after slaughter at 4 °C. Black circles in A - seaweed-covered salmon samples stored from day 5-21 after slaughter; gray squares in A - reference samples stored from day 5-14 after slaughter; gray triangles in A - reference samples stored until day 17-21 after slaughter. Correlation loadings marked with numbers in B represent chemical shifts of unknown origin in ppm. Abbreviations used in figure: Ala = alanine; Asp = aspartic acid; ATP = adenosine-5'-triphosphate; b-Ala = β-Alanine; Cr/PCr = creatine/phosphocreatine; Gly = glycine; Glyco = glycogen; Hx = hypoxanthine; IMP = inosine-5'-monophosphate; Ino = inosine; Ile = isoleucine; LA = lactic acid; Man = mannitol; Leu = leucine; Mal = maltose; NA = nicotinic acid; NAM = niacinamide; Phe = phenylalanine; Tau = taurine; TMA = trimethylamine; TMAO = trimethylamine oxide; Tyr = tyrosine; Ura = uracil; Val = valine.

Table 3.5. Summary of significant buckets ($p < 0.05$), relative signal assignments, significance levels and *F* values from ANOVA for storage of premium quality salmon.

Chemical shift of bucket (ppm)	Compound assignment	Sig. level	*F* value
Cluster of reference PQS			
0.96	Leucine	0.010	7.188
1.01	Isoleucine	0.009	7.488
1.04	Valine	0.000	19.307
1.71	-	0.009	7.457
2.14	Methionine	0.028	5.138
2.15	-	0.000	23.691
2.56	β-Alanine	0.000	38.144
2.78	-	0.031	4.937
2.90	TMA	0.007	8.018
2.93	-	0.000	416.485
3.04	Creatine/Phosphocreatine	0.000	1046.988
3.23	-	0.000	40.301
3.27	TMAO	0.000	115.006
3.42	Taurine	0.000	23.640
3.46	-	0.011	6.957
3.51	-	0.005	8.856
3.53	-	0.018	6.081
3.56	Glycine	0.000	35.586
3.90	-	0.000	27.242
3.92	-	0.000	25.875
4.05	-	0.002	11.365
4.07	-	0.000	30.336
4.12	Lactic acid	0.000	93.297
4.28	Inosine	0.000	33.667
4.33	-	0.000	30.980
4.37	-	0.001	13.929
4.65	-	0.009	7.439
5.24	-	0.018	6.013
5.38	Glycogen	0.020	5.792
5.40	Maltose	0.005	8.540
7.19	Tyrosine	0.000	17.817
7.43	Phenylalanine	0.001	11.838
7.55	Uracil	0.000	17.794
7.60	Niacinamide	0.000	29.455
8.20	Hypoxanthine	0.000	16.858
8.28	-	0.000	103.470
8.50	-	0.000	19.896
8.52	-	0.000	136.123
8.58	IMP	0.017	6.122
8.94	Nicotinic acid	0.000	734.601
Cluster of sugar kelp-treated PQS			
1.48	Alanine	0.013	6.731
2.80	Aspartic acid	0.038	4.578
3.80	Mannitol	0.000	73.052

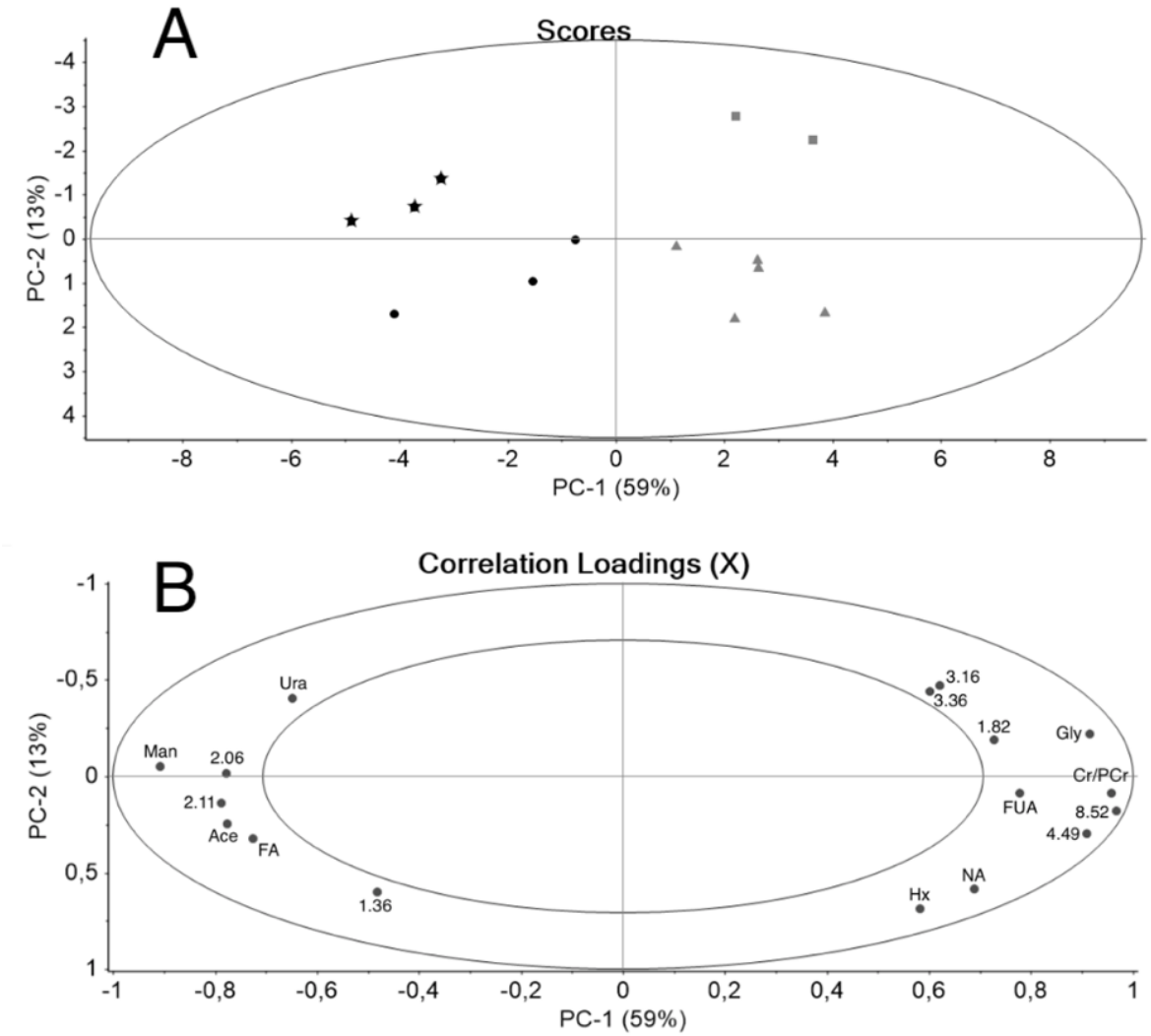

Figure 3.12. Scores plot (A) and correlation loadings (B) of mean-centered PCA performed using reduced bucket table ($p < 0.05$) of ^{1}H NMR spectra of standard quality salmon (SQS) stored from 7-21 days after slaughter at 4 °C. Black circles in A – sugar kelp-treated salmon samples stored until day 9-14 after slaughter; black stars in A – sugar kelp-treated salmon samples stored until day 17-21 after slaughter; gray squares in A - reference samples stored from day 7-9 after slaughter; gray triangles in A - reference samples stored until 11-21 days after slaughter. Correlation loadings marked with numbers in B represent chemical shifts of unknown origin in ppm. Abbreviations used in figure: Ace = acetoin; Cr/PCr = creatine/phosphocreatine; FA = formic acid; FUA = fumaric acid; Gly = glycine; Hx = hypoxanthine; NA = nicotinic acid; Ura = uracil.

Table 3.6. Summary of significant buckets ($p < 0.05$), relative signal assignments, significance levels and *F* values from ANOVA for storage of standard quality salmon.

Chemical shift of bucket (ppm)	Compound assignment	Sig. level	*F* value
Cluster of reference SQS			
1.82	-	0.009	9.958
3.16	-	0.042	5.317
3.36	-	0.009	9.928
3.57	Glycine	0.000	46.434
3.93	Creatine/Phosphocreatine	0.000	30.126
4.49	-	0.001	23.391
6.52	Fumaric acid	0.007	11.096
8.20	Hypoxanthine	0.016	8.040
8.52	-	0.000	30.181
8.94	Nicotinic acid	0.018	7.784
Cluster of sugar kelp-treated SQS			
1.36	-	0.045	5.133
2.06	-	0.037	5.614
2.11	-	0.031	6.134
3.82	Mannitol	0.000	66.786
4.42	Acetoin	0.016	8.137
5.81	Uracil	0.035	5.764
8.45	Formic acid	0.011	9.384

3.4.2 Diffusion of metabolites

During sampling of sugar kelp-treated salmon muscle of the PQS storage experiment, liquid originating from the package of sugar kelp was observed in the plastic zip-lock bags used for storage. The sugar kelp liquid had been transferred to the salmon muscle upon addition. The seaweed liquid can extract polar metabolites from the salmon tissue; this can influence the amount of metabolites extracted from the sugar kelp-treated salmon samples. Therefore, salmon muscle, seaweed tissue and dripped liquid from the seaweed represent a 3-component connected system. It is suggested water-soluble polar metabolites can diffuse from the original salmon muscle to sugar kelp tissue through sugar kelp liquid until equilibrium is reached.

The concentrations of lactic acid, creatine/phosphocreatine and anserine extracted from sugar kelp-treated PQS was significantly reduced compared to reference from the first timepoint of sampling. Comparison of ^{1}H spectra of the collected sugar kelp liquid from sampling of PQS at day 9 after slaughter with sugar kelp liquid from the original package of sugar kelp (figure 3.13) indicated transfer of lactic acid, creatine/phosphocreatine and anserine from salmon muscle into sugar kelp liquid. Doublet and quadruplet resonances at app. 1.33 and 4.12 ppm, respectively, indicate presence of lactic acid. Both resonances were observed in the spectrum of sugar kelp liquid from salmon storage. A doublet resonance signal at app. 1.33 ppm was observed in the spectrum of the sugar kelp liquid from the original package, at a reduced intensity compared to the other spectrum. It is suggested this can be resonance signals originating from the amino acid threonine, as no quadruplet

resonance was observed in the spectrum at 4.12 ppm. For the PQS sugar kelp liquid spectrum, singlet resonances of creatine/phosphocreatine were observed at 3.03 and 3.93 ppm. Multiplet resonance signal of anserine was observed at 2.67 ppm. As the mentioned metabolites were not observed in the reference spectrum of sugar kelp liquid, and creatine/phosphocreatine and anserine are not known to occur in seaweed tissue, these observations indicate diffusion of metabolites from salmon muscle into sugar kelp liquid.

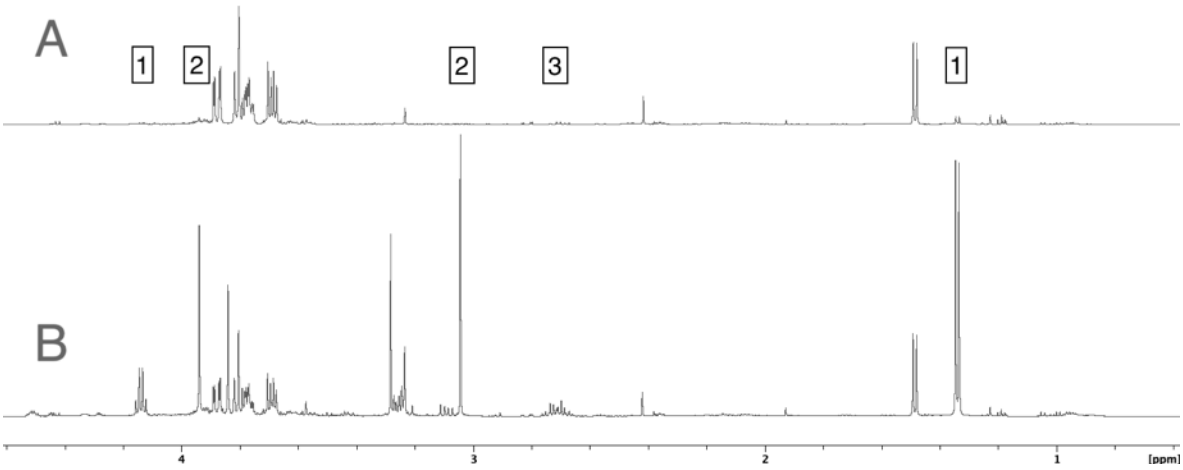

Figure 3.13. Comparison of ^{1}H NMR spectra of sugar kelp liquid from original package (A) and collected sugar kelp liquid from storage of PQS at 4 °C and sampled day 9 after slaughter (B). Boxes 1 mark expected chemical shifts of lactic acid resonance signals. Boxes 2 mark expected chemical shifts of creatine/phosphocreatine resonance signals. Box 3 marks an expected chemical shift of anserine resonance signals.

Sugar kelp tissue is another component of the system created upon addition of seaweed during storage of salmon. Comparison of ^{1}H spectra of collected sugar kelp tissue from sampling of PQS at day 9 after slaughter with sugar kelp tissue from the original package (figure 3.14) also indicated transfer of lactic acid, creatine/phosphocreatine and anserine from salmon muscle into the sugar kelp tissue, substantiating the indications of metabolite diffusion from salmon muscle into the 2 other system components during storage. Major reduction of metabolite concentrations in salmon muscle upon addition of seaweed during storage is therefore suggested to result from such diffusion.

