Master thesis in Biotechnology

Superchilling of Salmon

Changes due to Proteolytic Degradation

Gunn-Marit Erlanda Fosshaug

May 2010

Summary

Isolation and identification of peptides in muscle foods can help us to understand the complex reactions that occur during storage of food, and help us improve shelf life. This work is part of the project “Competitive Food Processing in Norway” (KMB project). The aim of this thesis is to investigate the changes that occur in the peptide fraction of muscle tissue extract during storage, so that the proteolytic changes that occur during storage can be better understood.

In this thesis changes in proteins and peptides during storage have been investigated in samples superchilled using air and liquid carbon dioxide (LIC) to two ice levels and chilling references. Measurements of drip loss, water content and liquid loss have been done for the chilled reference samples, while the measurements of the superchilled samples have been collected from previous work in the KMB project. The investigations of the proteins and peptides have been done by extracting water soluble proteins from muscle tissue, and isolate peptides by precipitation using ethanol. The proteins in the extracts have been quantified using Lowry’s method and they have been identified using gel filtration by fast protein liquid chromatography (FPLC) and mass spectrometry (MS).

There were no significant differences in drip loss between the different chilling methods during storage. However, the significantly higher drip loss in samples superchilled by air indicate some formation of large ice crystals in the muscle compared to superchilling using liquid carbon dioxide. The liquid loss was significantly higher for samples superchilled using air before sixteen days of storage. After this there were no significant differences between the chilling methods.

There were no significant changes during storage in the content of neither water nor ethanol soluble proteins on wet weight basis.

Gel filtration using FPLC was a good tool for fractionation and purification of the extracts. Seven peaks were compared during storage. Precipitating the extracts of water soluble proteins made the concentrations of large proteins and peptides > 80 000 g/mol decrease and mostly disappeared. The superchilled samples showed no significant changes during storage. A significant decrease in compounds with a molecular weight of approximately 970 g/mol and an increase in small compounds below 100 g/mol were observed for the chilled reference samples.

MS was used as a tool for isolation and identification of compounds in the extracts and FPLC fractions. Some changes were detected for certain compounds. However, the work of identifying these compounds was troublesome as the mass to charge ratio is not accurate enough to be certain of the compound. Further work in identifying compounds in salmon muscle should include the use of MALDI analysis, which is more accurate for determining correct molecular weights than MS.

Three of the compounds found by MS were identified as glycine, creatine and anserine.

The combined results from FPLC and MS show that the protein content and structure in the superchilled samples are generally more stable than the chilled reference samples. These results suggest that superchilled fish products have a more stable muscle quality than fish stored on ice, while the ice chilled reference samples indicate an increase of free amino acids during storage. This increase suggests increased proteolytic activity during storage on ice.

Seen together, the results suggest that there are some changes in the composition of the proteins around 10 to 14 days of storage. Further work should investigate this period of storage, as it could give answers about important reactions concerning shelf life and product quality.

Preface

This work is a part of the project Competitive Food Processing in Norway and the work was carried out at the Department of Biotechnology at the Norwegian University of Science and Technology in Trondheim, Norway.

First of all I would like to thank Professor Turid Rustad for excellent guidance throughout the working process, and her acquaintance during the years. Dr.ing. Anne Sissel Duun is also to be thanked for her insight and knowledge of the field. Inga Marie Aasen is thanked for running the MS analysis, explaining and being so enthusiastic about the field of superchilling and mass spectrometry. Per Egil Gullsvåg is thanked for being helpful and positive when vacuum packing the chilled reference samples. Reza and Diana are thanked for analyzing standards using FPLC. The lab interns Berit, Sirpa and Inesa are thanked for filleting, weighing and extracting the chilled reference samples. Siri is thanked for being a lab equipment-oracle.

To my fellow students and colleagues; these two years would not have been the same without you. The lunch, coffee and cake-breaks have been very welcome excuses to get out of the reading room.

I would also like to thank my mother and father for their encouragement and support. My brothers and sisters are thanked for their perspective, humour and weekend visits. At last, but not least, Runar is thanked for his love, patience and for making me laugh every day.

_____

Gunn-Marit Erlanda Fosshaug
Trondheim, 18th of May 2010

Table of Contents

1 Introduction 1
1.1 General aspects 1
1.2 Quality of fish 3
1.2.1 Muscles in fish 3
1.2.2 Changes in the muscle after death 6
1.2.3 Quality aspects *post mortem* 7
1.3 Shelf life 10
1.3.1 Quality aspects of cooling 10
1.4 Superchilling 13
1.4.1 Methods for superchilling 15
1.4.2 Effects of superchilling 16
1.5 Proteolytic enzymes in fish muscle 18
1.5.1 Cathepsins 20
1.5.2 Calpains 21
1.6 Isolation and characterisation of peptides 23
1.6.1 Precipitation 23
1.6.2 Quantification of proteins 24
1.6.3 Chromatography 25
1.6.4 Mass spectrometry 26
1.7 Competitive Food Processing in Norway 29
1.8 Purpose of this work 30
2 Materials and methods 32
2.1 Preparation of samples 32
2.2 Drip loss 34
2.3 Water content and liquid loss 35
2.4 Preparation of extracts 36
2.5 Ethanol precipitation 36
2.6 Lowry's method 36
2.7 Fast Protein Liquid Chromatography 37
2.8 Mass spectrometry 38

3 Results 39
3.1 Drip loss and water holding capacity 39
3.1.1 Drip loss 39
3.1.2 Water content 40
3.1.3 Liquid loss 41
3.2 Protein content 42
3.3 Gel filtration 43
3.3.1 Molecular weight distribution determined by gel filtration 44
3.3.2 Gel filtration by FPLC performed on water soluble proteins 45
3.3.3 Gel filtration by FPLC performed on ethanol soluble proteins 46
3.3.4 Changes in peaks during storage 48
3.4 Mass spectrometry 50
3.4.1 Changes in composition during storage 50
3.4.2 Relative abundance for m/z values 51
3.4.3 Composition of the peaks 55
4 Discussion 57
4.1 Drip loss and water holding capacity 57
4.2 Protein content 59
4.3 Gel filtration using fast protein liquid chromatography 60
4.4 Mass spectrometry 63
5 Conclusions and further work 67
6 References 69

Appendixes 77

1 Introduction

1.1 General aspects

The use of fish as a diet protein source has existed for several thousands of years. It is a protein source that has been available all over the globe, and as long as there have been fish, there has been fishing. The increasing demand for fish protein, has led to overexploitation of many fish species. This has forced the nations to regulate fishing, and find new ways to obtain fish. Fish farming have been able to increase the supply of fish for the market (Magnussen et al., 2008); however there are still concerns about how fish farming affects the ecosystem in the sea (Ervik et al., 2006).

Salmon is one of Norway's most important export articles. In 2009, Norwegian companies exported 2.6 million tons of seafood worldwide, with a combined value of 44.7 billion NOK. Farmed salmon amounted to 23.7 million NOK. The export to China alone, were above 23 000 tons in 2009, and an increase is expected in 2010. (Norwegian Seafood Export Council, 2010)

The fish muscle is among the more easily degradable substrates for protein hydrolysis processes, and the need for technologies to preserve this muscle food is increasing with the demand for fresh fish. As is to be explained in chapter 1.3.2 the shelf life of fish increases with decreasing temperature. Fresh fish is stored on ice at a temperature of about 4 °C. Frozen fish is kept at approximately -20 °C. The maximum recommended storage time for these temperatures is 14 days and 3-12 months respectively. For superchilled fish the shelf life have been reported to be doubled compared to fish stored on ice. (Huss, 1995, Ronsivalli and Baker, 1981, Duun and Rustad, 2008)

As is to be explained in chapter 1.4, superchilling means to keep products at temperatures on the borderline between chilling and freezing, which is 1 to 3 centigrades below their initial freezing point(Duun, 2002, Hemmingsen, 2002). The purpose is to prolong the shelf life of fresh food (Duun, 2008).

Transporting fresh fish demands efficient transport methods that preserve the products all the way from the sea to the consumer. The increasing environmental concerns are also a driving force towards more environmentally friendly transport and preservation methods. (Winther, 2009)

Increasing the proportion of superchilled products would increase the energy needed for chilling; however the demand for ice would decrease, and as a consequence the potential volume for transporting fish would increase. Because superchilled products will keep the same quality as fresh fish for a longer time, this makes it possible to transport and store fish more energy efficient. The fish that before were transported by airfreight, may now be shipped in containers or bulk. (Magnussen et al., 2008, Winther, 2009)

1.2 Quality of fish

The quality of fish is a very complex term, and what is considered a high quality fish differs between species and also between consumers. Quality does not only relate to the freshness of fish, how old it is and the appearance, it also relates to the service received by the costumer and the costumer's expectations and specifications. (Nortvedt et al., 2006) As a consequence of the demand of the consumers, there have been considerable research directed at prolonging shelf life while ensuring the safety of fish and seafood (Cortesi et al., 2009).

As is to be explained in chapter 1.3, prolonging shelf life means keeping the quality high for as long as possible. The quality of fish is related to a number of factors. Texture, water holding capacity, taste, smell and colour are a few of the parameters used to classify fish. All of these parameters may change after death (chapter 1.2.3), and some changes in the fish muscles may have a crucial effect on product quality (Michalczyk and Surowka, 2007).

Within one fish species the quality may vary with different genetic backgrounds of families and individuals (Bahuaud, 2009). By adjusting rearing conditions according to the fish's individual specifications, the fish farmer may achieve fish with desired quality characteristics. In this way fish farming allows the fish farmer to influence the quality of the fish and thereby prolong shelf life. (Hultmann, 2003)

1.2.1 Muscles in fish

All vertebrates have three different types of muscle; smooth muscles in viscera and blood vessels, heart muscles and the skeletal muscles. The skeletal muscle is controlled by brain impulse and is the motile apparatus of the fish. It is also this muscle we most often eat as meat. In fish this constitutes about 40-68 % (Rustad, 2005) of the body weight depending on species and fish size. Pelagic fishes have the highest proportion of skeletal muscles. (Lynum, 2005)

The main components in fish muscle are proteins, water, lipids and ash. In general, the fish muscles are composed of approximately 20 % protein and 80 % water and lipids combined. If the lipid content increases, the water content decreases. The protein content remains relatively constant. (Rustad, 2005)

As already mentioned, the proteins make up approximately 20 % of the muscle weight. The proteins are divided into three groups based on solubility. Sarcoplasmic proteins constitute about 20 % of the total protein in the muscles, and are soluble in water. These proteins are mostly enzymes and chromo proteins. The myofibrillar proteins make up about 65-70 % of the total protein in the muscles. They are soluble at ionic strengths higher than 0.4, and are extracted in buffers containing salt. These proteins are the most unstable proteins in cold water fish species. The myofibrillar proteins are also the most important proteins for the functional properties of the muscle, and thereby also quality. The third group of proteins constitutes less than 3 % of the proteins in fish. These proteins are called connective tissue proteins and are generally insoluble in neutral solutions; however, by adding acid or alkali to the solution they may be solubilised. (Rustad, 2005) Knowledge about the proteins solubility can be utilized in isolation and characterization of proteins and peptides as described in chapter 1.6.1.

Most of the muscles in fish are located as two fillets stretching from the head to the tail on each side of the fish (Rustad, 2005). The muscles are composed of myotomes, separated by myosepts. By looking at a cross-section, the muscle segments will show a ring-pattern (Figure 1.1). The muscle fibres are found in the myotomes, while the myosept consists of connective tissue. Collagen is the main constituent of the connective tissue. The connective tissue, myofibrillar and cytoskeleton proteins give the fish its texture. One of the reasons why fish is softer than mammalian meat is that the collagen in fish has a lower melting temperature and is less cross-linked than mammalian collagen. (Lynum, 2005)

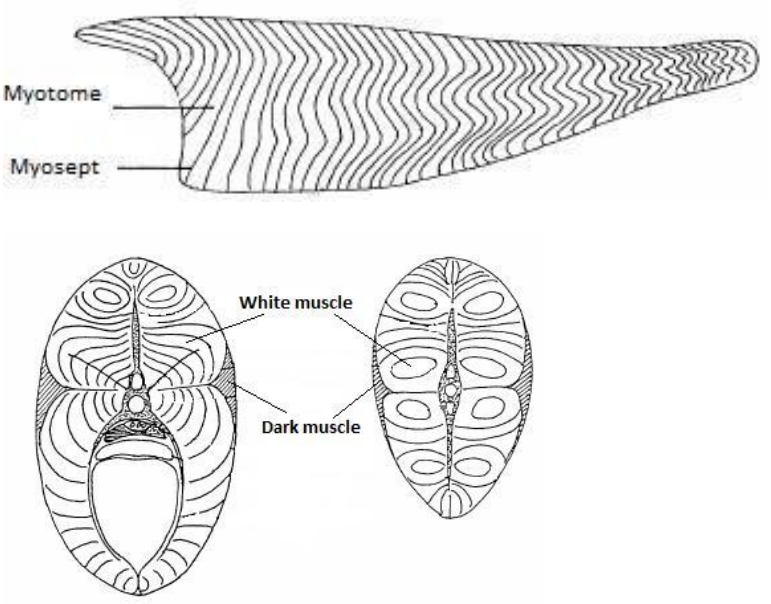


Figure 1.1: The metameric structure of fish muscle. (Lynum, 2005)

Figure 1.1 also illustrates that there are two different types of muscles in fish; the white muscle and the dark muscle, with the white muscle making up most of the muscle. The dark muscle is used for aerobic activity like constant swimming, while the white muscle is used for anaerobic activity during rapid bursts of swimming i.e. when escaping. The energy source of the dark muscle is oxidation of lipids, while the energy source of the white muscle is glycogen breakdown (Rustad, 2005). The dark muscle is made up of smaller bundles of muscle fibres and has a richer blood flow than white muscle. Compared to the dark muscle, the white muscle has a low fat content and is rich in glycolytic enzymes. The bundles in the dark muscle are constructed of several long muscle cells which consist of several myofibrils. (Lynum, 2005)

Myofibrils are bundles of the contractile proteins, actin and myosin. These proteins are responsible for contraction and relaxation of the muscle. This occurs by sliding action of the thin actin filaments and thick myosin filaments with the length of the filaments remaining the same. The contraction of the muscle starts by a nerve impulse which changes the permeability of the membrane in the muscle fibre. Ca-ions will leak into the myofibrils and the myosin will cleave the ATP. The energy released from this, will be utilized to make chemical connections between actin and myosin. The fibres are now

contracted. Subsequently the actin releases itself from the myosin and the muscle returns to its resting stage. (Rustad, 2005, Lynum, 2005)

1.2.2 Changes in the muscle after death

The changes in the muscle after death are divided in three steps; *pre-rigor*, *rigor* and *post-rigor*. When processed, the muscles behaviour is depending on the extent of the contraction.

In the *pre-rigor* state, the muscle is soft and elastic. After death the supply of oxygen in the muscle stops. This results in an incomplete decomposition of glycogen which is converted to lactic acid. Incomplete decomposition of glycogen result in less ATP formed pr mole of glucose broken down, and the total amount of ATP in the muscle is decreasing as shown in figure 1.2.

