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Determination of quality metabolites in different brands of salmon fillets

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Preface

This master´s thesis was written at the Department of Biotechnology at Norwegian University of Science and Technology (NTNU) in Trondheim, Norway. The thesis is the end of a 2-year master´s program in Cell and Molecular Biology at NTNU.

Firstly, I would like to thank to my co-supervisor Dr. Elena Shumilina, who has been always very helpful and patient with me. I would never have been able to finish my master´s thesis without her guidance, support and motivation to finish the research for writing this thesis.

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Finally, special thanks go to my beloved family for spiritually supporting and encouraging me throughout these years. I would not have been able to study in Norway and write this thesis without all the love and support they have given me daily.

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Abstract

Fish offer a multitude of nutritional benefits and its world´s food supply has grown rapidly in the last years. The quality of fish is an important factor for the fish industry and consumers, this field is required to be studied more for further improvement and development of the fish industry. Salmon nowadays is a popular food product and its aquaculture is one of the fastest growing food production systems in the world. In this thesis, fillets of Atlantic salmon (*Salmo salar*) stored at 4 °C for two weeks were studied. In literature reviews, post-mortem biochemical processes and their changes which can be related to autolysis or bacterial activity were summarized. The normal quality fish processing and high quality fish processing were compared and described. This thesis offers a method that can be used to monitor and determine the quality of salmon. The post-mortem biochemical processes in normal quality and high quality salmon were monitored and compared through the storage. NMR is a reliable and popular tool used for food quality control, and together, 13 water soluble metabolites belonging to amino acids, organic acids, sugars, alcohols, nucleotides, biogenic amines and vitamins were analyzed and quantified using NMR spectroscopy. Some of the most important metabolites were monitored using mass spectrometry. The obtained results showed significant difference between normal and high quality salmon.

Keywords: *NMR, fish quality, salmon, post-mortem biochemical processes.*

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Abbreviations

ADP	adenosine- 5´-diphosphate
AMP	adenosine- 5´-monophosphate
ATP	adenosine- 5´-triphosphate
BA	biogenic amines
BMRB	Biological Magnetic Resonance Data Bank
cfu	colony forming units
DMA	dimethylamine
D_2O	deuterium oxide
EM	electromagnetic
FA	formaldehyde
FIA	flow injection analysis
FIDs	Free Induction Decays
FWHM	full width at half maximum
HMDB	Human Metabolome Database
HMDS	high definition mass spectrometer
HQ salmon	high quality salmon
Hx	hypoxanthine
IMP	inosine-5´-monophosphate
Ino/HxR	Inosine
LA	lactic acid
LAB	lactic acid bacteria
MS	mass spectrometry

MVA	multivariate analysis
NAM	niacinamide
NaOH	Sodium hydroxide
NMR	nuclear magnetic resonance
NPN	non-protein nitrogen compounds
NQ salmon	normal quality salmon
NTNU	Norwegian University of Science and Technology
PCA	Principal component analysis
RF	radiofrequency
SIMCA	Soft Independent Modeling of Class Analogy
S/N	signal-to-noise ratio
TCA	trichloroacetic acid
TMA	trimethylamine
TMAO	trimethylamine oxide
TSP	3-trimethylsilyldeuterosodium propionate
U	uric acid

1 Literature

1.1 Post-mortem biochemical processes in fish

During storage, fish undergo several changes. In the beginning, it is changes that are perceived with senses, mainly appearance and texture. The biggest change starts with the onset of rigor mortis. After a fish is killed, its muscle has limp elastic texture and remains relaxed for several hours. With the onset of rigor mortis, muscle starts to become hard and stiffen. Rigor mortis can last for a day or more. The time span of rigor mortis can be affected by specie and several factors such as temperature, handling, size and physical condition of the fish, which influence the persistence of rigor mortis (Huss, 1995). Usually, rigor mortis in fish begins to set in 1 to 6 hours after death (Tejada, 2009), while in Atlantic salmon it begins 8 hours after death and is commonly resolved after 60 to 70 hours after death when stored at 0°C (Wang et al., 1998). According to another study, Atlantic salmon under stress has a later onset of rigor mortis, from 4 to 24 hours. While rigor mortis in electrically stimulated fish sets in about 2 to 4 hours after death (Roth et al., 2006). In general, it has been proposed that rigor mortis sets in more rapidly at higher temperatures (Poulter et al., 1982).

There have been accepted S-curve patterns for deterioration of chilled fish stored in ice which is divided in 4 phases (Huss, 1976a). The shape of this pattern, shown in Figure 1, indicates a fast degradation during Phase 1 and 4, and linear slower degradation during Phase 2 and 3. In Phase 1, a pleasant and sweet taste typical for fish is present, maximized during 2 to 3 days after harvesting. Past this period, the flesh becomes neutral and starts to lose its typical taste and odor due to autolysis. In Phase 3, approximately 6 days after harvesting, unpleasant volatile compounds like trimethylamine (TMA) are produced due to bacterial activity. For phase 3, it is typical that the fish off-flavour becomes sour, fruity and slightly bitter, which turns later to a sickly sweet, ammoniacal smell. According to Huss (1976a), fish is spoiled and not acceptable for human consumption after about 12 days.

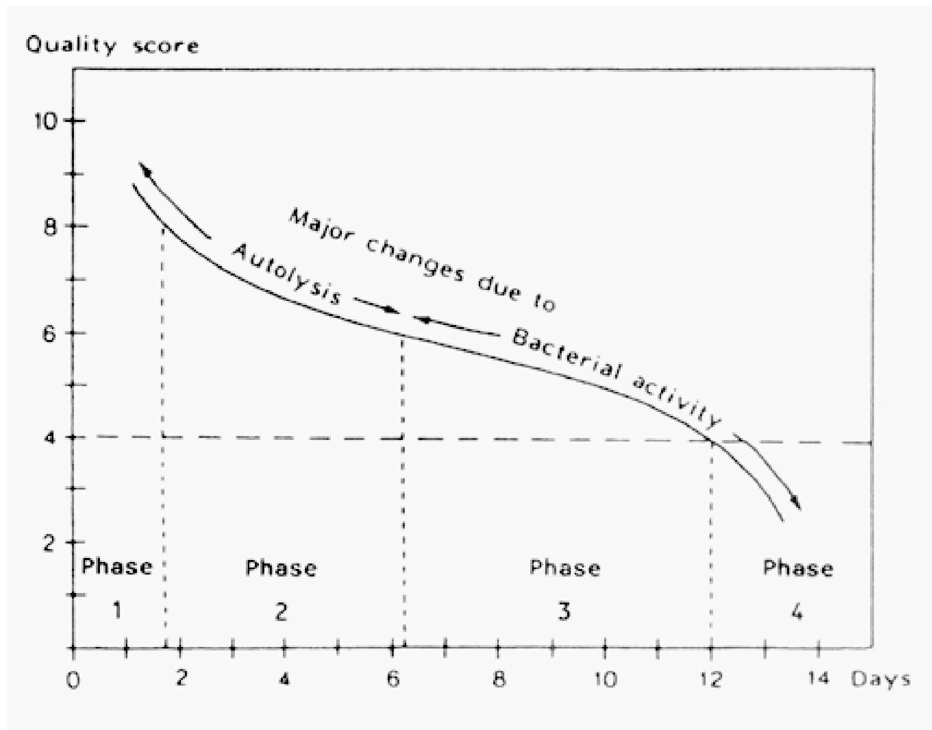

Figure 1: Major changes in quality of the fish occurs due to autolysis and bacterial activity. The quality score appropriates specific freshness of the fish. Quality score 10 at *y* axis indicates the maximum freshness, 8 indicates good quality and 6 it is for a neutral tasteless fish. The spoiled fish is at score 4. The graph is S-shaped and indicated fast degradation during Phase 1, linear rate during Phase 2 and 3, and high rate in Phase 4, when the fish is spoiled (Huss, 1976a).

All those major changes in quality resulting in fish spoilage occurs due to autolysis or bacterial activity.

1.1.1.1 Changes due to autolysis

Autolysis means "self-digestion" of its own cell and occurs due to the action of enzymes, therefore referred to as enzymatic activity. After death, the normal level of ATP in fish is depleted. These lower levels of ATP trigger rigor mortis. With onset of rigor mortis, the pH of the muscle drops and thus largely influences the physical properties of the muscle. The muscle loses its limp elastic texture and some of its water-holding capacity. One of the first autolytic changes, firstly observed by Saito et al. (1959), is degradation of ATP-related compounds. The degradation of ATP in fish muscle occurs by the action of enzymes: ATP-ase, myokinase, AMP deaminase, IMP phosphorylase, nucleoside phosphorylase, inosine nucleosidase and xanthine oxidase (Gill, 1992). Surette et al. (1988) confirmed by studies of sterile and non-sterile cod, that the degradation of ATP through to inosine occurs entirely due to autolytic changes. They

also proposed that handling process does not have any coherence with acceleration of autolytic changes.

There are also some proteolytic enzymes which are involved in autolytic changes. They are responsible for proteolytic enzymes resulting in softening or gapping of the muscle tissue. One of the main proteases are cathepsins, which become active and released into the cell juices upon physical abuse or upon freezing and thawing of post mortem muscle. The calpains in fish breaks down proteins of the myofibril that result in tenderization of the fillets (Muramoto M. et al., 1989, Tejada, 2009). The other enzymes, which change the structure of the fillets are collagenases which break down the myotome and cause “gapping” of the fillets. It usually happens if the fillets are stored at high temperature even for a short time, or stored on ice for too long (Sato et al., 1991).

1.1.1.2 Changes due to bacterial activity

The immune system of fish prevents the growth of bacteria in the flesh in newly-caught fish. There are many different microorganisms found on the outer surfaces, such as on the skin and gills, and as well as in the intestines. The microorganisms vary according to species, but also due to the environmental conditions the live fish were caught from. It has been stated by Liston (1980) that 10^2-10^7 cfu (colony forming units)/cm^2 is a normal range of organisms on the skin surface. When the immune system collapses after fish is killed, bacteria starts to spread freely and invade the flesh. As mentioned by Huss (1995), there should be counted on a difference between spoilage flora and bacteria. Spoilage flora represents only bacteria that is presented on the flesh when the fish spoils, but directly do not participate on the spoilage process, whereas the latter is involved in the production of off-odors and off-flavours during the spoilage. Each fish has its own spoilage bacteria and it requires better studying of sensory, microbiological and chemical properties to find out which of the bacteria is responsible for the spoilage of a particular fish.

Rasmussen et al. (2002) studied the shelf life of Atlantic salmon and proposed 6-7 days to be the time until bacteria reach 10^7 cfu/g, therefore the salmon is spoiled after this point. They observed *Pseudomonas* and *Moraxella* in aerobically stored salmon, and predominant colonies of Gram-positive bacteria such as lactic acid bacteria and coryneform, were observed in salmon stored under anaerobic conditions. In other studies, by Leroi et al. (1998) who studied smoked

Atlantic salmon, Gram-negative bacteria, mainly *S. putrefaciens*, was observed immediately after the smoking process. During storage *S. putrefaciens* has decreased, while Gram-positive bacteria, mostly LAB, increased (Leroi et al., 1998). As reviewed by the same authors, the packaging of smoked salmon in vacuum seems to inhibit *S. putrefaciens* and *Pseudomonas spp*., but increase CO^2-resistant bacteria such as *P. phosphoreum. S. putrefaciens* might be involved in spoilage in smoked salmon. As mentioned in the review, the domination of LAB, which grows well under anaerobic conditions, was observed by several authors who connected LAB with spoilage and off-odors. In the study by Leroi it was unlikely, but they suggested *Brochotrix spp*. to be involved in smoked salmon spoilage with strong sour and cheesy odors, however further studies were suggested.

1.1.2 ATP degradation

In first hours after fish is killed, adenosin-5'-triphosphate (ATP) in the fish muscle is degraded to its catabolites by endogenous enzymes. Therefore, there is a formation of adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), inosine-5′-monophosphate (IMP), inosine (Ino or HxR) and hypoxanthine (Hx) that degrades to xanthine (Xa) and uric acid (U) (Tejada, 2009).

$$ATP \rightarrow ADP \rightarrow AMP \rightarrow IMP \rightarrow Ino \rightarrow Hx \rightarrow Xa$$ (Howgate, 2006)

Nucleotides determine taste and flavour of the meat of the fish. IMP is one of the products of ATP degradation, it is responsible for the fresh flavour in the meat of the fish. It has a very sweet and pleasant taste. These pleasant properties are decreased with the degradation of IMP to Ino and Hx. Subsequently, bitter flavours are presented (Tejada, 2009). It was reported by Spinelli (1965), that there were no observed changes in bitterness with increasing quickly concentrations of Ino and Hx. It has been proven that ATP is degraded to IMP very quickly, while degradation of IMP occurs at a slower rate (Tejada, 2009).

Concentrations of ATP and following IMP may vary and are dependent upon factors such as harvest season as well as fish rearing methods. ATP concentration maybe often affected by aquaculture cultural practices or genetic fish selection. With higher concentrations of ATP,

there are also higher concentrations of IMP during the time of consumption. It is these higher levels of IMP that cause the more pleasant taste in the fish to be present (Howgate, 2006).

The products of ATP degradation are often used for quantification and evaluation of the quality loss and freshness of fish during storage above freezing temperatures. The *K-index* based on these products seems to be assumed as one of the best techniques to grade fish freshness (Tejada, 2009). High resolution NMR is a very useful tool for the determination of metabolites needed for calculation of the *K-index* (Shumilina et al., 2015). The *K-index* is defined as (Saito et al., 1959):

$$K - index\ (\%) = \frac{\mathrm{HxR + Hx}}{\mathrm{ATP + ADP + AMP + IMP + HxR + Hx}}\ x\ 100$$

K-index shows linear responses with time during storage (Tejada, 2009). Increasing values are reported at very early stages of storage. With increasing *K-index*, the freshness of the fish decreases. The *K-index* varies also with fish species. It has been shown that ATP, ADP and AMP concentrations are not required for other indices which also evaluate fish freshness (Tejada, 2009). This is because ATP is immediately and highly degraded to IMP after death and shows very low concentrations during storage. The *K-index* is also used as a criteria for consumption of fish in raw form (Tejada, 2009, Tejada et al., 2007). Japanese researchers evaluated the limit of the *K-index* for raw fish consumption ('sashimi grade') equal to 20 % (Hamada-Sato et al., 2005). In previous studies by Erikson et al. (1997) salmon with *K-index* lower than 70-80 % was proposed as an indicator for "good quality" fish, while *K-index* of 40-50 % is marked as "excellent quality" fish.

1.1.3 Lactate and glucose

Glucose is a monosaccharide with six carbons and is one of the smallest units which belongs to carbohydrates. Finfish muscle involving salmon normally contains less than 1 % of the carbohydrates (Haard, 1992, Sikorski, 1990). The content of carbohydrates in fish muscle may be influenced by several factors. Food intake, spawning or water temperature may also have an impact on the glycogen content (Haard, 1992). According to a study conducted by Huss

(1995), stressed fish have higher hyperactivity once caught and they might experience a rapid decline of glycogen.

Glucose serves as energy source for plants and animals, and provides energy that is further utilized by cells used in critical life processes such as protein synthesis, growth and repair of cells, and others. The stored form of glucose is called glycogen, which is normally stored in the liver and skeletal muscle of the fish. For fish in good biological conditions, it is typical to have an adequate source of glycogen in the skeletal muscle and a source of glycogen and lipid in the liver. The amount of glycogen present in the fish's muscle, at the moment a fish is killed, is an important factor for determining of meat quality (Haard, 1992). It provides information about the pH of meat and the glycolytic potential. Fish in good biological conditions show a low post-mortem pH and their meat has a firm texture. Fish in poor biological conditions, however, show only a small resource of glycogen in its muscle. The flesh texture of poor fish is very soft after cooking and contains high pH levels (Huss, 1995).

In living fish, oxygen is normally circulated through the gills and carried by blood to muscle tissue. The aerobic degradation of glucose in fish muscle follows the Embden-Meyerhof-Parnas pathway (Figure 2) (Sikorski, 1990), where one molecule of glucose is broken down to 2 molecules of pyruvate. The created pyruvate is then required for other processes in cellular respiration. Most of the energy from this reaction is used to break down ADP to ATP. This aerobic reaction produces carbon dioxide, water and energy represented by 36 molecules of ATP in the mitochondrion in the living fish. When the fish is killed, the heart ceases to pump blood and oxygen is no longer supplied to the fish's muscle anymore. Therefore, there is a need for glycolysis as it is the only way the energy can be produced if there is no oxygen available. The production of energy without oxygen is far less effective than as with

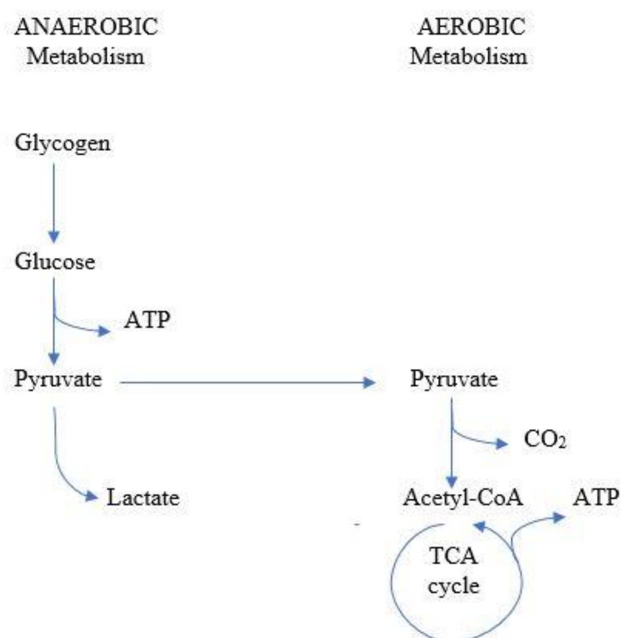

Figure 2: Aerobic and anaerobic break down of glycogen in fish muscle (Sikorski, 1990).

oxygen, because only 2 molecules of ATP are produced. Dead fish without supplementation of oxygen cannot keep its normal level of ATP and muscles enters rigor mortis.

This process under anaerobic conditions results in accumulation of lactic and pyruvic acids, decreasing the pH levels of the muscle. There is also a relation between post-mortem production of lactic acid and stored glycogen in the living muscle. Increased level of lactic acid is one of the indicators related to stress in fish. The process of capturing may be very stressful for the fish, because of its period, struggling and other negative effects. This can cause an increase in anaerobic glycolysis which might result in higher lactic acid production, followed by lower pH in muscle (Poli et al., 2005). If the fish has time for a recovery from the stressful conditions, the muscle and blood will be cleaned from formation of lactic acid (Milligan, 1996). This is possible only during fish rearing, when the fish is moved to a stock with lower density, better water quality or the oxygen level. The slaughtering of fish occurs quickly, so there is no time for this kind of recovery, and thus pH of muscle remains low (Poli et al., 2005).

It has been observed that final product lactic acid has tendency to increase when glycogen is completely degraded, and maltose decreased in post mortem. This increase in lactic acid was observed in Atlantic salmon fillets after about 7-8 days of storage at 4 °C (Shumilina et al., 2015).