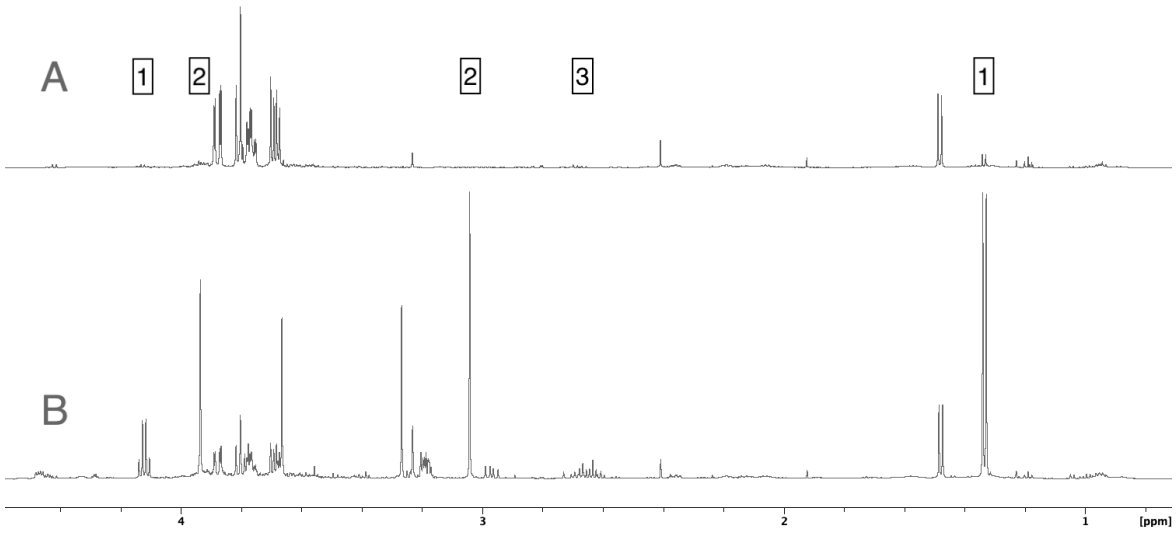

Figure 3.14. Comparison of ^{1}H NMR spectra of reference sugar kelp tissue (A) and collected sugar kelp tissue from storage of PQS at 4 °C and sampled day 9 after slaughter (B). Boxes 1 mark expected chemical shifts of lactic acid resonance signals. Boxes 2 mark expected chemical shifts of creatine/phosphocreatine resonance signals. Box 3 marks an expected chemical shift of anserine resonance signals.

For the SQS storage experiment, the amount of seaweed liquid present in the plastic bags after storage was observed to be very small compared to the amount in the PQS storage experiment. The same trends of metabolite diffusion as observed for PQS was also observed for SQS. Comparison of ^{1}H spectra of collected sugar kelp tissue from sampling of SQS at day 9 after slaughter with sugar kelp tissue from the original package (figure 3.15) indicated transfer of lactic acid, creatine/phosphocreatine and anserine from salmon muscle into the sugar kelp tissue. The reduced amount of seaweed liquid used in the storage of SQS might have influenced the 3-component system by hampering the metabolite exchange between salmon muscle and seaweed tissue. Major reduction of metabolite concentrations in salmon muscle upon addition of seaweed during storage is therefore suggested to result from metabolite diffusion within the 3-component system of salmon muscle, sugar kelp tissue and liquid.

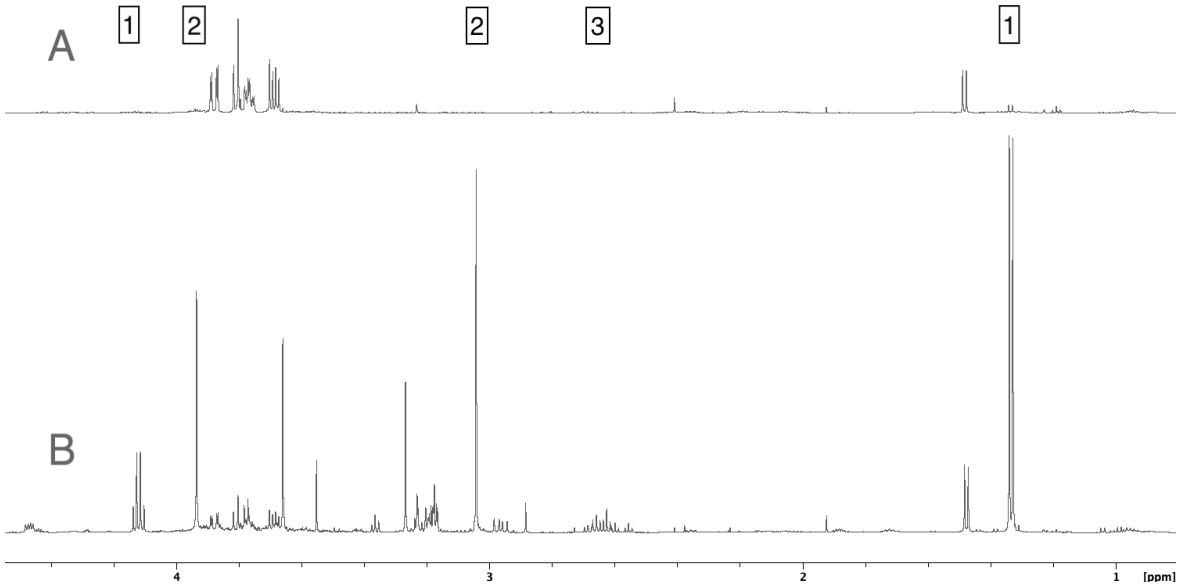

Figure 3.15. Comparison of ^{1}H NMR spectra of reference sugar kelp tissue (A) and collected sugar kelp tissue from storage of SQS at 4 °C and sampled day 9 after slaughter (B). Boxes 1 mark expected chemical shifts of lactic acid resonance signals. Boxes 2 mark expected chemical shifts of creatine/phosphocreatine resonance signals. Box 3 marks an expected chemical shift of anserine resonance signals.

3.4.3 ATP catabolites

To calculate K_1 and *H* values (equations IV and V, section 1.5.2.1), and thus evaluate salmon freshness upon addition of sugar kelp during storage, the amount of IMP, inosine and hypoxanthine was quantified in PQS and SQS samples. K_1 values of both PQS and SQS did not yield any significant differences between reference and sugar kelp-treated samples. The *H* values for the sugar kelp-treated PQS was lower compared to the reference samples at day 17-21 after slaughter (figure 3.16). This can suggest the addition of sugar kelp to salmon during storage can slow down the conversion of ATP catabolites to hypoxanthine. However, there was no significant difference between the *H* values of reference and sugar kelp-treated SQS. It is suggested this was a result of the SQS being at a more advanced stage in ATP degradation, making addition of sugar kelp less effective for inhibiting ATP degradation.

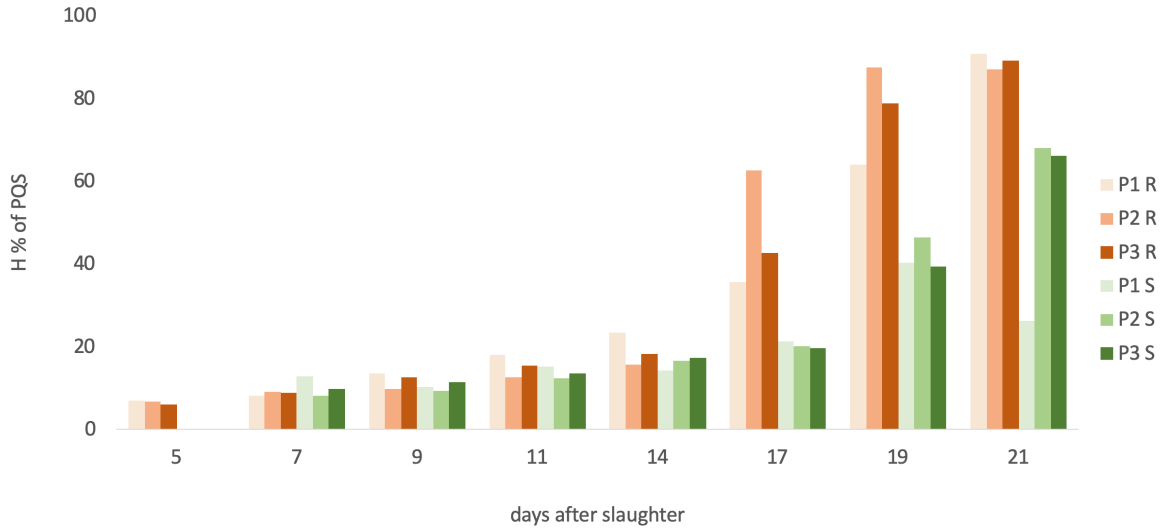

Figure 3.16. Calculated *H* values in percentage for reference and sugar kelp-treated PQS stored at 4 °C for day 5-21 after slaughter.

3.4.4 Formation of fermentation products

Microorganisms can utilize sugars for fermentation, which results in production of fermentation products. Acetoin and formic acid occurred at higher concentrations in sugar kelp-treated samples compared to reference during storage of SQS (figures 3.17 and 3.18). Acetoin and formic acid are potential products of mixed acid fermentation and 2,3-butanediol fermentation (7, 8). It is suggested the addition of sugar kelp upon storage of SQS provided more favorable conditions for producing these fermentation products, compared to storage of salmon without sugar kelp (reference). However, the concentration of 2,3-butanediol was reduced in sugar kelp-treated salmon compared to reference samples (figure 3.19). Increased concentration of acetoin in salmon muscle can result in enhanced yoghurt odor and creamy butter taste (9). Increased concentration of formic acid in salmon muscle can result in improved retainment of nutritive value and hampering of degradation processes (11). Addition of sugar kelp to salmon also increased the concentration of mannitol (figure 3.20), a sugar that can be used for fermentation. However, no accurate assessment of mannitol being used as substrate for fermentation producing acetoin and/or formic acid could be done, as there was no trend for mannitol concentrations in reference samples to compare with.

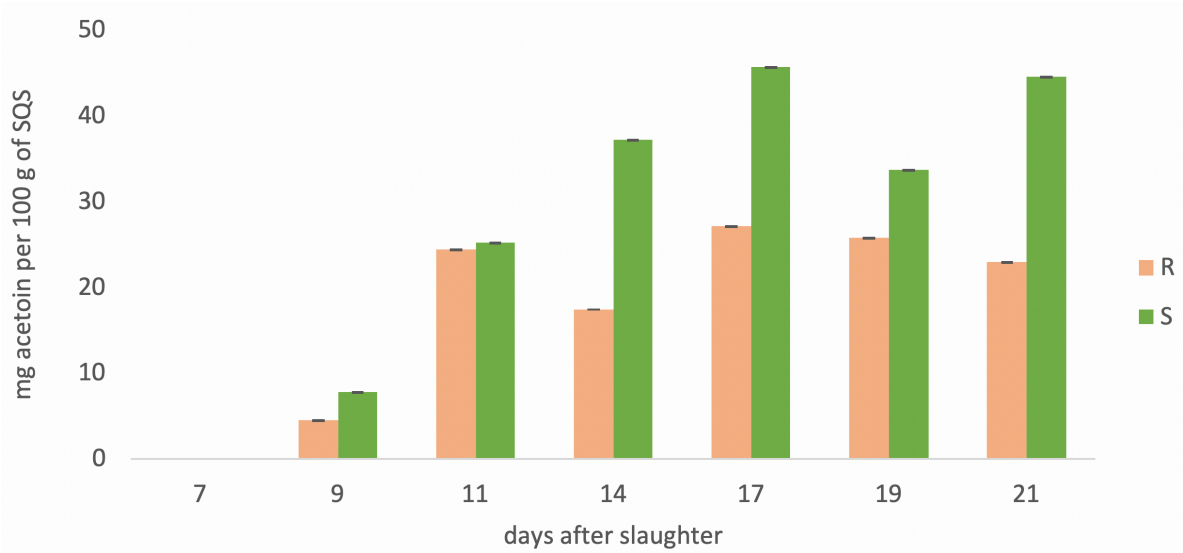

Figure 3.17. Concentrations of acetoin in reference and sugar kelp-treated standard quality salmon (SQS) stored at 4 °C from day 7-21 after slaughter. Abbreviations: R = reference; S = sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