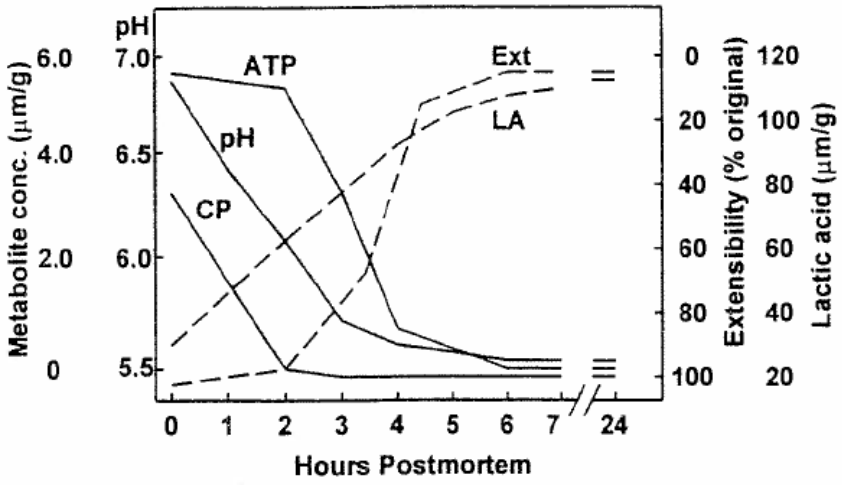


Figure 1.2: Chemical and physical changes in muscle post-mortem. The pattern shown would be typical for pig muscle undergoing normal metabolism. (Greaser, 2001)

In order to contract and relax, the muscle needs ATP. When the ATP level decreases below a certain threshold, the muscle becomes stiff and inelastic, it enters the *rigor mortis* state (Rustad, 2005, Lynum, 2005). *Rigor mortis* is, among other factors, influenced by killing method and storage temperature. When the fish is stressed or the temperature is

low, the *rigor mortis* period will start earlier and last longer (Erikson et al., 2006, Rustad, 2005).

The increasing concentrations of lactic acid in the muscle lead to decreasing pH in the muscle (figure 1.2). In fish, the pH usually decreases to about 6. The decrease in pH varies with species, size, feed and *pre-mortem* conditions. (Rustad, 2005) The pH value affects water holding capacity (chapter 1.2.3), texture and fillet gaping (Larsen, 2007).

In *rigor mortis* the muscle will be stiff and inelastic. This is also when the muscles water holding capacity is at its minimum. As the muscle pass out of *rigor*, the water holding capacity will increase. (Rustad, 2005)

Fish in the *post rigor* state is soft.(Lynum, 2005) In this state the muscle gradually gets more tender as a result of the decomposition of myofibrillar tissue. Low concentrations of O_2 or low pH may cause the lysosomal membranes to burst, releasing enzymes that decompose the muscle (chapter 1.5.1). (Rustad, 2005, Lynum, 2005)

1.2.3 Quality aspects *post mortem*

Post mortem the muscle contracts uncontrolled (chapter 1.2.2) because the ATP level in the muscle is below a certain threshold. Even though the ATP content in the muscle is decreasing below the threshold for uncontrolled contraction *post mortem*, the degradation of ATP continues. ATP is hydrolyzed to ADP and AMP, which in turn is deaminated to IMP. IMP is dephosphorylated to inosine which is decomposed to ribose and hypoxanthine. Hypoxanthine accumulates in the muscle. These different compounds can be used to determine the freshness of fish by the K-value. (Lynum, 2005, Rustad, 2005)

As explained in chapter 1.2.2, the muscle pH decreases after death. Both the rate of pH decrease and the final pH is of importance when it comes to quality aspects such as the texture and water holding capacity of the muscle. (Rustad, 2005)

By subjecting the fish to pre-slaughter crowding stress, Bahuaud (2009) found that the production of lactic acid reduced the muscle pH immediately *post-mortem*. As will be explained in chapter 1.5.2 cathepsins are acidic proteases and the activity is dependent on pH. The reduction in pH *post mortem* increased the activity of muscle cathepsin B and L earlier than if the pH was higher. The higher enzymatic activity may directly influence quality by accelerating the decomposition of myofibrillar tissue. Muscle pH was significantly correlated with the decomposition of myofibrillar tissue, fillet firmness and the total activity of cathepsin B and L. The effect of starving fish before slaughter may have the same impact on pH as stress. (Bahuaud, 2009)

The pH in the muscle will also affect the water holding capacity of the muscle. The water holding capacity of the muscle is important for the appearance and textural qualities of the muscle. At the isoelectric point (pI) of a protein, the net charge is zero. This means that there are equal amounts of negative and positive charges. The room for water in between the proteins decrease as the charges attract each other and the space between the charges decrease. (Rustad, 2005)

The most loosely bound water or liquid is lost as drip loss. Drip loss happens without the influence of any outer forces. Liquid loss is the loss of liquid in connection with the water holding capacity. This is loss due to the muscle being under the influence of outer sources e.g. centrifugation. The level of the liquid loss has to be seen in context with the amount of drip loss. Samples with high drip loss are more likely to be able to hold better on to the remaining water during the centrifugation analysis of water holding capacity. (Duun, 2008)

The drip loss and water holding capacity is among other factors influenced by muscle structures and activity of enzymes. Drip loss may influence the eating quality of the product as it reflects how the product retains water. Because of this, products with a high liquid loss may be perceived as dry and tough. (Duun, 2008) Some loss of nutrients in the liquid may lead to a product of lower nutritional value.

Reactions leading to degradation or oxidation of the lipid fraction, and thereby causing changes in smell and taste of the fish, are lipid reactions. Here there are differences between the white and the dark muscle. The two types of muscle have different oxidation reactions adjusted to each muscle. The dark muscle does normally have a higher content of lipids as well as a higher lipase activity than the white muscle. Because of this the oxidation level is higher. (Rustad, 2005) Lipases and phospholipases in the muscles catalyze the hydrolysis of fats to free fatty acids and may cause desirable or undesirable changes in taste (Tironi et al., 2007).

Proteinases and peptidases are enzymes which hydrolyze peptide bonds and thereby deteriorate the muscle (chapter 1.5). A previous study concluded that a higher number of microbes present on the skin did not lead to leakage of peptidases into the fillets through the surface. Therefore microorganisms on the skin are not important for changes in salmon texture during storage. (Hultmann and Rustad, 2004) However; the ice-crystals formed during the freezing process or frozen storage may disrupt the lysosomes, and release lysosomal enzymes which may deteriorate the muscle texture (chapter 1.3.2). (Hultmann, 2003)

1.3 Shelf life

Fish is an easily perishable food, and to obtain a long shelf life it is crucial that the initial product quality is high (Fernandez et al., 2009), and that the processing and packaging is carried out according to good manufacturing practice (GMP), to maintain shelf life (Leistner, 1999, Cortesi et al., 2009). The most important factor for prolonging shelf life of easily perishable products, disregarding GMP, is temperature (chapter 1.3.1) (Magnussen et al., 2008, Ronsivalli and Baker, 1981, Duun and Rustad, 2008).

To preserve the quality of fish, it is important to maintain an undisrupted cold chain. The cold chain should have a sufficiently low and stable temperature all the way from slaughter to the consumer. (Hemmingsen, 2002) If the cold chain is not well functioning, however, there is a need for other preservation factors to prolong shelf life. There are several methods of preserving muscle foods; salting, dehydration, thermal treatment, freezing and refrigeration. In addition there are different packaging systems such as vacuum packaging and modified atmosphere packaging (MAP), which all have become more popular in prolonging the shelf life of muscle foods (Cortesi et al., 2009, Strasburg et al., 2008).

The preservation methods mentioned above can be used in combination in what is called hurdle technology. In hurdle technology each preservation principle (e.g. low or high temperature) is called a hurdle. (Hemmingsen, 2002, Leistner, 1999)

1.3.1 Quality aspects of cooling

Refrigeration and freezing are the most effective ways to inhibit microbial growth (Magnussen et al., 2008), as well as chemical and biochemical deterioration in muscle foods (Strasburg et al., 2008). Already at catch, the fish is cooled down to prolong the shelf life. Live chilling may prolong the *pre-rigor* period to about 24 hours, which enables *pre-rigor* processing (Erikson et al., 2006). Erikson et al.(2006) found that the

fish that had been cooled was less stressed, and thereby the quality of the end-product was improved.

Figure 1.3 shows that the enzymatic activity is the most important cause of quality deteriorations during low-temperature storage. However physical and chemical reactions like denaturation of proteins and oxidation of lipids might still occur. This may e.g. result in rancidity. (Strasburg et al., 2008)

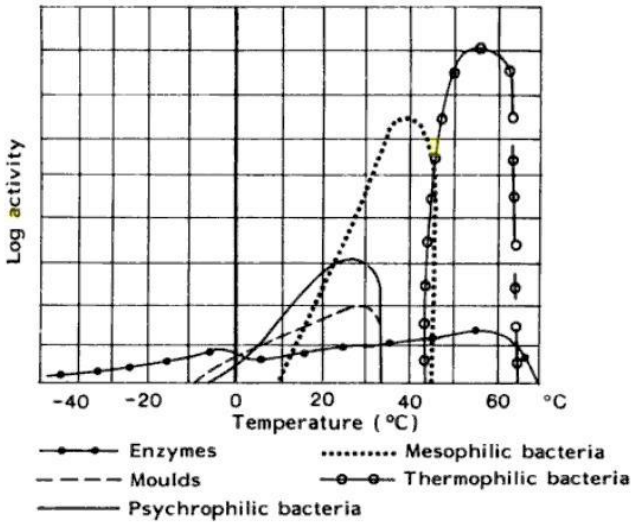


Figure 1.3: Relative enzyme activity and microbial growth rate in relation to temperature (Andersen et al., 1965)

Hultmann and Rustad (2004) suggested that most of the proteolysis during refrigerated storage is due to endopeptidases cleaving peptide bonds distant to the termini of polypeptide chains.

The freezing rate is also important for the product quality. Slow freezing results in formation of a few, but large ice crystals, mainly extracellularly. Fast freezing promotes formation of small ice crystals distributed both inside and outside the muscle cells. (Strasburg et al., 2008) Formation of ice crystals and withdrawal of water from the protein matrix caused by freezing of tissue may cause protein denaturation called freeze denaturation (Duun, 2008, Mackie, 1993). Freeze-generated product degradations appear more rapidly at fluctuating storage temperatures. Such temperature may cause different

crystallization rates and sizes which may lead to enzymatic leakages and an increase in microbial growth after thawing (Hemmingsen, 2002).

Nilsson and Ekstrand (1993) studied membrane integrity in muscle tissue of rainbow trout, during various freezing treatments and storage on ice, by measuring enzymatic activity. They concluded that storing on ice induced enzyme leakage into the centrifuged tissue fluid, however, not to the level found in frozen samples. Membrane integrity was not significantly affected by storage on ice for different durations. With a freezing process following ice storage, changes in membrane permeability was indicated due to increased enzyme activity in the press juice. (Nilsson and Ekstrand, 1993) Enzyme activity may also increase due to an increasing concentration of substrate in the solute as the water crystallizes.

For fish that have been frozen *pre rigor* a phenomenon called thaw *rigor* might take place. This condition may result in a shortening of the muscle, an excessive drip loss and a substantial loss of water holding capacity. This is due to the structural damage of membranes as a result of ice crystal formation. Because the membrane is no longer intact, there is an influx of Ca^{2+} to the sarcoplasm. This triggers the muscle contraction as the ATP levels are sufficiently high for shortening to occur. (Strasburg et al., 2008) It is recommended that fish is cooled to an intermediate temperature (0-4°C) prior to freezing (Lynum, 2005) or heated to a temperature of -3- -5 °C before thawing to avoid thaw rigor (Rustad, 2010b).

The thawing process may also contribute to enzymatic leakage from the lysosomes in fish muscle (Rehbein and Cakli, 2000). The studies of Nakai et al. (1995) suggested that the proteolysis was faster during the storage of previously frozen beef at 4 °C than in unfrozen beef stored at the same temperature.

1.4 Superchilling

Partial freezing and shell freezing is together with superchilling, terms which explain the principle of keeping products at temperatures on the borderline between chilling and freezing. The purpose is to prolong shelf life of fresh food as the lowered temperature slows the microbial metabolism and spoilage reaction rates further down. This is explained further in chapter 1.3.1. (Ronsivalli and Baker, 1981, Duun, 2008)

Frozen foods are usually stored at -17 °C or below. Chilled foods are stored at 0 °C to 8 °C. Superchilled or partially frozen food is stored at -1 °C to -4 °C, which is 1 to 3 centigrades below their initial freezing point as seen in figure 1.4. (Hemmingsen, 2002, Ronsivalli and Baker, 1981, Duun and Rustad, 2008) There are several definitions of superchilling. Olafsdottir et al. (2006) state that superchilling is lowering the temperature of the fillets quickly to -1 °C.

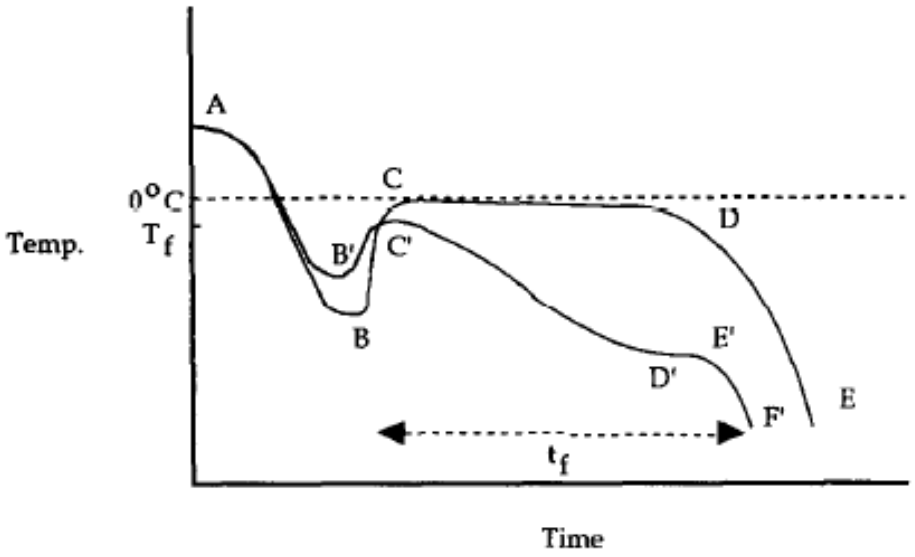


Figure 1.4: A temperature-time curve of water (ABCDE) and an aqueous solution, e.g. 10 % sugar (AB'C'D'E'F') during freezing. Points B and B' represents the superchilling temperature. T_f represents the initial freezing point of the solution, while t_f represents the freezing time as determined by such an experiment. (Goff, 1992)

The rate of freezing and the size of the ice crystals are important factors concerning the product quality (Olafsdottir et al., 2006). In the superchilling process, the surrounding temperature is set below the temperature of the initial freezing point. This causes some ice formation in the outer few millimetres of the product, depending on which method is used, and this ice stores the refrigeration capacity inside the product. The amount of ice formation is very dependent on the temperature around the initial freezing point as seen in figure 1.5. The refrigeration capacity of the product makes it possible to maintain a low temperature in the product during transport and storage, without using ice. (Duun, 2008, Hemmingsen, 2002, Hansen et al., 2009, Magnussen et al., 2008)

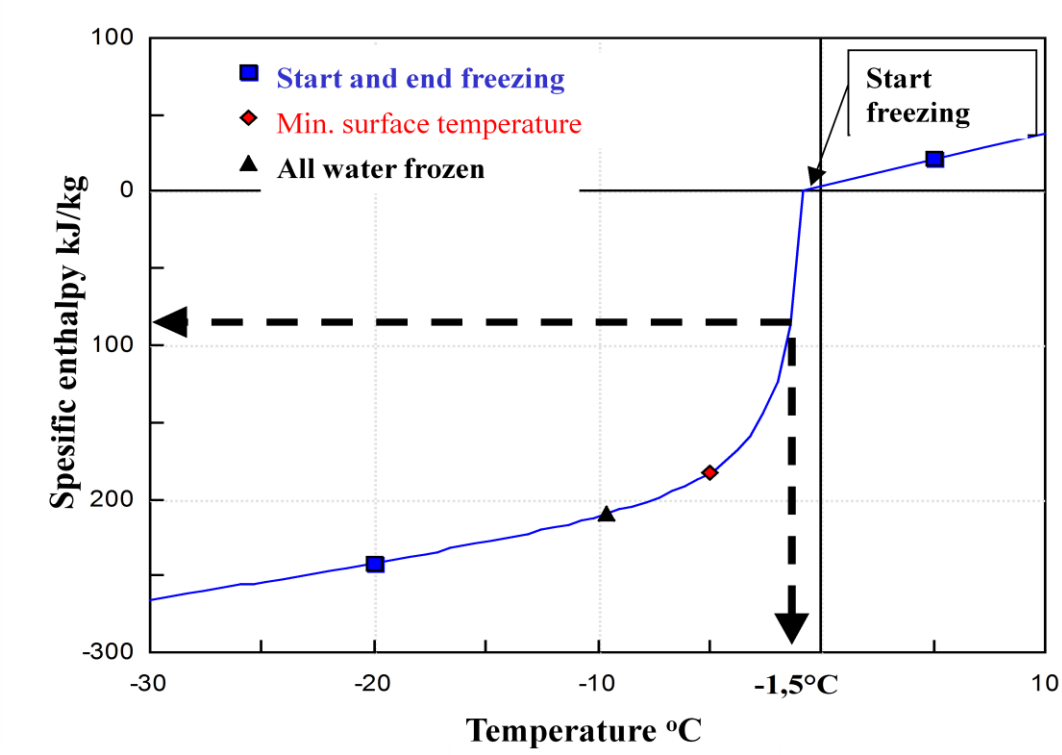


Figure 1.5: Specific enthalpy kJ/kg relative to temperature. At -1.5 °C the amount of ice in the product corresponds to approximately 30 % (Rustad, 2010b).