1.1.4 Proteolysis and amino acids

The texture of fish muscle is one of the important properties for fish industry. The texture might be influenced by many factors, and of them can be the amount and properties of proteins. The rate and extent of proteolysis, that cause fragmentation of myofibrils and connective tissues, is also important post-mortem factor (Hultmann and Rustad, 2002).

Proteins in fish muscles can be degraded post-mortem by tissue or bacterial proteases. The protein degradation leads to slow changes in physical properties of fish. The adjacent myofibrils lose adhesion between themselves and mechanical fragmentation occurs (Sikorski, 1990). Fish muscle after endogenous proteases undergoes textural changes and gaping (Hultmann and Rustad, 2002). This myofibrils fragmentation decreases if the fish muscle is stored in ice. Proteolytic breakdown mainly depends on the activity of the enzymes, pH in the muscle, properties of the connective tissues, and the presence of proteinase inhibitors (Sikorski, 1990).

Fish muscle also contains non-protein nitrogen compounds (NPN) such as free amino acids, peptides, guanidine compounds, trimethylamine oxide, urea, betaines, nucleotides and nucleic

acids. Wild fish normally has a higher content of NPN than farmed fish (Haard, 1992). It has been studied that fish with higher content of NPN has a rapid loss of quality of the flesh due to the rapid enzymatic and/or bacterial decomposition of these metabolites, such as metabolism of amino acid to amines. The content of amino acids and their related compounds effects taste and flavour properties (Haard, 1992).

In general, wild fish appears to have more free amino acids than cultured fish (Haard, 1992). Taurine is the main free amino acid in salmon (Haard, 1992). The most abundant amino acids found in Atlantic salmon by Shumilina et al. (2015) were creatine, taurine, lysine and threonine. The other identified amino acids were glycine, alanine, serine and threonine. They contribute to sweet taste (Sikorski, 1990). The amino acids which contribute to bitter taste (Sikorski, 1990) were leucine, valine, methionine, phenylalanine and histidine (Shumilina et al., 2015).

Amino acids play a very important role in the building blocks of proteins and are also very important intermediates in metabolism. 18 amino acids are needed for human nutrition and some of them, known as essential amino acids, cannot be synthesized by humans and must be included in the diet.

Fish is rich in essential amino acids such as lysine, methionine, cystine, threonine and tryptophan (Sikorski, 1994).

The post-mortem metabolism of non-protein compounds, such as amino acids, can lead to the production of aldehydes, sulfides, mercaptans, and short-chain fatty acids, with its typical undesirable, putrid odors. Some amino acids can be decarboxylated, mainly due to the action of bacteria, and forms biogenic amines, which in higher concentrations are dangerous for health. The breakdown of these compounds is responsible for the development of odors, for the gradual loss of the fresh appearance of fish and the development of spoilage. In the beginning storage in ice, autolysis with own enzymes (endogenous) occurs and lead to the spoilage. The breakdown of amino acids can also occur by bacterial enzymes (exogenous). Some breakdowns of amino acids can occur both by endogenous or exogenous enzymes, thus why it is impossible to distinguish between them (Sikorski, 1990).

1.1.5 Biogenic amines

Biogenic amines are non-volatile amines with a low molecular weight and can have aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine) structures (ten Brink et al., 1990). Biogenic amines have

been found at low concentrations in non-fermented food such as fruits, vegetables, meat, milk and fish, and may be of endogenous origin.

Special emphasis is given on fermented foods where biogenic amines have been observed at high concentrations (Önal, 2007). Biogenic amines are mainly produced by microbial decarboxylation of free amino acids or by the amination and transamination of aldehydes and ketones during storage or processing of the products (Önal, 2007). Enterobacteriaceae and Enterococcus are the most common bacteria growing on fish, meat and their products, that are involved in decarboxylation of free amino acids (Önal, 2007). The most common biogenic amines and their amino acid precursors are listed in Table 1.

Table 1: The most common biogenic amines and their amino acids precursors (Önal, 2007) .

Biogenic amines	Precursor
Monoamines	
Histamine	Histidine
Tyramine	Tyrosine
Tryptamine	Tryptophan
Diamines	
Putrescine	Ornithine
Cadaverine	Lysine
Polyamines	
Spermidine	Putrescine
Spermine	Putrescine

Even though some of the biogenic amines such as histamine, tyrosine and putrescine play an important role in human and animal physiological functions, it can be toxic if food with such high concentrations of these amines was consumed (ten Brink et al., 1990).

Some amines carry out vasoactive effects (tyramine), psychoactive effects or combination of both (histamine) (ten Brink et al., 1990). Excessive oral intake of biogenic amines can cause symptoms such as nausea, respiratory distress, hot flush, sweating, heart palpitations, headache, bright red rash, oral burning, and hyper- or hypotension (RICE and KOEHLER, 1976). The most food intoxications occurred by biogenic amines are related to histamine, therefore its

effects are well known. Histamine poisoning is often linked to “scromboid fish poisoning” because of high intake of scromboid fish, such as tuna, mackerel and sardines (ten Brink et al., 1990). As it has been reviewed by Shumilina et al. (2016) from previous studies, there are suggested maximum tolerable levels for each biogenic amine in fish and fishery products. Level of tyramine is tolerable to be 95 mg/100g (Paulsen et al., 2012, Rauscher-Gabernig et al., 2012). The maximum tolerable levels of histamine were limited to 10-20 mg/100g by the European legislation (EC, 2005). In Austria putrescine was limited to 17 mg/100g and 51 mg/100g for cadaverine in fish products (Rauscher-Gabernig et al., 2012).

Most amines are heat-resistant, with ongoing decarboxylases even after pasteurization, and the amounts of histamine, putrescine and cadaverine might even increase during storage and spoilage by microorganisms in fish, whereas the amounts of spermine and spermidine decrease (ten Brink et al., 1990). There are also a few amines, such as putrescine and cadaverine, which can react with nitrite and form carcinogenic nitrosamines (ten Brink et al., 1990) and beside this function cadaverine and putrescine can potentiate the toxicity of histamine (Önal, 2007). Synthesis of biogenic amines are shown in Figure 3.

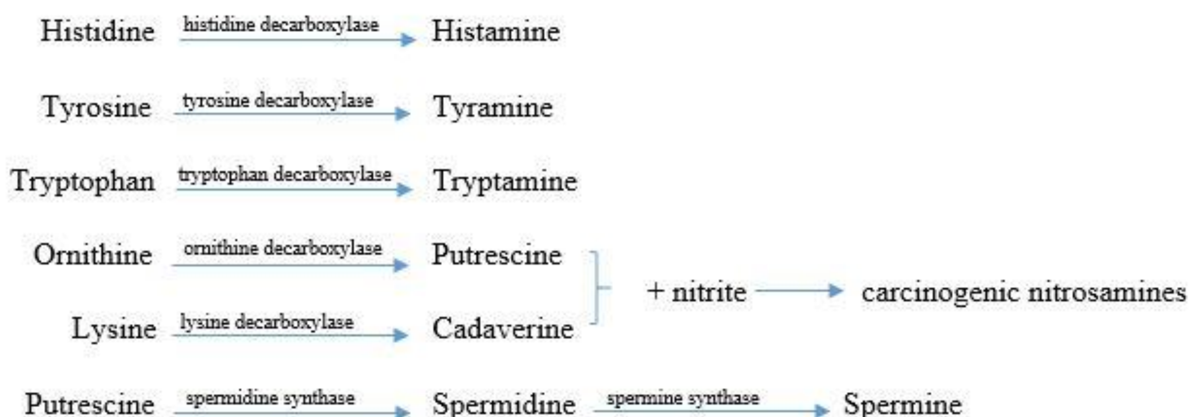

Figure 3: Synthesis of biogenic amines (Prester, 2011).

Studies oriented towards the determination and monitoring of biogenic amines in foods are not only performed because of their potential toxicity, but also to use biogenic amines as food quality markers (Önal, 2007).

Shumilina et. all found (2015) the formation of tyramine and cadaverine in the fillets of Atlantic salmon due to decarboxylation of their precursors tyrosine and lysine, respectively. The biogenic amines have been monitored qualitatively by NMR and observed after about seven days of storage at 4 °C.

The formation of biogenic amines upon storage was observed also in other research of Shumilina et al. (2016) focusing on salmon by-products. Cadaverine and putrescine were found in viscera and minced/whole heads. Histamine was observed only in viscera and tyramine was found in all by-products.

1.1.6 TMA/TMAO

Trimethylamine oxide (TMAO) is part of the non-protein N-fraction of the fish muscle. TMAO is chiefly presented with high levels in marine species, and in freshwater species it is found in small concentrations (Rehbein and Schreiber, 1984, Van Waarde, 1988). There are large number of factors which influence the content of TMAO, mainly it is in consideration of species, environment, age, diet, salinity, season and size of the fish. The difference is usually from a few to several hundred mg of TMAO-nitrogen/100g of fish muscle (Bystedt et al., 1959). TMAO increases concurrently with size and age, and decreases when the fish migrates from seawater to freshwater, this occurs due to the drop in the salinity of the new environment. It was also reviewed by Van Waarde (1988), that distribution of TMAO over the fish body is unequal; a high level is observed in the fin muscles, the superficial layer of the body musculature and the head and end of the fish myotome.

TMAO is also responsible for osmoregulation in fish and is interestingly used as a freshness indicator (Chung and Chan, 2009). Not all the fish species are able to undergo biosynthesis of trimethylamine N-oxide, therefore TMAO must be obtained from diet. Some species, which were exposed to TMAO-free diet, did not contain TMAO in their muscle (Van Waarde, 1988). Benoit and Norris (1945) showed that TMAO in young salmon is of exogenous origin.

During bacterial spoilage of fish, trimethylamine (TMA) can be formed by the bacterial reduction of TMAO caused by bacterial enzymes. Another possibility is that TMA may be created by the breakdown of choline or other trimethyl alkylammonium compounds, such as carnitine or betaine (Hebard et al., 1982, Sikorski, 1990, Van Waarde, 1988). TMAO degradation seems to take place only in dead fish muscle, and the content of the created TMA is widely used as an index of the spoilage of fresh fish (Bystedt et al., 1959). TMAO may be

demethylated to equimolar formaldehyde (FA) and dimethylamine (DMA) by enzymatic action of the endogenous enzyme, trimethylamine oxide aldolase (TMAOase) (Castejón et al., 2016). Formed formaldehyde causes cross-linking of proteins with a relative decrease of extractable protein nitrogen. It influences the fish meat properties, and due to the release of large amounts of moisture, the fish meat becomes dry (Van Waarde, 1988).

Shewanella putrefaciens, Photobacterium phosphoreum, Vibrionaceae are the spoilage bacteria which are capable to utilize TMAO as the electron acceptor and result in TMA formation (Gram and Huss, 1996). The spoiled fish products start to have an unpleasant "fishy" smell and taste. Amine compounds with a very low odor threshold when combined with particular lipids result in an especially distasteful flavor in fish products (Van Waarde, 1988).

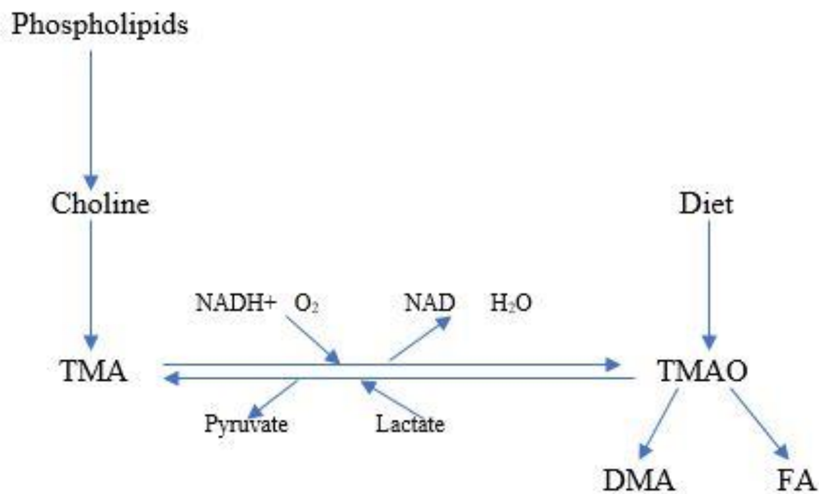

Figure 4: Metabolism of trimethylamine oxide (TMAO) and its derivatives (Van Waarde, 1988).

Based on different range of TMA levels fish is characterized in to I grade, II grade, or not acceptable for human consumption (Hebard et al., 1982). I grade is attached to fish of premier quality with TMA levels in the range of 0-4.2 mg/100g. To be categorized as II grade equal to acceptable quality, the fish must have levels between 4.25-29.5 mg/100 g of TMA level. If the TMA level is higher than 29.5 mg/100g then the fish is not acceptable for human consumption (Hebard et al., 1982).

1.1.7 Fermentation

Fermentation is a metabolic process by which sugars are degraded in the absence of oxygen. The products of this anaerobic and chemical degradation are usually organic acids, gases, or alcohol. Fermentation can occur in yeast, bacteria or in oxygen starved muscle. There are several types of fermentation pathways, which are usually named after their main products (Gottschalk and Peinemann, 1992). They vary by involving different microorganism and forming different end products. The major fermentation pathways shown in Figure 5 follow Embden–Meyerhof–Parnas pathway (glycolysis) by which pyruvate is formed.

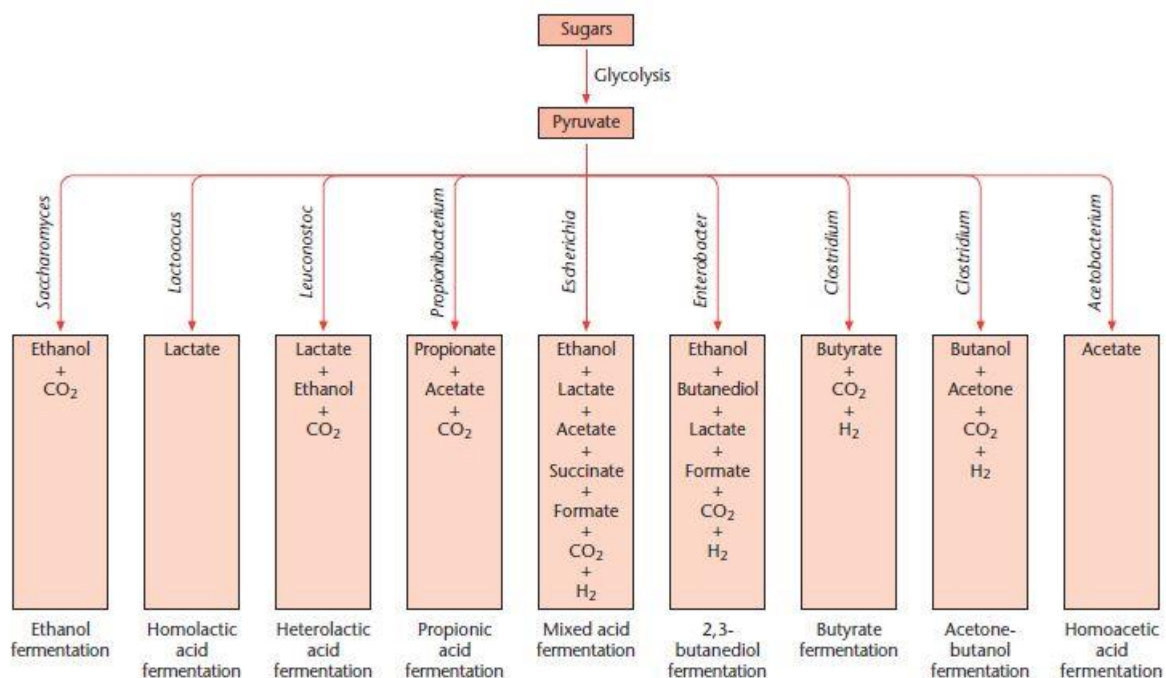

Figure 5: Major fermentation pathways of sugars involving different organisms and with formed end products (Müller, 2001).

The other type of fermentation does not form pyruvate by glycolysis, but it follows Entner-Doudoroff pathway involving *Zymomonas* species (Müller, 2001).

1.1.7.1 Ethanol fermentation

Ethanol is formed by yeast or by Z*ymonomas* species. In both cases, ethanol is formed by fermentation of glucose, but by different pathways. Yeast (*Saccharomyces* spp.) degrades

glucose by glycolysis to pyruvate, whereas *Zymomonas* species follows Entner-Doudoroff pathway. *Zymomonas* lives in environments with high sugar concentrations (Müller, 2001).

Glucose $\longrightarrow$ 2 CO_2 + 2 ethanol

Ethanol is a byproduct of various fermentation occurring by many lactic acid bacteria, enterobacteria and clostridia.

1.1.7.2 Mixed acid and 2,3-butanediol fermentation

Both fermentation types are carried out by the anaerobic enterobacteria. Mixed acid fermentation does not produce butanediol, but ethanol, carbon dioxide and mixture of acids such as acetic, lactic ad formic acids. Mixed acid fermentation involves *Salmonella*, *Escherichia*, *Citrobacter*, *Shigella* and *Proteus,* and glucose is converted by glycolysis (Müller, 2001).

Glucose $\longrightarrow$ ethanol + lactate + acetate + succinate + formate + CO_2 + H_2

Butanediol fermentation involves *Klebsiella*, *Enterobacter*, *Serratia*, *Erwinia* and *Hafnia*. As is evident from the name of the fermentation, the butanediol and ethanol are formed. However, fewer acids are formed from pyruvate than they are formed in mixed acid fermentation (Müller, 2001).

Glucose $\longrightarrow$ ethanol + butanediol + lactate + formate + CO_2 + H_2

1.1.7.3 Homoacetate fermentation

The end product of this fermentation is acetate and conversion of glucose to pyruvate happens by glycolysis. The fermenters involved in this chemical process are microorganisms such as *Moorella thermoacetica* and *Acetobacterium woodii* (Müller, 2001).

Glucose $\longrightarrow$ 3 acetate

1.1.8 Niacinamide

Vitamin B3 is an important water-soluble vitamin that can be found in fish. Vitamin B3 is also called niacin and niacinamide (NAM) and is a part of the vitamin B complex. Vitamin B3 can be found in different forms such as nicotinic acid, niacinamide and inositol hexaniacinate. Mainly it is important for balancing blood cholesterol levels, for maintaining a healthy

cardiovascular system and metabolism, helps with brain function, helps with treating diabetes, and healthy skin formation.

The niacinamide might be an important indicator for determination of fish species, as its amount among fish varies (Table 2).

Table 2: Content of niacin reported in fish by several studies.