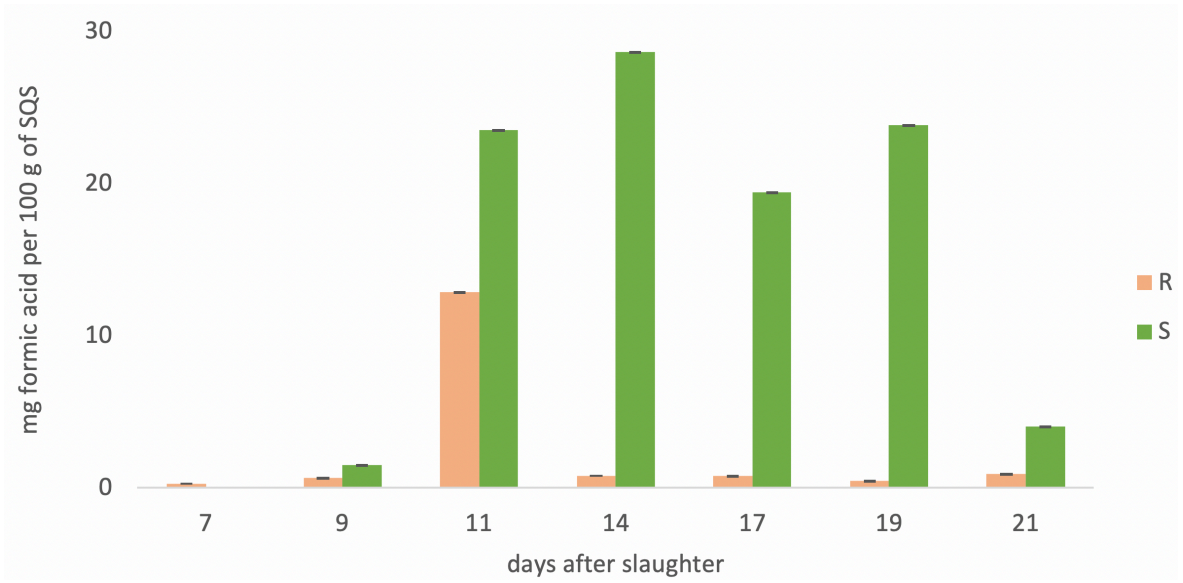

Figure 3.18. Concentrations of formic acid in reference and sugar kelp-treated standard quality salmon (SQS) stored at 4 °C from day 7-21 after slaughter. Abbreviations: R = reference; S = sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

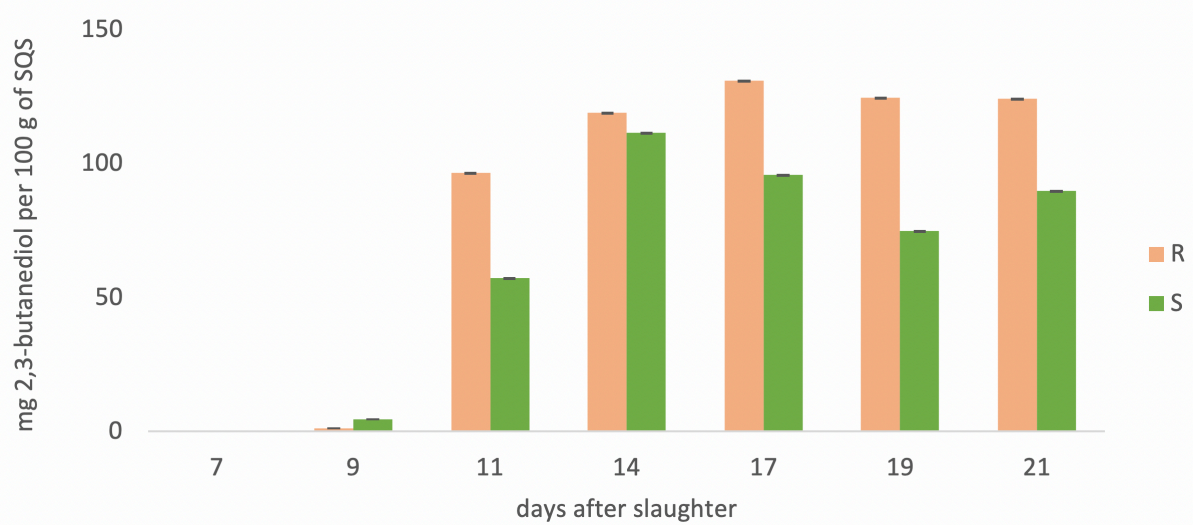

Figure 3.19. Concentrations of 2,3-butanediol in reference and sugar kelp-treated standard quality salmon (SQS) stored at 4 °C from day 7-21 after slaughter. Abbreviations: R = reference; S = sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

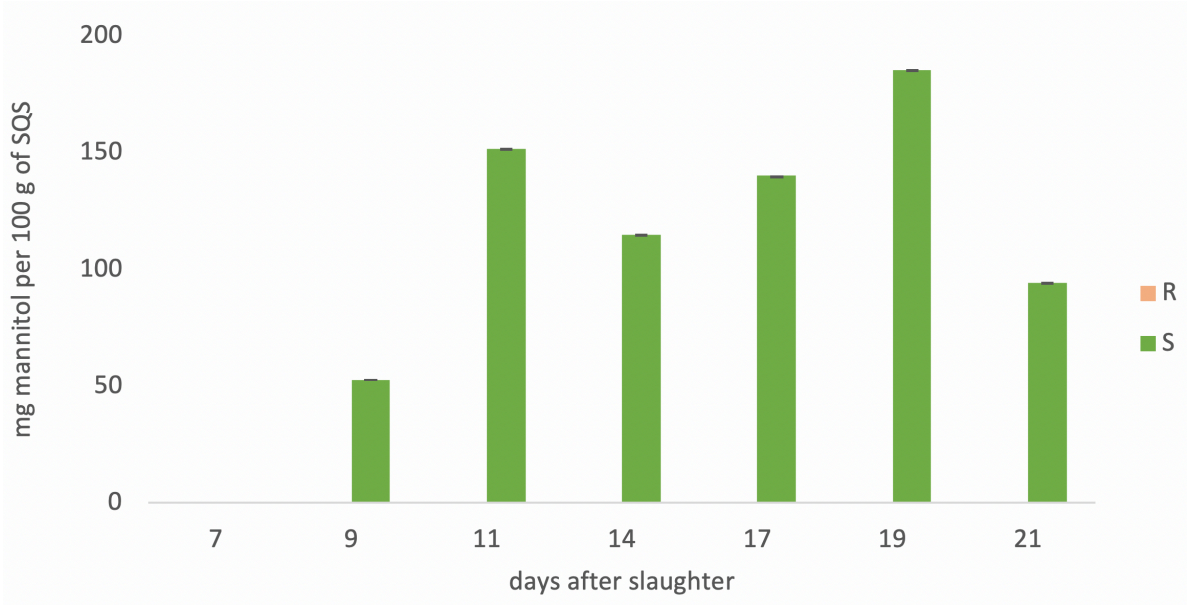

Figure 3.20. Concentrations of mannitol in reference and sugar kelp-treated standard quality salmon (SQS) stored at 4 °C from day 7-21 after slaughter. Abbreviations: R = reference; S = sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

Succinic acid is an umami taste related compound (45), which can change taste properties of foods. It is a potential product of mixed acid fermentation. The concentration of succinic acid in PQS was increased in sugar kelp-treated samples until day 14 after slaughter (figure 3.21). From day 17-21 after slaughter the succinic acid concentration in reference pieces was

increased compared to sugar kelp-treated. As the concentration of succinic acid was generally stable after addition of seaweed upon the storage, this indicates diffusion from sugar kelp is the reason for increased amounts of succinic acid. The significant increase of succinic acid from 17-21 days after slaughter in PQS reference pieces is suggested to result from bacterial fermentation. As this occurred after the normal shelf life of salmon, it will not represent a favorable way of increasing the succinic acid concentration in salmon to increase umami taste. However, as concentrations for succinic acid were increased from the first timepoint of sampling, it suggests addition of sugar kelp upon storage can change taste properties of salmon muscle within the commercial shelf life of salmon. The trends for succinic acid concentrations in SQS (figure 3.22) were not the same as for PQS. Within 14 days after slaughter, addition of sugar kelp upon storage did not indicate significant differences in concentrations of succinic acid between reference and sugar kelp-treated SQS. From day 17-19 the concentration of succinic acid was increased in sugar kelp-treated samples compared to reference, but this was outside of the commercial shelf life of salmon, and it was suggested to result from bacterial fermentation. As the SQS was thought to be at a more advanced stage of bacterial spoilage than the PQS, the increase concentrations of succinic acid could be a result mainly of bacterial fermentation and not diffusion from sugar kelp. It is suggested that improving taste of salmon by increasing the concentration of succinic acid can be done through metabolite diffusion by addition of sugar kelp upon storage of fresh salmon.

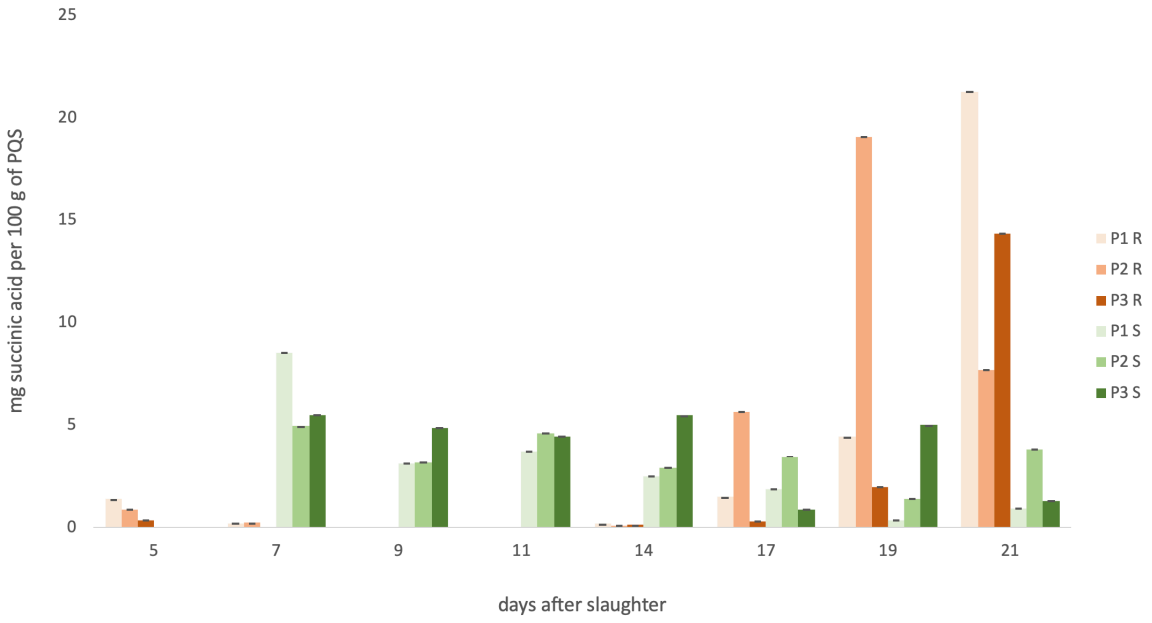

Figure 3.21. Concentrations of succinic acid in reference and sugar kelp-treated PQS stored at 4 °C from day 5-21 after slaughter. Abbreviations: P1 R = parallel 1 reference; P1 S = parallel 1 sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

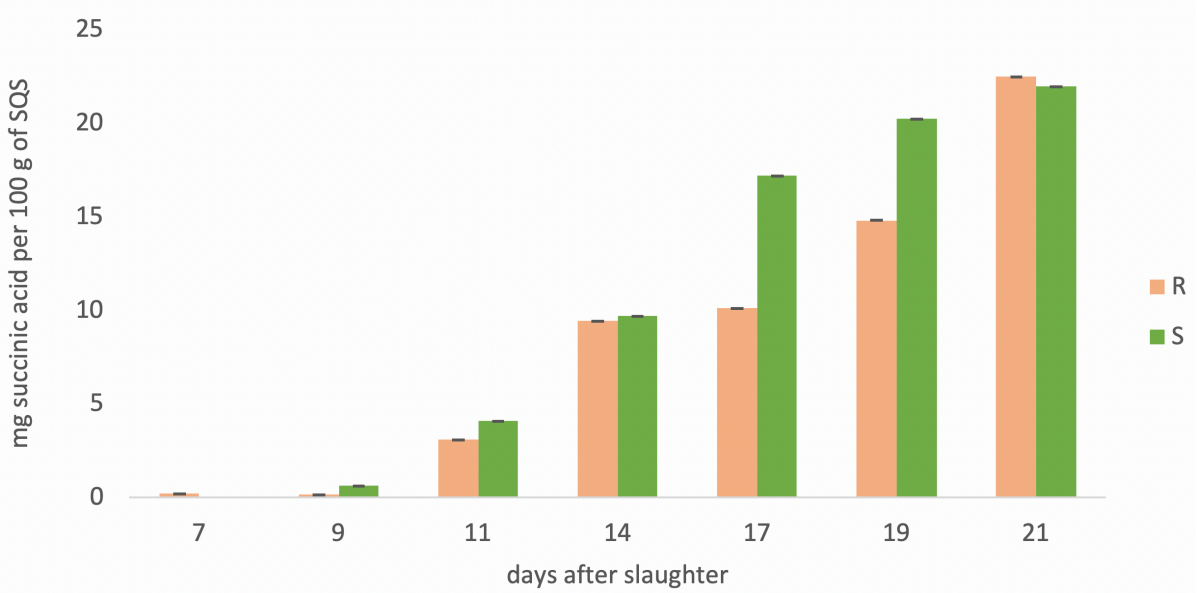

Figure 3.22. Concentrations of succinic acid in reference and sugar kelp-treated standard quality salmon (SQS) stored at 4 °C from day 7-21 after slaughter. Abbreviations: R = reference; S = sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

3.4.5 Formation of trimethylamine

The influence of sugar kelp addition on TMA formation and if/when the compound's concentration in salmon muscle exceeded allowed threshold levels was examined. Salmon quality can be graded as prime quality at TMA concentration less than 4.2 mg/100 g; acceptable between 4.2-29.5 mg/100 g and a maximum rejection level at 29.5 mg/100 g of fish (30). The TMA levels in the 3 parallels of sugar kelp-treated PQS samples were within prime quality during the whole storage period from 7-21 days after slaughter (figure 3.23). The 3 parallels of the reference PQS samples had TMA levels within premium quality from 5-14 days after slaughter. After this the TMA levels significantly increased. During 17-21 days after slaughter some parallels of the reference PQS samples reached concentrations of TMA that surpassed the suggested TMA rejection level. However, commercial shelf life of salmon stored at 4 °C is generally set to 14 days. TMA levels in salmon muscle surpassing rejection levels after 14 days will therefore have no commercial impact. The results suggest that addition of seaweed to PQS during storage significantly reduces the formation of TMA. The same trend of seaweed addition reducing TMA formation during storage was also observed for the storage experiment of SQS. The TMA levels for both reference and sugar kelp-treated samples had exceeded premium quality within day 9 after slaughter, and by day 14 after slaughter the reference sample had exceeded the rejection level of TMA in fish (figure 3.24). The TMA level of the sugar kelp-treated samples remained below the rejection level during the entire storage period. The packaging of PQS salmon within 4 hours after slaughter might be the reason for reduced TMA formation in these samples. As the SQS samples were not packed after slaughter and not covered in seaweed before day 7 after slaughter, it is suggested the process of bacterial spoilage have gotten further in the SQS fillet at the start

of the experiment compared to the PQS fillets.