1.4.1 Methods for superchilling

Many studies have been carried out on superchilling from the sixties and till now. The work has expanded from herring to other fish species, poultry, and red and white meat. (Magnussen et al., 2008) There are several methods used to superchill foodstuffs.

Superchilling by using refrigerated air may be the easiest method. There are several methods (sharp, jacketed, blast and fluidized bed freezers etc.) which cannot all be mentioned here. When using a tunnel blast freezer, cold air is circulated through an evaporator and around the products by one or more fans. The air saturated with water makes it unnecessary to pack the products beforehand to prevent drying. (Ronsivalli and Baker, 1981)

Refrigerated sea water (RSW) is an effective medium for fish chilling (Hemmingsen, 2002). RSW is a cooling medium, which has a high cooling rate. Some of the disadvantages is that there could be an excessive uptake of salt (or water by species with a low fat content), loss of protein and problems with anaerobic spoilage bacteria. The RSW medium has been used for several fish species. For salmon, using this method for storing and transportation is advantageous over iced storage (e.g. bulk chilling). (Graham et al., 1992)

Adding carbon dioxide to RSW has been used as an extra preservative. This is because the carbon dioxide lowers the pH which has a beneficial effect on the fish's microbial quality. Cryogenic liquid like liquid nitrogen or carbon dioxide may be used in brines used for immersion freezing or superchilling. In spray freezing carbon dioxide is sprayed over the product. (Ronsivalli and Baker, 1981)

For superchilling of packed products air freezing (i.e. impingement freezing or blast freezing without evaporated air) seems to be promising, while for unpacked fish fillets, superchilling by combined methods (contact, evaporated air and slurry) seems to be promising. (Magnussen et al., 2008)

1.4.2 Effects of superchilling

As explained in chapter 1.4 the purpose of superchilling as a preservation method, is to improve the biochemical, microbial and sensorial quality of fresh food, so that the shelf life is prolonged.

Bahuauds (2009) work on superchilling of Atlantic salmon to a fillet core temperature of -1.5 °C, resulted in the formation of intra- and extra-cellular ice crystals in the upper layer of the fillets. The ice crystals seemed to cause lysosomal breakages which probably increased the release of lysosomal cathepsins into the cytosol. This release started already 12 h after treatment, and continued during the whole period (four weeks) of subsequent storage on ice.

The most important cause of deterioration in products stored below 0 °C is enzymatic activity (chapter 1.3.1). As explained in chapter 1.5 the activity of enzymes are generally very low at temperatures below 20 °C (Hultmann, 2003). However, the work done by French et al. (1988) and Rehbein and Cakli (2000) found that there were faster enzymatic reactions in partially frozen salmon than frozen or ice chilled salmon. This could lead to a significant increase in hydrolysis of soluble muscle proteins of superchilled salmon. Duun and Rustad (2008) found that activities of cathepsins B and B+L at 5 °C and 20 °C were stable throughout the storage period in all storage groups. Softening caused by enzymes may therefore occur during subsequent chilled storage.

Even if the pre-slaughter crowding stress accelerated fillet softening in the first phases of chilled storage, after 24 h post-mortem no significant impact on fillet texture was noticed. The texture of the salmon was not affected by the superchilling treatment measured one week post-mortem. (Bahuaud, 2009)

Hemmingsen (2002) reported that the moisture content and the water holding capacity of superchilled fillets were as good as or even better in superchilled fillets than in the chilled salmon fillets. In addition the superchilled storage gave lower bacterial numbers than ordinary chilled storage.

As mentioned in chapter 1.3 hurdle technologies will increase shelf life. In this way superchilling can be combined with other hurdles. Combining superchilling and MAP doubled the shelf life of salmon compared to the control sample (Fernandez et al., 2009).

The combination of modified atmospheric packaging and superchilling shows promising results (Sivertsvik et al., 2003). MAP containing high carbon dioxide levels have shown to inhibit the normal spoilage flora in fish (aerobic, gram negative, psycrotrophic species), and may therefore prolong shelf life. Toxin producing anaerobic microorganisms like *Clostridium* or *Vibrio* and facultative aerobic species may still be able to grow in MAP packages. Therefore low temperatures and strict processing hygiene is of great importance. In addition, the elimination of ice in these packages, also has both economic and sanitation advantages. (Hemmingsen, 2002)

By using MAP, the liquid loss may be higher because of the decreasing pH at high CO_2 levels (Hansen et al., 2009). Hansen et al. (2009) combined short-term superchilling and MAP with a CO_2 emitter. This prolonged the shelf-life of pre-rigor salmon fillets. Sivertsvik et al. (2003) found that combining MA and superchilling gave a product of high quality after 24 d of storage at -2 °C with almost total bacterial growth inhibition (<1000 colony-forming units (cfu) /g). In addition no negative texture differences were observed in the superchilled salmon. The increase in drip loss was also insignificant.

1.5 Proteolytic enzymes in fish muscle

The proteins and peptides in food have a range of functional properties e.g. hydrational, solubility and interfacial, flavour binding, gelling and textural properties. In addition, they are important for the nutritional value of the product. The quality and deterioration of proteins are therefore very important for the quality of muscle foods, both sensory and nutritional. (Strasburg et al., 2008)

As the fish dies and the blood flow stops, the hormonal regulation of enzymatic processes in the fish also stops. In addition to this, the low pH may lead to release of enzymes from the lysosomes, however, the proteolytic enzymes involved in the generation of peptides can also be affected by the pH decline (Moya et al., 2001). The lysosomal enzymes, such as calcium-activated calpains and lysosomal acidic cathepsins (Bauchart et al., 2007), break down the components of the cell, which leads to autolysis of the muscle. The defence mechanism against microorganisms in the muscle tissue is also deactivated. (Rustad, 2005)

In the work of Benjakul et al. (1997) denaturation and degradation of muscle proteins became obvious within 8 days of iced storage due to proteolytic activity in the muscle. Denaturation of the myofibrils and connective tissue makes the proteins more susceptible to the hydrolytic action of cathepsin B. (Hultmann and Rustad, 2004)

The work of Bahuaud (2009) indicates that formation of ice crystals, oxidative stress, muscle pH and genetic background can play a role in the degradation of the muscle (chapter 1.3.1). Season and maturity may also be important (Søvik, 2005). The proteolytic enzymes may in addition to this be affected by smoking and irradiation (Hultmann et al., 2004). The results of Stoknes (1994) indicated that free fatty acids and heating for a short period of time may activate enzymes. Ionic strength may have different impacts for different enzymes in fish. Free fatty acids, heating and ionic strength may also cause denaturation.

Sarcoplasmic proteins are also experiencing some proteolytic degradation although this does not contribute to increase tenderness (Moya et al., 2001). Collagenase-like enzymes, however, may cause textural changes (Hultmann et al., 2004). According to the results of Bahuaud (2009), the activities of cathepsins and/or gene expressions were strongly correlated with the degradation of the extra cellular matrix. Increasing cathepsin activity corresponded to an increase in muscle degradation. (Bahuaud, 2009)

It is not unusual to find a general increase in free amino acid concentrations (Moya et al., 2001, Bauchart et al., 2007) and free fatty acids during post mortem fish storage (Stoknes, 1994). Disappearance of large protein compounds and appearance of smaller compounds have also been reported, suggesting some post mortem proteolysis (Bauchart et al., 2007).

Along with microbial degradation, enzymatic processes are the most important quality deteriorating processes at low temperature storage (chapter 1.3.1) (Søvik, 2005). There are differences in enzymatic activities and processes between species. E.g. fish muscle and liver have been reported to contain about 5-10 times more cathepsin than mammalian muscle per gram muscle tissue (Siebert, 1958).

Fish muscle contains several proteinases capable of causing autolytic degradation of the muscle. Cathepsins are activated by decreasing pH after death (chapter 1.5.1). Different cathepsins may act in concert to autolyze fish muscle (Hultmann and Rustad, 2004). Calpain is an enzyme which is activated by Ca^{2+} and may also contribute to autolytic degradation in the early stages of muscle deterioration (Benjakul et al., 1997). Working with dry-cured ham, Mora et al. (2009) concluded that calpains were responsible for the extensive hydrolysis of proteins during this process. The proteolytic systems considered most important by scientists today are the combination of cathepsins and calpains (Herrera-Mendez et al., 2006).

1.5.1 Cathepsins

Cathepsins are lysosomal cysteine proteinases and according to Barret and Kirschke (1981) these proteinases may be the most active proteinases in the human body as they hydrolyze more peptide bonds than the other proteinases. Because cathepsins are acidic proteases, their main pH range is 4-6 and they are irreversibly inactivated above pH 7 (Barrett and Kirschke, 1981). In fish they become more active as the pH decreases to about 6 *post mortem* (chapter 1.2.2).

The lysosomal system including catheptic proteases could play an important role in muscle degradation and quality both at the myofibrillar and connective tissue levels and both *pre* and *post mortem* (Bahuaud, 2009). Hultmann and Rustad (2004) found that lysosomal catheptic enzymes are involved in deterioration of muscle texture, and different cathepsins may act together in autolysis of fish muscle. The most important cathepsins in fish are cathepsin B and L (Bahuaud et al., 2010).

The softening phenomenon of *post mortem* muscle of salmon is probably caused by the lysosomal cysteine proteases cathepsins B and L. Cathepsin B and L activities are known to be able to degrade myofibrillar proteins, and could play an important role in fillet firmness by hydrolyzing collagen. Collagen is a major constituent of the connective tissue in fish (Lynum, 2005), thus, it plays an important role in the physical properties of the muscle tissue.

Cathepsin L is described as a powerful proteolytic enzyme which cleaves the proteins in the vicinity of hydrophobic residues. Cathepsin B has only a small prosthetic group and cleaves c-terminal dipeptides sequentially from some polypeptides, with broad specificity. Many proteins are degraded by cathepsin B, however, the bonds cleaved have not been identified. (Barrett and Kirschke, 1981)

Yamashita and Konagaya (1991) found that cathepsin B do not hydrolyze collagen, while cathepsin L appeared to be able to split native collagen at the non helical region. The enzyme was found to hydrolyze connectin and/or nebulin, however this was a very slow process compared to cathepsin L. The physical structure of the myofibril was more or less

destroyed by the cathepsin L activity. The work also suggested that myosin molecules underwent limited proteolysis, accompanied by the disappearance of the myosin light chain. These findings suggest that cathepsin L must be responsible for the extensive softening of the muscle having high catheptic activity. (Yamashita and Konagaya, 1991)

Cathepsins B and L showed significantly greater activity toward the denatured proteins than toward native proteins. It is therefore possible that autolysis may be enhanced if the muscle proteins undergo denaturation, resulting from exposure of the *post mortem* muscle to high temperature and low pH due to glycolysis, during storage and processing. Cathepsin B and L relative gene expressions do not always correlate with cathepsin B and L activity in the muscle. However Bahuaud (2009) found that the gene expression always correlated positively with muscle degradation and/or fillet softness.

There are several uncertainties concerning how the cathepsin system works. Bahuaud (2009) experienced this and suggested that cathepsin B might act in the first stages *post mortem*, while cathepsin L might take over later in the storage period.

In the work done by Hultmann (2003) activities of specific proteolytic enzymes were detected throughout the storage period, and it seemed that cathepsin B-like activities are the most important in salmon. Cathepsin B contribute to both the early and late stages of lysosomal breakdown, thus it has an important role in hydrolysis of tissue proteins (Søvik, 2005).

1.5.2 Calpains

Calpains are calcium-activated proteases. After slaughter, the integrity of the cell membranes is reduced, leading to a leakage of free calcium ions in the cytosol, eventually activating calpains. (Koohmaraie, 1996)

Bahuaud (2009) found no significant correlation between calpain activity in the muscle and fillet texture of salmon. These results may be due to the measurements being carried out 2 days *post mortem*. Calpains are known to degrade rapidly, and the initial activity of

the enzyme might not be reflected. However; the importance of calpains for texture development is still inconclusive.

Calpains only cause a limited proteolysis as they cleave at specific sites on myofibrillar proteins. The proteins susceptibility to other proteinases (e.g. cathepsins) is enhanced as the proteins are degraded into large fragments and more cleaving sites become available. (Hultmann, 2003)

Both cathepsin B and L are endopeptidases. Cathepsin activity may be measured as total activity in muscle extracts (Bahuaud, 2009, Duun and Rustad, 2008, Stoknes, 1994) or in the lysosomal fraction. This can be done by measuring specific substrate for the enzyme or colorimetric methods to find the total protein content. Measuring this immediately *post mortem* may indicate the level of potential proteolysis exciting *in vivo*, before death. The measurements can then be continued throughout the storage period. (Bahuaud, 2009)

According to Søvik (2005) the enzymatic activity is positively correlated to the amount of free amino acids in viscera from cod.

1.6 Isolation and characterisation of peptides

There are several analytical methods for isolation, quantification and characterisation of proteins and peptides in foodstuffs. Non-specific methods provide information about the total protein content. However these methods do not characterize the peptides during storage. (Piraino et al., 2007) Characterization of these peptides is important for understanding what happens during proteolysis and finding which proteases that are active during storage.