Amount	Fish	
1.36-3 mg/100g	Atlantic salmon	(Shumilina et al., 2015)
8.8 mg/100g	Salmonidae	(Brækkan, 1959)
0-23.4 mg/100g	Tuna	(Sikorski, 1990)
0.6-9.6 mg/100g	Herrings	(Sikorski, 1990)
2.8-14.2 mg/100g	Halibuts	(Sikorski, 1990)
0.2-6.7 mg/100g	Codfish	(Sikorski, 1990)

1.2 Salmon packaging

There is a long range of biochemical and physical changes which occurs from the time when the fish is slaughtered till the fish is eaten. Within a few hours after the fish is slaughtered, rigor mortis occurs. In salmon, rigor mortis sets in around 8 hours after death (Wang et al., 1998). Therefore, first, the fish must be stored for three to five days, and then the post-rigor processing can be done, according to normal and traditional processing (Figure 5) of the fish, which is a common procedure (Hoholm, 2011). However, post-rigor processing seems less prevalent in Norway. Often the storing of the fish for three to five days is done during transit for example to Denmark or France. Besides that, the quality of the fish which is processed during post-rigor is lower than the fish processed in pre-rigor (Heggestad, 2010, Hoholm, 2011).

Normal quality processing => post - rigor

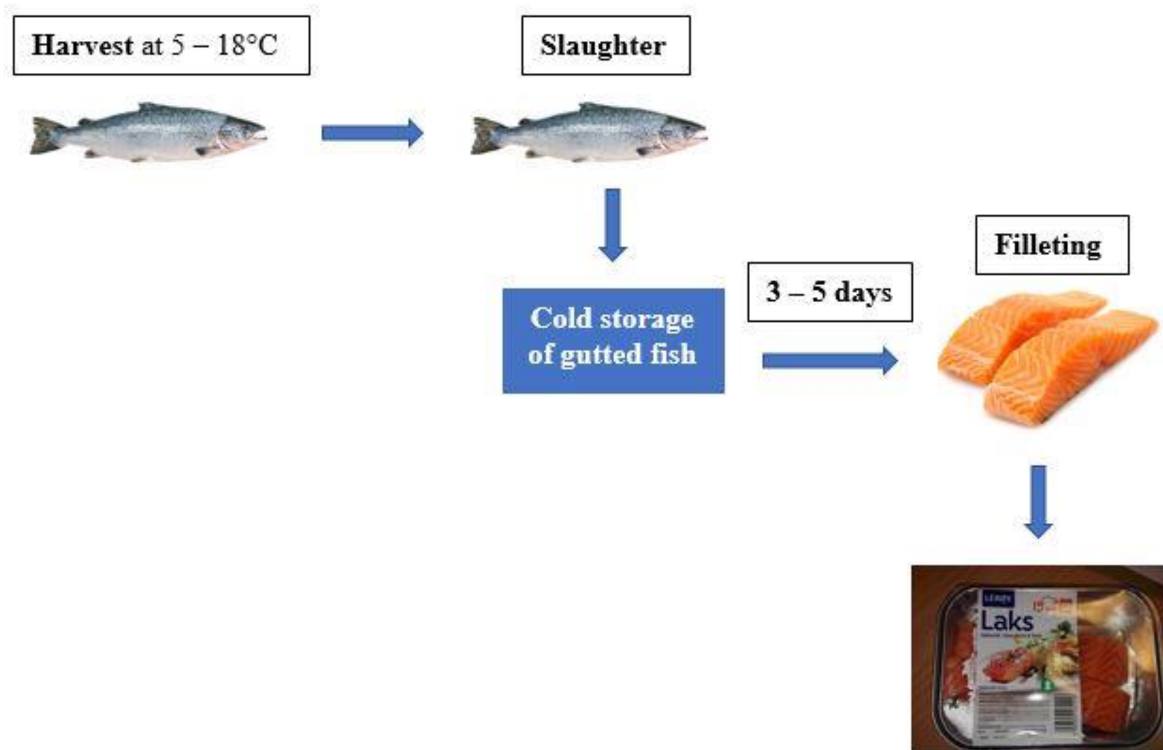

Figure 6: Traditional processing of the fish in post-rigor. The fish is harvested, slaughter, and kept gutted in a cold storage for 3 to 5 days, before further processing and filleting is done

There has been newly invented and developed method called the Bremnes´s method (Figure 7), which was an outcome of a doctoral thesis of the researcher Per Olav Skjervold, who studied prospects and effects of chilling and processing fish before the onset of rigor mortis (Skjervold et al., 1996). This new method is based on an extension of the pre-rigor time needed for pre-rigor processing, which gives very fresh fillets of extraordinary high-quality. This was a key element and the scientific basis for establishing SALMA.

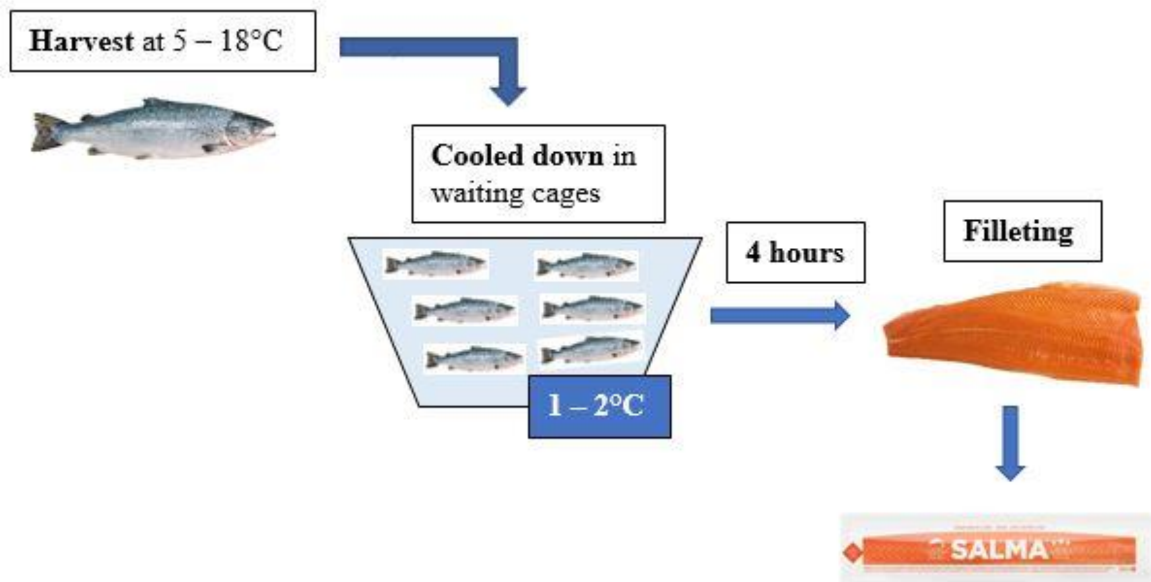

Figure 7: The processing of the fish in pre-rigor, which is typical for SALMA. Fresh fish is harvested and cooled down in waiting cages up to 4 hours in temperature approximately 1 - 2°C. After 4 hours, further processing of the fish is done. This is typical procedure for filleting the fish in pre-rigor, which cause that fish is less stressed during the slaughter.

The main idea of the Bremne´s method is that after the fish has been harvested, it is transferred into waiting-cages for a couple of hours. In these waiting-cages the fish is cooled down approximately at 1°C and by this procedure ongoing rigor mortis is postponed. Within four hours after harvesting, the fish is filleted from bones and skin while it is still in pre-rigor period. In pre-rigor period, the muscle is very soft and elastic, which causes difficulties during filleting. Therefore, new fillet cuts free of skin and bone have been proposed, these fillets are known among Norway´s producers as SALMA belly loin and SALMA back loin. By using this new method of processing and cooling down the fish, the fish is less stressed during slaughter and gives higher quality fillets.

It has been also documented by Skjervold et al. (2001) that pre-rigor processing has positive effects on some quality parameters of the fish. The research focused on chilling salmon prior to

harvest and filleting of salmon in different time periods, pre-rigor (2 hours after harvest), in-rigor (1 and 2 days after harvest) and post-rigor (5 days after harvest). The results showed positive effects in salmon, which was filleted in pre-rigor. The fillets from pre-rigor processing were thicker, had brighter colour and firmer texture than the fillets from post-rigor processing. Gaping was significantly increased with later filleting. After 14 days of storage, the fillets processed in pre-rigor had almost no gaping, while the fillets from post-rigor processing had significant gaping. There was no significant difference between the experimental groups regarding to filleting yield, water binding capacity and fillet weight loss. Due to this study, it has been suggested to start production of fish as early as possible after harvesting.

1.3 Liquid state high resolution NMR and Metabolomics

Metabolomics is a discipline which gives a comprehensive analysis of the metabolome (all metabolites) of a biological system (cell, tissue, organ, biological fluid, or organism). Metabolomics has been utilized in many different fields such as human disease, drug discovery, plant physiology, and in recent years it has become very useful in both the food industry and for nutrition scientists.

Food is an important source of essential body nutrients such as proteins, lipids, carbohydrates, vitamins and minerals; which are imperative to produce energy, stimulate growth and maintain life. Each foodstuff contains a complexity of many metabolites with different chemical properties, which are obtained in different concentrations. The metabolites occurring in food may be present naturally but also can be attributed to storage and processing. Or it can be added in order to preserve the food or for different nutritional purposes (Mannina et al., 2012). The metabolomic analysis of those metabolites give us an opportunity not only to understand what is important for aroma, taste, texture, or colour of foodstuff, but also to understand the nutritional properties of foodstuff and its effect on human health (Tomassini et al., 2013).

The most often used analytical techniques are mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy. Both techniques provide a high throughput of data, and by applying of other analysis, it is able to get a wide range of data and comprehensive information about metabolome within a biological system (Tomassini et al., 2013).

1.3.1 NMR

NMR spectroscopy has become a remarkable tool among the other analytical techniques, because of its advantages such as simplicity of quantification, straightforward metabolite identification, and ability to determine unexpected metabolites (Mannina et al., 2012). By NMR spectroscopy, it is possible to detect low molecular weight metabolites in a solution at a concentration beyond 10^{-6} M. However, in comparison with MS technique, there are some limitations in NMR spectroscopy. Nevertheless, NMR spectroscopy is still one of the best suitable techniques for food quality control and assurance (Tomassini et al., 2013). It is non-invasive and non-destructive for the sample, reproducible and it can analyze foodstuff sample without or with minimal chemical treatment (Erikson et al., 2012, Tomassini et al., 2013).

1.3.1.1 Theoretical basis of NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is based on using radiofrequency waves to discover magnetic nuclei. NMR signal can detect only nuclei that are magnetic and has non-zero spin by applying the electromagnetic (EM) radiation. Those magnetic nuclei with non-zero spin move from one spin state to another by radiofrequency (RF) waves with specific frequency for each chemical element. This property of magnetic nuclei is called as nuclear induction and it was first observed by Isidor Rabi´s group at Columbia University (Rabi, 1937, Rabi et al., 1938), later is was named as nuclear magnetic resonance, or NMR (Gorter and Broer, 1942) in clinical uses is often called as MRS.

As reviewed by Tomassini et al. (2013), the sample is prepared, usually as a solution in a narrow-walled glass tube and inserted in high-field electromagnet. Its larger coil generates B_0 field and the smaller coil, known as the probe coil, is where the sample sits. This probe coil generates RF waves, B_1 field, that irritates the sample and flip nuclei in the sample from one spin state to another. Later, RF waves are turned off and the nuclei goes back to its relaxation ground state, and emits RF waves whose frequencies provide the information about the sample. These emitted RF waves are represented as Free Induction Decays (FIDs) in the Time domain and transferred as Frequency domain. Afterwards, they are presented as one-dimensional (1D) or two-dimensional (2D) spectrum. Each signal or peak corresponds to the amount of energy necessary to bring nuclei into resonance.

The emitted RF radiation has four parameters such as intensity, frequency, half-life, and phase, which provide a lot of information about the sample. NMR is a highly quantitative and

discriminatory analytical tool, which can distinguish among metabolites obtained in the sample, recognize individual amino acids in protein. Besides that, NMR can provide information about how much of the metabolite is present, what metabolite is it, where is it or what is its function.

1.3.1.2 ^{1}H experiment

The chemical shift in NMR expresses the energy of the emitted EM radiation. Some of the resonances shown by a chemical shift can be split in two, three or more sub-peaks. These sub-peaks are called as doublets, triplets and multiplets, respectively.

NMR can be used for a sample consisting of any spin-active nuclei. The most used nuclei are ^{1}H and ^{13}C. ^{1}H is presented in organic materials with high 99.98 percentage, and ^{13}C only with 1.1 percentage. Therefore, it allows higher sensitivity using ^{1}H. However, the data produced by ^{1}H NMR are often overlapped and more difficult to be analyzed. The reason behind is that its chemical range is very small, even smaller than ^{13}C (Lenz and Wilson, 2007). ^{1}H NMR signals cover only a 10 ppm range, while ^{13}C signals cover a 200 ppm range (Markley et al., 2017).

1.3.1.3 Quantitative NMR

To be able quantify absolute concentrations of metabolites, there is a need for NMR standard to be added in a sample. If 1D ^{1}H NMR spectra has a suitable resolution and sufficient signal-to-noise, the intensity of the peaks usually correspond to their relative concentration (Markley et al., 2017). The chemical shift reference often used for ^{1}H NMR spectra is 3-trimethylsilyl-deuterosodium propionate (TSP) (Harris et al., 2002).

1.3.1.4 Metabolic fingerprinting

The concentration of the nuclei that produces signal and the area under partially saturated peak has a linear relationship that give us a characteristic metabolic phenotype, like a barcode or a snapshot. If this snapshot comes from the preparation of a cell, tissue or organism it is called metabolic fingerprinting. If it looks at cell media or excretory products, it is called metabolic footprinting (Allen et al., 2003). Metabolic fingerprinting is a rapid and noninvasive analytical approach which helps us to characterize phenotypes and distinguish between metabolic states caused by environmental changes (Liang et al., 2015). 1D spectra of the frequency domain carries qualitative information about a sample, and metabolic fingerprint corresponds to a quantity to each peak (Tomassini et al., 2013).

1.3.2 Metabolites and metabonomics/metabolomics

Metabolomics and metabonomics are fields which both study metabolites of a sample. Both studies belong to the same category, however, metabonomics is a subcategory of metabolomics. Metabolomics, or metabolic phenotyping, studies the quantitative description of all low-molecular-weight (<1 kDa) components in a sample (Holmes et al., 2008, Nicholson, 2006). Each cell and tissue have a characteristic metabolic fingerprint which can be changed due to external stimuli, such as physiological or environmental changes. Metabolomics studies the response of metabolites to those stimuli. It often tracks the development of the response at a given time. Metabolomics has been already used in drug toxicity studies, disease modeling, and diagnostics. In the future, it appears to be a promising study that might be integrated with genomics and proteomics in order to provide better understanding of systems, pathways and their interactions (Kosmides et al., 2013).

Metabonomics is a subcategory of metabolomics and it studies the interaction of metabolites in a sample over time (Holmes et al., 2008, Nicholson, 2006).

1.3.2.1 NMR metabolomics

Nuclear magnetic resonance (NMR) spectroscopy is a common technique to produce metabolomic data. It is high-throughput technique, which takes only few minutes and it does not require any knowledge of studied metabolite. The databases, such as HMDB, BMRB and others, which are free to use, nowadays offer standard NMR spectra and relevant information on small biological molecules. However, it is still not sufficient and there is a need for higher standards in NMR metabolomics (Markley et al., 2017).

1.4 Mass spectrometry

Mass spectrometry (MS) is a technique used in analytical chemistry, where chemical compounds are ionized and these created ions are further sorted based on their mass-to-charge ratio (Finehout and Lee, 2004). A sample, might be liquid, solid, or gas, and the separation is procced by accelerating and placing them to a magnetic or electric field (Guo, 1999). Results might be analyzed by looking at known masses of atoms or molecules then comparing them with masses displayed as spectra in MassLynx software. Mass spectrometry is a useful tool to find a chemical compound contained in a sample by its known mass, however, we are unable to calculate exact amount of this chemical compound.

1.5 Statistics

Metabolomics offers a global snapshot of all small-molecule metabolites in cells and biological fluids. However, this is a large cache of information, which is challenging and requires further relevant data analysis. One way to find meaning in metabolomics datasets is using multivariate analysis (MVA) methods. One of the most used multivariate analysis method for metabolic fingerprinting is PCA (Worley and Powers, 2013).

1.5.1 PCA

Principal component analysis (PCA) shows a variability in data which are shown on a PCA map as clusters. It helps to reduce high-dimensional data in smaller dimensions. Each cluster represents a different metabolic fingerprint. The aim of PCA is to find potential biomarkers-metabolites, which differ most between classes and how certain metabolites are influenced (Kosmides et al., 2013).

1.5.2 SIMCA models

The SIMCA (Soft Independent Modeling of Class Analogy) is an approach which is used to build the models of classes or processes and to predict a probable class membership for new samples. This procedure is included:

i) a PCA of all NMR data in order to identify if data clustering is possible, ii) a reduction of the number of variables in order to increase the models stability; iii) a development of reference models; iv) a classification of a new set of samples into the established models (Manual, 2017, Wold and Sjöström, 1977).

2 Aim of this study

The main goal of this thesis is to propose method/protocol that can be used to monitor and determine quality of salmon. The method will be based on monitoring and comparing the post-mortem biochemical processes. Monitoring and quantification of water soluble metabolites will be done by NMR method. The results of this thesis can be used for further applications in the food industry to monitor quality of different food products, not only in fish.

3 Material and methods

3.1 Atlantic salmon

Eight species of farmed Atlantic salmon (Salmo salar) from the same Norwegian supplier SalMar Nutrimar were bought 1 day after they had been harvested. On the same day, these salmon species were brought to the laboratory at the Department of Biotechnology, Norwegian University of Science and technology (NTNU), where they have been stored for 21 days. Back loin parts of these fillets were cut into pieces of around 100 g and stored in plastic bags (reduced oxygen) at 4 °C.

Three packages of premium quality salmon fillets, brand "Salma back loin" were received from Salmar (Norway). All three replicas were stored at 4°C.

Two premium quality fillets (brand "Frøya, mid loin" and "Salma, rich belly loin" were purchased at a local store and stored at 4°C for 21 days.

3.2 Experiment design

To be able to monitor the post-mortem biochemical processes, the fillets were stored and analyzed for approximately 21 days. The first sample was analyzed within a few hours on the same day, as it has been brought to the lab (1 day after harvesting). T_0 corresponds to the time, when the samples arrived in the lab. Sampling of salmon was carried out at: 1 (T_0), 2 (T_2), 3 (T_3), 5 (T_5), 7 (T_7), 9 (T_9), 11 (T_{11}), 14 (T_{14}), 17 (T_{17}), 21 (T_{21}) days of storage at 4 °C. Together, the samples were analyzed at 10 different time periods. The samples were cut in 5 g sections of muscle from the top loin at each time period. For calculating errors, these sections were weighted twice, before and after cutting.

3.3 Chemicals

Deuterium oxide (D_2O, 99.9%) was purchased 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* (Shumilina et al., 2015). Sodium hydroxide (NaOH) from *Sigma–Aldrich* was used for pH adjustments.