The amount of formed TMA should correlate to the reduced amount of TMAO if it is a result of bacterial reduction in the salmon samples (31). The TMAO resonance signal in the spectra was observed to be overlapped with resonance signal from betaine. TMAO quantification (figure 3.25) was therefore performed by subtracting the integral value of betaine from the TMAO integral. Good correlation was shown for conversion of TMAO to TMA in the reference samples for both PQS and SQS, and the sugar kelp-treated samples of SQS. However, the TMA/TMAO correlation between the PQS sugar kelp-treated samples showed an unstable trend for TMAO concentration levels that was not paired with the expected change in TMA levels (figure 3.26). Formation of dimethylamine and formaldehyde, which are possible conversion steps for TMAO (31), was not observed in the spectra. It is suggested metabolite diffusion to the other system components (sugar kelp liquid and tissue) caused the fluctuations in concentration. As less sugar kelp liquid was applied to the 3-component system during the storage of SQS, it is suggested diffusion of metabolites from the salmon muscle was reduced compared to the PQS storage experiment. Figure 3.25 shows stable concentration levels of TMAO in PQS samples after addition of sugar kelp and the proposed metabolite diffusion. It is suggested the system of salmon muscle, sugar kelp liquid and tissue provided a condition not favorable for TMA formation, as the remaining TMAO levels in the sugar kelp-treated PQS samples was stable and not converted to TMA.

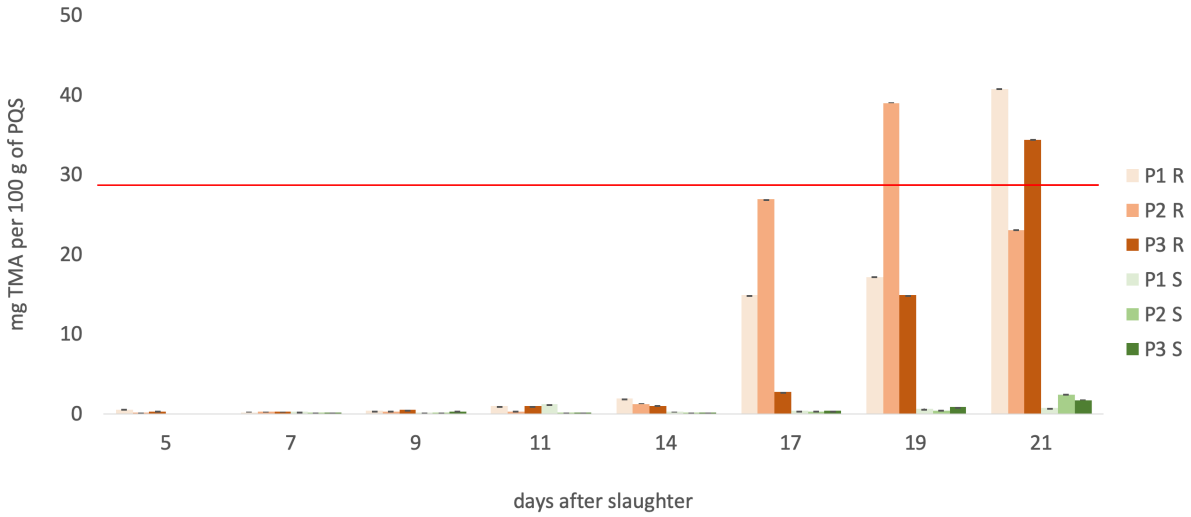

Figure 3.23. Concentrations of trimethylamine (TMA) in reference and sugar kelp-treated premium quality salmon (PQS) stored at 4 °C from day 5-21 after slaughter. Red line indicates rejection level for TMA in foods. Abbreviations: P1 R = parallel 1 reference; P1 S = parallel 1 sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

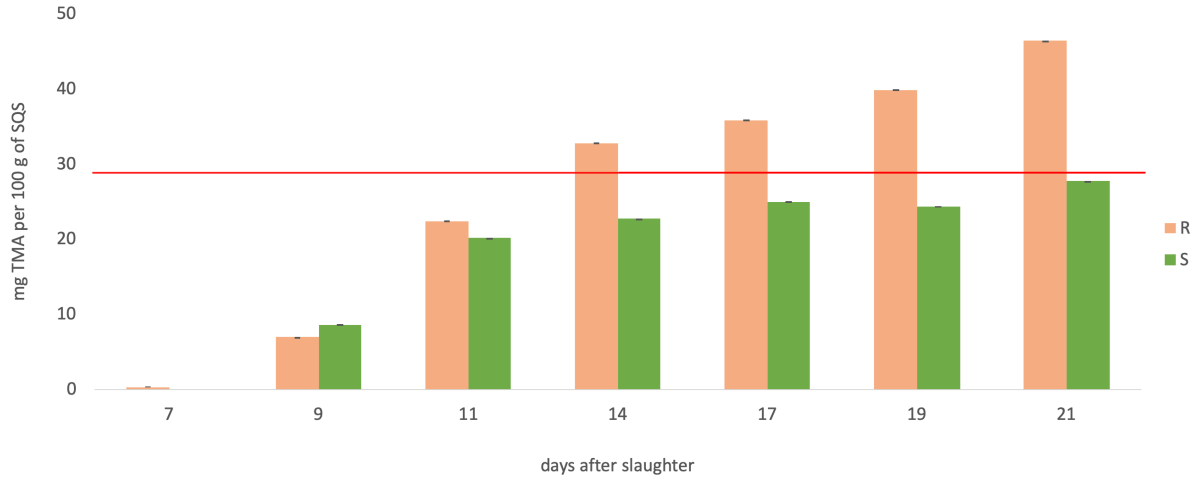

Figure 3.24. Concentrations of trimethylamine (TMA) in reference and sugar kelp-treated standard quality salmon (SQS) stored at 4 °C from day 7-21 after slaughter. Red line indicates rejection level for TMA in foods. Abbreviations: R = reference; S = sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

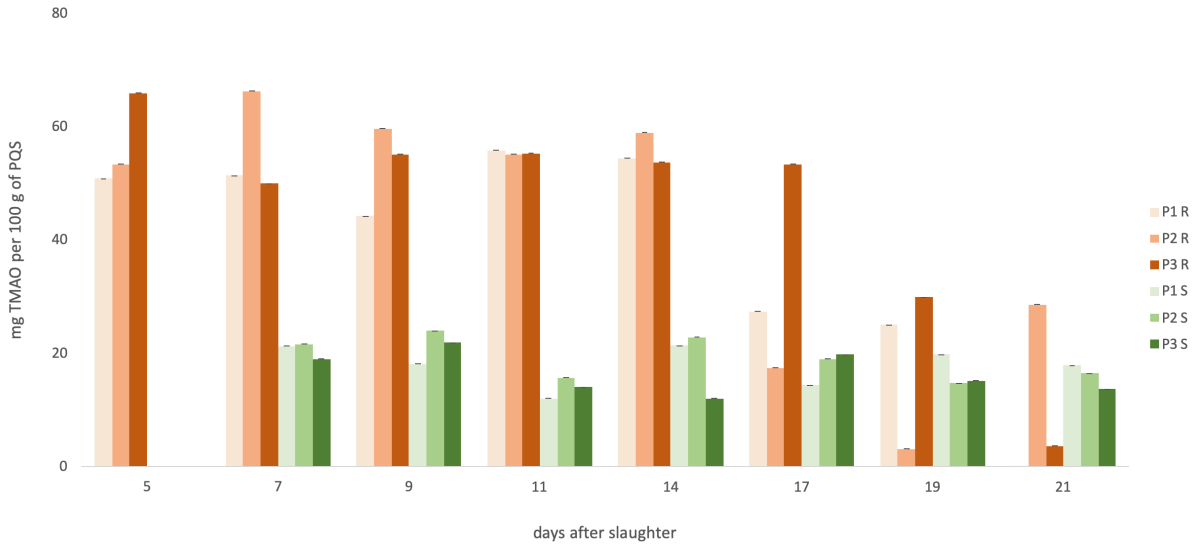

Figure 3.25. Concentrations of trimethylamine oxide (TMAO) in reference and sugar kelp-treated PQS stored at 4 °C from day 5-21 after slaughter. Abbreviations: P1 R = parallel 1 reference; P1 S = parallel 1 sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

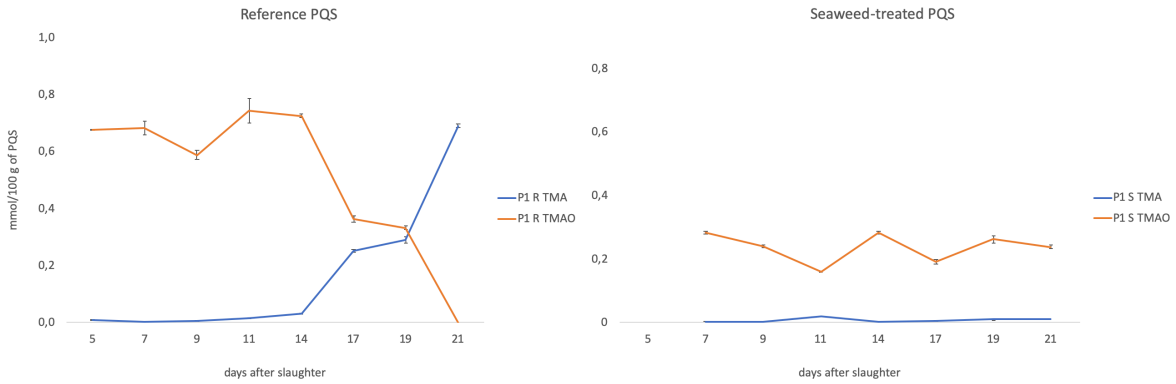

Figure 3.26. Comparison of trimethylamine oxide (TMAO) and trimethylamine (TMA) concentrations in reference (left) and sugar kelp-treated (right) premium quality salmon (PQS) for parallel 1 stored at 4 °C from day 5-21 after slaughter. TMAO – orange; TMA – blue. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

3.4.6 Formation of biogenic amines

Although biogenic amines are needed for many essential functions in humans and animals, consumption of food containing high amounts of these amines can have toxicological effects.

The level of tyramine in PQS and SQS did not exceed the proposed maximum tolerable level of 95 mg/100 g in all salmon samples (32). Formation of tyramine in PQS was only detected in parallel 3 of the reference sample. Formation of tyramine in SQS was observed from day 11-21 after slaughter in both sugar kelp-treated and reference samples (figure 3.27). The addition of seaweed increased the formation of tyramine in salmon samples on days 14-19 after slaughter compared to reference. The concentration of tyramine at day 19 after slaughter in sugar kelp-treated SQS was 5.0 mg/100 g.