1.6.1 Precipitation

As mentioned in chapter 1.2.1, the proteins are divided into three groups based on solubility. The water soluble proteins are Sarcoplasmic proteins and consist mainly of enzymes. Salt soluble proteins can be solubilised in buffers with added salts, while insoluble proteins can be soluble in acids or alkali. (Rustad, 2010a)

The solubility of the protein can change during storage and processing, and will among other factors affect functional properties such as the proteins emulsifying and gelling properties. Measurements of solubility can therefore be used to study changes in protein structure and functional properties caused by denaturation. By using chromatographic or mass spectrometry methods, precipitation of protein is a valuable tool for further studies of peptides found in lower concentrations. (Damodaran, 2008, Rustad, 2010a)

These different degrees of solubility decide what method to use to precipitate a protein. Protein precipitates are aggregates of protein molecules large enough to be visible and to be collected by centrifugation. The solubility of the protein is determined by the distribution of hydrophilic and hydrophobic residues on the proteins surface and the size of the protein. When a protein precipitates it is a result of attraction and repulsion forces between the protein and the solute. (Rosenberg, 1996)

Proteins can also be fractionated by using organic solvents such as ethanol. The organic solvents decrease the solubility because the permittivity of the aqueous solution is

lowered (Damodaran, 2008). As a general rule; the larger the molecule, the lower the solvent concentration required for precipitation (Rosenberg, 1996). Overrein (2010) found that hydrophobicity is important for the solubility of peptides in TCA. The solubility of the protein in organic solvents decreases markedly with decreasing temperature, and makes the temperature control important for this reaction (Marshall, 1986). Skistad (2008) suggested that precipitation using 75 % ethanol excludes proteins > 90 kDa as their molecular weight is too high to stay dissolved in ethanol.

1.6.2 Quantification of proteins

Non specific methods for quantifying proteins include nitrogen determination, amino acid analysis and colorimetric, spectrophotometric and fluorimetric methods.

The most commonly used nitrogen determination method is Kjeldahl. This method involves complete destruction of the protein using sulphuric acid and catalysts followed by addition of sodium hydroxide (NaOH) and titration. The method is widely used in the food industry, but in protein chemistry it has been replaced by more sensitive analysis such as amino acid analysis. After complete hydrolysis the protein can be subjected to amino acid analysis e.g. HPLC. Fluorimetric analysis is a highly sensitive method for determining amino acids. Spectrophotometric methods are not as sensitive as colorimetric methods because they rely on a specific wavelength of λ= 280 nm. (Darbre, 1986)

There are several colorimetric methods for determination of soluble protein. The standard used is usually bovine serum albumin (BSA). This may give some erroneous results if the test protein has a different structure to BSA. The biuret (using various cupric reagents) and Bradford (using Coomassie Brilliant Blue G-250) methods are two examples of colorimetric methods. (Darbre, 1986, Rustad, 2010a)

The most widely used colorimetric method is Lowry's method. (Darbre, 1986) This method is 10 or 20 times more sensitive than spectrophotometric analysis (λ= 280 nm) and 100 times more sensitive than the biuret reaction. It is simple and easy to adapt for small scale analysis, unlike the two methods mentioned above which only determine

peptide chains above a certain length (Rustad, 2010a). One of the disadvantages is that the color intensity varies with different proteins and it is not strictly proportional to concentration. It is also highly pH dependent and reducing agents may interfere (Rustad, 2010a). The method is however suitable to measurement of mixed tissue proteins or protein during enzyme fractionations. (Lowry et al., 1951)

Lowry's method consists of two steps. First there is a reaction with copper in alkali followed by a reduction of the Folin reagent by the copper-treated protein. Free amino acids will give less color than proteins and this may influence the results. In addition, the concentration is not strictly proportional to the standard curve. (Lowry et al., 1951, Rustad, 2010a) Cross reactions between free amino acids and dipeptides with copper complexes have been reported to contribute to color formation using this method (Hortin and Meilinger, 2005).

1.6.3 Chromatography

As mentioned in chapter 1.5 the proteins in fish have different properties such as hydrophobicity, charge and size. These properties can be taken advantage of, when analysing proteins and peptides. Chromatography is a technique which involves the isolation of components in a mixture of pores. This is done by the flow of a liquid or a gas through the mixture. The flow is driven by pressure or gravity and it cause differential migration of the components. (Rosenberg, 1996) Chromatography can be used to improve the understanding of proteolysis, and there are several types of chromatography (Piraino et al., 2007).

The primary objective of gel filtration chromatography is to achieve rapid separation of molecules based on size. The gel consists of an open cross-linked three dimensional network of pores. Molecules of less than maximum pore size may penetrate the pores. The separation depends on the difference in the ability of various molecules to enter these pores. The results are monitored as the liquid exits the column, by monitoring the UV absorbance at 280 nm. Because the molecules larger than the weight exclusion limits

cannot enter any pores, they come through the column as a sharp peak in the void volume. (Rosenberg, 1996)

In the work by Skistad (2008) fast protein liquid chromatography (FPLC) using a Superdex column was found to be a good tool for fractionation and purification of extracts, however, it had a limited accuracy to determine the molecular weight of small peptides. HPLC is a good method for identifying amino acids and detecting changes in composition during storage (Alomirah et al., 1998, Michalczyk and Surowka, 2007, Nakai et al., 1995, Skistad, 2008), however, it is hard to identify di- or tri-peptides if you do not run standards as it only compares the chromatogram with a measurement of standards. (Skistad, 2008)

1.6.4 Mass spectrometry

Mass spectrometry is an important analytical technique for studying bio-active peptides, proteins and other bio molecules. Mass spectrometers are either used to measure the molecular weight of the molecule or to determine other features such as amino acid sequences. (Domon and Aebersold, 2006, Rustad, 2010a)

The sample used is applied to the apparatus and subjected to ionization, acceleration, deflection and detection as showed in figure 1.6 below.

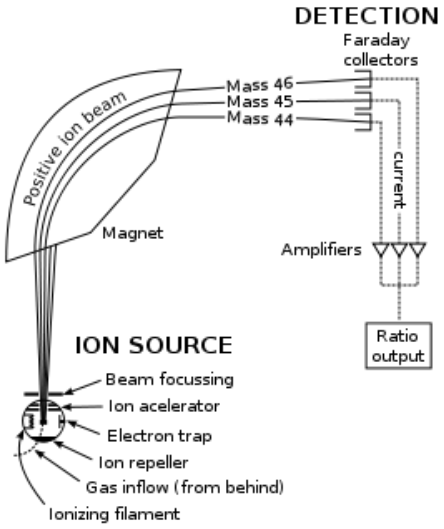


Figure 1.6: The components of a mass spectrometer. The sample is ionized by an ion source e.g. electrons, accelerated by an ion accelerator, deflected through a magnet and detected by faraday collectors. The signal is then amplified before the ratio output is measured. (Fyson, 2008)

The results are presented in a mass spectrum, where relative intensity (%) makes the y-axis, and the mass to charge (m/z) ratio makes the x-axis. An example of a mass spectrum is shown in figure 1.7 below.

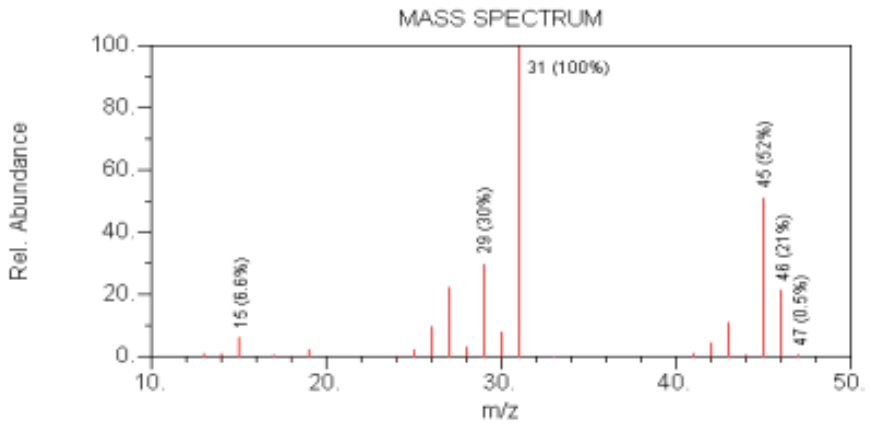


Figure 1.7: The mass spectrum of ethanol. (The University of Arizona, 2010)

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) is widely used for the analysis of large biomolecules, and can obtain results for peptide masses in the presence of salts and other buffer components. This makes it possible to obtain accurate molecular masses of peptides. (Piraino et al., 2007)

To get a even better sensitivity than MALDI, one can add a column used for HPLC. This gives a good platform for Liquid Chromatography Mass Spectrometry (LC-MS). The data from this analysis are more detailed and can be compared to databases for protein identification. However, the method is limiting concerning the amount of different proteins present in the sample, and the sample should not contain more than 100 proteins. Samples containing more than 100 proteins will require additional separation. (Virginia Bioinformatics Institute, 2010)

Previous work using Gas Chromatography Mass Spectrometry (GC-MS) showed that it was an advantageous method when trying to identify proteins, however, small peptides or proteins not present in a library is troublesome to identify (Bauchart et al., 2007). It was also found that it was more useful to use MS analysis after fractionating the samples first. (Skistad, 2008) This may give valuable information about the function of the peptides, the quality of the food and the enzymes that are active during storage (Parkin, 2008).

1.7 Competitive Food Processing in Norway

The main objective of the project Competitive Food Processing in Norway (KMB project) (178 280/ I 110) is to improve and secure the competitive marine and agricultural food processing industry in Norway, by addressing and developing critical knowledge and technologies for more effective processing through coordinated efforts by industry and research institutes.

One of the sub objectives of the KMB project is to achieve improved knowledge regarding the governing mechanism related to superchilling, and to define critical process design parameters for the future implementation.

The work involve several research partners, NTNU, SINTEF Fisheries and Aquaculture and SINTEF Materials and Chemistry. It is divided into six work packages, where superchilling of fresh food is one of them. The objectives of this work package is to develop a thermodynamic basis and data for superchilling process design, study the dynamic influence of temperature, ice growth and ice fraction on product quality and develop a quality model for selected products, develop efficient, labour saving and environment friendly processing and preservation lines and find optimal storage and distribution methods and arrangements for safe food for the consumers.

The work in this package started in January 2006 and aims to be completed in December 2010. The expected results is production of products with prolonged shelf life as fresh food, engineering and management tools for the development and control of superchilling processing, storage and distribution, and as a result a more flexible Norwegian food industry and extended understanding of ice growth and crystallization in food tissue. The KMB project aims to get a better understanding of how superchilling at different conditions will influence the quality and shelf life of products posterior to thawing as well. Further, the project aims towards finalizing the thermodynamic basis for recommending product specific superchilling conditions.

1.8 Purpose of this work

This work is part of the KMB project. As described in chapter 1.7 the project aims to obtain a better understanding of how superchilling at different conditions will influence the quality and shelf life of products.

Several superchilling experiments have been carried out as a part of the KMB project. The work in this thesis is based on one of these experiments where the aims were to study superchilling of salmon, with three different superchilling methods and ice levels, and to follow changes in different biochemical parameters such as proteolytic activity. Results from this experiment have been used to explain some of the results in this thesis.

The purpose of this thesis is to study the changes due to proteolytic degradation during storage by determining the concentration of proteins, isolating the peptides and by analyzing the peptide fraction for two superchilling methods at two ice levels and chilled reference samples.

The raw material used was Atlantic salmon (*Salmo salar*) stored on ice or superchilled by using two different methods for superchilling, chilling by air tunnel and LIC-cabinets. The proteolytic changes were studied by analyzing the peptide fraction. This has been done by isolating the peptides, first by extraction and then by precipitation. After this the peptides have been analyzed by using FPLC and GC-MS.

2 Materials and methods

2.1 Preparation of samples

The experiment was carried out in May 2008. Whole gutted salmon (3-4 kg) from Lerøy Midnor, Hitra, were used for this study. The salmon arrived as fillets and two equal pieces of approximately 280-300 g from each fillet were vacuum packed as shown in figure 2.1.

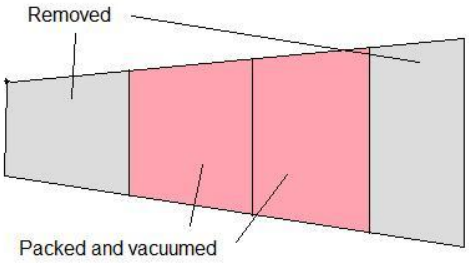


Figure 2.1: Utilisation of fillet. The ends of the fillets were cut of, and the mid-pieces were packed and vacuumed.

The methods used for superchilling the products was liquid carbon dioxide and air. In the air freezing tunnel, the temperature used was -25 °C, air speed 2 m/s and the time was 10 and 20 minutes. The salmon was superchilled to two ice levels of 10 % and 20 % and stored at -1.5 °C and -1.7 °C, respectively.

A set of samples were superchilled using liquid ice carbon dioxide (LIC) cabinets (borrowed from Yara) to an ice level of 20 % and the samples were stored at -1.7 °C. The temperature of the liquid ice carbon dioxide was approximately -78 °C. The salmon that were superchilled using air were one day old, while the salmon that were superchilled using LIC cabinets were four days old. All had been stored on ice, prior to the superchilling. Day zero is the day the salmon was superchilled. The reference samples were collected at day zero, prior to superchilling.

The superchilled fillets were stored for four weeks (28 days), and samples were collected 5-8 times during this period at 3-5 days intervals. The quality of the superchilled products

were measured by chemical, physical and microbiological methods during storage. Drip loss, liquid loss, and texture were measured 5 times during storage, while microbial growth was measured eight times. The ice-fraction was also measured, but at day zero only.

The fillets were frozen at -40 ºC until further analysis could be performed. The part reported in this thesis has been analysing the contents and composition of proteins and peptides during storage time. The samples analysed were the reference samples, the salmon chilled by air and 10 % ice stored at -1.5 °C for one and four weeks, and the salmon chilled by using LIC cabinets and 20 % ice stored at -1.7 °C for one, two and four weeks.

In addition, storage of a chilled reference was carried out. Five whole, gutted, salmon (approx. 4-5 kg) from SalMar, Frøya, were filleted two days after slaughter. Three equal pieces were cut of each fillet and vacuum packed. The pieces were stored on ice for 20 days. Samples were collected 5 times at 4-6 days intervals. The quality of the ice-chilled products was measured during storage. Drip loss was measured 4 times and liquid loss 5 times during storage.

The samples were labelled as shown in appendix A and the analyses were carried out as shown in figure 2.2.

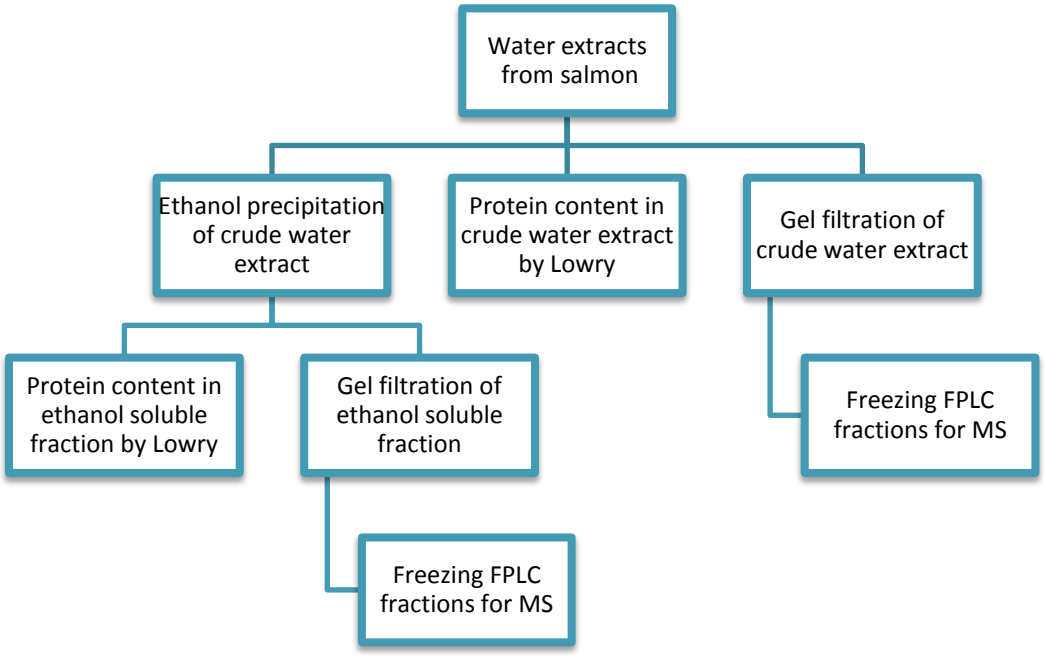


Figure 2.2 Flow scheme for the procedures carried out in this thesis

2.2 Drip loss

Measurements of drip loss were carried out for the chilled references using the same method as Duun (Unpublished Results). The samples were taken out of the ice container, dried with paper towels, and weighed. The piece of salmon was taken out of the bag, and the liquid on the outside of the fish and the inside of the bag were carefully wiped off. The fish and the bag were then weighed again. The difference in the weights was set as the amount of drip loss in grams.