3.4 Trichloroacetic acid extraction

The water soluble polar metabolites were extracted from the fish samples using trichloroacetic acid. The 5 g samples were homogenized in 30 ml of 7,5 % TCA using a horizontal homogenizer (Retsch). The homogenate was filtrated using a filter paper and pH of this filtrate was adjusted to pH 7.0 by using 9 M NaOH (Sodium hydroxide). The samples with filtrate were stored at -20 °C until NMR was proceed.

3.5 NMR sample preparation

495 µL of the TCA extract was mixed with 55 µL of 1mM TSP in 20mM sodium phosphate buffer, pH 7.0 in an Eppendorf tube. The samples were centrifuged at 20000xg for 10 minutes. 530 µL of this centrifugated mix was transferred in a standard 5mm NMR tube and stored in -20 °C before further analysis.

3.6 NMR spectroscopy

1D ^{1}H NMR spectra were taken at 300 K using a Bruker 600 MHz Avance III HD equipped with a 5-mm SmartProbe z-gradient probe and SampleCase at the NMR center of the Faculty of Natural Sciences and technology at the Norwegian University of Science and Technology in Trondheim, Norway. The NMR experiments were acquired with the pulse sequences noesygppr1d. For metabolites quantification of the 1D ^{1}H experiments the following setting were used: number of scans ns = 48 and RG = 12.7.

All the NMR data were processed with the TopSpin 3.5.b.88pl7 (Bruker, Germany. NMR data were assigned by using the reference standard form the published data at HMDB (Human Metabolome Database) and BMRB (Biological Magnetic Resonance Data Bank), freely accessible databases and previous laboratory data (Shumilina et al., 2015).

3.7 Mass spectrometry

1 ml of TCA extracts of the normal quality salmon at 4 different time points (T0, T7, T14 and T21) were analyzed. Analyses were performed with an ACQUITY I-class UPLC system

coupled to a Synapt G2Si high definition mass spectrometer (HDMS) mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI source operating in negative or positive mode. FIA– qTOF data were acquired and processed using MassLynx software (v4.1).
MS analyses were performed under constant ESI conditions. The capillary voltage, cone voltage and source offset voltage in negative and positive mode were set at -2.5 kV/3.0 kV, -30 V/35 V and – 40 V/35 V, respectively. The source temperature maintained at 120 °C, desolvation gas temperature 200 °C, and desolvation gas flow rate was set at 800 L/h. The latter to match the low flow rate in FIA mode. The cone gas flow rate was fixed at 50 L/h and the nebulizer gas flow maintained at 6 bar.

The instrument was operated in high resolution scan mode with a scan time of 0.5 s. Interscan delay was 0.015s. Mass range was set to 50-2000 Da, the same range as the valid calibration performed with Na-formate immediately before analysis. The instrument was calibrated with a mass accuracy of 1 ppm and deliver a resolution of 40 000 full width at half maximum (FWHM) in high resolution mode. Data was recorded in continuum.

Samples were introduced to the mass spectrometer in flow injection analysis (FIA) mode. To accomplish this, the UHPLC was set to bypass, to direct the flow passed the column, directly to the mass spectrometer. In this case, a mobile phase consisting of methanol/acetonitrile (50/50) + 0.1% formic acid was used, and a flow gradient was programmed as shown in Table 3. The injection volume was set to 5 µL. Water/acetonitrile (9/1) + 0.1 % formic acid was used as wash solvent for the UHPLC system.

Table 3: Flow gradient for FIA analysis.

Step	**Total time (min)**	**Flow rate (mL/min)**	**A (%)**	**B (%)**	**Curve initial***
1	Initial	0.150	0	100	6
2	0.09	0.150	0	100	6
3	0.10	0.100	0	100	6
4	1.50	0.200	0	100	6
5	1.60	0.800	0	100	6
6	1.85	0.800	0	100	6

7	1.95	0.150	0	100	6
8	2.00	0.150	0	100	6

*a curve initial of 6 refers to a linear gradient

During the FIA analysis, a lockmass flow of 10 µL/min leucine enkephalin (1 ng/mL) was infused into the ion source to correct the mass axis on the fly. The lockmass flow from a separate capillary, and the capillary voltage was set to -3.0kV/2.5kV (negative or positive depending on the operation mode).

3.8 Statistics

3.8.1 PCA

^{1}H NMR spectra were normalized to the average mass (5 g) of the extracted salmon tissue and volume (30 ml) of extraction. The processing of all NMR spectra was done using TopSpin 3.5.b.88pl7 (Bruker, Germany). The region from 9.005 to 5.185 ppm and from 4.305 to 0.755 ppm of all ^{1}H NMR spectra was binned using 0,01 ppm bucketing. This bucketing was done by AMIX 3.9.14 Bruker Software. A total of 737 buckets with integral values were analyzed. The table of integrals were used as an input for Unscrambler, version 10.4, CAMO Software AS Oslo, Norway. Later, the non-significant regions were removed and only 199 buckets were analyzed by PCA. For further analyzing of the sample, the SIMCA model was performed.

4 Results and Discussion

4.1 Uncertainty of methods

4.1.1 Weighting and extraction

The mass was weighed before (m_1) and after (m_2) cutting it into small sections. The uncertainty for mass was estimated to 0.85 %. It has been calculated by following formula:

$$\% \Delta = [\Delta (m_1, m_2) * 100] / m_1$$

Volume accuracy was estimated to 4,16 % (1.25 ml). The smallest unit of our Falcon tubes was 2.5 ml.

4.1.2 TSP (external standard) concentration

TSP is a common external standard used in NMR analysis. The pipetting error was estimated as 1.32 % based on standard deviation of the TSP peak integral values.

$$\text{Pipetting error} = (SD * 100) / \text{mean of TSP integral values}$$

4.1.3 Calculation of total method uncertainty

Total method uncertainty was estimated as 6.33 %. It was calculated by summing up uncertainties for mass (0.85 %), for volume (4.16 %) and pipetting error (1.32 %).

4.1.4 NMR – S/N measurement

The signal-to-noise ratio (S/N) is an important criterion for accurate integrations. Detection of signal-to-noise ratios (S/N) was done by TopSpin software. S/N ratio was calculated by following formula (Maniara et al., 1998):

$$S/N = Imax / (2 * \sigma noise)$$

Imax expresses the maximum amplitude of the peak and σnoise represents the standard deviation of the noise region (12-11 ppm).

It has been known that if S/N ratio is lower than 3, the composition of samples cannot be analyzed. Detection of metabolites is possible only if S/N ratio is greater than 3, and quantification of these metabolites can be done if S/N ratio exceeds 10 (Maniara et al., 1998).

The number of scans of NMR experiment were optimized to have the S/N ratio for the metabolite with lower concentration higher than 10.

4.2 Monitoring of post-mortem biochemical processes by quantitative 1H NMR

The post-mortem biochemical processes change over the time when fish is stored. To monitor these changes, some of the most important fish metabolites were monitored. Based on previous studies (Shumilina et al., 2015), the metabolites shown in Table 4 were chosen to be monitored. Most of those metabolites are part of the post-mortem biochemical processes and their concentrations changed during storage. However, some metabolites such as niacinamide had stable concentration during the storage.

Table 4: Metabolites of our interest, used for monitoring of biochemical processes post-mortem.

ATP	Valine	Trimethylamine
AMP	Alanine	Trimethylamine oxide
IMP	Lysine	2,3-butanediol
Inosine	Cadaverine	Ethanol
Hypoxanthine	Tyramine	Acetic acid
Glucose	Tyrosine	Niacinamide

To simplify the results presentation, only two normal quality and two high quality salmons will be used. Principal component analysis (PCA) was performed on all salmon samples.

As it has been mentioned in the literature part of this thesis, the major changes in post-mortem biochemical processes occur between 2 to 12 days of storage (Huss, 1976b). It may differ by fish species. According to the previous studies performed by Huss (1976b), the final monitoring was done between day 3 and day 14 of storage at 4 °C. During this period, the graphs exhibit linear S-shape, this was the time when the concentrations of the specific metabolites changed the most.

4.2.1 ATP degradation and K-index

K-index has been suggested as one of the best indicator of freshness (Tejada, 2009). In the literature review it was mentioned, that fish with K-index around 70-80 % is considered as good

quality salmon, 40-50 % as excellent quality and less than 20 % belongs to fish with very high quality (´sashimi grade´), suitable for raw consumption.

The results show that only one HQ salmon stored 1 day is suitable for raw consumption. The K-index between NQ and HQ salmons does not differ very much during first days of storage. The emphasis is given on later days of storage, when the difference between the quality of the salmon is more significant. According to K-index, all salmon samples seem to be of an excellent quality up to 3 days (T3) of storage. NQ salmon reaches the maximum level of K-index after the third day (T3) of storage, while HQ salmon reaches the maximum level approximately by the ninth day (T9) of storage. Passing this period, salmon is not fresh anymore and needs to be rejected for human consumption.

Table 5: The table shows K-index of the NQ salmon (NQ1, NQ2) and the HQ salmon (HQ1, HQ2). Orange color indicates fresh and higher quality fish, with a possibility to be eaten in raw form. Dark blue shows excellent quality, light blue shows good quality, and white color indicates fish that are no longer considered as fresh and are not recommended for human consumption.

Storage	**K-index (%)**			
time	**NQ1**	**NQ2**	**HQ1**	**HQ2**
T0	33,1	24,4	20,3	34,0
T2	56,2	51,8	31,5	47,2
T3	67,1	59,9	-	52,1
T5	100,0	100,0	55,6	66,7
T7	100,0	100,0	68,6	77,2
T9	100,0	100,0	80,7	88,2
T11	100,0	100,0	90,9	91,2
T14	100,0	100,0	98,4	98,6
T17	100,0	100,0	99,3	100,0
T21	100,0	100,0	100,0	100,0

Nucleotides and their catabolism are very important indicators for the quality of fish fillets. High concentration of IMP, which has a very sweet and pleasant taste, is related to a good quality fish. Whereas inosine and hypoxanthine are related to bad quality and unpleasant taste

and flavors. Hypoxanthine is a metabolite which indicates loss of freshness with its increasing concentration during the storage. The differences are visible between NQ and HQ salmon. In NQ salmon, hypoxanthine was rapidly increasing from its third (T3) day of storage. After this timepoint, the IMP was completely degraded to hypoxanthine. After 14 days of storage, hypoxanthine in NQ salmon starts to decrease, this is most likely connected to ongoing degradation of hypoxanthine to xanthine and uric acid. This requires further research, such as using a different method, as xanthine was not found by NMR in our samples.

Since the K-index is higher than 70% after 3 days in NQ salmon and after 9 days in HQ salmon, the shelf-life of Atlantic salmon stored at 4 °C is proposed to be 3 days for NQ salmon, and 9 days for HQ salmon.

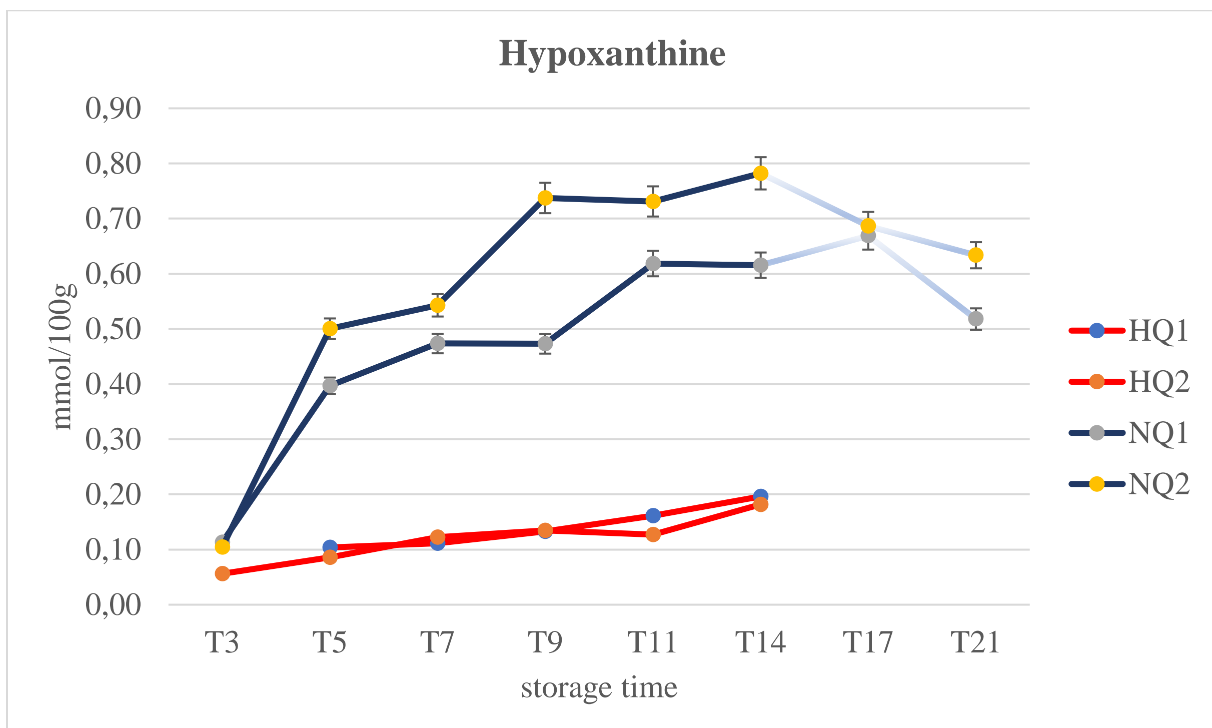

Figure 8: The graph shows ongoing development of hypoxanthine, which correlates to the loss of quality in the salmon. The blue lines show NQ salmon (NQ1, NQ2), while the red lines (HQ1, HQ2) show HQ salmon. The rapid increasing in the concentration of hypoxanthine was observed only in NQ salmon. After two weeks of its storage, hypoxanthine decreases again, which is probably correlated to degradation of hypoxanthine to xanthine and uric acid.

4.2.2 Proteolysis and amino acids

There were not any observed significant differences in concentration of amino acids in NQ and HQ salmon. The concentrations of amino acids, such as valine (Figure 9) and alanine (Figure 10), increased during the storage time at 4 °C, because of proteolysis, but the changes in concentrations were not as rapid.

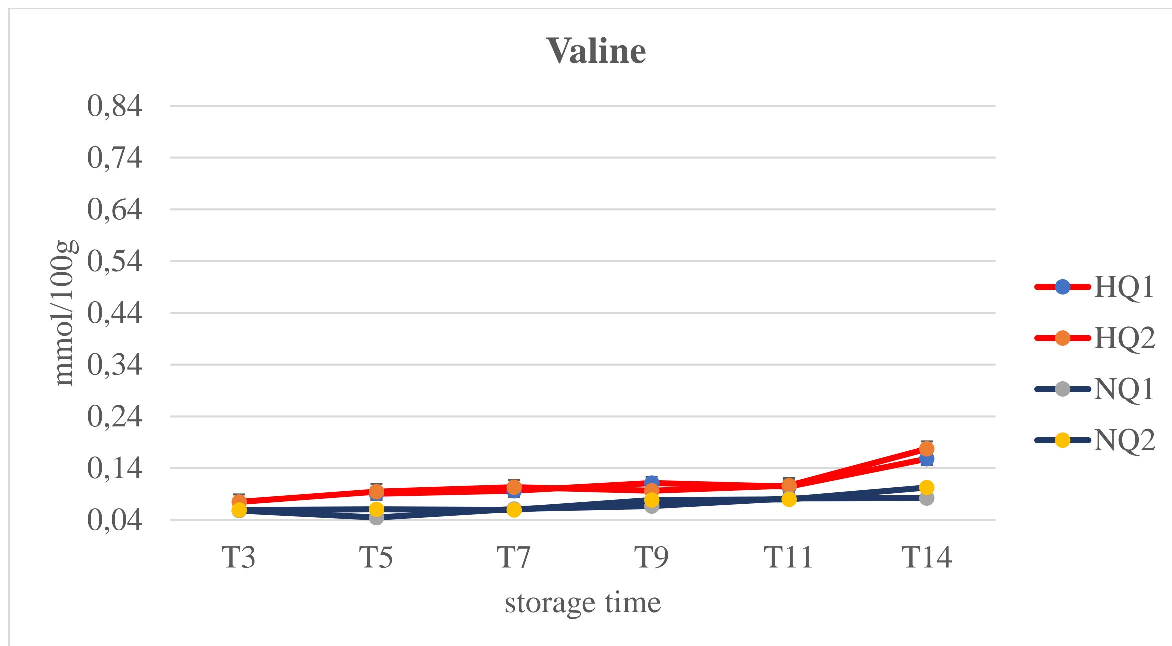

Figure 9: The concentration of valine between HQ and NQ salmon.

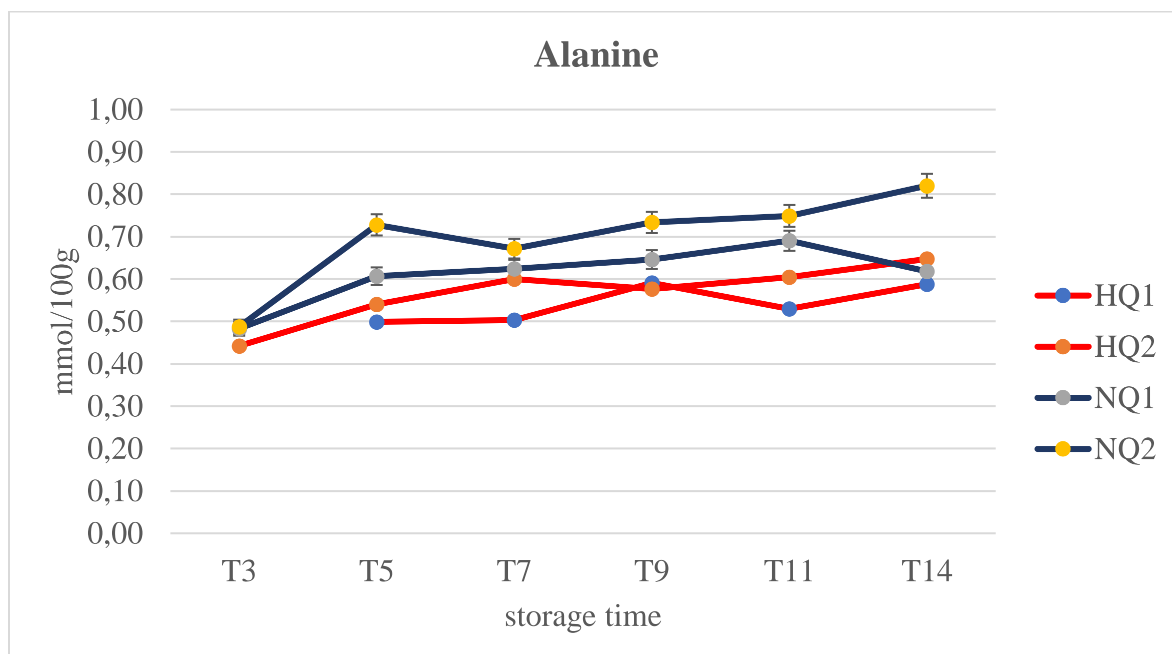

Figure 10: The concentration of alanine between HQ and NQ salmon.