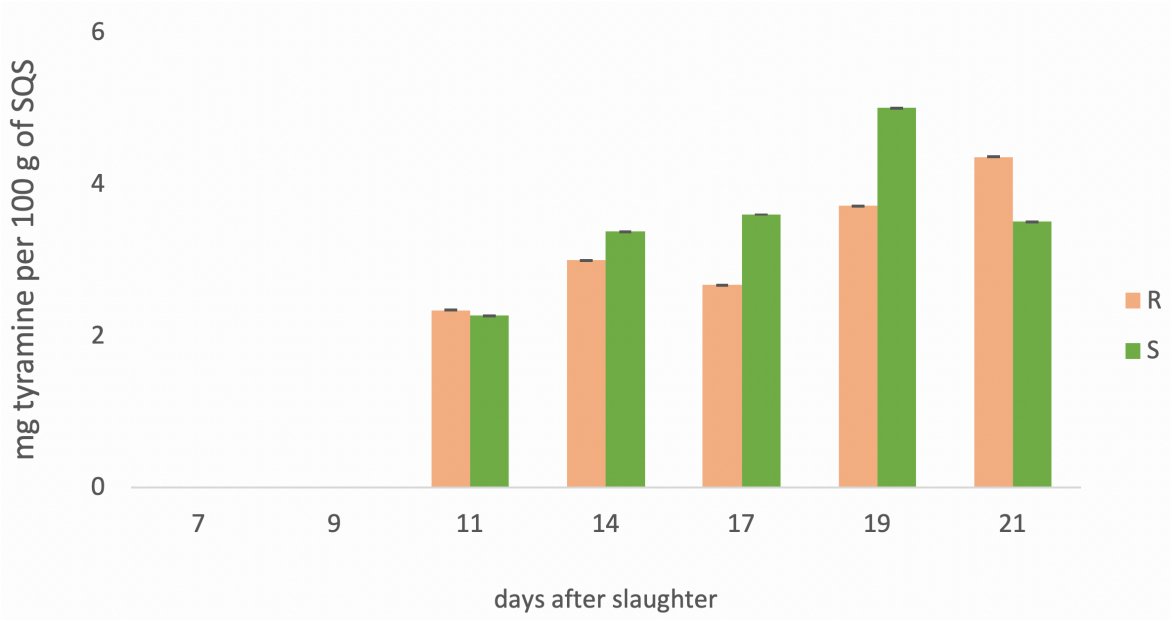

Figure 3.27. Concentrations of tyramine in reference and sugar kelp-treated SQS stored at 4 °C from day 7-21 after slaughter. Abbreviations: R = reference; S = sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

All quantified concentrations of putrescine in the SQS storage was below the rejection level of 17 mg/100 g (33), and the concentrations did not exceed 9 mg/100 g of salmon (figure 3.28). The formation was observed from day 14 after slaughter. The formation of putrescine in sugar kelp-treated samples was reduced compared to the reference samples. Due to overlapping of putrescine resonance signals with cadaverine, amount of putrescine was not quantified for the PQS storage experiment. However, it was qualitatively observed in the spectra that putrescine formation only was present in reference samples from day 17 after slaughter. Figure 3.29 shows formation of putrescine 19-21 days after slaughter in reference PQS. No formation of putrescine was observed in the PQS sugar kelp-treated samples. Formation of putrescine in reference PQS was observed in samples stored until day 17-21 after slaughter.

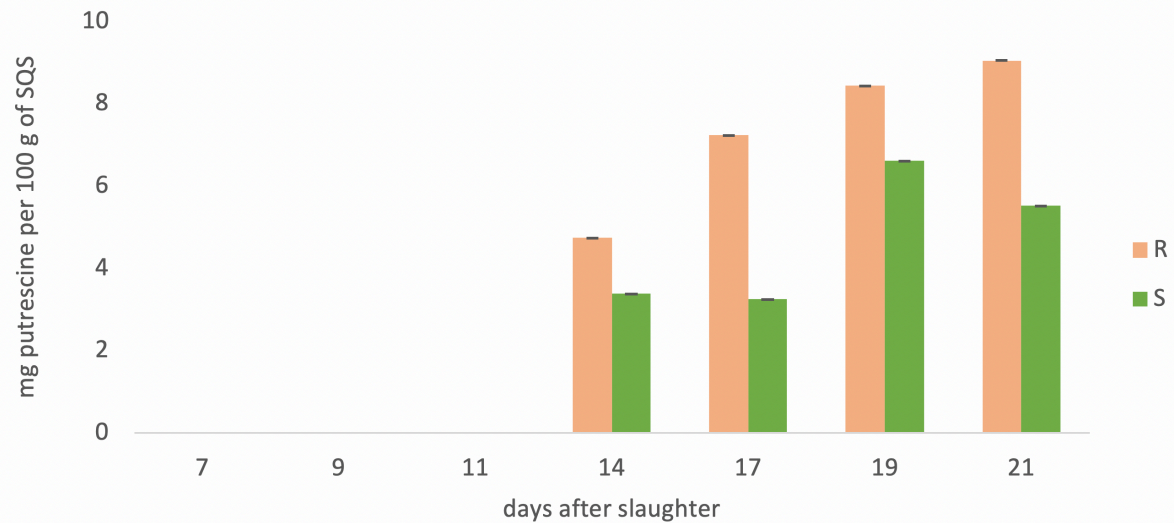

Figure 3.28. Concentrations of putrescine in reference and sugar kelp-treated SQS stored at 4 °C from day 7-21 after slaughter. Abbreviations: R = reference; S = sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

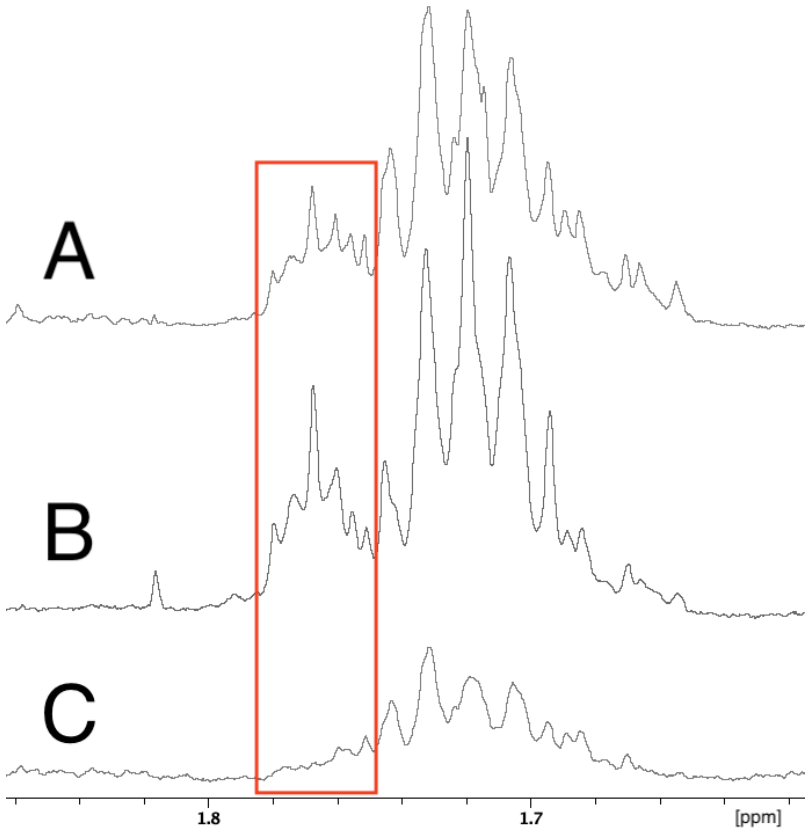

Figure 3.29. Comparison of ^{1}H NMR spectra of TCA extracted PQS muscle stored at 4 °C and sampled day 19-21 after slaughter. The picture shows the ppm range of putrescine signals. Spectrum A: reference PQS day 19 after slaughter; spectrum B: reference PQS day 21 after slaughter; spectrum C: sugar kelp-treated PQS day 21 after slaughter.

Cadaverine and its amino acid precursor lysine have overlapping signals in NMR spectra, therefore no quantification of the compounds was performed. However, it was qualitatively observed in the spectra that reference PQS samples had resonance signals at 1.46 ppm for samples stored until day 17-21 after slaughter (figure 3.30). These were overlapped by doublet alanine resonance signals at 1.48 ppm. It is suggested the resonance signals at 1.46 ppm originated from cadaverine. The resonance signals at 1.46 ppm were not observed for the sugar kelp-treated PQS samples. This suggests a reduction in formation of cadaverine in sugar kelp-treated PQS samples during storage. In the SQS samples, the resonance signals at 1.46 ppm suggested to be cadaverine were observed in both reference and sugar kelp-treated samples from day 11 of storage.

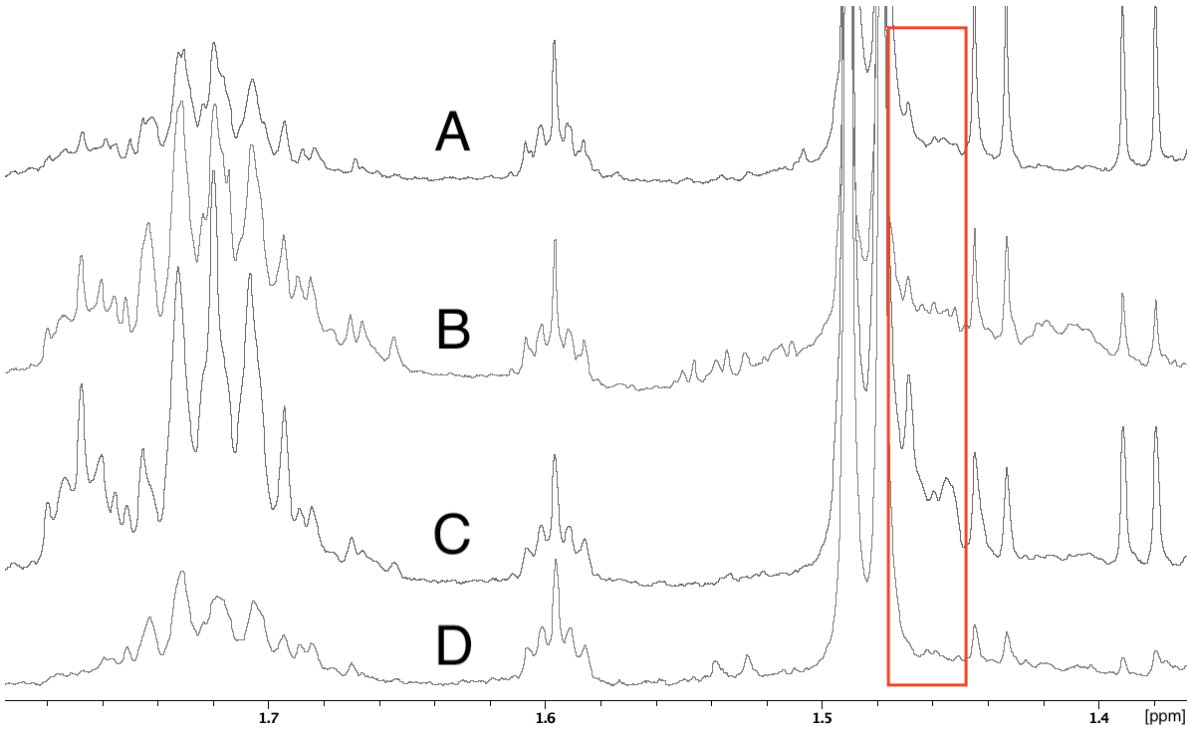

Figure 3.30. Comparison of ^{1}H NMR spectra of TCA extracted PQS muscle stored at 4 °C sampled at day 17-21 after slaughter in the ppm range of cadaverine signals. Spectrum A: reference PQS day 17 after slaughter; spectrum B: reference PQS day 19 after slaughter; spectrum C: reference PQS day 21 after slaughter; spectrum D: sugar kelp-treated PQS day 21 after slaughter.

Formation of histamine was observed during storage for both reference and sugar kelp-treated SQS. From day 11 after slaughter the quantified concentrations of histamine (figure 3.31) exceeded the proposed rejection level of 10-20 mg/100 g (34) for safe consumption for both reference and sugar kelp-treated SQS. The concentration of histamine at day 11 after slaughter was 31.99 and 22.07 mg/100 g for reference and sugar kelp-treated SQS, respectively. This result suggests the standard assigned salmon shelf life of 14 days (2 weeks) may need to be reduced to insure safe levels of histamine in standard quality salmon for human consumption. Addition of seaweed upon storage of SQS was shown to decrease the formation of histamine compared to reference.

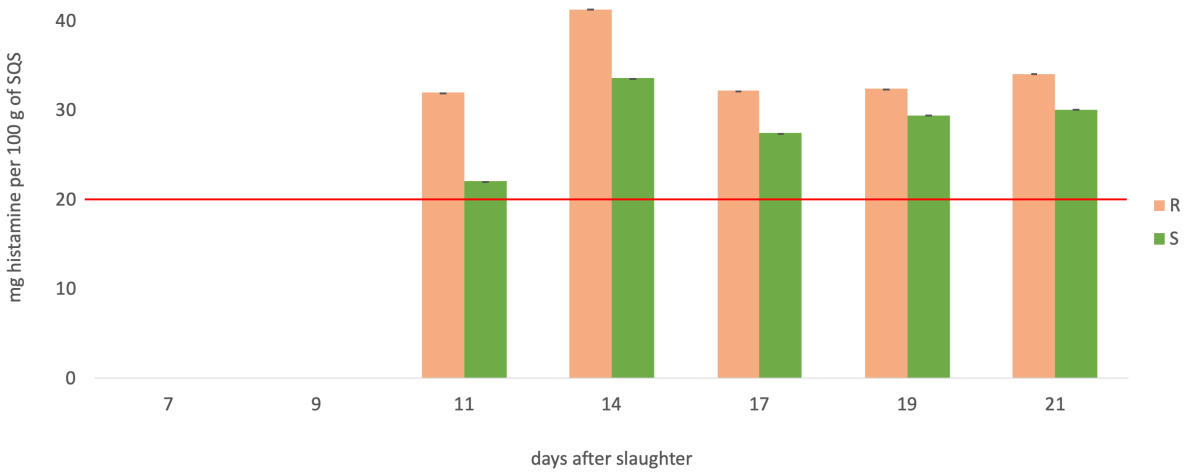

Figure 3.31. Concentrations of histamine in reference and sugar kelp-treated SQS stored at 4 °C from day 7-21 after slaughter. Red line indicates upper limit for rejection level of histamine in foods. Abbreviations: R = reference; S = sugar kelp-treated salmon. Error bars represent the standard deviation of the mean integral value used to calculate the metabolite concentration.

The difference in amount of produced biogenic amines between PQS and SQS samples is suggested to be a result of pre-rigor and post-rigor processing of the salmon after slaughter, resulting in different stages of bacterial spoilage of the PQS and SQS at the start of the experiment.