Results of drip loss from the superchilled salmon are taken from Anne Sissel Duuns measurements in 2008. (Duun, Unpublished Results)

2.3 Water content and liquid loss

The measurements of water content and liquid loss were carried out for the chilled references. A piece of fish of about 10 g was minced in a small food processor.

For the water content measurements the heat resistant glass test tube was weighed before adding approximately 2 g of minced fish. The tubes were incubated at 105 °C for 24 hours. After cooling in a desiccator, the tubes were weighed again. The difference in the weights was set as the water content in grams. The water content measurements were carried out in duplicates.

The liquid loss measurements were carried out according to Eide et al. (1982). Approximately 2 g of minced fish was added to the pre-weighed test tube with a membrane bottom. The tube was placed in a centrifuge tube with glass beads in the bottom and centrifuged at 210 g (1 500 rpm) for 5 min. The tubes were weighed before and after centrifugation. The difference in the weights was set as the amount of liquid lost in grams. The liquid loss measurements were carried out in duplicates.

Results of water content and liquid loss from the superchilled salmon are taken from Anne Sissel Duuns measurements in 2008. (Duun, Unpublished Results)

2.4 Preparation of extracts

The extractions were made from fillets thawed over night at 4 °C. 10 g of salmon fillet was homogenized in 80 mL ice-cold distilled water, by using Ultra Turax T25 basic. Extracts were held on ice during the procedure. Centrifugation was carried out by using a GSA rotor and a Sorvall Centrifuge at 4 ºC and 12 000 g for 20 min. Supernatant was filtered through glass wool, and the volume made up to 100 mL with distilled water. The extracts were stored at -80 ºC in 4 aliquots. The thawed extracts were later used as described in figure 2.2 above.

2.5 Ethanol precipitation

11.2 mL of crude water extract was transferred to a large centrifuge tube, and 40 mL 96 % ethanol was added to a final concentration of 75 %. The tube was centrifuged by using a GSA, Sorvall centrifuge at 4 ºC and 15 000 g for 20 min. The supernatant was decanted into a round bottom flask with a glass plug. The ethanol was evaporated on a rotavapor until no smell of ethanol could be detected. After evaporation the round bottom flasks were put in freezer at -20 ºC prior to lyophilisation. The samples were freeze dried. Before further analysis the samples were redissolved in 2.8 mL distilled water.

2.6 Lowry's method

The protein concentration in the extracts was determined using the Lowry method (Lowry et al., 1951). Bovine serum albumin (BSA) was used as standard with concentrations 12.5, 25, 50, 100, 150, 200 and 300 µg/mL. For the spectrometric measurements acrylic cuvettes (Sarstedt) were used. Measurements were carried out on an Ultrospec 200 UV/ visible Spectrophotometer from Alpha Biotech. Measurement of the standard and the samples were carried out in triplicates.

2.7 Fast Protein Liquid Chromatography

Fast Protein Liquid Chromatography (FPLC) carried out by using Äkta FPLC with a Superdex™ peptide 10/300 GL column and a detection wavelength of 280 nm. The column is a pre-packed glass column for high performance filtration of natural recombinant or synthetic peptides and other small biomolecules. The gel in the superdex column consists of a three-dimensional molecular network of cross-linked agarose and dextran pores into which molecules of less than maximum pore size may penetrate. The maximum limit for molar weight of the peptides in an aqueous buffer is 20 000 g/mol for the Superdex™ column. The column is most reliable at molecular weights between 100 and 7 000 g/mol. (GE Healthcare, 2006)

The mobile phase was 50 mM imidazole buffer at pH 7.0. The solution was prepared by dissolving 68.08 g imidazole in deionised water, adjusting the pH to approximately 7 using 1 M HCl. The volume was adjusted to 2 L and the solution was filtered through a 0.2 μm membrane filter.

The standards used were cytochrome C, aprotinine, vitamin B12, the tripeptide tyr-tyr-tyr and the amino acids L-lysine and L-cysteine. In addition a mixed protein standard was used. The molecular weight of the proteins in this mixture varied from 14 400 g/mol to 97 000 g/mol. The content of this standard is found in appendix B.

The samples were filtered through 0.2 μm syringe filters prior to injection in the column. The sample volume used was 200 μL.

The liquid from peaks were collected in fraction collector tubes. The fractions were transferred to and frozen in eppendorf tubes marked with the tube position and frozen at -80 ºC for later MS analysis of the peptides.

2.8 Mass spectrometry

The fractions and extracts were freeze dried and redissolved in 50µL distilled water, and frozen at -20 °C.

The frozen fractions and extracts were delivered to Inga Marie Aasen at SINTEF, who carried out the MS analysis.

For the MS analysis an Agilent Inc. Model 1100 LC-MS-system was used. The system consisted of a degassing unit for mobile phases, an autosampler, pumps, column oven and a single quadrupole mass spectrometer. All the analyses were done without column separation, with electrospray ionization (ESI) in a positive and negative method and a mixture of 10 mM ammonium acetate (pH 6.7) in 90 % isopropanol as a mobile phase. A mass area of m/z 100 to m/z 1500 was investigated.

3 Results

3.1 Drip loss and water holding capacity

3.1.1 Drip loss

The drip loss were found as described in chapter 2.2, and calculated as described in appendix C.2. All measured and calculated data, including standard deviations, are given in appendix D.1.

The drip loss of vacuum-packed salmon fillets as a function of storage time is given in figure 3.1.

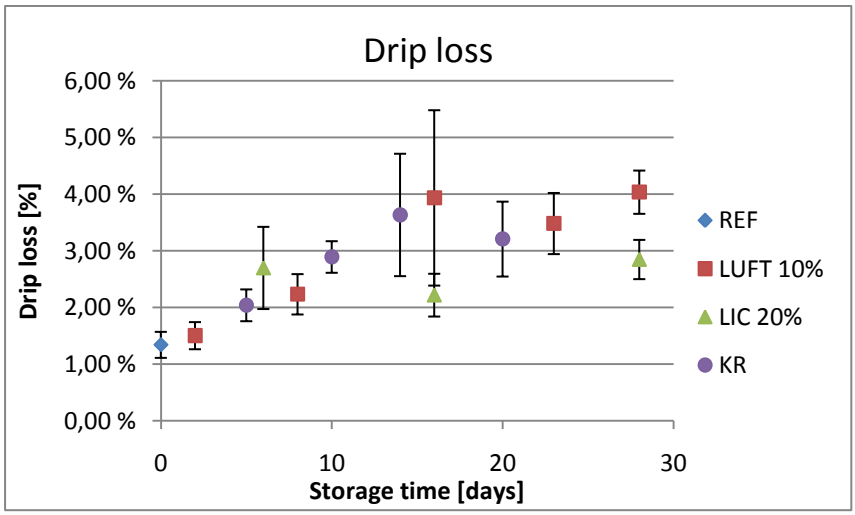


Figure 3.1: Comparison of drip loss [%] relative to storage time [days] of salmon stored on ice (KR and REF) and superchilled using air (LUFT 10 %) and liquid CO_2 (LIC 20 %). Mean values of drip loss are given in percent of product weight. Y-error bars represent the standard error of the mean. (n=2-5) Results of REF, LUFT 10 % and LIC 20 % were found from analysis done by Anne Sissel Duun (Duun, Unpublished Results).

There is a slight increase in drip loss during the storage time. The current work shows that there is no significant difference in drip loss between the salmon stored on ice compared to superchilled salmon. However, at the end of the storage period, salmon superchilled by air show a tendency to a higher drip loss than salmon superchilled by liquid carbon dioxide.

3.1.2 Water content

The water content in all samples were found as described in chapter 2.3, and calculated as described in appendix C.3. All measured and calculated data, including standard deviations, are given in appendix D.2.

The water content of vacuum-packed salmon fillets as a function of storage time is given in figure 3.2.

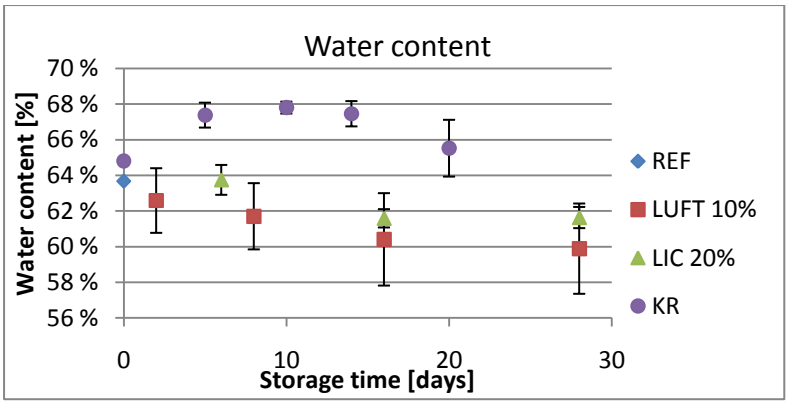


Figure 3.2: Comparison of water content [%] relatively to storage time [days] of salmon stored on ice (KR and REF) and superchilled using air (LUFT 10%) and liquid carbon dioxide (LIC 20%). Mean values of water content are given in percent of product weight. Y-error bars represent the standard error of the mean. (n=2-5) Measurements of REF, LUFT 10 % and LIC 20 % were carried out by Anne Sissel Duun (Duun, Unpublished Results).

The water content in samples stored on ice is significantly higher than the superchilled samples throughout the storage period. There is no significant difference between the superchilling methods. The samples superchilled by air have larger standard deviations than the other samples.

3.1.3 Liquid loss

The liquid loss in all samples were found as described in chapter 2.3, and calculated as described in appendix C.4. All measured and calculated data, including standard deviations, are given in appendix D.3.

The liquid loss of vacuum-packed salmon fillets as a function of storage time is given in figure 3.3.

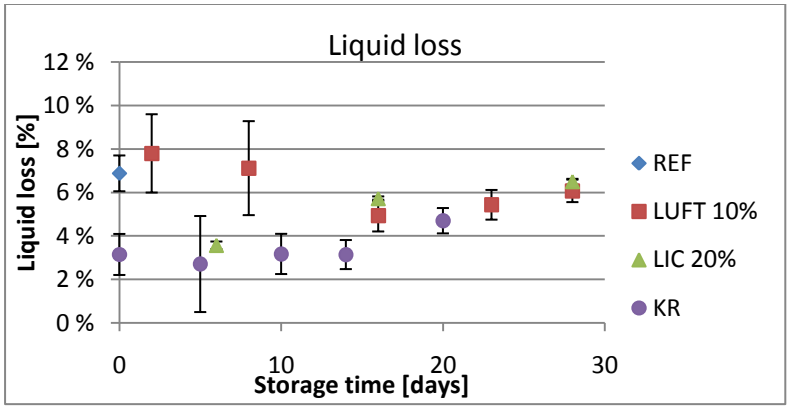


Figure 3.3: Comparison of liquid loss [%] relative to storage time [days] of salmon stored on ice (KR and REF) and superchilled using air (LUFT 10%) and liquid carbon dioxide (LIC 20%). Mean values of water holding capacity are given in percent of product weight. Y-error bars represent the standard error of the mean. (n=2-5) Results of REF, LUFT 10 % and LIC 20 % are found from analysis done by Anne Sissel Duun (Duun, Unpublished Results).

The current work shows that the liquid loss is lower in salmon stored on ice than superchilled salmon throughout the storage period. The samples superchilled using air show significantly higher liquid loss than the other chilling methods before 10 to 16 days of storage. The difference is however not significant after 16 days of storage. The samples superchilled using air, have a somewhat higher liquid loss than the ice chilled references until 10 to 14 days of storage.

3.2 Protein content

The total protein content in all extracts were found using Lowry's method as described in chapter 2.6 using extracts prepared as described in chapter 2.4 and 2.5. Calculations of protein content by Lowry's method are given in appendix C.5. An assembled table of data including the standard deviations are given in appendix E.1.

The total concentration of protein on wet weight basis in the extracts as a function of storage time is given in figure 3.4.

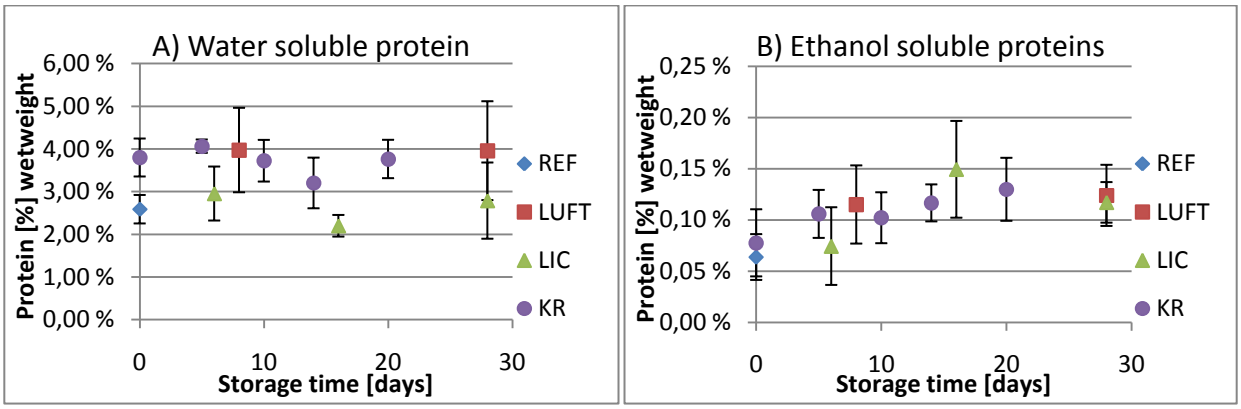


Figure 3.4: A) Content of water soluble protein [% wet weight] relative to storage time [days] of salmon stored on ice (REF and KR) and superchilled using air (LUFT 10 %) and liquid carbon dioxide (LIC 20 %). Mean values of protein content are given in protein % wet weight. Y-error bars represent the standard error of the mean. B) Content of proteins soluble in ethanol relative to storage time (days) of salmon stored on ice (REF and KR) and superchilled using air (LUFT 10 %) and liquid carbon dioxide (LIC 20 %). Mean values of protein content are given in protein % wet weight. Y-error bars represent the standard error of the mean.