4.2.3 Biogenic amines

The biogenic amines were detected by 1D ^{1}H NMR spectra, but some peaks were overlapped and their quantification was difficult. No BA were detected in HQ during the storage. The presence of lysine in HQ salmon was detected at 1.71 ppm, however, the cadaverine was not detected (Figure 11). In NQ salmon, decarboxylation of lysine occurred, and subsequent formation of cadaverine was observed at 1.71 ppm (Figure 12). The formation of cadaverine started around the fifth day of storage (T5) at 4 °C.

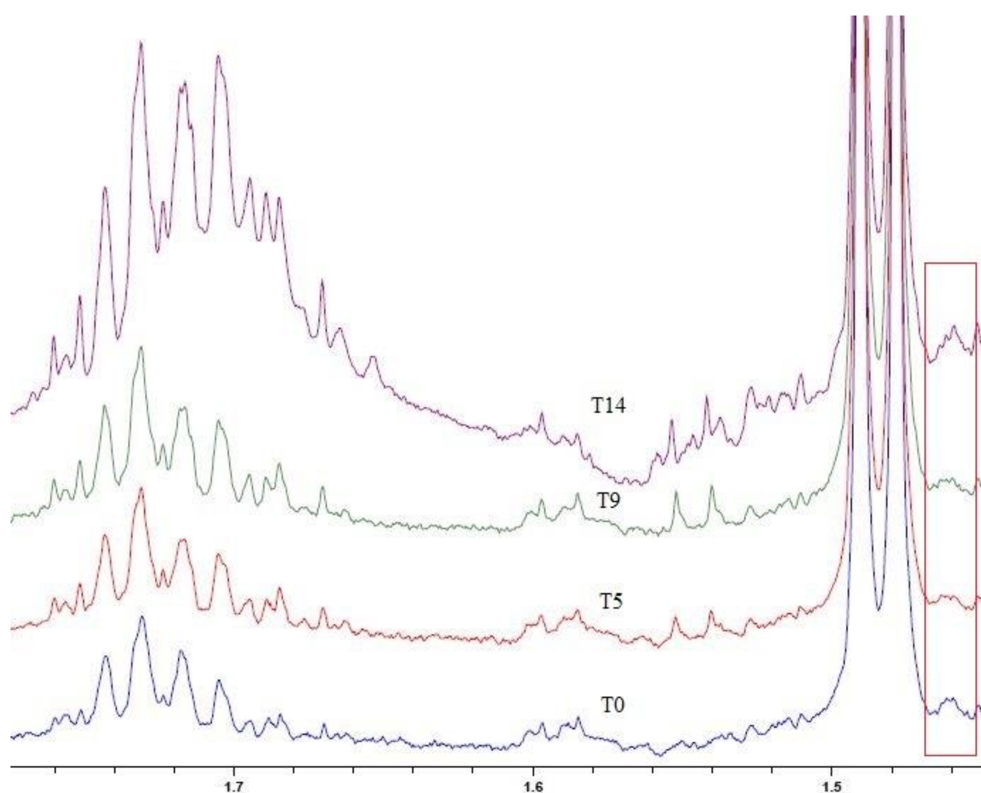

Figure 11: The picture shows lysine at 1.71 ppm, the precursor of biogenic amine cadaverine. However, the formation of cadaverine at 1.46 ppm in HQ salmon was not observed.

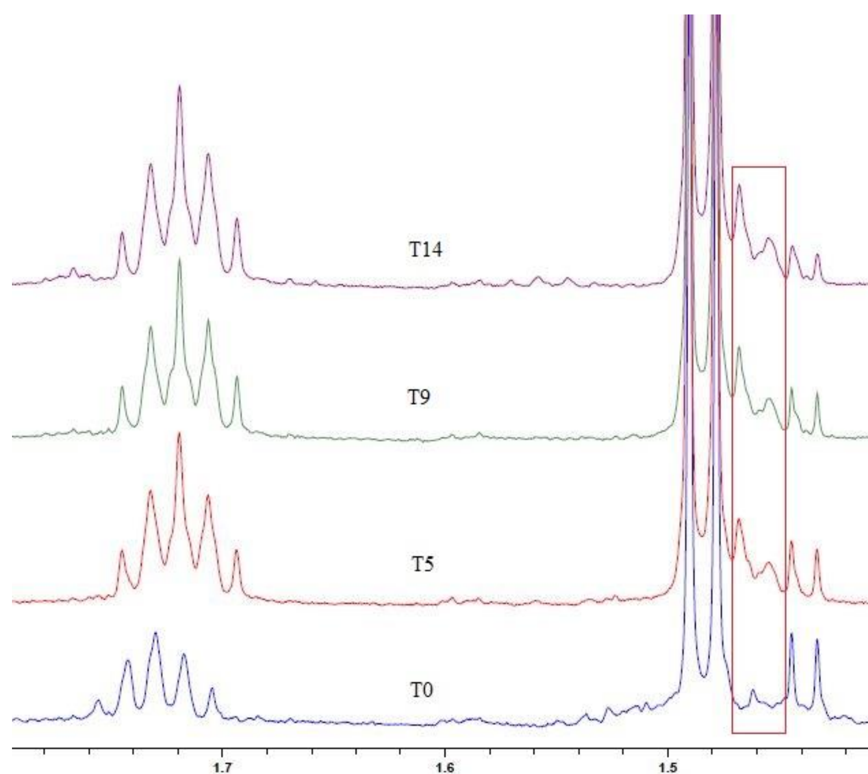

Figure 12: The picture shows peaks of lysine at 1.71 ppm in NQ salmon. The formation of cadaverine at 1.46 ppm (red frame) from its precursor lysine is shown for the first two week of storage in NQ.

The presence of non-essential amino acid tyrosine, which is a precursor for biogenic amine tyramine, was observed in HQ salmon at 6.88 ppm. However, the formation of tyramine from its precursor was not observed in HQ salmon within the first two weeks of storage at 4 °C (Figure 13).

The different case was NQ salmon, where the formation of tyramine and its precursor tyrosine at 6.88 ppm were observed. The formation of tyramine started around the fifth day of storage (T5) at 4 °C (Figure 14).

Even though, the exact concentrations for biogenic amines were not calculated, they were used for further PCA analysis. The difference in biogenic amines between NQ and HQ salmon was shown here. The formation of biogenic amines occurred only in NQ salmon, while in HQ only precursors of biogenic amines were detected.

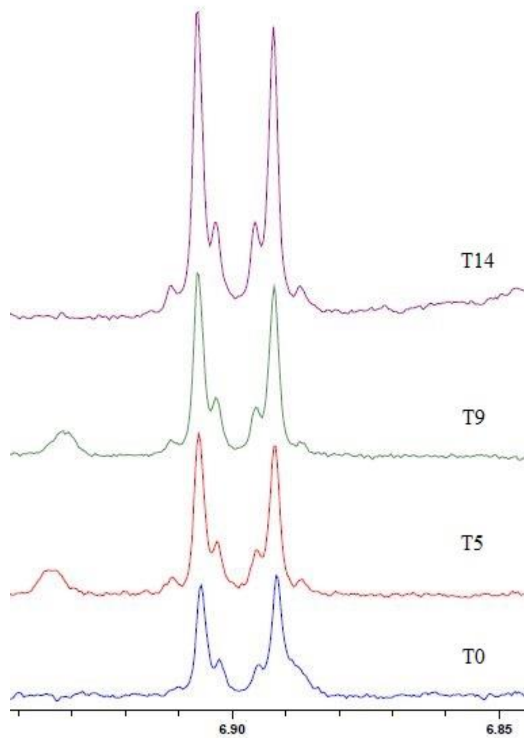

Figure 13: There is a presence of non-essential amino acid tyrosine at 6.88 ppm, which is a precursor for the formation of biogenic amine – tyramine. The formation of tyramine does not occur for the first two weeks in HQ salmon.

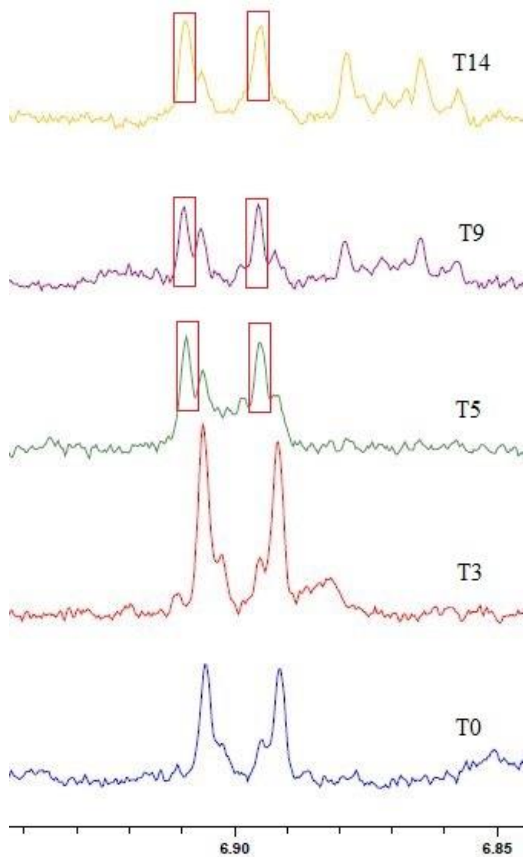

Figure 14: The presence of tyrosine at 6.88 ppm, which is a precursor for biogenic amine – tyramine. The picture shows the formation of tyramine (red frame) after fifth day in NQ salmon and its continuance for the first two weeks of storage.

4.2.4 TMA/TMAO

The results show a difference in rate of formation of TMA in NQ and HQ salmon. The NQ salmon belongs to I grade (0-4.2 mg/100g), described in the literature section, up to first 3 days of storage at 4 °C with the levels of TMA up to 0.28 mg/100g. At the fifth day, there is a rapid increase of TMA in NQ salmon up to 27.50 mg/100g, which corresponds to values II grade (4.25-29.5 mg/100g) for acceptable quality. After seven days (there is no data on day 6), TMA level in NQ salmon exceeds a maximum acceptable level for consumption (29.5 mg/100g), and fish is not acceptable for human consumption.

There is slower formation of TMA in HQ salmon than in NQ salmon. The level of TMA is lower than maximum acceptable level (29.5 mg/100g) even after 21 days of storage. The highest level of TMA in HQ salmon was measured at the last day of storage and it was only 10.15 mg/100g. Since the TMA level are so low, HQ salmon is still acceptable for consumption and even before 14 days of storage belongs to I grade (0-4.2 mg/100g).

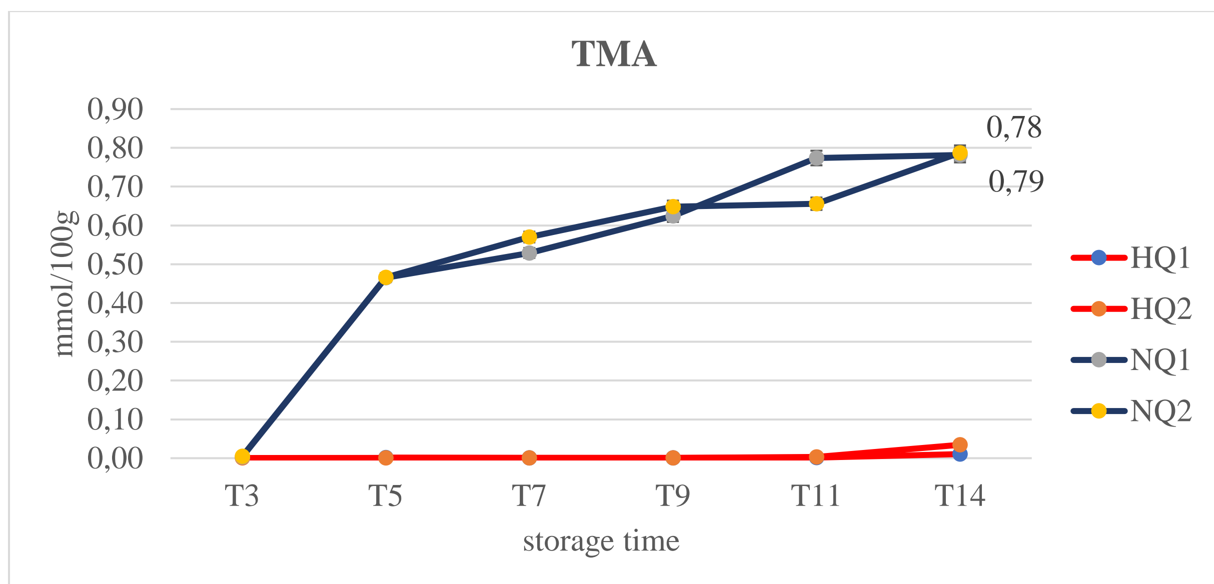

Figure 15: Formation of TMA in high quality (HQ) salmon and normal quality (NQ) salmon compared. In the graph, the final concentrations of TMA after 2 weeks of storage at 4 °C are shown. The final concentration of TMA for NQ1 is 0.78 mmol/100g, and for NQ2 is 0.79 mmol/100g.

As shown in the graph (Figure 15), TMA does not appear in HQ salmon before 11-14 days of storage. TMA formation in NQ starts to develop rapidly by the third day of storage. This rapid increasing may be correlated to the reduction of TMAO into TMA. The initial concentrations

of TMAO 0.89 mmol/100g (NQ1) and 0.76 mmol/100g (NQ2) are close to the final concentrations of TMA at 14 days – 0.78 mmol/100g (NQ1) and 0.79 mmol/100g (NQ2). There still may be minimal differences, as the monitoring of TMA formation was done only until 14 days. However, almost the complete amount of TMAO was converted to TMA in both samples of NQ salmon. TMAO was most likely degraded by bacterial enzymes. In NQ1 salmon, not all TMAO was degraded into TMA, therefore TMAO still might be observed after 14 days. The bigger concentration of TMA was observed in NQ2, than the initial concentration of TMAO was. It seems that TMA was formed not only by degradation of TMAO by bacteria, but most likely other precursors, such as choline, carnitine or betaine, contributed to the formation of TMA (Van Waarde, 1988) and thus more TMA was formed.

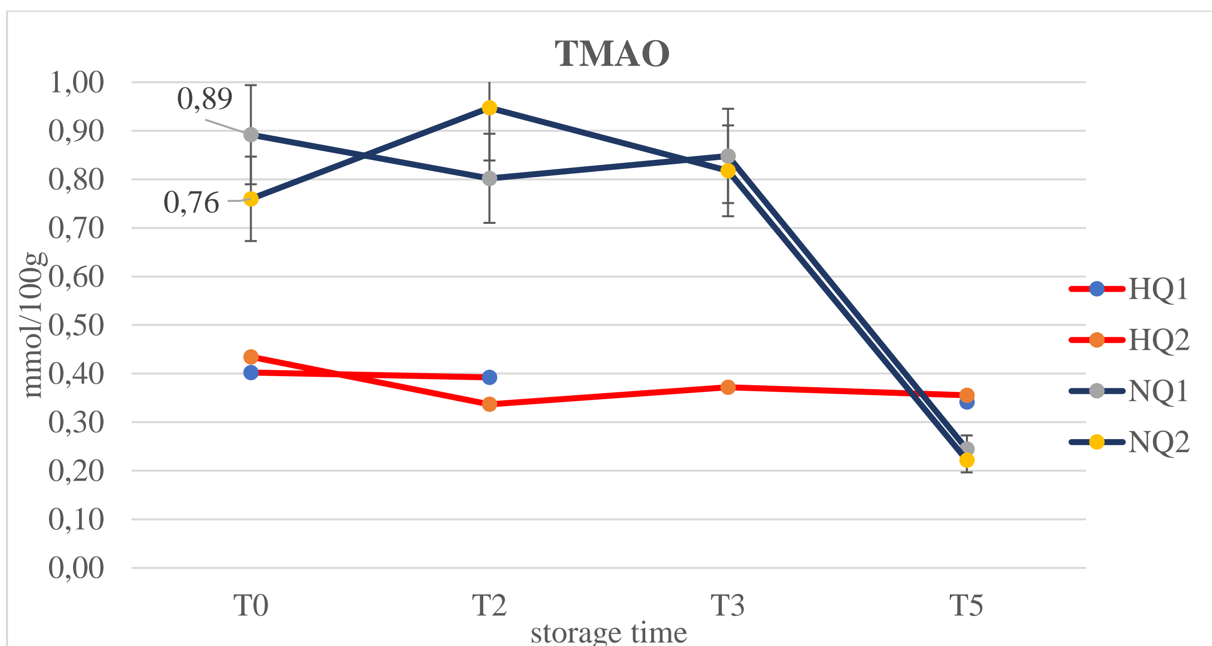

Figure 16: The graph shows the quantified concentrations of TMAO in HQ and NQ salmon before its degradation to TMA. In the graph, we can see initial concentration of TMAO in NQ salmon. The initial concentration for NQ1 is 0.89 mmol/100g, and for NQ2 is 0.76 mmol/100g.

The results showed that concentration of TMAO in NQ salmon is twice as high as the concentration in HQ salmon, which is a very interesting fact. The reason behind this might be due to differences in the diet of the fish; however, it requires further research with more statistical data.

4.2.5 Fermentation

Fermentation is a chemical process involving various types of bacteria that ferment sugars and produce different products, such as ethanol, 2,3-butanediol, acetic acid, and many others. There were consequential differences in the observed concentrations of ethanol, 2,3-butanediol and acetic acid between NQ and HQ salmon. HQ salmon shows a very stable concentration for these metabolites, while NQ salmon shows rapid increasing for all these metabolites. This rapid increasing indicates that some type of bacteria was involved in the fermentation process. There are several types of bacteria which are involved in fermentation, unfortunately, this was not researched in this thesis. It is suggested that bacteria such as *Zymonomas* spp., LAB, Enterobacteria, Clostridia, Escherichia may be involved in the production of ethanol; whereas *Moorella thermoacetica*, *Acetobacterium woodii* may be involved in the production of acetate; and *Klebsiella*, *Enterobacter*, *Serratia*, *Erwinia* and *Hafnia* may be involved in the production of 2,3-butanediol.