3.4.7 Carotenoid composition of salmon during storage

A distinct color change and flaky texture of homogenate was observed in sugar kelp-treated PQS at day 19 and 21 after slaughter (figure 3.32). The homogenate of the sugar kelp-treated samples was observed to have a pale white color, contrasted to the pink color of the homogenate from the reference samples. Acetone extraction of non-polar metabolites was performed for the SQS storage experiment to identify and confirm differences in carotenoid composition upon addition of seaweed in storage of salmon muscle. Figure 3.33 shows the 6.0 – 6.8 ppm range (^{1}H NMR spectral range of carotenoids) of ^{1}H NMR spectra of sugar kelp-treated (A and B) and reference (C) SQS sampled at day 17-21 after slaughter. This region contains the resonances of polyenic chains of different carotenoids. Differences in signals between spectra A and B compared to C can be observed, most prominent in the region around 6.6 ppm. It suggests that storage of salmon with the addition of seaweed influences the carotenoid composition in the salmon muscle. Resonance signals of an astaxanthin reference is also shown in figure 3.33 spectrum D, as astaxanthin is known to influence the color of salmon muscle (35, 36). Spectra A-C in figure 3.33 does not match the resonance signals of spectrum D perfectly; it is suggested other carotenoids than astaxanthin are present in the salmon samples. The difference in appearance between sugar kelp-treated and reference samples was more prominent in the storage of PQS compared to the storage of SQS. It can be results of different amounts of seaweed liquid added upon sample preparation of SQS samples. It was observed less in the SQS compared to the PQS storage

experiment, as no excess liquid was collected from the plastic zip-lock bags used to store the SQS samples in. This can suggest seaweed liquid contained compounds that were responsible for the change in carotenoid composition or loss of carotenoids in the salmon samples.

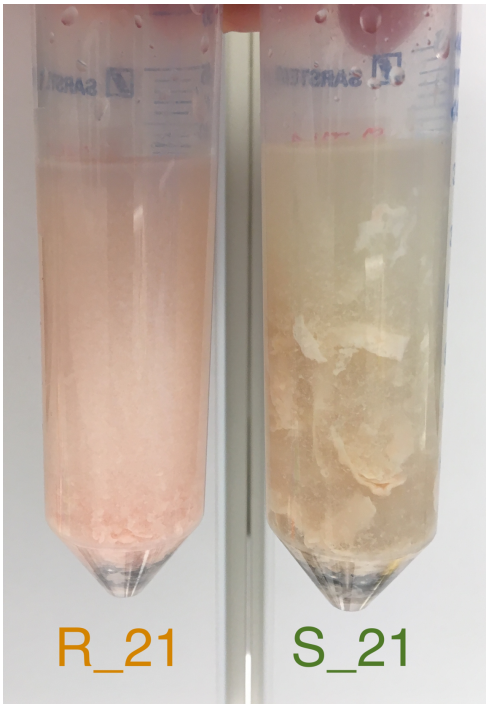

Figure 3.32. Photo of salmon homogenates during TCA extraction of reference (left) and sugar kelp-treated (right) PQS stored at 4 °C until day 21 after slaughter.

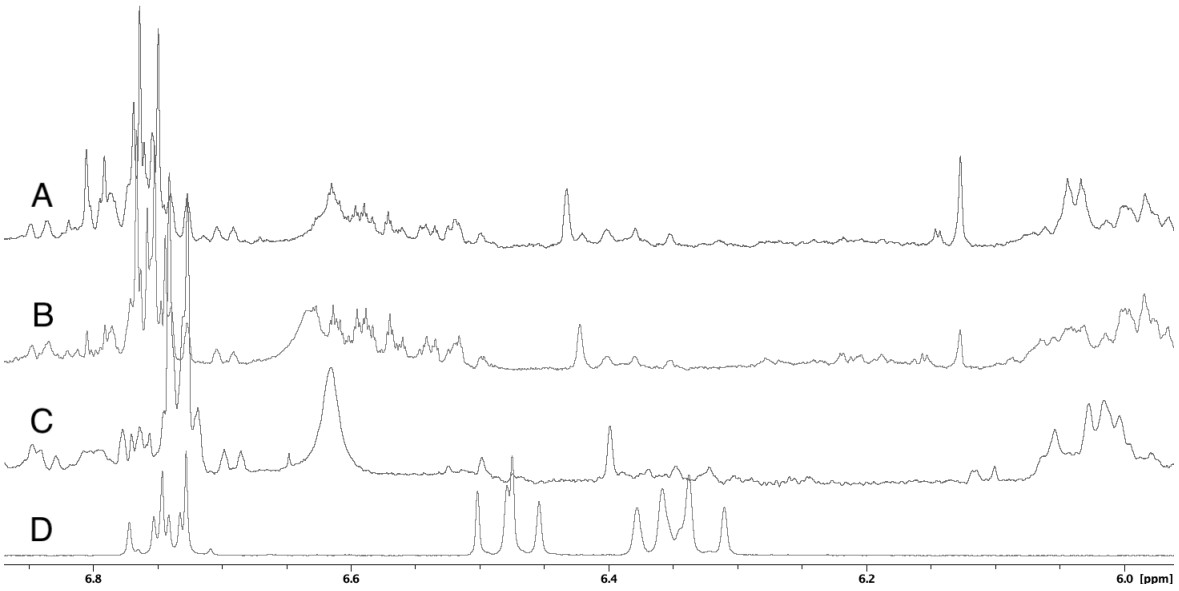

Figure 3.33. Comparison of ^{1}H NMR spectra of acetone extracted SQS muscle sampled at day 17-21 after slaughter in the ppm range of carotenoid signals. Spectrum A: sugar kelp-treated SQS day 17 of storage; spectrum B: sugar kelp-treated SQS day 21 of storage; spectrum C: reference SQS day 21 of storage; spectrum D: astaxanthin reference.

3.5 Error analysis

As the resonance signals of all spectra originating from TCA extractions are relative to the integral of the TSP signal (internal standard) of its respective spectrum, the pipetting error from addition of TSP during the experiments can be estimated. A high standard deviation from the mean TSP integral value for an experiment indicates high relative pipetting error, and it will influence quantification of metabolites. Table 3.7 summarizes selected NMR acquisition parameters used for obtaining spectra and TSP mean integral values and relative errors.

Table 3.7. Summary of selected NMR spectra acquisition parameters and calculated TSP error for the performed experiments. Abbreviations: NS = number of scans; RG = receiver gain.

Experiment	NS	RG	Mean TSP integral value	Relative TSP error (%)
Storage of sugar kelp	48	50.8	8437089.99	8.0
Fermentation of sugar kelp	48	50.8	10055240.13	4.2
Storage of premium quality salmon	48	144	20785354.15	3.5
Storage of standard quality salmon	48	144	19253595.75	2.4

4 Reflections and future prospects

4.1 Addition of sugar kelp upon storage of Atlantic salmon

Addition of sugar kelp upon storage of salmon demonstrated the creation of a 3-component system constituting of salmon muscle, sugar kelp tissue and liquid. This was not taken into account when creating the experimental design prior to carrying out the experiments. Standard amounts of added sugar kelp tissue and liquid was not applied to the salmon during sample preparation prior to storage. This made it difficult to do an accurate quantification the diffusion of metabolites between the system components. It also made the amount of applied sugar kelp tissue/liquid parameters that have had unknown effects on the metabolic changes in the salmon muscle during storage. To improve the experimental design and prevent metabolite diffusion between salmon muscle and sugar kelp components, it is suggested dried sugar kelp material could be applied to salmon muscle upon storage. This would show post-mortem metabolic changes occurring in salmon that not are results of metabolite diffusion.

However, applying sugar kelp tissue and liquid showed metabolites also were transferred from kelp/liquid and into the salmon muscle. For instance, increased concentrations of the umami-related compound succinic acid and amino acids aspartic acid and alanine in sugar kelp-treated salmon muscle within the commercial shelf life was observed. This demonstrates the possibility to change taste properties and nutritive value of salmon muscle by addition of sugar kelp upon storage. Also, though reduced concentrations of TMA and biogenic amines might have solely resulted from metabolite diffusion to other system components, it demonstrates the potential of a treatment for salmon during storage that can reduce levels of compounds that decrease salmon quality. The drawback of such a treatment would be unwanted diffusion of metabolites from the salmon muscle that reduces nutritive value and its overall quality. It should be noted that storage of salmon with sugar kelp over an extended period of time influenced sensory properties of the salmon muscle, such as color and texture. These parameters also influence salmon quality. Adding sugar kelp upon storage of salmon for shorter amounts of time is suggested to retain color and texture in salmon muscle. Even though the discoloration of SQS was less compared to the PQS, it might be correlated with the amounts of sugar kelp and liquid applied upon storage being reduced in SQS. The experimental design of the PQS storage experiment could have been improved by also carrying out acetone extraction, as the discoloration of the salmon muscle was most prominent here. The suggested anti-bacterial effects sugar kelp had on PQS were less prominent in the SQS experiment. This might suggest addition of sugar kelp is less effective when the bacterial spoilage of the salmon muscle is more advanced, which it was thought to be for the SQS fillet. Procuring salmon fillets that were fresh caught (slaughter date as close to experimental startup as possible) for the storage experiments could have given better insight on how addition of sugar kelp affected ATP degradation and if sugar kelp inhibits bacterial growth on salmon muscle during storage. As sugar kelp contains sugars such as mannitol that diffused into the salmon muscle, it can be used as substrate for fermentation by microorganisms and support growth and production of fermentation products. Increased formation of acetoin and formic acid in sugar kelp-treated SQS was observed, which can yield improved taste and arrest of degradation processes. These results substantiate the potential for a sugar kelp-treatment of salmon upon storage

to improve taste properties. Having done microbial tests during the salmon storage experiment could have been beneficial to more accurately assess how addition of sugar kelp influences bacterial growth.

4.2 Processing and fermentation of sugar kelp

The algal water extracts used in the storage experiment of sugar kelp was sent to our lab from SINTEF. They were by-products from an experiment to assess the effect of different storage conditions and processing of sugar kelp, which it experiences on ships after harvest. The experimental design used to produce the algal water extracts could have been improved to better fit the design for our lab's experiment. Exact information on weight of sugar kelp used for extraction and resulting volume of extracts was not available. Therefore, no normalization of spectra was done prior to producing bucket tables and importing them for PCA. Normalization of spectra before PCA removes differences in spectra due to weight and volume and makes them more comparable, and normalization of spectra from the salmon storage produced improved PCA results compared to non-normalized. Also, the sampling intervals between the different treated sugar kelp samples was different. Cold-stored sugar kelp was sampled at 2, 5 and 12 days of storage, while room temperature-stored samples were sampled at 2, 5 and 8 days of storage. Having the sampling intervals 2, 5, 8 and 12 days of storage for all sugar kelp samples would have made it easier to compare results. It is also suggested that more frequent sampling within 12 days of storage could have been done. This would have made it easier to accurately distinguish when post-harvest changes were results of autolysis or bacterial activity. If microbial tests were included to the experimental design, presence of microorganisms that perform mixed acid and butanediol fermentation could have been determined. This would have substantiated the indication of increased concentrations of organic acids being a result of bacterial fermentation.

As the storage of cut sugar kelp at room temperature (C-RT) gave significant metabolic changes after 5 days of storage, this information can be valuable to sugar kelp cultivators and producers within the food industry. Transport and storage of sugar kelp on board ships can influence the quality of the seaweed. It might be more cost and space effective with C-RT treatment and storage of sugar kelp, but the results from this thesis demonstrate it can significantly influence the metabolic composition of sugar kelp after several days of storage. If the goal was to have the least amount of metabolic changes in the product during longer transport and storage, C-RT sugar kelp would not be preferable. However, if C-RT storage and treatment of sugar kelp yielded improved taste and nutritive value, it could be a beneficial way for storage and treatment. For C-RT treatment of sugar kelp, microbial tests would have to be carried out to assure safety for food consumption. As fermented foods often have increased shelf life due to conditions not favorable for pathogenic microorganisms, this could be the case for C-RT sugar kelp. Increased concentrations of organic acids were observed, which could make the product acidic. This could also affect the taste of the sugar kelp product, which might be unwanted. Also, increased concentrations of amino acids might not directly improve the nutritional value. If they are just released from the algal cells due to autolysis, the same amount would also be consumed when eating whole sugar kelp. This now becomes a question of bioavailability. More research on how amino acids are absorbed in the intestine of humans would benefit this thesis. It is however

suggested that C-RT sugar kelp makes nutrients more bioavailable, as sugar kelp consists of cellulose, which humans are unable to digest. When it comes to improved taste of sugar kelp, increased concentrations of amino acids, even though it just could be a result of autolysis, is suggested to enhance taste as the amino acids will be more available to taste receptors during consumption.

4.3 Fermentation of sugar kelp

The lactic acid bacteria (LAB) fermentation of sugar kelp by different strains demonstrated production of different fermentation products. Even though fermentation by all strains increased the concentrations of some essential amino acids compared to reference, production of compounds such as acetone and inosine can negatively influence taste and odor of the fermented sugar kelp product, and they may overpower contributions from umami taste related compounds such as glutamic acid and succinic acid. To determine which strain yielded the LAB fermented sugar kelp best suited for human consumption, in terms of taste and odor, an actual taste evaluation would have to be performed. As this thesis only reports on concentration changes in compounds known in literature to influence taste and odor properties of food, the interaction of all taste components within LAB fermented sugar kelp and the overall taste of the product might differ from the suggestions made in this thesis. This also applies for the storage experiments of salmon and sugar kelp.