The content of extractable protein in salmon is not changing significantly during the storage time. There is no significant difference between the chilling methods. The protein content was also calculated as percent of dry matter. These results did not show any significant changes during storage time or between the chilling methods.

3.3 Gel filtration

Gel filtration using fast protein liquid chromatography (FPLC) was performed on the precipitated samples as described in chapter 2.7. An example of a chromatogram of water soluble proteins with indicated peaks is given in figure 3.5 below. The chromatograms from the gel filtration using FPLC are given in appendix F.

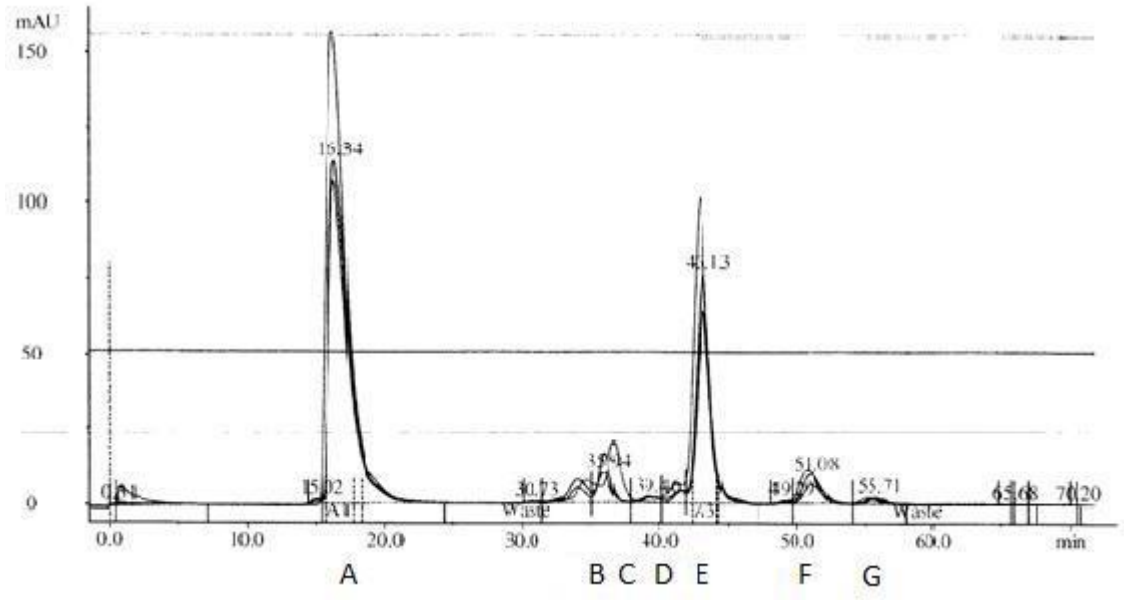


Figure 3.5: Chromatogram of water soluble proteins showing seven indicated peaks. A at 16 min, B at 35 min, C at 37 min, D at 40 min, E at 43 min F at 51 min at G at approx. 55 min retention time.

An example of a chromatogram of ethanol soluble proteins is given in figure 3.6 below. As seen in figure 3.5 the peak area of peak A has decreased considerably.

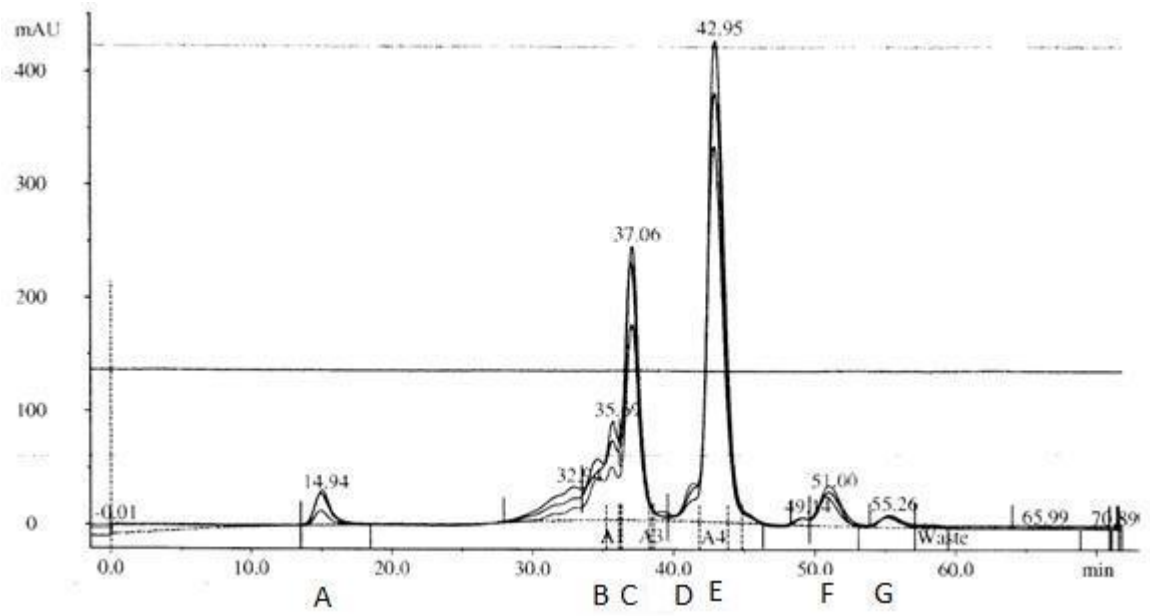


Figure 3.6: Chromatogram of ethanol soluble proteins showing seven indicated peaks. A at 16 min, B at 35 min, C at 37 min, D at 40 min, E at 43 min F at 51 min at G at approx. 55 min retention time.

3.3.1 Molecular weight distribution determined by gel filtration

The protein size distribution was found by running standards as described in chapter 2.7. The distribution was calculated as given in appendix C.6.2. Table 3.1 below shows the protein size distribution for the seven selected peaks, A-G, with retention time and molecular weight. The column is regarded as most accurate for the molecular weights in the range of peak B-E (GE Healthcare, 2006).

Table 3.1: Protein size distribution for the seven peaks A-G with retention time and molecular weight. The values marked in green is where the column (Superdex peptide 10/300GL) is regarded as most accurate (GE Healthcare, 2006).

Peak	Retention time [min]	Molecular Weight [g/mol]
A	16	80200
B	35	1400
C	37	970
D	40	510
E	43	250
F	51	45
G	55	20

3.3.2 Gel filtration by FPLC performed on water soluble proteins

In order to follow the changes in the fish, the seven peaks which were found in all chromatograms were selected. The areas of the seven largest peaks were compared relative to the total areas during storage time as shown in figure 3.7. This was calculated as given in appendix C.6.1. An assembled table of measured and calculated data is given in appendix F.2.2.

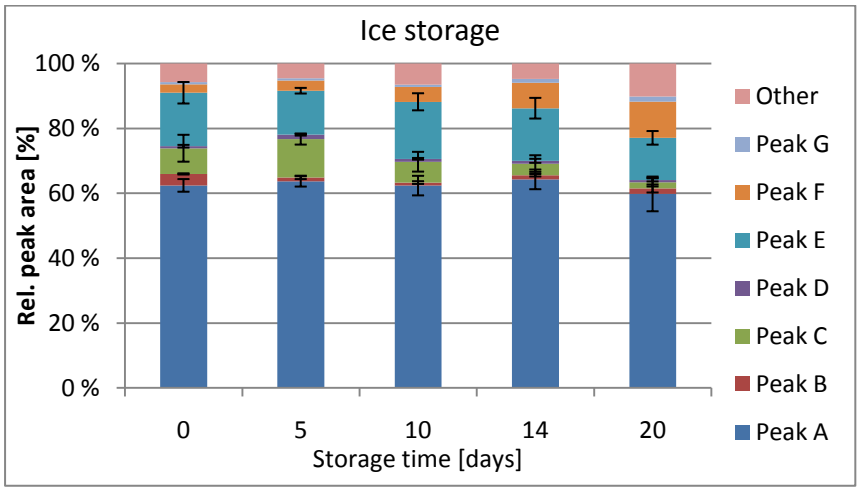


Figure 3.7: Comparison of relative peak areas [%] of water soluble proteins from chilled reference samples (KR) of salmon. Y-error bars represent the standard error of the mean. (n=3-4)

According to these results there are some changes in the peak areas during storage. To get a better picture of this, the proteins were precipitated in ethanol to exclude some of the larger proteins mainly in peak A. This was done for all the samples.

3.3.3 Gel filtration by FPLC performed on ethanol soluble proteins

The areas of the seven largest peaks were compared relative to the total areas during storage time as shown in figure 3.8. This was calculated as given in appendix C.6.1. An assembled table of measured and calculated data is given in appendix F.3.2.

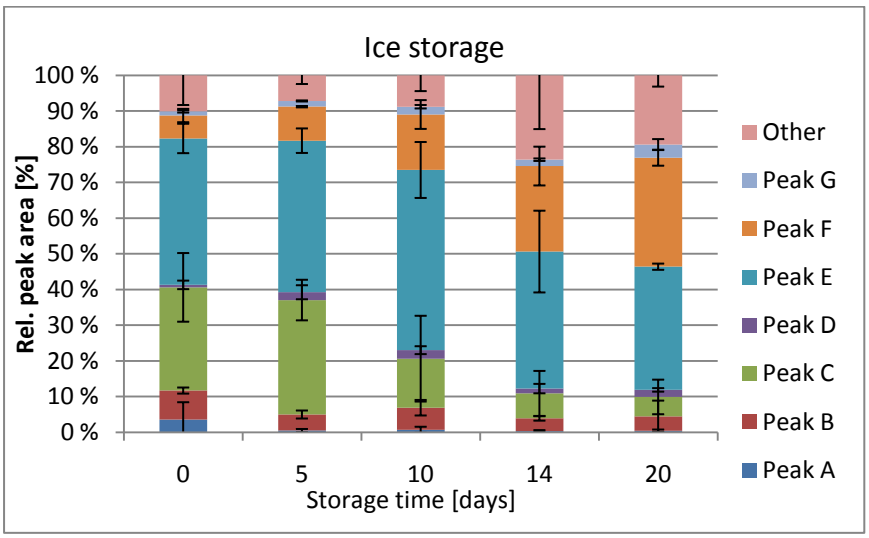


Figure 3.8: Comparison of relative peak areas [%] of ethanol soluble proteins from chilled reference samples (KR) of salmon. Y-error bars represent the standard error of the mean. (n=3-4)

As storage time progresses there are some changes in the relative area of the different peaks. The largest differences are found in peaks C, D, E and F. These indications are also found for the samples superchilled using air in figure 3.9 and liquid carbon dioxide in figure 3.10 below.

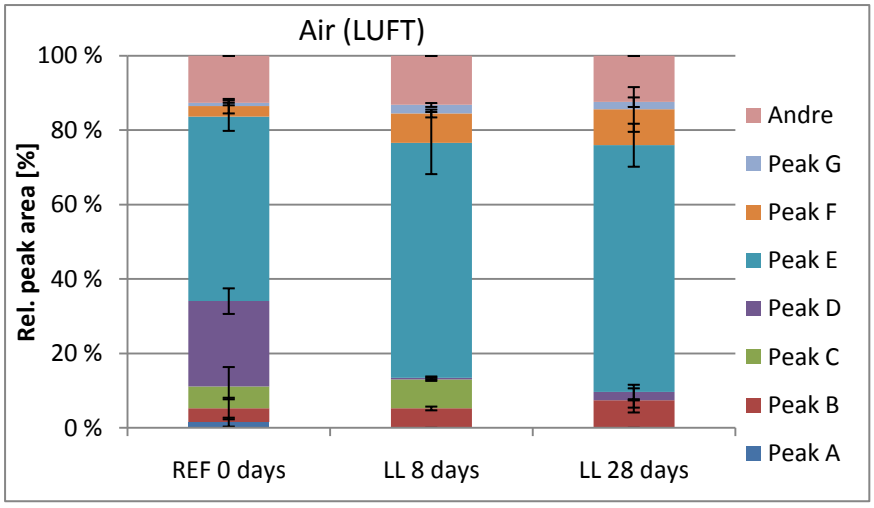


Figure 3.9: Comparison of the relative peak areas [%] of ethanol soluble proteins from samples superchilled using air (LUFT) and stored for 8 and 28 days. The sample REF was withdrawn at day 0 prior to superchilling. Y-error bars represent the standard error of the mean. (n=3-5)

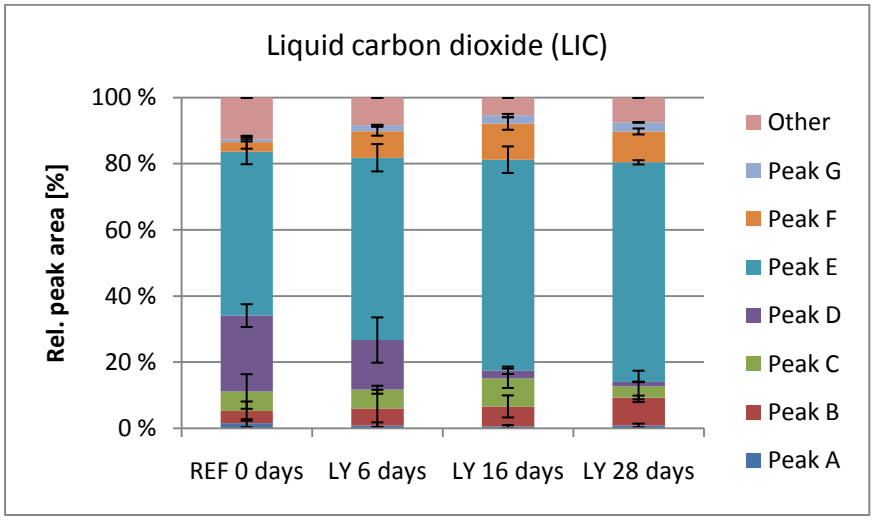


Figure 3.10: Comparison of the relative peak areas [%] of ethanol soluble proteins from samples superchilled using liquid carbon dioxide (LIC) and stored for 6, 16 and 28 days. The sample REF was withdrawn at day 0 prior to superchilling. Y-error bars represent the standard error of the mean. (n=3-5)

3.3.4 Changes in peaks during storage

To get a closer look at the changing peaks found by FPLC, the changes in the different peaks were plotted relative to storage time for each storage method as shown in figure 3.11. All calculations are found in appendix F.4.