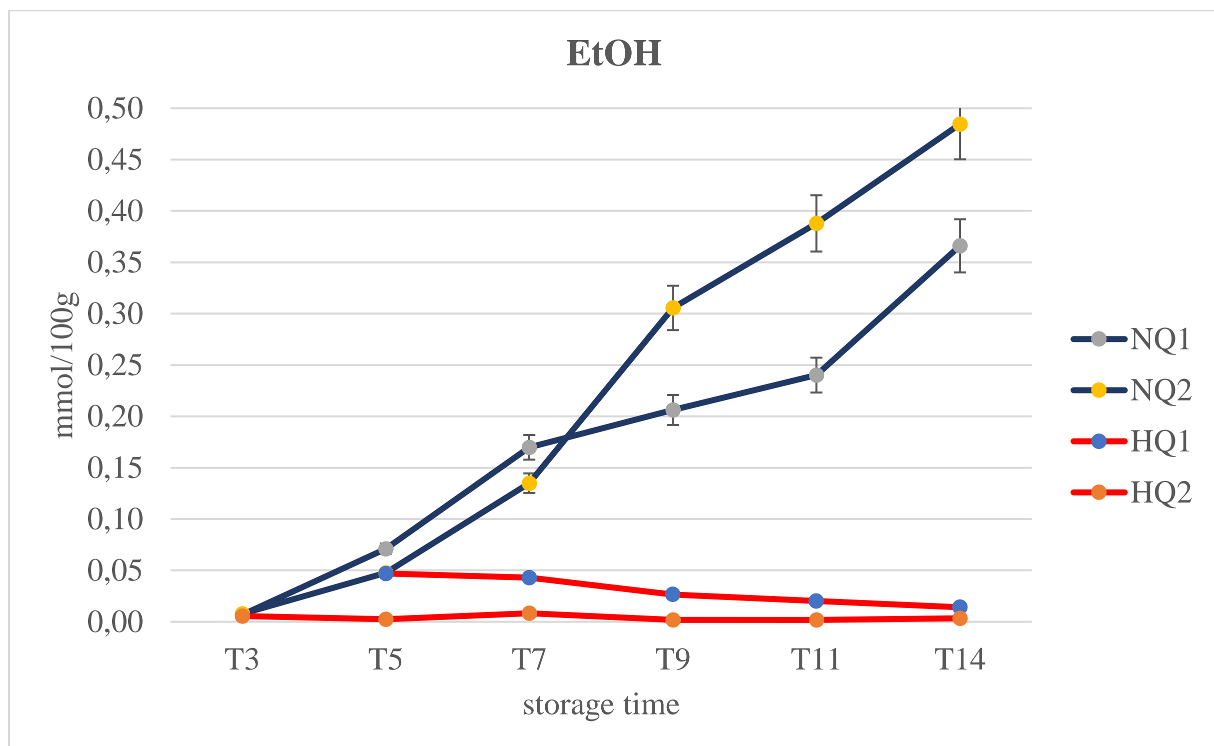

Figure 17: The concentration of ethanol between NQ and HQ salmon.

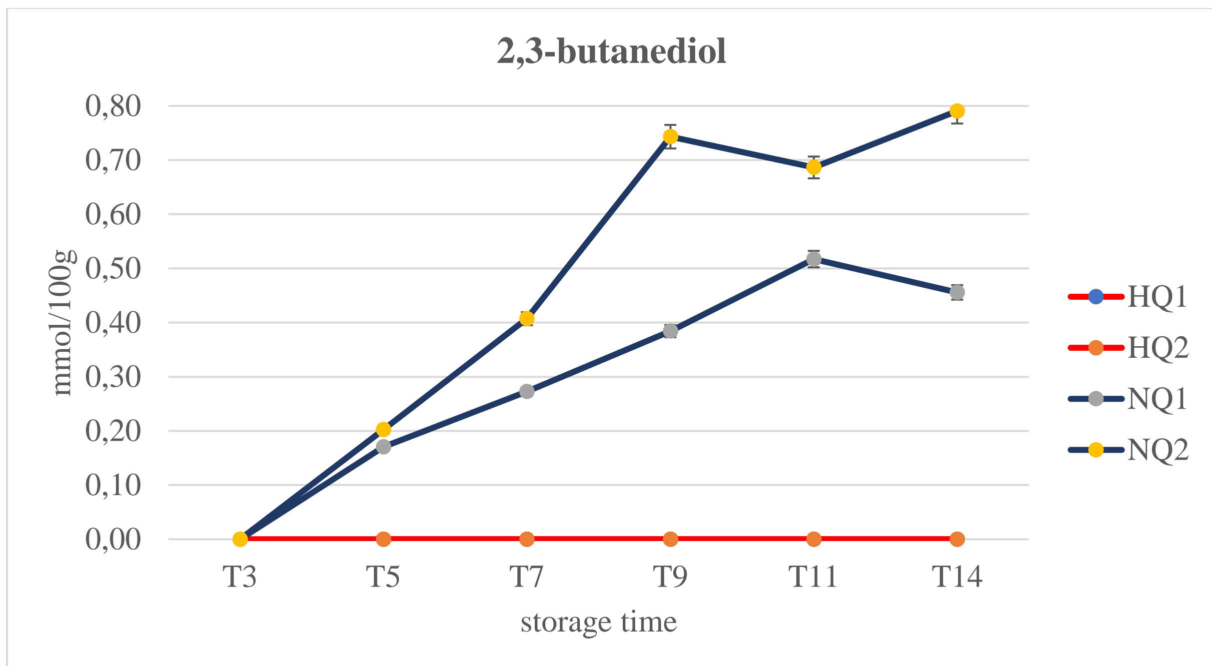

Figure 18: The concentration of 2,3-butanediol between NQ and HQ salmon.

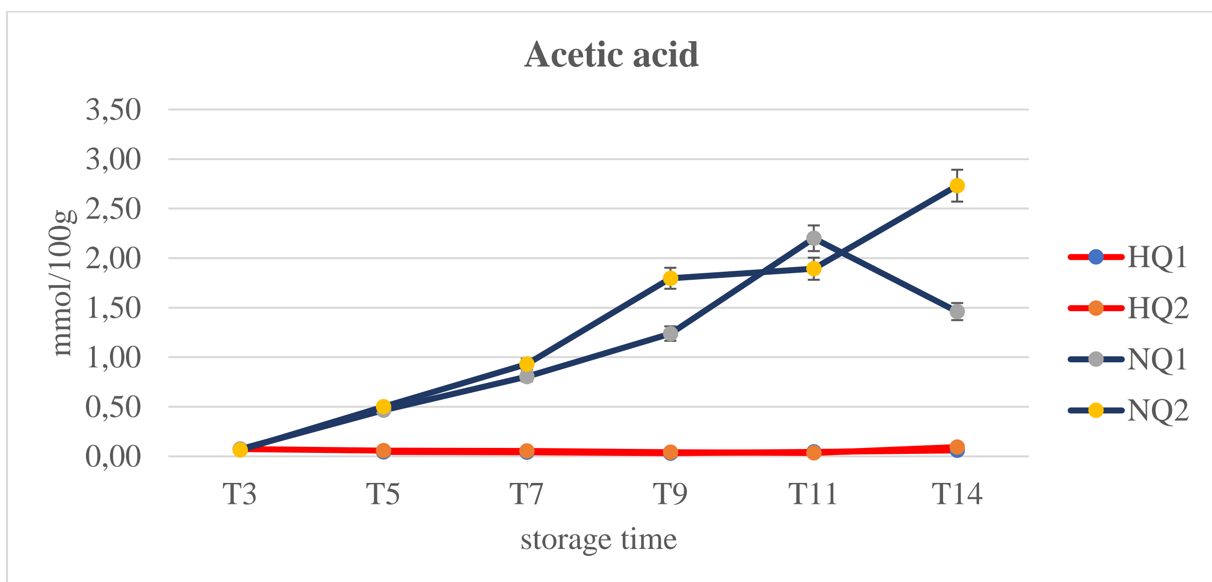

Figure 19: The concentration of acetic acid between NQ and HQ salmon.

4.2.6 Niacinamide

The niacinamide was detected at all time points in all samples stored at 4 °C. The concentration was almost stable in both HQ and NQ salmon without any significant difference (Figure 20). In both salmon, detected amount of niacinamide was 5-9 mg/100g, which is within the range of data found in the literature.

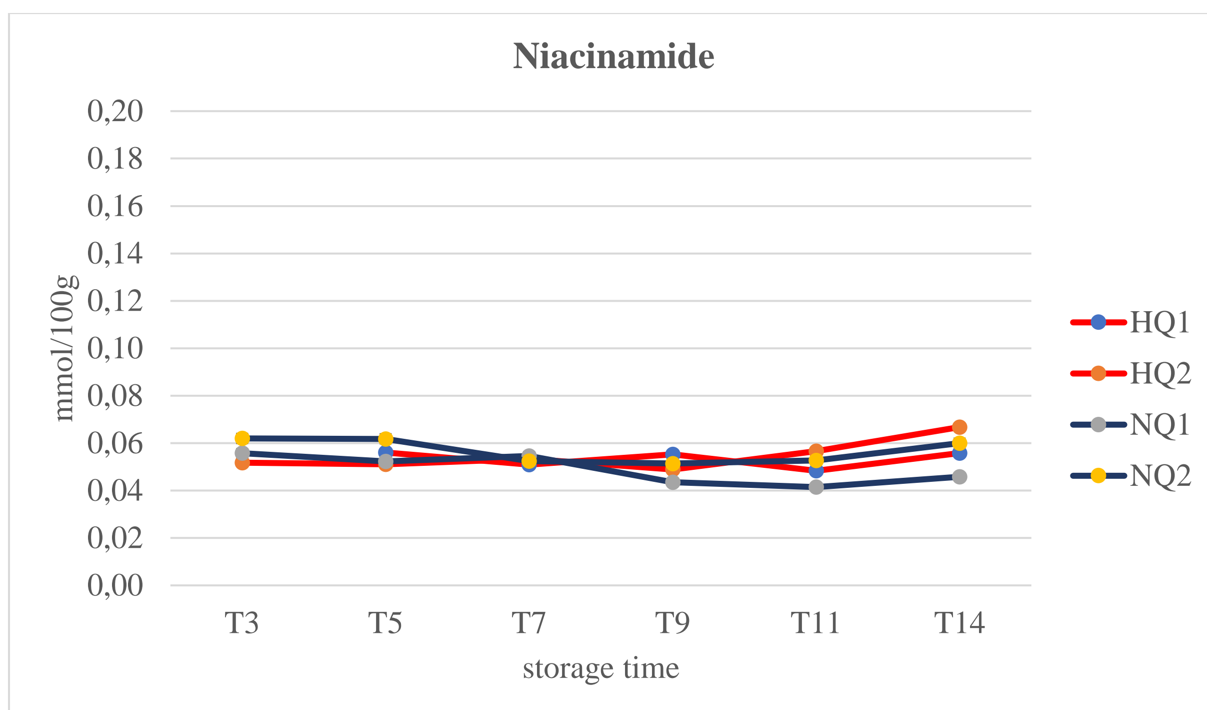

Figure 20: The concentration of niacinamide in Atlantic salmon stored at 4 °C. The red lines correspond to HQ salmon and the blue lines correspond to NQ salmon.

4.3 Mass spectrometry

TCA extracts (1 ml) of the same normal quality fish were analyzed at 4 different time points (T0, T7, T14 and T21). TCA extracts were previously adjusted by using sodium phosphate, therefore, we were expecting to see adducts of sodium with the chemical compounds we were looking for. The metabolites of the main interest were lactic acid, creatine, anserine, acetic acid, glucose, ethanol, trimethylamine, and trimethylamine oxide. All these metabolites were chosen to be analyzed because of their largest changes between different time points. In table 6, known masses of these metabolites are listed. The table contains also masses at their protonated stage (H^+, Na^+). It must be noted that all these metabolites may be seen only at protonated stage, either with positive or negative charge.

Table 6: The table shows expected monoisotopic molecular masses of the metabolites of our interest, including the masses of the abducts with H^+ and Na^+ (Huang et al., 1999). The results are shown on the right side of the table, marked with a specific sample, where the adducts of metabolite were observed.

	Molecular weight	Adduct with H^+	Adduct with Na^+	**Results**			
				Adduct with H^+	Sample	Adduct with Na^+	Sample
Lactic acid	90.03	91.03	113.02	90.05	T0, T7, T14, T21	-	-
Creatine	240.12	241.12	263.11	241.12	T0, T7, T14, T21	263.11	T7, T14
Anserine	131.06	132.07	154.05	132.07	T0, T7, T14, T21	154.05	T7, T14
Acetic acid	60.02	61.02	83.01	-	-	-	-
Glucose	180.06	191.07	203.05	-	-	-	-
Ethanol	46.04	47.04	69.03	-	-	-	-
TMA	59.07	60.08	82.06	-	-	-	-
TMAO	75.06	76.07	98.05	-	-	-	-

Only adducts of the main metabolites such as lactic acid, anserine and creatine were observed, both as adducts with H^+ and Na^+ (Figure 22). The real mass values of H^+ adducts with lactic acid, anserine and creatine were 90.05, 241.12, 132.07, respectively. The real mass values of Na^+ adducts with creatine and anserine were 263.11, 154.05, respectively. The adduct of Na^+ with lactic acid was not observed.

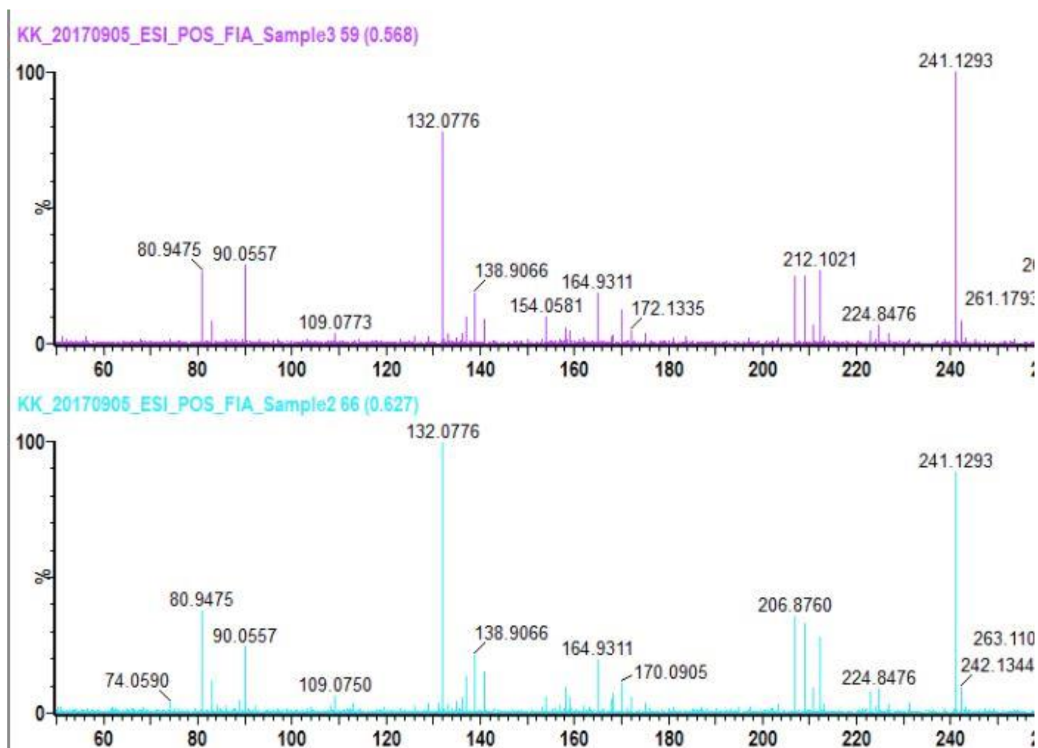

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Figure 21: Mass spectrum of a salmon at 7 (below) and 14 (above) days of a storage at 4°C. In the spectrum, the adducts of lactic acid (90.0557), anserine (241.1293, 263.1103) and creatine (132.0776, 154.0581) can be observed.

4.4 Statistics

4.4.1 PCA

In the beginning, ^{1}H NMR spectra were normalized to the average mass (5g) of the extracted salmon tissue and volume (30 ml) of extraction using TopSpin 3.5.b.88pl7. Normalization is an imperative step before loading our data into Amix for Principal component analysis (PCA). There is a probability of sample to sample variability due to variety of sample concentrations, normalization helps to reduce this variability and makes spectra comparable. After normalization, all the ^{1}H NMR spectra, from 9.005 to 5.185 ppm and from 4.305 to 0.755 ppm, were binned with the simple standard size bucketing (0.01 ppm).

A total of 737 buckets were analyzed. Integrals were calculated and the bucket table was produced. The table of integrals was used as an input for Unscrambler (version 10.4, CAMO Software AS Oslo, Norway). This data was loaded into Unscrambler and was analyzed by performing PCA. Figure 23 illustrates the distribution of salmon samples.

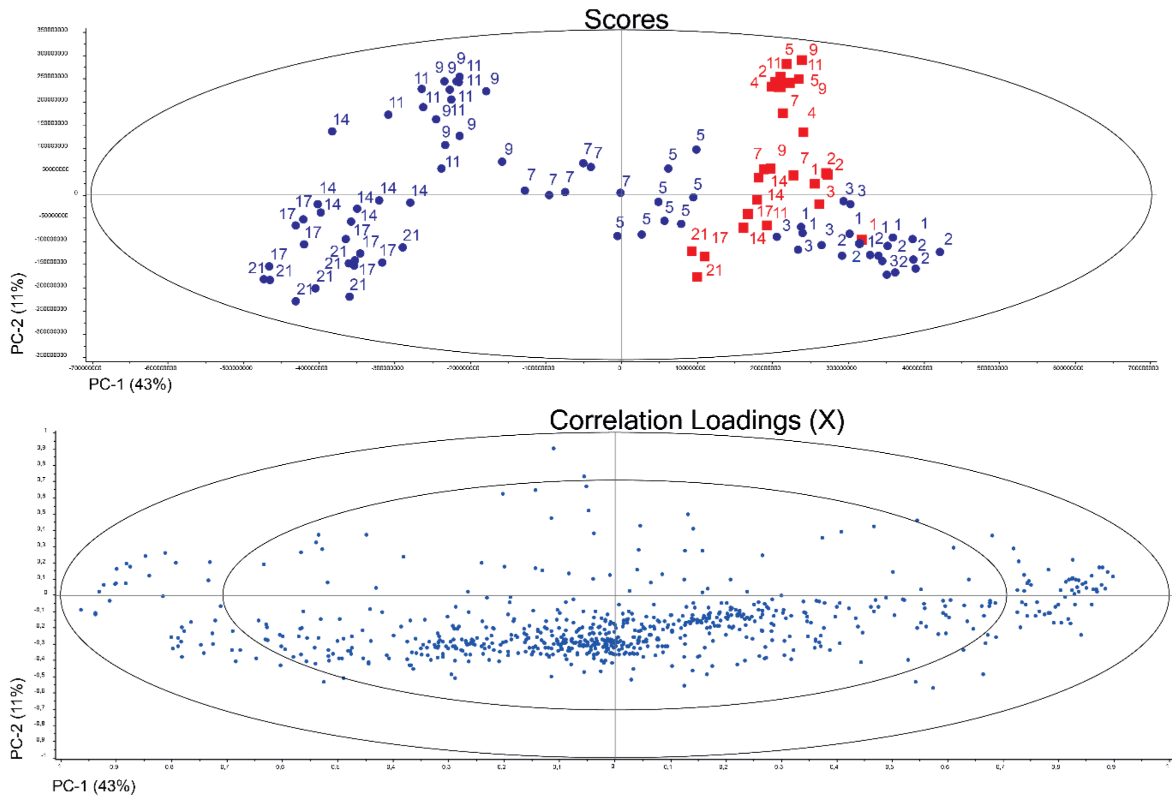

Figure 22: Scores and loading plots of PCA, where PC1 explains 43% and PC-2 – 11% of variables. Red squares – HQ salmon; blue dots - NQ salmon.