5 Concluding remarks

During the work on this thesis, metabolic post-harvest and post-mortem changes in sugar kelp and Atlantic salmon were characterized by NMR spectroscopy after different processing and storage conditions. Changes in metabolites which influence taste, nutritional value and shelf life of sugar kelp/salmon were used for quality determination.

Addition of sugar kelp upon storage of muscle tissue from Atlantic salmon created a 3-component system in both premium (PQS) and standard quality salmon (SQS) experiments. It constituted of salmon muscle, sugar kelp tissue and liquid. The system allowed diffusion of water-soluble metabolites between salmon muscle and sugar kelp tissue, which was facilitated by sugar kelp liquid. Diffusion between the system components in sugar kelp-treated salmon generally resulted in decreased concentrations of water-soluble metabolites. This included trimethylamine oxide (TMAO), a precursor for trimethylamine (TMA), which decrease salmon quality at too high concentrations. Reduction of concentrations of the biogenic amines putrescine and cadaverine in sugar kelp-treated salmon is suggested to result from diffusion or anti-bacterial effect of sugar kelp. However, the concentration of the biogenic amine histamine in both reference and sugar kelp-treated SQS exceed the upper rejection limit of 20 mg/100 g for food consumption at 11 days after slaughter. Addition of sugar kelp upon storage of salmon muscle also allowed succinic acid to diffuse into the salmon muscle within the commercial shelf life, which can enhance umami taste. In storage of standard quality salmon, the concentrations of acetoin and formic acid were increased compared to reference, which indicated addition of sugar kelp during storage also can promote bacterial fermentation and alter taste and odor properties of salmon. The carotenoid composition of salmon muscle was also influenced by addition of sugar kelp upon storage.

Storage of cut sugar kelp at room temperature (C-RT) had the most significant effect on the metabolome of sugar kelp during storage. The concentrations of amino acids valine, isoleucine, tyrosine and phenylalanine were increased, indicating metabolic change due to autolysis of sugar kelp tissue. The concentrations of formic acid, pyruvic acid, acetic acid and 2,3-butanediol were increased, indicating metabolic changes due to bacterial fermentation of the sugar kelp. Lactic acid bacteria fermentation of sugar kelp by strains *Lactobacillus paracasei* LUHS244, *Pediococcus acidilactici* KTU05-7 (Pa7) and *Pediococcus pentosaceus* KTU05-9 (Pp9) demonstrated sugar kelp to be a good substrate for LAB fermentation. Concentrations of essential amino acids valine and phenylalanine in the LAB fermented sugar kelp were increased compared to non-fermented sugar kelp. The concentration of glutamic acid was highest in Pp9 fermented sugar kelp. The concentration of succinic acid was highest in the LUHS244 fermented sugar kelp. Compared to non-fermented sugar kelp, the concentrations of glutamic and succinic acid in LAB fermented sugar did not increase more than 1.2 times. The strain Pp9 yielded 151.0 mg/100 g of glycerol in fermented sugar kelp, and an acetone concentration 31.3 times higher compared to non-fermented sugar kelp. The strain LUHS244 yielded 2.6 mg/100 g of inosine in fermented sugar kelp.

The performed research overall showed a high potential in using NMR for metabolic characterization of food systems. This can provide knowledge on sustainable and efficient storage of salmon using seaweeds, or optimal storage conditions for seaweeds after harvest.

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Appendix A

Table A1. Concentrations of extracted polar metabolites from processed *Saccharina latissima* stored 2-12 days at different temperatures. Metabolite concentrations are calculated based on intensity of assigned resonance signals from ^{1}H NMR spectra. Abbreviations: C-4 °C = cut sugar kelp stored at 4 °C; C-RT = cut sugar kelp stored at room temperature; W-4 °C = whole sugar kelp stored at 4 °C; W-RT = whole sugar kelp stored at room temperature.

Concentration (mg/100 g)																	
Storage time (days)																	
Compound		2				5				8				12			
	Parallel	W-4 °C	W-RT	C-4 °C	C-RT	W-4 °C	W-RT	C-4 °C	C-RT	W-4 °C	W-RT	C-4 °C	C-RT	W-4 °C	W-RT	C-4 °C	C-RT
2,3-butanediol	1	0.91	0.09	0.18	0.36	0.00	0.04	0.19	0.78	-	0.03	-	0.75	0.29	-	0.28	-
	2	2.32	0.06	0.13	0.51	0.34	0.02	0.22	1.22	-	0.05	-	1.91	0.38	-	0.41	-
	3	0.07	0.06	0.63	0.41	0.00	0.05	0.37	0.80	-	-	-	1.36	0.11	-	0.42	-
Acetic acid	1	1.23	3.00	0.41	6.75	6.91	4.34	1.00	28.61	-	8.41	-	29.81	13.15	-	32.97	-
	2	1.19	1.14	5.28	3.91	2.20	18.47	0.41	21.36	-	7.21	-	22.52	3.01	-	18.02	-
	3	0.18	1.61	1.05	4.96	1.26	25.20	0.70	18.98	-	-	-	32.35	19.97	-	11.98	-
Alanine	1	12.50	10.39	16.53	26.07	32.24	10.85	9.95	35.82	-	4.76	-	32.58	16.58	-	24.59	-
	2	16.68	16.24	13.54	11.31	14.56	6.15	11.49	25.39	-	1.06	-	14.83	13.24	-	11.39	-
	3	33.74	19.82	12.97	24.30	26.52	16.50	12.50	28.86	-	-	-	21.73	3.97	-	16.97	-
Formic acid	1	0.25	0.19	0.28	0.33	*	0.82	0.09	6.03	-	2.63	-	4.76	0.13	-	0.09	-
	2	0.10	0.07	0.09	0.20	*	4.53	0.08	3.66	-	1.04	-	0.18	*	-	0.14	-
	3	0.10	*	0.06	0.22	*	2.62	0.05	3.75	-	-	-	0.13	2.05	-	0.07	-
Glutamic acid	1	3.80	13.77	9.95	11.42	4.80	5.79	3.58	8.74	-	8.21	-	9.75	4.81	-	-	-
	2	7.11	12.87	8.61	9.19	5.99	13.84	2.18	9.12	-	5.64	-	4.72	3.41	-	3.93	-
	3	11.04	12.51	3.13	10.64	3.55	12.17	2.44	9.00	-	-	-	5.55	8.82	-	3.90	-
Isoleucine	1	0.82	0.28	0.22	0.37	0.45	0.21	0.35	2.08	-	0.09	-	2.03	0.49	-	0.20	-
	2	0.00	0.47	0.28	0.24	0.44	0.62	0.19	1.15	-	0.34	-	0.61	0.34	-	0.14	-
	3	0.39	0.62	0.49	0.64	0.51	0.32	0.29	1.91	-	-	-	1.30	0.66	-	0.26	-
Mannitol	1	2665.81	2818.46	1624.59	2186.45	549.40	68.85	1447.90	481.72	-	11.88	-	480.16	358.07	-	661.26	-
	2	957.98	401.25	2935.03	1344.64	3109.08	18.01	928.36	1608.49	-	0.00	-	1138.70	796.16	-	676.45	-
	3	1811.51	334.27	3459.69	1190.72	50.19	62.64	670.16	1011.51	-	-	-	587.87	13.19	-	1179.95	-
Phenylalanine	1	2.37	1.07	0.84	1.18	0.84	0.85	0.87	4.54	-	0.33	-	6.33	1.16	-	0.74	-
	2	1.03	1.26	0.63	0.64	0.78	1.18	0.44	2.83	-	0.68	-	1.67	0.65	-	0.28	-
	3	0.52	1.58	0.80	1.02	0.85	2.59	0.39	3.84	-	-	-	3.98	1.35	-	0.57	-

Table continued

		2				5				8				12			
	Parallel	W-4 °C	W-RT	C-4 °C	C-RT	W-4 °C	W-RT	C-4 °C	C-RT	W-4 °C	W-RT	C-4 °C	C-RT	W-4 °C	W-RT	C-4 °C	C-RT
Pyruvic acid	1	0.86	0.95	0.70	7.96	0.48	0.17	1.08	2.39	-	0.32	-	3.83	1.29	-	1.46	-
	2	0.74	0.28	0.76	4.59	0.60	0.04	0.50	0.90	-	0.17	-	12.49	0.93	-	1.77	-
	3	0.46	0.23	0.78	4.56	0.95	0.22	0.54	1.89	-	-	-	13.79	0.05	-	2.58	-
Tyrosine	1	1.02	0.31	0.47	0.58	0.47	0.35	0.41	2.96	-	0.26	-	2.93	0.64	-	0.54	-
	2	1.11	0.23	0.25	0.35	0.47	0.51	0.25	2.28	-	0.26	-	1.38	0.70	-	0.44	-
	3	0.31	0.68	0.81	0.91	0.61	1.51	0.00	2.66	-	-	-	2.44	0.69	-	0.60	-
Valine	1	1.82	0.73	1.02	1.47	1.00	0.58	0.69	4.62	-	0.23	-	4.86	0.99	-	0.82	-
	2	2.33	0.94	1.11	0.67	1.12	1.02	0.48	3.17	-	0.36	-	2.99	0.84	-	0.32	-
	3	1.02	1.27	1.52	1.83	1.16	1.41	0.80	3.39	-	-	-	4.05	1.26	-	0.82	-

** Signal-to-noise ratio (S/N) < 10*

Appendix B

Table B1. Concentrations of TCA extracted metabolites from Atlantic salmon (*Salmo salar*) muscle stored at 4 °C from day 5-21 after slaughter. Metabolite concentrations are calculated based on intensity of assigned resonance signals from ^{1}H NMR spectra. PQS and SQS: Storage experiments of premium quality salmon and standard quality salmon, respectively. Abbreviations R and S represent reference and sugar kelp-treated samples, respectively.

Concentration (mg/100 g)																	
Days after slaughter																	
Compound																	
		5		7		9		11		14		17		19		21	
PQS	Parallel	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Alanine	1	35.82	-	39.76	74.68	40.45	48.85	45.89	49.65	35.12	75.45	39.96	62.81	32.73	31.62	58.30	42.69
	2	28.70	-	32.22	48.95	32.82	43.70	39.71	50.02	31.67	58.00	39.31	57.76	46.83	50.77	55.80	55.74
	3	38.78	-	42.63	50.95	48.96	81.42	43.20	58.45	43.65	73.09	25.99	61.51	39.84	87.92	62.04	39.03
Anserine	1	671.55		728.22	304.35	714.22	319.10	700.59	223.65	572.00	292.02	631.43	194.52	580.65	216.93	554.70	244.89
	2	652.74		565.03	252.94	575.58	305.21	617.29	218.53	539.32	263.32	570.32	220.17	593.39	127.99	520.58	200.97
	3	774.76		751.42	341.72	757.25	347.63	687.16	205.95	647.99	198.59	637.31	194.94	574.51	208.04	600.39	195.33
Aspartic acid	1	0.00	-	0.00	4.17	0.00	3.30	0.00	1.67	0.00	3.79	0.00	3.19	1.61	0.00	0.00	0.00
	2	0.00	-	0.00	3.37	0.00	2.78	0.00	3.34	0.00	5.47	0.00	3.54	0.00	0.96	3.20	0.00
	3	0.00	-	0.00	2.60	0.00	2.14	0.00	3.64	0.00	6.06	0.00	0.89	0.00	1.91	0.00	0.64
β-Alanine	1	3.35	-	5.00	4.48	12.45	2.87	19.48	2.30	18.97	3.68	14.36	8.99	14.27	5.62	22.51	4.17
	2	2.58	-	4.77	1.08	6.61	3.37	9.04	2.37	13.17	4.09	11.73	5.16	8.53	4.44	12.97	4.58
	3	2.57	-	4.95	1.52	9.32	4.31	11.85	1.99	16.77	3.65	6.88	5.89	9.99	4.55	14.46	4.85
Betaine	1	8.58	-	10.00	2.41	10.46	4.46	12.52	3.68	14.42	4.10	22.06	3.86	29.49	7.70	28.82	8.92
	2	7.21	-	6.81	2.31	7.18	3.62	7.14	2.41	12.82	4.01	27.85	3.40	36.83	2.59	47.02	9.89
	3	8.02	-	10.06	3.42	10.26	5.29	9.84	2.53	10.16	2.75	38.93	6.80	32.35	4.73	40.16	9.16
Creatine/ phosphocreatine **(mmol/100 g)**	1	3.77	-	3.99	1.48	3.96	1.57	3.37	1.07	3.09	1.41	3.58	1.15	3.38	1.32	3.02	1.31
	2	3.63	-	3.51	1.36	3.41	1.59	3.43	1.09	3.32	1.46	3.43	1.19	3.70	0.74	3.01	1.22
	3	3.47	-	3.76	1.52	3.97	1.62	3.55	0.96	3.43	0.95	3.18	1.00	3.04	1.03	2.95	1.01
Glycine	1	12.92	-	15.00	3.88	14.25	3.27	13.46	3.78	11.09	5.47	11.48	2.98	11.14	3.38	15.31	3.97
	2	11.94	-	12.49	3.80	11.18	4.86	14.36	4.31	10.75	4.72	11.90	3.82	16.00	1.85	25.42	4.23
	3	14.42	-	16.17	4.79	16.06	6.57	16.40	2.64	13.95	4.62	13.94	3.97	16.31	5.02	21.84	3.62
Hypoxanthine	1	7.16	-	20.00	6.57	15.00	6.14	20.54	5.98	20.82	6.70	34.04	8.26	49.65	9.64	62.02	7.42
	2	7.62	-	18.52	7.55	10.32	5.73	13.68	5.28	15.00	8.86	55.79	8.04	76.23	9.71	53.53	17.47
	3	7.72	-	22.87	5.92	17.03	7.60	18.61	4.85	20.17	6.45	32.88	4.24	63.48	10.65	62.30	12.48