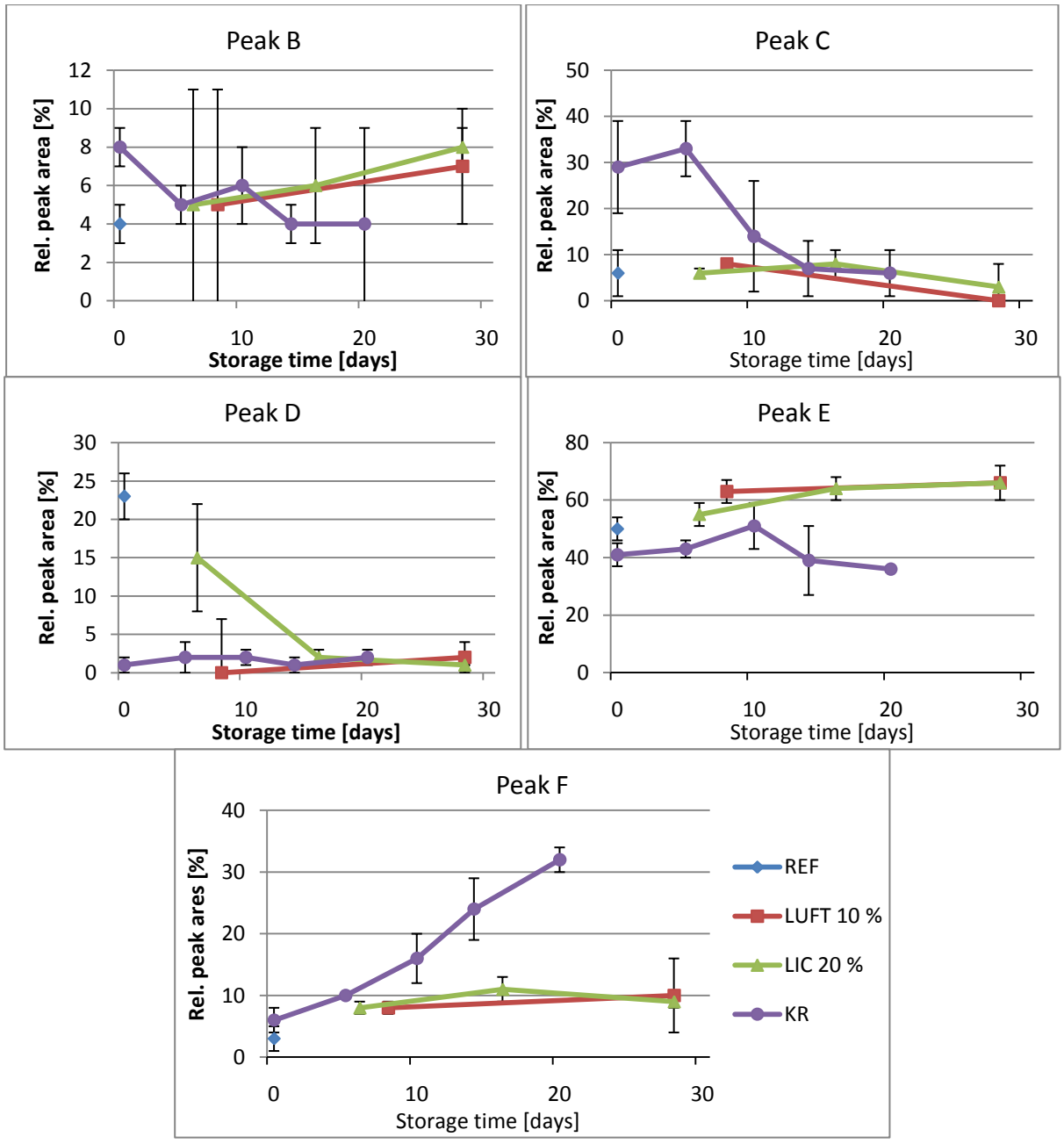


Figure 3.11: Comparing changes in relative peak areas [%] relative to storage time [days] of ethanol soluble proteins from salmon stored on ice (REF and KR) and superchilled using air (LUFT 10 %) and liquid carbon dioxide (LIC 20 %). Mean values of relative area content is given in % total area. Y-error bars represent the standard error of the mean. (n=3-5)

Peak B showed no significant differences in relative area between storage methods during storage time.

Peak C had a significantly higher relative area in the chilled reference samples during the first week of storage. After 10 days there was no significant difference between the methods.

Peak D had a significantly higher relative area in salmon superchilled using liquid carbon dioxide (LIC) during the first week of storage. After 10 days there was no significant difference between the methods.

Peak E had a significantly lower relative area in the chilled reference samples during the entire storage period compared to the two superchilling methods. The two superchilling methods were not significantly different for peak E during storage.

Peak F had a relative area which was significantly higher in the chilled reference samples during the entire storage period compared to the two superchilling methods. The two superchilling methods were not significantly different for peak F during the storage period.

3.4 Mass spectrometry

The next step was indentifying the compounds in the samples and in the peaks by mass spectrometry analysis. The mass spectrometry analysis was carried out as described in chapter 2.8. The calculations are given in appendix C.7, and the assembled tables for the measured and calculated data are given in appendix G.1 and G.2.

3.4.1 Changes in composition during storage

The abundances (for each m/z values) were sorted and all values below 5000-10000 were disregarded. The abundances left were used to calculate means and standard deviations for each group of withdrawal. The means for each group were used to calculate relative numbers compared to day zero as shown in appendix C.7.1. The samples composition was found by plotting the relative abundance for each m/z value compared to day zero, relative to storage time. In figure 3.12 this has been calculated for the ice chilled reference samples.

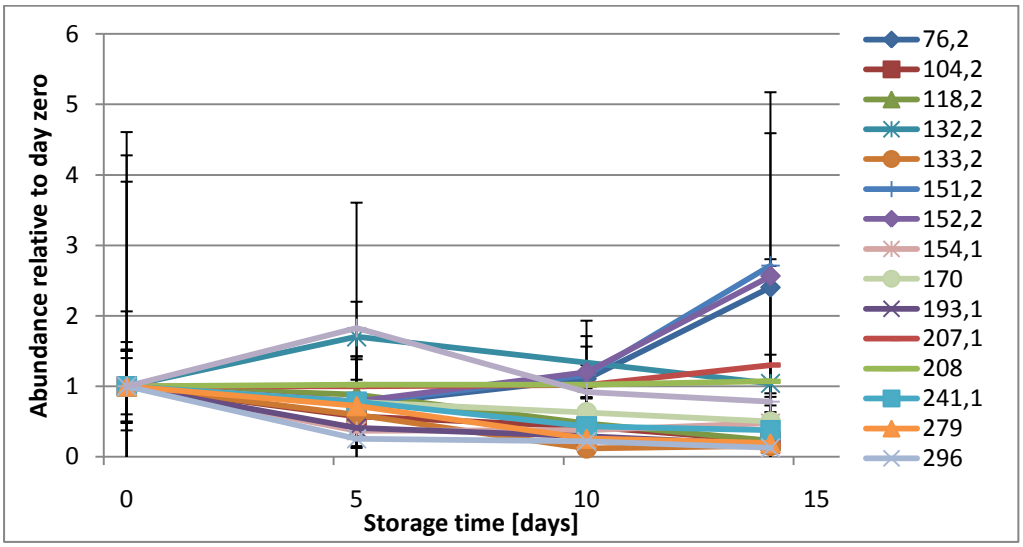


Figure 3.12: Changes in composition of reference samples during storage on ice. The abundance of the m/z values are plotted as a function of storage time. The means are calculated for each withdrawal and the y-bars represent the standard deviation of the mean. (n=3-4)

The results show that most of the abundances decrease during storage, while three of them (151.2, 152.2 and 76.2) increase between day 10 and 14. This was also done for the superchilled samples; however, they did not show any changes during storage.

To see if the composition of each sample changed relative to the largest compound during storage time, the abundance of all m/z values in percent of the highest abundance (151.2) was calculated as shown in figure 3.13.

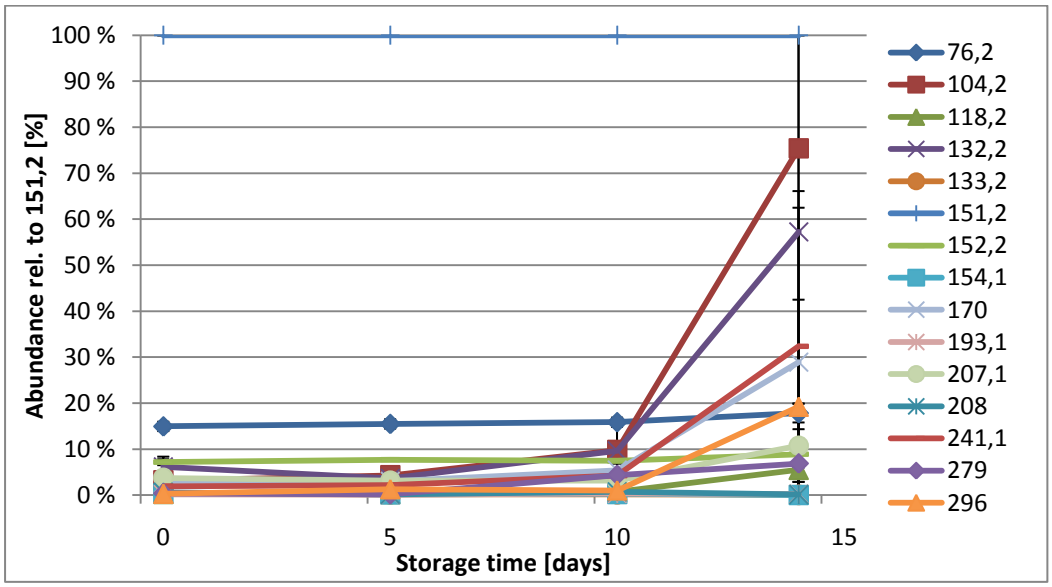


Figure 3.13: Relative abundance of the m/z values [%] compared to the abundance of m/z=151.2 relative to storage time [days] for the chilled reference samples. The points represent the mean values, while the y-bars represents the standard deviations of the means. (n=3-4)

The results show that most of the m/z values are increasing between day 10 and 14 of storage. The relative abundance of the three samples increasing the most is 104.2, 132.2 and 241.1. By looking at the standard deviations, they also increase at the end of the period. Similar calculations were done for the superchilled samples; however, they did not change during storage.

3.4.2 Relative abundance for m/z values

To compare the superchilling methods, the next step was carried out for selected m/z values. The relative abundances in % of the total abundance and compared to the abundance at day zero were plotted relative to storage time as shown in figures 3.14-3.19 below.

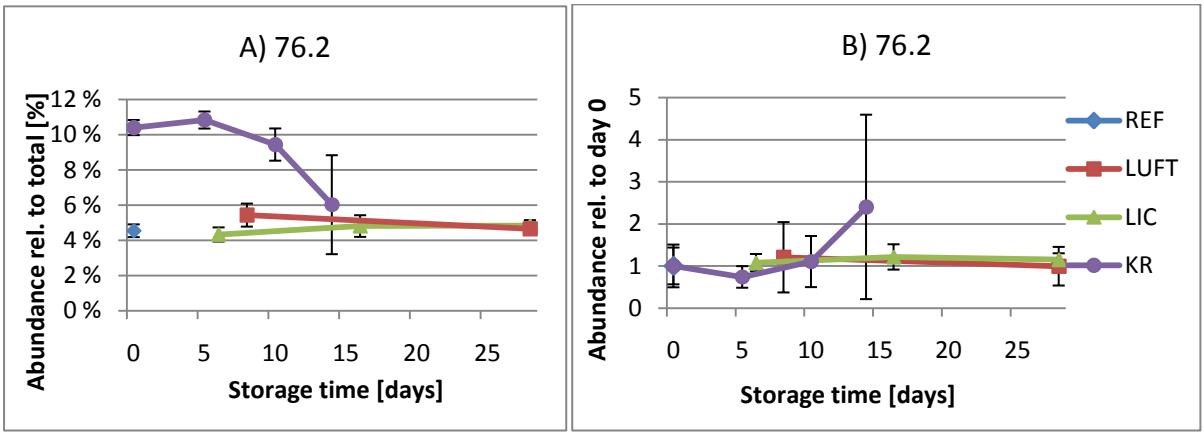


Figure 3.14: A) Abundance relative to total abundance [%] relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=76.2. Y-error bars represent the standard error of the mean. (n=3-5) B) Abundance relative to day 0 relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=76.2. Y-error bars represent the standard error of the mean. (n=3-5)

The results show that the superchilled samples are relatively stable while there are changes in the chilled reference samples. Relative to the total abundance 76.2 decreases during storage, while relative to day zero it increases. The largest changes appear at 10-14 days.

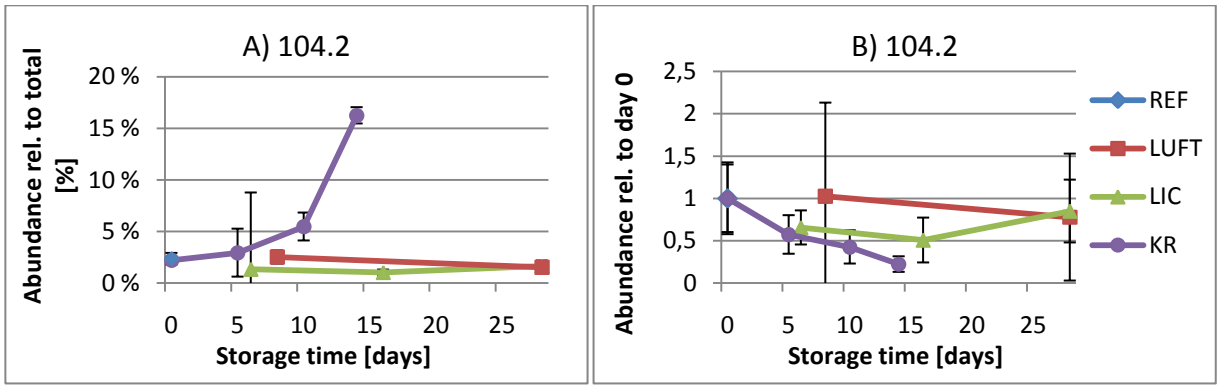


Figure 3.15: A) Abundance relative to total abundance [%] relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=104.2. Y-error bars represent the standard error of the mean. (n=3-5) B) Abundance relative to day 0 relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=104.2. Y-error bars represent the standard error of the mean. (n=3-5)

The results show that the superchilled samples are relatively stable while some changes appear at 10-14 days for the chilled reference samples. The abundance of (the m/z value) 104.2 increases relative to the total abundance and it decreases relative to day zero.

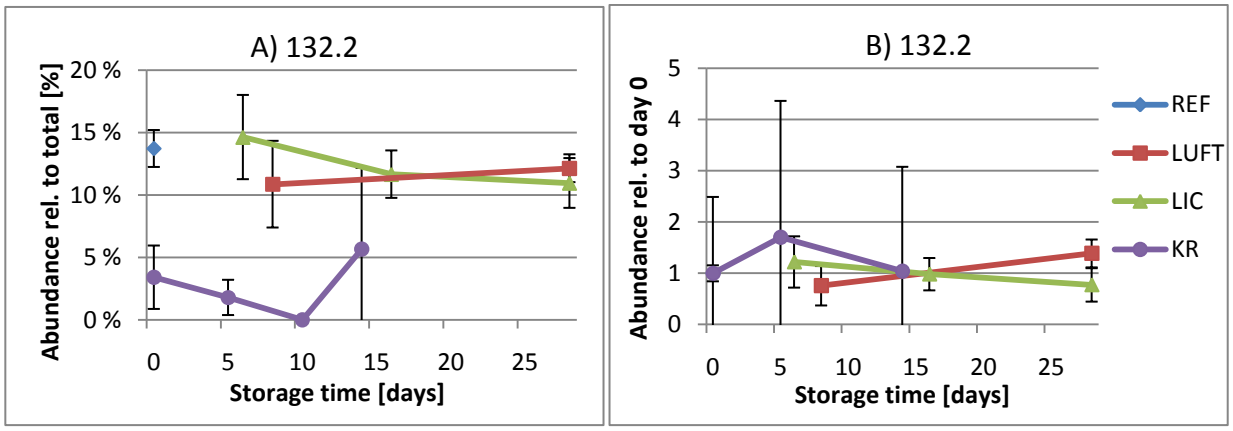


Figure 3.16: A) Abundance relative to total abundance [%] relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=132.2. Y-error bars represent the standard error of the mean. (n=3-5) B) Abundance relative to day 0 relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=132.2. Y-error bars represent the standard error of the mean. (n=3-5)

The results from figure 3.16 show that the superchilled samples are relatively stable. The chilled reference samples have a significantly lower abundance relative to the total abundance at 132.2 than the superchilled samples until day 14 when it increases. Relative to day zero there is no significant differences.