The representative points of the salmon samples are mapped in the space spanned by the first two principal components (PC1 versus PC2) that explains 43 % and 11 % of variables, respectively. The points which are closer to the center, in the lower part of the figure, are less significant. The points between the two circles are the ones which are significant and influence the distribution of variables most.

After, the non-significant regions were removed and only regions of metabolites shown in Table 7, which corresponds to 199 buckets, were analyzed by PCA. The main difference between the two different quality of salmon occurs due to the metabolites shown in Table 7. This score plot, shown by Figure 23, illustrates a reasonable clustering appearing according the different quality of salmon samples. The illustrating by the first two principal component analysis (PC1 versus PC2) explains 88 % and 6 % of variables, respectively.

Table 7: List of the most important metabolites used in PCA with their ppm spectral width.

Metabolite	δ, ppm	
Leu, Ile, Val	0,795	1,085
Ethanol, 2,3-butanediol, cadaverine	1,125	1,775
Acetate	1,905	1,935
Acetone, other	2,115	2,245
GABA	2,325	2,245
Succinic acid	2,405	2,425
TMA	2,895	2,915
Phenylalanine	7,325	7,455
Formic acid	8,455	8,475

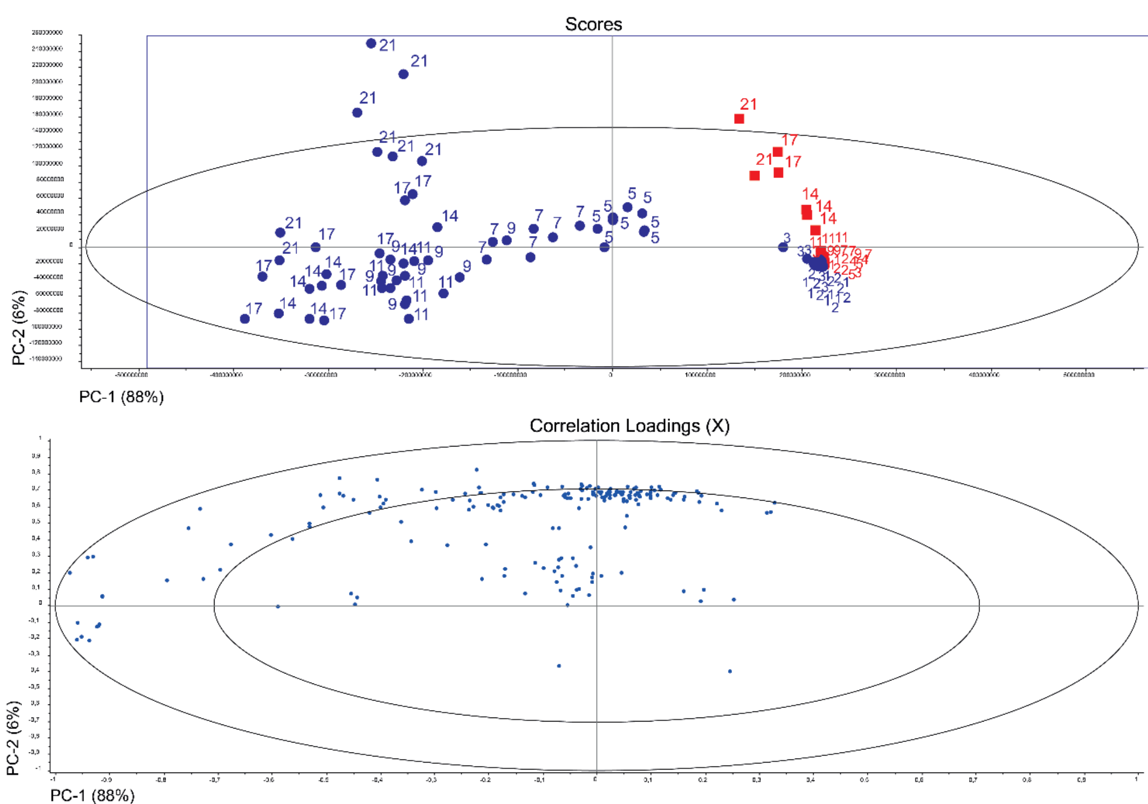

Figure 23: Score and loading plots of PCA of reduced data. It illustrates salmon samples distribution by PCA, where PC1 explains 88 % variables and PC2 explains 6 % of variables. The clustering shown in upper part of the figure corresponds to two different quality of the salmon samples. Red squares – HQ salmon; blue dots – NQ salmon.

The clustering seen in upper part of the Figure 23 corresponds to two different quality of the salmon samples and therefore SIMCA analysis could be performed by using SIMCA approach for further analyzing of the samples.

4.4.2 SIMCA model

Using SIMCA approach, SIMCA classification were performed and PCA was done only for HQ salmon. By performing this high quality SIMCA model, the classification table was produced, shown in Table 8, where it is a possibility to see which sample from NQ salmon belong and corresponds to the high quality SIMCA model.

Table 8: The classification table according to High quality SIMCA model is shown. The star is shown next to those samples, which correspond or belong to the high quality SIMCA model.

Sample - Class membership 5%, high quality model

1_St1	*	1_St2	*	1_St3	*	1_St4	*	2_St5	*	1_St7	*	1_St8	*	1_St9	*
2_St1	*	2_St2	*	2_St3	*	2_St4	*	5_St5		2_St7	*	2_St8	*	2_St9	*
3_St1	*	3_St2	*	3_St3	*	5_St4		7_St5		3_St7	*	3_St8		3_St9	*
5_St1		5_St2		5_St3		9_St4		9_St5		5_St7		5_St8		5_St9	
7_St1		9_St2		7_St3		11_St4		11_St5		7_St7		7_St8		7_St9	
9_St1		11_St2		9_St3		17_St4		14_St5		9_St7		9_St8		9_St9	
14_St1		14_St2		11_St3				17_St5		11_St7		11_St8		11_St9	
17_St1		17_St2		14_St3				21_St5		14_St7		14_St8		14_St9	
21_St1		21_St2		17_St3						17_St7		17_St8		17_St9	
				21_St3						21_St7		21_St8		21_St9	

In Figure 24, Coomans plots of SIMCA classification is shown. The big difference between NQ and HQ salmon can be seen. This figure shows a sample distance to the High quality SIMCA model. All the samples from NQ salmon stored more than 3 days at 4 °C are far from SIMCA

model which means that these samples are very different quality than those which belongs to SIMCA model.

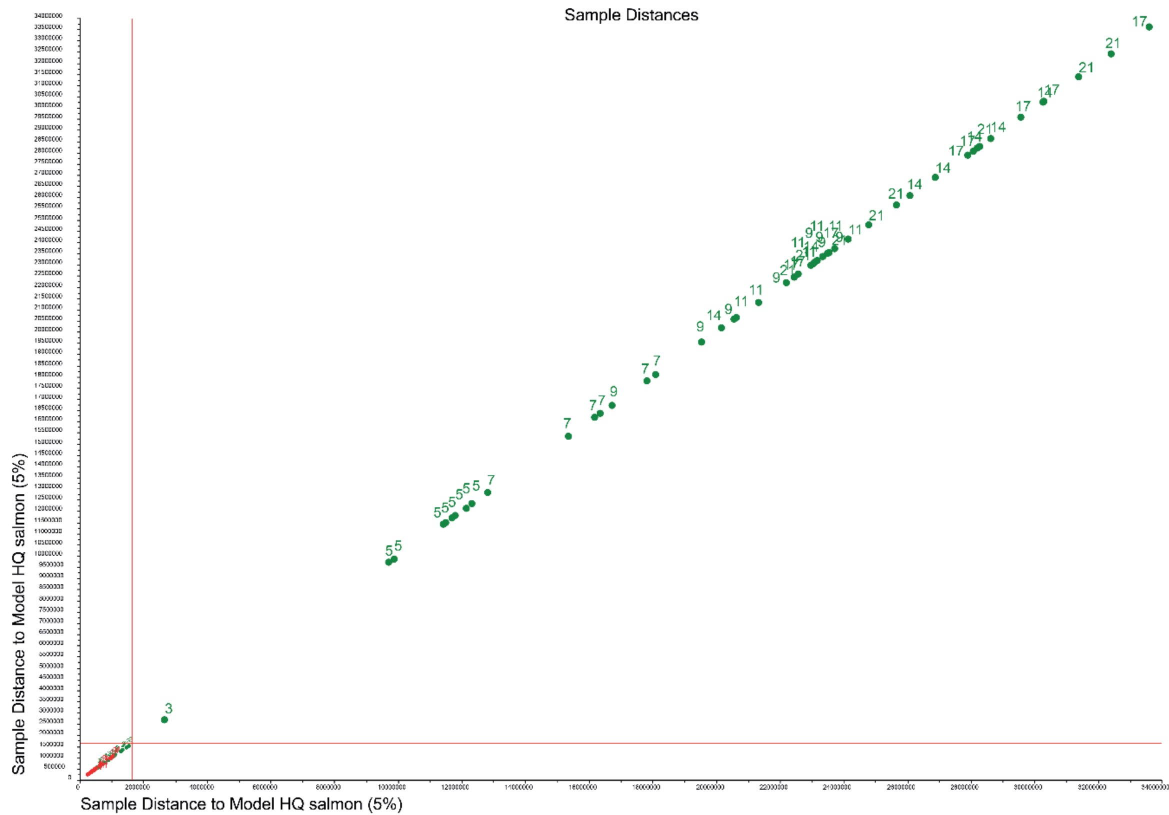

Figure 24: Coomans plot of SIMCA classification.

Figure 25 shows a zoomed version of the Coomans plot of SIMCA classification with emphasis on SIMCA model. This model includes all the samples from HQ salmon, and only samples up to 3 days of storage from NQ salmon. Those first 3 days of storage are the most valuable days according to quality. However, it is important to note, that to be able to monitor post-mortem biochemical processes their changes by time, a fish should be stored up to 7 days. During this first week, the most changes in biochemical cycles occur.

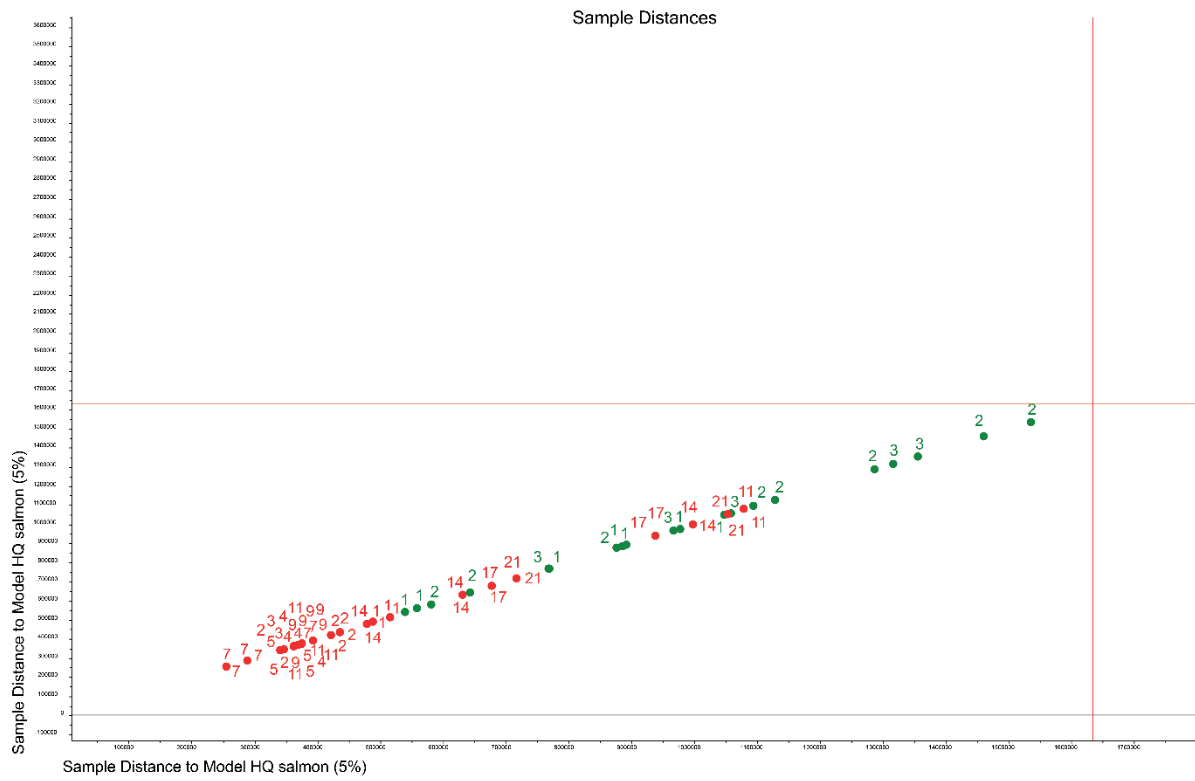

Figure 25: High quality SIMCA model (zoom-in version).

5 Conclusion

The most important fish metabolites were monitored by NMR. Most of those metabolites were part of the post-mortem biochemical processes and their concentrations changed during the storage. Some of them were stable during storage and any significant changes were observed. One of those metabolites was niacinamide, which is proposed to be a good indicator of fish species, as its concentration varies greatly between fish species. It was evident that the post-mortem biochemical processes change over the time when fish is stored. Their changes were significantly different between normal and high quality salmon. The major changes occurred between three days and up to two weeks of storage at 4 °C. It has been proposed by this thesis that fish should be stored for a minimum one week to be able to see differences between the quality of the salmon.

The concentrations of metabolites such as hypoxanthine, inosine and some of the other ATP degradation products were used for calculation of K-index. Only one high quality salmon was proposed to be of excellent quality and suitable for human consumption. Normal quality salmon reached the maximum tolerable concentration for consumption at the third day of storage, while high quality salmon was proposed to be edible after up to nine days of storage. Hypoxanthine is a metabolite which with its increasing concentration values corresponds to loss of freshness. In normal quality salmon, a rapid increase of hypoxanthine from the third day of storage was observed. After two weeks of storage, hypoxanthine was observed with increasing values, which is most likely connected to ongoing degradation to xanthine and uric acid. However, xanthine was not found in any of our sample, therefore, it requires further research with using a different method.

It has been known that after fish enters rigor mortis, its proteins start to degrade and muscle tissue changes its structure. Amino acids, such as valine and alanine, were monitored in all our samples, which indicated previous break down of proteins. However, any significant changes were observed in concentration between normal and high quality fish.

Biogenic amines are important metabolites which in higher concentrations may be dangerous for humans, due to of their toxicity. It is important to monitor these metabolites. The formation of biogenic amines was not observed in high quality fish, only the precursors of biogenic amines

were observed. It was a different case with normal quality fish, where the formation of tyramine and cadaverine occurred during the fifth day of storage.

Another, important metabolite, is TMA whose formation decrease the quality of the fish and it is most likely connected to degradation of TMAO by bacterial enzymes. While normal quality salmon had a rapid increase of TMA concentration during its first week of storage, high quality salmon showed very low concentration of TMA. The levels of TMA in high quality salmon did not exceed the maximum acceptable level for human consumption, while the normal quality fish was with maximum level after a week of storage.

Another process, where bacteria might be involved is fermentation. Bacteria eats the sugar and may produce different products such as organic acids, alcohols and others. The formation of ethanol, 2,3-butanediol and acetic acid were observed only in normal quality fish with very high concentrations. There are several types of bacteria which might be involved in this fermentation process, however, some hypothetical suggestions were made in this thesis, but it requires further analysis.

In this thesis, also NMR data were compared with mass spectrometry data. However, there was only the ability to monitor the adducts with H^+ and Na^+. The observed metabolites by mass spectrometry were only lactic acid, creatine and anserine.

In the end of this thesis, High quality SIMCA model was created. This model included all samples from high quality salmon and was compared to normal quality salmon samples. It has been found that while high quality salmon is a high quality for two weeks of storage, the normal quality salmon belonged to the SIMCA model only up to three days of storage.

The results of this thesis can be used for further applications in food industry to monitor quality of different food products, not only fish.

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Appendix

Table 9: Weighting of salmon and error calculation.

Error analysis Sample Name	Mass (m1) before (g)	Mass (m2) after (g)	Δ	% Δ
TSalm_1_T0	4,92	-	-	-
TSalm_1_T2	5,04	-	-	-
TSalm_1_T3	5,02	4,92	0,10	2,05
TSalm_1_T5	5,02	4,89	0,13	2,61
TSalm_1_T7	5,08	5,08	0,00	0,06
TSalm_1_T9	5,01	4,99	0,02	0,40
TSalm_1_T11	5,09	5,08	0,01	0,26
TSalm_1_T14	5,02	5,00	0,02	0,32
TSalm_1_T17	5,02	5,01	0,02	0,30
TSalm_1_T21	5,02	5,01	0,01	0,28
TSalm_2_T0	4,98	-	-	-
TSalm_2_T2	5,09	-	-	-
TSalm_2_T3	5,08	5,01	0,07	1,46
TSalm_2_T5	5,02	4,89	0,13	2,53
TSalm_2_T9	5,06	5,04	0,02	0,34
TSalm_2_T11	5,03	5,00	0,02	0,42
TSalm_2_T14	5,06	5,05	0,01	0,20
TSalm_2_T17	5,06	5,04	0,01	0,28
TSalm_2_T21	5,02	4,99	0,02	0,44
TSalm_3_T0	5,06	-	-	-
TSalm_3_T2	4,99	-	-	-
TSalm_3_T3	5,04	4,96	0,07	1,45
TSalm_3_T5	5,01	4,89	0,12	2,32
TSalm_3_T7	5,05	4,95	0,10	1,98
TSalm_3_T9	5,04	5,03	0,01	0,28
TSalm_3_T11	5,05	5,03	0,02	0,38
TSalm_3_T14	5,05	5,02	0,03	0,53
TSalm_3_T17	5,01	5,00	0,01	0,28
TSalm_3_T21	5,03	5,01	0,02	0,38
TSalm_4_T0	5,05	-	-	-
TSalm_4_T2	5,03	-	-	-
TSalm_4_T3	5,03	4,96	0,07	1,29
TSalm_4_T5	5,03	4,92	0,12	2,32
TSalm_4_T7	5,07	4,96	0,11	2,07