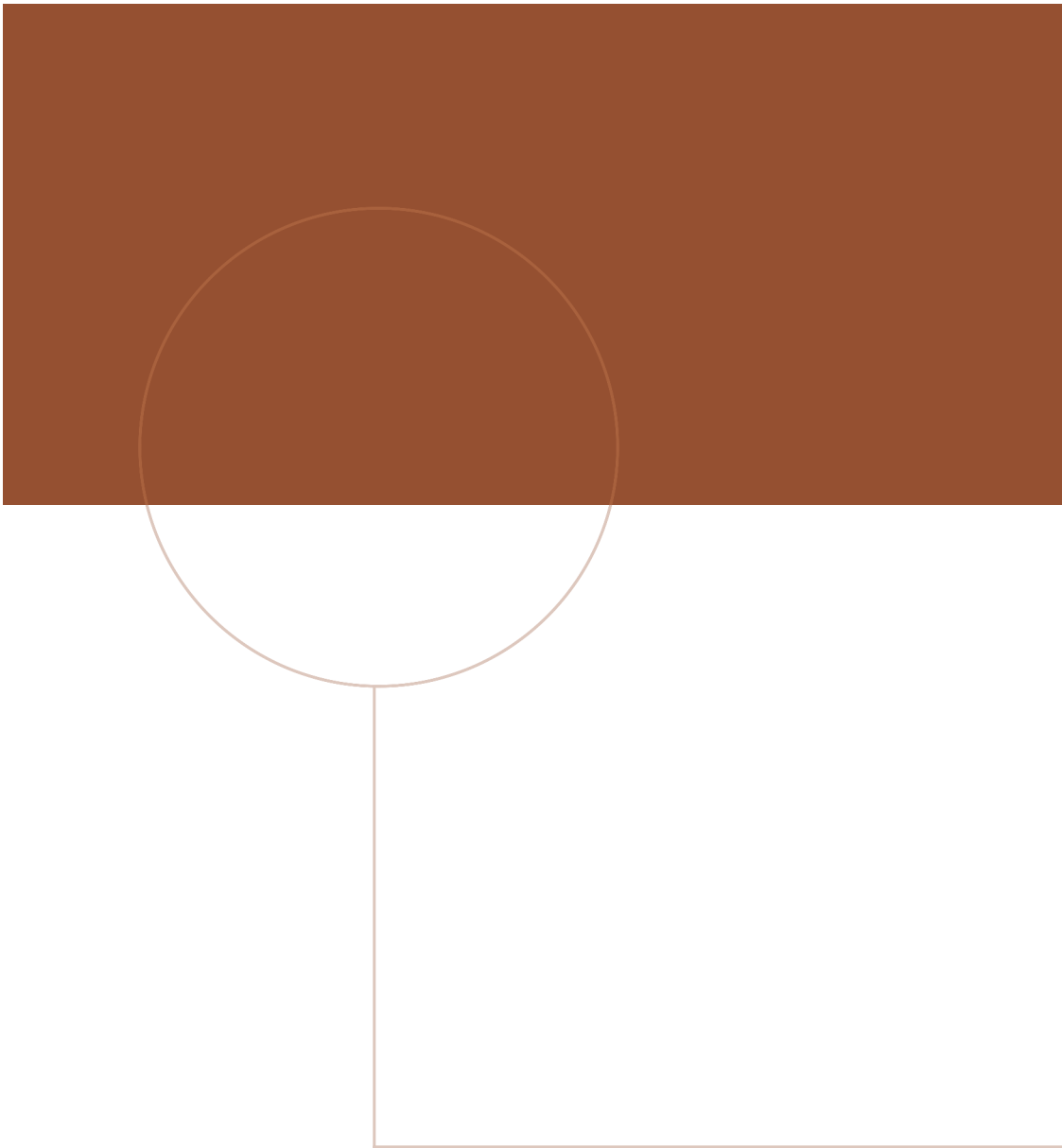
Table continued

Compound		5		7		9		11		14		17		19		21	
	Parallel	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Inosine	1	110.71	-	117.85	81.51	181.74	89.96	177.02	62.72	128.49	79.13	115.07	59.66	50.35	28.15	10.27	41.06
	2	128.95	-	136.13	69.73	159.97	103.49	184.19	72.14	159.10	87.23	65.76	62.77	21.41	22.00	15.50	16.20
	3	141.51	-	140.81	88.91	193.67	109.70	195.66	61.00	171.78	60.51	80.21	34.09	30.13	32.20	12.57	12.54
IMP	1	102.70	-	132.37	8.43	8.07	20.04	7.43	4.13	7.77	0.00	7.72	0.00	6.11	0.00	2.50	0.00
	2	103.38	-	58.67	19.10	34.40	7.13	5.56	1.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3	120.59	-	116.16	24.71	47.29	8.39	6.28	0.00	7.03	0.00	8.37	0.00	4.66	0.00	3.00	0.00
Lactic acid	1	576.51	-	613.29	219.11	652.26	250.87	643.03	182.97	468.45	225.64	527.62	179.59	304.69	116.81	360.98	146.79
	2	588.61	-	506.47	180.55	545.75	253.77	568.22	178.19	485.83	221.04	454.54	167.49	365.82	102.09	159.40	143.21
	3	633.48	-	578.85	207.80	674.83	278.22	593.79	147.24	530.04	153.36	218.87	102.29	163.72	137.20	280.87	48.65
Mannitol	1	0.00	-	0.00	303.00	0.00	142.25	0.00	147.36	0.00	223.74	0.00	642.18	0.00	112.66	0.00	144.45
	2	0.00	-	0.00	257.48	0.00	168.25	0.00	229.00	0.00	513.98	0.00	514.37	0.00	382.38	0.00	236.53
	3	0.00	-	0.00	125.65	0.00	219.55	0.00	366.80	0.00	459.45	0.00	172.09	0.00	530.34	0.00	257.82
Nicotinic acid	1	6.89	-	7.14	2.79	7.93	3.26	8.57	2.46	7.16	3.87	7.69	2.52	7.12	2.05	7.14	2.82
	2	7.26	-	6.32	2.75	7.03	3.87	8.34	2.80	7.46	3.69	7.98	2.87	7.89	1.50	6.82	2.80
	3	7.93	-	8.02	3.05	8.03	3.55	7.82	2.31	8.02	1.94	7.84	2.45	6.32	2.10	6.94	1.74
Phenylalanine	1	3.63	-	5.46	2.34	4.39	3.63	5.81	3.38	5.53	5.38	6.40	2.99	11.67	4.24	11.34	6.02
	2	2.98	-	6.03	3.01	5.00	3.01	6.59	3.51	6.15	4.53	8.81	3.69	17.10	2.08	27.77	4.42
	3	4.66	-	5.23	3.29	5.23	3.57	6.45	2.46	5.54	3.82	7.21	2.80	13.36	3.89	18.58	4.92
Succinic acid	1	1.37	-	0.18	8.51	0.00	3.11	0.00	3.70	0.17	2.49	1.47	1.86	4.41	0.34	21.26	0.92
	2	0.86	-	0.22	4.94	0.00	3.17	0.00	4.60	0.08	2.90	5.64	3.43	19.07	1.39	7.68	3.82
	3	0.36	-	0.00	5.48	0.00	4.86	0.00	4.44	0.11	5.46	0.29	0.87	1.98	4.99	14.35	1.29
TMA	1	0.52	-	0.15	0.22	0.36	0.11	0.95	1.17	1.90	0.23	14.83	0.37	17.18	0.61	40.75	0.72
	2	0.08	-	0.24	0.06	0.29	0.09	0.28	0.05	1.22	0.10	26.85	0.30	38.96	0.40	23.07	2.39
	3	0.26	-	0.20	0.13	0.47	0.28	0.90	0.13	1.01	0.12	2.70	0.34	14.82	0.81	34.35	1.72
TMAO	1	50.85	-	51.30	21.25	44.16	18.08	55.81	12.02	54.43	21.28	27.32	14.38	24.97	19.72	0.00	17.87
	2	53.33	-	66.23	21.56	59.66	23.96	55.07	15.66	58.93	22.83	17.42	19.00	3.04	14.68	28.60	16.46
	3	65.95	-	49.95	18.93	55.08	21.84	55.27	13.99	53.69	11.97	53.36	19.77	29.86	15.13	3.63	13.61
Tyrosine	1	6.41	-	8.26	2.65	6.88	5.24	8.01	3.77	7.35	6.04	8.51	2.81	11.75	4.76	12.82	6.32
	2	6.64	-	6.46	3.95	6.93	4.11	8.87	4.13	7.50	4.69	10.50	3.85	18.82	2.15	30.84	4.30
	3	7.98	-	8.44	4.80	10.25	5.97	10.00	3.74	8.57	4.12	10.24	3.73	11.19	4.24	8.29	4.92
Valine	1	8.19	-	9.76	5.07	9.39	5.16	12.16	4.73	8.95	7.84	9.98	7.03	15.58	6.86	14.50	8.59
	2	9.50	-	10.32	5.34	9.83	6.01	11.87	5.41	9.89	7.21	13.29	6.08	23.15	4.04	33.22	8.51
	3	11.17	-	10.94	5.62	12.37	7.57	12.11	5.12	11.16	6.31	12.79	6.38	18.18	7.07	22.43	8.71

Table continued

				7		9		11		14		17		19		21	
SQS	Parallel			R	S	R	S	R	S	R	S	R	S	R	S	R	S
2,3-butanediol	1	-	-	0.12	-	1.27	4.72	96.78	57.37	119.28	111.50	131.18	95.74	124.69	74.89	124.23	90.04
Acetoin	1	-	-	0.00	-	4.47	7.78	24.47	25.18	17.39	37.21	27.14	45.75	25.78	33.76	22.95	44.56
Alanine	1	-	-	65.42	-	71.11	76.36	88.59	76.97	86.78	84.91	82.55	83.95	85.54	87.61	87.10	84.53
Anserine	1	-	-	580.57	-	488.55	556.42	603.14	477.29	679.32	540.87	596.26	433.81	618.26	423.81	646.31	378.07
β-Alanine	1	-	-	19.78	-	18.61	19.46	24.97	18.80	27.23	24.24	24.81	22.29	24.12	22.62	25.81	25.63
Betaine	1	-	-	12.04	-	14.61	11.34	13.48	8.47	13.30	9.96	13.53	8.21	11.54	7.50	12.72	10.76
Ethanol	1	-	-	2.64	-	1.75	2.03	15.73	3.83	20.18	5.20	15.46	6.99	20.36	9.59	21.87	8.75
Formic acid	1	-	-	0.26	-	0.65	1.50	12.87	23.55	0.82	28.67	0.81	19.47	0.50	23.84	0.95	4.07
Fumaric acid	1	-	-	0.43	-	*	*	0.34	*	0.47	*	0.49	*	0.50	*	*	*
Glycine	1	-	-	47.41	-	45.61	38.73	52.61	33.37	50.40	40.72	52.27	36.42	54.39	34.79	55.81	44.09
Histamine	1	-	-	0.00	-	0.00	0.00	31.99	22.07	41.32	33.61	32.18	27.47	32.41	29.47	34.10	30.07
Hypoxanthine	1	-	-	17.23	-	26.54	28.76	88.21	45.95	93.83	42.26	80.06	21.36	82.82	26.51	85.22	28.13
Inosine	1	-	-	116.04	-	121.68	116.28	8.05	6.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IMP	1	-	-	70.35	-	1.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mannitol	1	-	-	0.00	-	0.00	52.41	0.00	151.47	0.00	114.94	0.00	139.94	0.00	185.30	0.00	94.09
Nicotinic acid	1	-	-	5.69	-	4.99	5.80	6.98	5.17	8.18	6.68	7.03	5.37	6.91	5.17	7.82	5.37
Putrescine	1	-	-	0.00	-	0.00	0.00	0.00	0.00	4.74	3.38	7.22	3.24	8.43	6.60	9.04	5.53
Succinic acid	1	-	-	0.21	-	0.13	0.61	3.10	4.09	9.46	9.68	10.09	17.19	14.81	20.24	22.49	21.96
TMA	1	-	-	0.28	-	6.91	8.57	22.38	20.14	32.75	22.69	35.84	24.96	39.85	24.25	46.36	27.69
TMAO	1	-	-	17.43		14.08	9.07	0.00	1.49	0.00	0.00	0.46	0.21	0.08	0.63	0.00	0.40
Tyramine	1	-	-	0.00	-	0.00	0.00	2.34	2.27	3.00	3.39	2.68	3.61	3.72	5.00	4.36	3.51
Tyrosine	1	-	-	7.00	-	5.82	7.60	0.95	1.36	0.75	0.84	1.32	1.11	1.01	1.14	1.07	1.03
Uracil	1	-	-	0.67	-	0.58	0.70	0.00	0.00	0.00	0.53	0.36	1.14	0.82	1.45	1.13	1.18

** Signal-to-noise ratio (S/N) < 10*



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