TSalm_4_T9	5,03	5,02	0,01	0,24
TSalm_4_T11	5,06	5,05	0,01	0,22
TSalm_4_T14	5,04	5,03	0,02	0,32
TSalm_4_T17	5,09	5,08	0,01	0,29
TSalm_4_T21	5,02	4,99	0,03	0,66
TSalm_5_T0	5,00	-	-	-
TSalm_5_T2	5,05	-	-	-
TSalm_5_T3	5,04	5,02	0,02	0,38
TSalm_5_T5	5,07	4,99	0,08	1,62
TSalm_5_T7	5,05	5,03	0,03	0,49
TSalm_5_T9	5,01	5,00	0,02	0,36
TSalm_5_T11	5,08	5,06	0,02	0,33
TSalm_5_T14	5,08	5,06	0,02	0,32
TSalm_5_T17	5,04	5,03	0,01	0,28
TSalm_5_T21	5,04	5,00	0,04	0,89
TSalm_7_T0	5,05	-	-	-
TSalm_7_T2	5,01	-	-	-
TSalm_7_T3	5,05	5,02	0,03	0,59
TSalm_7_T5	5,06	4,97	0,09	1,82
TSalm_7_T7	5,03	4,92	0,12	2,28
TSalm_7_T9	5,02	5,00	0,02	0,48
TSalm_7_T11	5,02	5,00	0,02	0,48
TSalm_7_T14	5,08	5,05	0,03	0,55
TSalm_7_T17	5,01	5,01	0,00	0,04
TSalm_7_T21	5,02	4,99	0,03	0,58
TSalm_8_T0	5,04	-	-	-
TSalm_8_T2	5,01	-	-	-
TSalm_8_T3	5,02	5,00	0,02	0,42
TSalm_8_T5	5,01	4,91	0,11	2,09
TSalm_8_T7	5,07	4,97	0,10	2,01
TSalm_8_T9	5,07	5,03	0,03	0,65
TSalm_8_T11	5,01	4,98	0,03	0,66
TSalm_8_T14	5,00	4,99	0,01	0,28
TSalm_8_T17	5,08	5,06	0,02	0,37
TSalm_8_T21	5,03	4,99	0,04	0,80
TSalm_9_T0	5,01	-	-	-
TSalm_9_T2	5,02	-	-	-
TSalm_9_T3	5,05	4,96	0,09	1,74
TSalm_9_T5	5,03	4,93	0,10	2,01
TSalm_9_T7	5,00	4,91	0,09	1,86
TSalm_9_T9	5,03	5,01	0,02	0,38
TSalm_9_T11	5,04	5,03	0,01	0,20
TSalm_9_T14	5,01	4,98	0,03	0,50

TSalm_9_T17	5,05	5,02	0,03	0,50
TSalm_9_T21	5,02	4,97	0,04	0,88
mean (%)				**0,85**

Table 10: TSP integral – error calculation

Error analysis	**TSP Integral**
Sample Name	
TSalm_2_T0	-
TSalm_2_T2	-
TSalm_2_T3	1730311,00
TSalm_2_T5	1765398,00
TSalm_2_T9	1700743,00
TSalm_2_T11	1746468,00
TSalm_2_T14	1714596,00
TSalm_2_T17	1726561,00
TSalm_2_T21	-
mean	1730679,50
SD	22919,96
%	**1,32**

Metabolite (Integrals) / Sample Name	Leu	Val	2,3-BDL	EtOH	LA	Ala	Acetic acid	Acetone	Glutamate	Succinic acid
TSalm_1_T0	1,50	7,27	-	9,49	1,06	1,09	16,18	-	1,45	1,13
TSalm_1_T2	0,62	4,04	-	38,89	0,51	4,64	16,20	-	2,55	2,44
TSalm_1_T3	2,49	0,68	-	5,88	0,58	2,82	0,47	-	2,09	5,00
TSalm_1_T5	1,71	4,20	3,45	2,63	0,10	3,27	3,76	1,50	3,04	2,29
TSalm_1_T7	0,84	2,65	3,51	2,96	0,48	1,95	3,18	1,63	2,45	0,19
TSalm_1_T9	0,83	0,79	3,14	0,51	0,48	2,48	1,71	4,70	1,23	1,85
TSalm_1_T11	3,07	0,37	3,39	0,33	0,53	1,48	0,95	1,24	5,25	1,43
TSalm_1_T14	4,15	1,98	3,76	0,38	0,40	5,32	2,07	6,98	42,58	1,73
TSalm_1_T17	1,18	1,27	3,52	1,41	0,40	3,66	1,70	1,40	7,45	0,44
TSalm_1_T21	4,07	9,97	3,17	1,35	0,47	8,49	0,17	4,10	0,70	0,13
TSalm_2_T0	0,26	0,82	-	3,76	0,62	2,01	18,22	-	1,17	1,06
TSalm_2_T2	1,37	0,46	-	2,86	0,43	3,59	10,41	-	0,87	5,79
TSalm_2_T3	1,48	3,36	-	7,11	0,80	1,62	18,44	3,30	0,71	1,03
TSalm_2_T5	0,93	0,56	3,25	2,96	0,37	6,67	3,42	5,45	3,65	0,22
TSalm_2_T7	-	-	-	-	-	-	-	-	-	-
TSalm_2_T9	0,53	2,82	3,21	1,14	0,33	5,90	3,00	2,52	0,59	0,50
TSalm_2_T11	0,85	2,69	3,00	0,17	0,12	3,41	1,63	11,78	36,23	0,16
TSalm_2_T14	53,63	1,81	3,43	0,41	0,38	5,43	1,54	2,83	5,68	0,10
TSalm_2_T17	4,58	3,15	2,76	1,47	0,55	4,73	1,69	1,08	0,86	1,54
TSalm_2_T21	1,17	10,41	3,05	1,60	0,28	6,90	3,11	1,03	31,52	0,30
TSalm_4_T0	0,66	7,14	-	-	0,32	2,11	13,59	-	3,07	1,81
TSalm_4_T2	0,85	6,20	-	0,80	0,24	1,82	26,02	-	0,31	1,06
TSalm_4_T3	0,21	3,35	-	13,07	0,27	1,07	17,26	-	0,73	-
TSalm_4_T5	0,66	1,60	3,32	4,21	0,33	1,40	4,13	0,78	6,03	0,95
TSalm_4_T7	3,01	2,34	3,98	2,59	0,16	1,28	0,67	0,53	9,52	0,62
TSalm_4_T9	3,05	2,49	2,92	2,95	0,42	1,03	0,79	2,64	4,74	1,90
TSalm_4_T11	3,84	5,05	2,60	0,66	0,09	2,31	1,02	2,53	9,37	1,31
TSalm_4_T14	5,57	7,64	2,40	0,83	0,16	3,12	1,37	0,92	-	1,17
TSalm_4_T17	6,67	14,82	2,76	3,21	0,57	1,14	1,48	0,87	-	6,09
TSalm_4_T21	1,18	13,09	2,98	2,52	0,38	6,73	0,94	4,09	12,67	0,24
TSalm_5_T0	3,34	65,63	-	-	0,26	0,97	17,77	-	1,44	24,08
TSalm_5_T2	1,74	5,20	-	33,45	0,18	2,36	23,50	-	2,61	4,52
TSalm_5_T3	1,66	3,82	-	23,72	0,09	3,38	20,78	-	1,63	4,44
TSalm_5_T5	7,11	1,17	0,19	7,68	0,07	1,62	1,26	0,63	0,24	0,09
TSalm_5_T7	3,04	1,68	3,39	2,85	0,72	1,61	0,35	0,20	0,55	0,49

TSalm_5_T9	1,48	0,55	2,76	1,49	0,33	2,77	0,38	11,81	0,03	0,74
TSalm_5_T11	3,46	0,83	3,12	1,09	0,17	2,04	0,76	1,49	4,24	0,50
TSalm_5_T14	1,44	7,85	3,23	0,92	0,37	1,61	0,77	1,32	0,00	1,32
TSalm_5_T17	42,85	2,08	3,20	0,37	0,29	1,71	0,87	0,39	4,20	0,48
TSalm_5_T21	2,33	12,48	2,41	1,18	0,65	6,32	1,28	1,69	3,30	0,19
TSalm_7_T0	1,77	1,81	-	-	0,48	1,78	27,90	-	0,41	2,37
TSalm_7_T2	0,78	0,74	-	61,76	0,04	2,58	31,21	-	0,51	9,45
TSalm_7_T3	3,35	1,63	-	36,22	0,33	1,04	10,12	-	0,75	1,25
TSalm_7_T5	3,14	0,06	3,36	2,36	0,11	4,50	2,57	2,49	0,95	0,63
TSalm_7_T7	1,78	3,41	3,20	5,31	0,37	1,30	0,52	0,96	3,47	1,69
TSalm_7_T9	2,78	1,57	2,74	3,33	0,47	3,32	0,98	7,32	6,97	0,23
TSalm_7_T11	2,14	3,48	2,88	19,78	0,41	2,19	0,90	7,78	3,14	0,52
TSalm_7_T14	0,84	2,07	3,11	1,37	0,39	3,64	0,56	0,89	4,76	0,35
TSalm_7_T17	3,22	3,01	2,89	3,39	0,59	0,97	0,33	0,99	3,51	0,26
TSalm_7_T21	0,95	10,49	3,27	2,80	0,72	3,71	0,78	0,47	2,63	0,22
TSalm_8_T0	0,52	2,81	-	-	0,19	0,88	14,03	-	1,12	7,54
TSalm_8_T2	0,27	0,97	-	-	0,40	1,45	18,74	-	0,82	3,27
TSalm_8_T3	1,12	1,61	-	11,18	0,90	1,38	9,19	16,52	2,72	2,49
TSalm_8_T5	1,07	1,60	3,17	7,46	0,72	5,57	1,69	0,25	2,77	2,23
TSalm_8_T7	0,99	0,46	2,19	2,61	0,72	5,01	0,67	0,48	1,52	0,30
TSalm_8_T9	4,10	2,27	0,69	1,08	0,23	2,08	0,12	2,09	4,37	1,15
TSalm_8_T11	0,52	0,30	2,74	1,88	0,46	3,81	1,04	1,64	17,36	0,99
TSalm_8_T14	0,58	2,12	2,80	1,23	0,75	3,61	1,74	1,55	4,85	0,20
TSalm_8_T17	0,73	6,86	2,72	0,63	0,57	2,07	1,11	1,10	-	0,70
TSalm_8_T21	2,15	8,68	2,24	3,38	0,84	4,93	1,05	0,50	32,59	3,11
TSalm_9_T0	1,25	5,33	-	-	0,69	2,85	15,20	-	0,53	6,34
TSalm_9_T2	0,57	2,68	-	12,61	0,69	3,15	15,16	-	0,68	3,33
TSalm_9_T3	2,12	0,53	-	59,69	0,40	1,80	3,71	-	1,40	1,98
TSalm_9_T5	1,22	2,57	3,24	3,84	0,61	3,02	0,84	2,21	2,27	0,88
TSalm_9_T7	1,55	1,26	3,07	2,40	0,21	5,44	0,72	7,55	2,13	0,17
TSalm_9_T9	5,15	1,57	2,81	1,59	0,82	5,91	0,69	3,02	4,40	0,43
TSalm_9_T11	3,78	1,56	2,89	2,58	0,97	5,82	0,43	1,63	11,32	0,34
TSalm_9_T14	4,85	4,10	2,99	1,40	1,19	5,39	0,23	1,93	13,25	0,40
TSalm_9_T17	2,70	1,19	2,86	0,35	1,18	6,03	0,63	1,28	1,89	0,83
TSalm_9_T21	3,91	13,66	3,01	6,37	1,27	19,62	0,63	0,74	0,78	0,54
Error calculation (%)	**3,47**	**4,56**	**2,93**	**7,07**	**0,47**	**3,43**	**5,89**	**2,86**	**5,28**	**1,96**

Table 11: Error calculation for integrals of each signal (part 2).

Metabolite (Integrals) / Sample Name	TMA	TMAO	Anserine	P-Crt	Maltose	Hx	Inosine	Formic acid	IMP	Niacinamide
TSalm_1_T0	3,13	0,13	3,07	0,41	11,14	4,55	4,11	-	3,72	1,79
TSalm_1_T2	30,55	6,83	0,40	0,13	3,71	9,99	2,85	4,95	7,30	5,66
TSalm_1_T3	10,56	2,12	1,95	0,33	2,19	2,00	0,74	3,15	6,22	5,39
TSalm_1_T5	3,23	6,14	0,79	0,57	1,65	0,68	1,60	3,31	-	5,95
TSalm_1_T7	2,07	45,49	1,24	0,79	8,19	2,49	1,55	1,93	-	2,25
TSalm_1_T9	1,22	23,32	2,28	1,39	28,46	1,98	4,95	1,92	-	0,79
TSalm_1_T11	0,79	14,61	3,97	0,71	-	3,95	-	1,95	-	0,82
TSalm_1_T14	1,63	1,84	1,09	1,73	-	4,21	-	2,98	-	9,16
TSalm_1_T17	0,98	11,59	0,41	1,23	-	0,71	-	0,92	-	1,29
TSalm_1_T21	1,48	12,68	0,77	1,38	-	3,06	-	1,36	-	-
TSalm_2_T0	-	1,70	1,21	0,68	7,53	1,27	4,46	-	3,03	1,19
TSalm_2_T2	9,67	3,03	1,56	0,19	12,31	4,47	4,29	-	6,20	1,01
TSalm_2_T3	4,09	2,85	1,66	0,74	6,29	1,70	5,61	2,80	1,87	0,20
TSalm_2_T5	0,31	2,69	1,30	0,05	4,39	8,23	4,08	0,55	20,74	0,89
TSalm_2_T7	-	-	-	-	-	-	-	-	-	-
TSalm_2_T9	1,26	20,35	2,65	0,58	7,28	1,39	3,52	0,66	-	1,18
TSalm_2_T11	1,26	22,62	0,69	1,01	-	1,91	9,93	0,27	-	3,43
TSalm_2_T14	0,81	6,27	0,25	1,65	-	0,90	-	1,26	-	0,71
TSalm_2_T17	0,37	1,36	0,90	1,81	-	1,07	-	3,32	-	2,00
TSalm_2_T21	0,55	50,99	1,48	1,86	-	0,81	-	2,09	-	4,41
TSalm_4_T0	-	1,76	0,26	4,97	18,96	9,22	2,49	-	0,97	1,78
TSalm_4_T2	8,44	1,49	1,46	1,40	2,71	0,71	0,85	-	0,04	0,74
TSalm_4_T3	8,21	0,66	1,19	0,27	2,26	3,17	0,31	-	2,07	0,18
TSalm_4_T5	0,72	3,35	0,64	2,12	2,80	2,38	1,83	1,00	-	1,29
TSalm_4_T7	0,66	26,41	0,52	0,43	15,16	1,52	2,56	1,64	-	11,63
TSalm_4_T9	0,38	4,11	0,10	3,05	8,95	1,48	-	0,52	-	5,28
TSalm_4_T11	0,50	4,81	0,21	2,84	-	1,13	-	0,37	-	0,48
TSalm_4_T14	0,73	36,25	0,43	2,40	-	0,28	-	5,26	-	2,94
TSalm_4_T17	0,30	28,97	0,07	2,43	-	1,32	-	0,62	-	1,15
TSalm_4_T21	0,20	21,32	0,67	1,91	-	0,97	-	1,03	-	-
TSalm_5_T0	-	1,47	0,36	0,36	1,18	4,45	1,51	-	0,81	2,20
TSalm_5_T2	0,78	1,27	0,21	0,20	1,41	12,76	0,32	1,52	0,32	7,00
TSalm_5_T3	1,65	0,78	0,46	0,23	16,73	-	0,85	-	3,88	2,32
TSalm_5_T5	0,67	2,77	1,79	0,40	16,80	3,48	2,19	1,96	3,28	6,55
TSalm_5_T7	0,32	4,70	0,65	0,13	-	6,46	2,72	1,67	-	0,33
TSalm_5_T9	0,11	7,07	1,39	3,48	-	0,56	6,41	0,63	-	0,40

TSalm_5_T11	0,12	19,78	1,08	4,05	-	2,92	0,63	0,67	-	2,67
TSalm_5_T14	0,46	20,18	0,30	3,17	-	0,61	-	1,52	-	0,59
TSalm_5_T17	0,28	13,50	0,34	2,14	-	0,73	-	5,50	-	3,11
TSalm_5_T21	0,09	14,30	1,39	2,89	-	0,97	-	5,62	-	1,53
TSalm_7_T0	-	2,81	1,57	5,53	-	31,82	0,75	-	1,78	2,29
TSalm_7_T2	3,68	1,26	1,94	1,35	7,00	3,98	0,91	4,59	0,93	1,35
TSalm_7_T3	0,18	0,87	2,44	0,86	2,96	4,54	3,59	17,33	2,83	2,05
TSalm_7_T5	0,31	0,06	0,38	0,20	15,87	0,35	5,32	0,08	-	1,26
TSalm_7_T7	0,42	25,02	0,80	4,75	2,36	27,05	4,82	1,79	-	0,20
TSalm_7_T9	0,48	20,03	0,32	3,54	-	1,84	3,21	0,55	-	4,12
TSalm_7_T11	2,58	3,37	0,18	3,35	-	1,78	5,34	0,57	-	3,36
TSalm_7_T14	1,71	1,95	0,23	3,17	-	1,30	-	0,61	-	2,77
TSalm_7_T17	1,49	12,39	0,33	3,03	-	1,22	-	4,82	-	2,10
TSalm_7_T21	1,39	17,40	0,38	2,27	-	0,64	-	0,76	-	3,93
TSalm_8_T0	4,83	1,81	0,53	3,21	8,99	3,59	1,62	-	1,24	3,68
TSalm_8_T2	4,46	3,06	0,30	0,46	0,62	6,04	1,74	-	5,60	0,97
TSalm_8_T3	1,03	1,29	0,54	0,87	3,99	4,32	2,37	12,31	7,87	4,05
TSalm_8_T5	0,96	6,30	0,61	5,28	17,04	1,70	3,14	1,36	-	8,68
TSalm_8_T7	0,81	18,74	1,00	5,08	16,82	1,71	10,71	1,72	-	2,66
TSalm_8_T9	0,51	7,45	3,47	0,39	-	2,62	87,24	0,01	-	2,42
TSalm_8_T11	0,07	8,90	0,51	2,07	-	2,69	-	0,27	-	3,34
TSalm_8_T14	0,67	22,35	2,14	1,91	-	1,04	-	1,78	-	7,37
TSalm_8_T17	0,86	9,49	0,65	2,24	-	7,23	-	10,32	-	7,84
TSalm_8_T21	0,40	11,88	0,68	2,20	-	0,45	-	2,79	-	3,93
TSalm_9_T0	9,56	2,51	1,01	2,79	1,01	24,09	2,58	2,42	2,17	7,85
TSalm_9_T2	19,42	1,48	1,87	5,40	3,55	4,12	1,87	-	0,08	5,46
TSalm_9_T3	0,83	0,16	0,99	4,63	17,36	0,64	0,61	15,23	1,04	7,84
TSalm_9_T5	0,53	80,00	1,20	2,97	10,50	2,67	1,56	0,45	-	5,64
TSalm_9_T7	0,29	15,68	0,88	2,84	-	0,74	5,54	2,65	-	2,91
TSalm_9_T9	0,84	18,20	2,55	2,40	-	0,66	-	4,07	-	1,08
TSalm_9_T11	0,54	21,85	0,49	0,19	-	1,49	-	1,20	-	3,60
TSalm_9_T14	0,30	1,72	0,60	0,21	-	0,82	-	6,42	-	5,39
TSalm_9_T17	0,09	13,71	0,94	0,18	-	1,93	-	3,87	-	2,01
TSalm_9_T21	0,29	6,00	0,85	1,14	-	0,87	-	2,09	-	-
Error calculation (%)	**2,43**	**11,44**	**1,04**	**1,89**	**8,48**	**3,74**	**5,08**	**2,86**	**3,65**	**3,19**