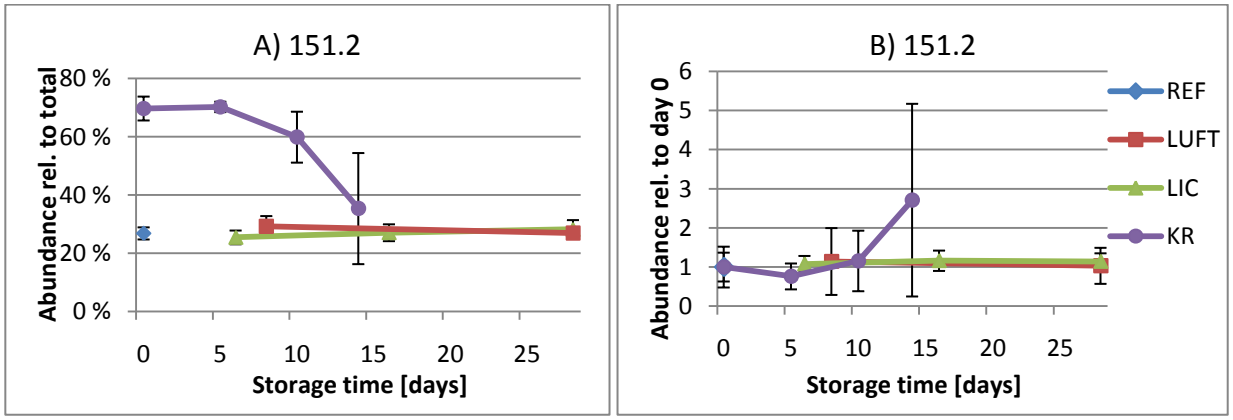


Figure 3.17: A) Abundance relative to total abundance [%] relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=151.2. Y-error bars represent the standard error of the mean. (n=3-5) B) Abundance relative to day 0 relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=151.2. Y-error bars represent the standard error of the mean. (n=3-5)

The results from figure 3.17 show that superchilled samples are relatively stable while the chilled reference samples change during storage time. The samples decrease relative to the total abundance and increase relative to day zero. The largest changes are found between 10 and 14 days of storage.

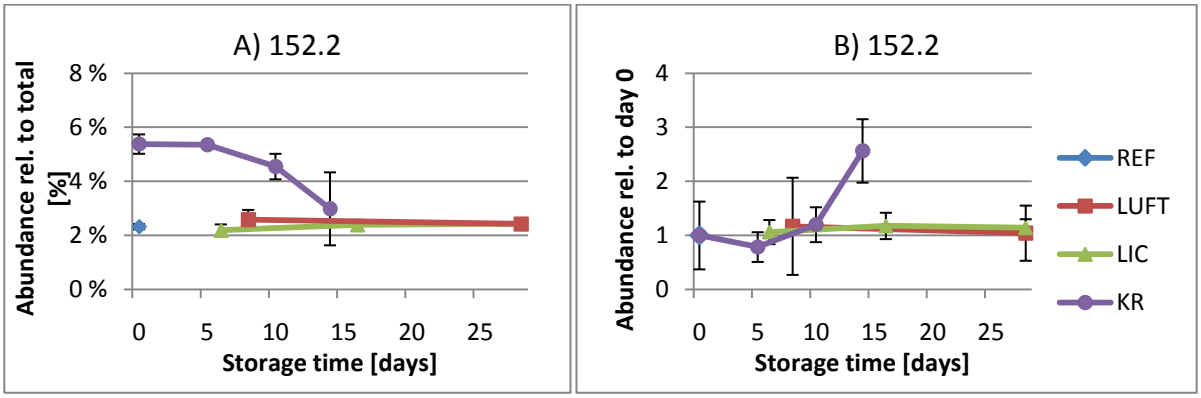


Figure 3.18: A) Abundance relative to total abundance [%] relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=152.2. Y-error bars represent the standard error of the mean. (n=3-5) B) Abundance relative to day 0 relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=152.2. Y-error bars represent the standard error of the mean. (n=3-5)

The results show that the superchilled samples are relatively stable while the chilled reference samples are decreasing relative to total abundance and increasing relative to day zero.

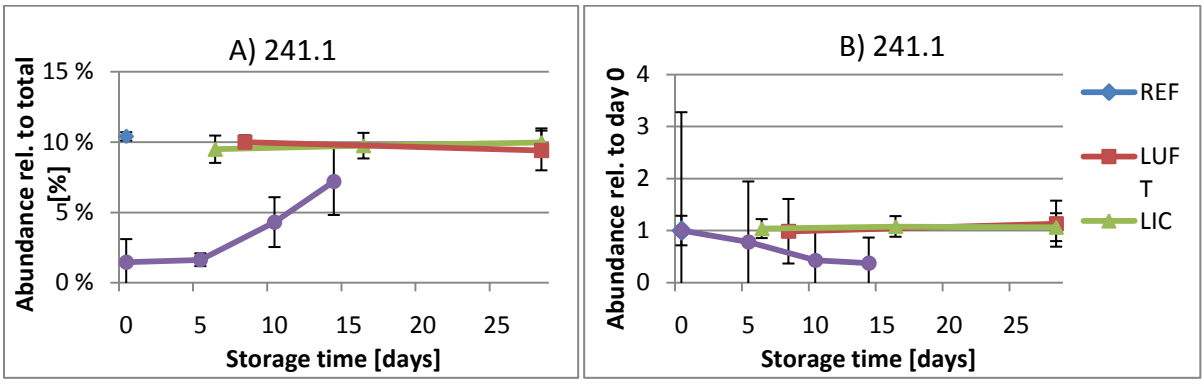


Figure 3.19: A) Abundance relative to total abundance [%] relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=241.1. Y-error bars represent the standard error of the mean. (n=3-5) B) Abundance relative to day 0 relative to storage time [days] for salmon stored on ice (KR and REF) and superchilled using air (LL) and liquid CO_2 (LY) m/z=241.1. Y-error bars represent the standard error of the mean. (n=3-5)

The results show that the superchilled samples are relatively stable while the chilled reference samples are increasing relative to total abundance and decreasing relative to day zero.

3.4.3 Composition of the peaks

Fractions of an extract of ethanol soluble proteins from salmon superchilled using air and stored for one week, were prepared as described in chapter 2.8 to find the composition of the peak fractions. This extract was made from salmon superchilled by air and stored for one week. The composition of the peaks was found by plotting the relative abundance for each peak as seen in figure 3.20.

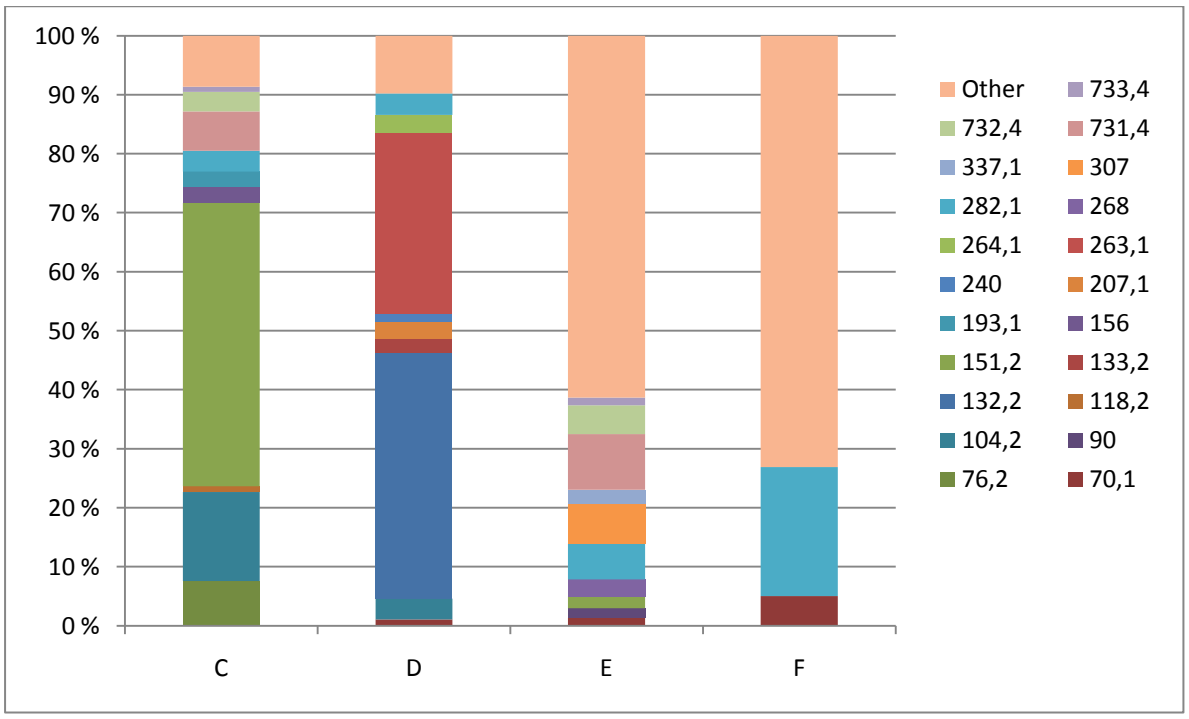


Figure 3.20: Composition of peaks in % for samples superchilled by air and stored for one week.

The results show that the level of proteins and peptides in the samples decreases for each peak. The constituents in the highest amount (%) of total are 151.2, 104.2 and 76.2 for peak C, 263.1, and 132.2 for peak D, 731.4, 307, and 282.1 for peak E and 282.1 and 70.1 for peak F.

4 Discussion

4.1 Drip loss and water holding capacity

The most loosely bound water or liquid is lost as drip loss. Drip loss is an important parameter as salmon is sold by weight. Loss of water will lead to a lower price of the product. In addition, it might lead to a deterioration of appearance and reduced eating quality. As explained in chapter 1.2.3 the drip loss and water holding capacity is among other factors influenced by muscle structures and activity of enzymes (Bahuaud, 2009, Duun and Rustad, 2008, Hultmann and Rustad, 2004, Rustad, 2005). Drip loss and water holding capacity reflects the moisture content, and may therefore influence the eating quality of the product (Duun and Rustad, 2008). Some loss of nutrients in the drip may lead to a product of lower nutritional value.

Drip loss was calculated as percent of weight loss. As seen in chapter 3.3.1 the drip loss during storage on ice (REF and KR) and thawing after superchilling (LUFT 10 % and LIC 20 %) increased somewhat throughout the period. Both the superchilled samples and the samples stored on ice lost 1-4.5 % of their weight as drip loss during storage. The standard deviations were largest at 14 to16 days of storage which may indicate some changes in the muscles capability to retain water at this time.

According to Duun and Rustad (2008), a drip loss above 3 % may be considered as high. Even if the results in this study show a drip loss above 3 %, they confirm the development of drip loss during storage found by Duun and Rustad (2008). There were no significant differences between the storage methods. However, the results show that salmon chilled by air had a significantly higher drip loss after 28 days of chilling, than salmon superchilled by liquid carbon dioxide (LIC). The salmon superchilled by LIC, have probably had a shorter chilling time than the salmon superchilled by air. The difference in

chilling time and temperature could cause larger ice crystals in the samples superchilled by air, than by LIC which is a fast freezing method and promotes the formation of small ice crystals (Strasburg et al., 2008). An increased drip loss could be due to cell damage caused by ice crystallization as described by Ronsivalli and Baker (1981).

As shown in chapter 3.3.2 the water content in the salmon stored on ice (64-68 %) were significantly higher than that of the superchilled salmon (57-65 %). This may be because the chilled reference samples were collected from a different batch than the superchilled samples. In addition, it was harvested at a different time of year. The high water content could be explained by a leaner fish. The water content of the chilled reference samples have a slight increase during the first 14 days of storage, before it decreases. The difference in water content between the chilled reference samples and the superchilled samples is in accordance with the results of Hemmingsen (2002). Due to the high water content in the samples, there is more loosely bound water in the chilled reference samples. This might have given a higher drip loss for the chilled reference samples than the superchilled samples with lower water content.

The liquid loss was lower in salmon stored on ice compared to the samples superchilled by air as shown in chapter 3.3.3. After 14 days, however, the differences were no longer significant. These results are supported by several findings (Duun and Rustad, 2008, Morkore et al., 2002, Nilsson and Ekstrand, 1993) which all have no significant difference in liquid loss between storage on ice or storage by superchilling.

The level of the liquid loss has to be seen in context with the amount of drip loss. Duun and Rustad (2008) suggested that samples with high drip loss are more likely able to hold better on to the remaining water during the centrifugation procedure of the liquid loss analysis. The results in this study do not show any connection between the drip loss and the liquid loss.

4.2 Protein content

Protein degradation may enhance extractability, when caused by proteolytic activity. Protein extractability is used as a tool to monitor protein denaturation. The knowledge on the effect of process and storage on textural properties and activities of cathepsins can be valuable for understanding the relationship between protein stability and superchilling.

The muscle proteins can, as mentioned in chapter 1.2.1, be divided into three classes based on solubility properties. The water soluble sarcoplasmic proteins consist mainly of enzymes and chromo proteins.

As shown in chapter 3.2 the content of water soluble protein (Sarcoplasmic proteins) is between 2 % and 5 % on wet weight basis. These results confirm Rustads (2005) statement that one fifth of the proteins are sarcoplasmic proteins and soluble in water. There are no significant differences between the storage methods or throughout the storage period. These findings confirm the results of Duun and Rustad (2008).

The findings concerning proteins solubilities are not supported by Skistads work on chilled salmon (2008), who found that proteins soluble in ethanol increased 2-3-fold after one week of storage, while the water soluble proteins had a slight decrease. The reasons for this may be that according to Lowry et al. (1951) and Hortin and Meilinger (2005) amino acids and peptides may give inconsistent results. Amino acids may give less color than proteins; however they may also cross-react with peptides and give more color than amino acids. An increase in free amino acids as the muscle degrades may therefore not be detected by the Lowry method. In addition, the amount of color is not strictly proportional with the concentration (Rustad, 2010a). Because of the relative constant level of water soluble proteins found in this study, it may be reasonable to not see any significant changes in the ethanol soluble proteins as well.

4.3 Gel filtration using fast protein liquid chromatography

Gel filtration using fast protein liquid chromatography (FPLC) was performed to find the content of different molecular weight proteins and peptides in the extracts. Imidazolebuffer was used as an eluent in the FPLC-fractionation. Standards were run, and the molecular weight distribution was calculated as shown in the results in chapter 3.3.1.

In chapter 1.6.2 it is described that the exclusion limit of the column for a protein in an aqueous solution is 20 000 g/mol, and that it is most accurate between 100 and 7 000 g/mol. This indicates that the column is not reliable outside the range described in chapter 3.3.1, which corresponds to peak B-E.

All the water soluble extracts had seven peaks in common as shown in chapter 3.3.2, while the ethanol precipitated extracts had six (3.3.3). The reason for this was that the largest molecules, calculated to about 80 200 g/mol, precipitated in ethanol. This is in accordance with the theory presented by Rosenberg (1996) which stated that large molecules have a tendency to aggregate and precipitate when adding ethanol to the solution. The results in this theses confirms the findings by Skistad (2008) that proteins >90 kDa are precipitated when using a solvent of 75 % ethanol.

As described in chapter 3.3.3 and 3.3.4, there were some changes during storage for the different chilling methods. This was seen best when comparing the relative area of the peaks during storage time for the different chilling methods.

Peak B, with a retention time of approximately 35 minutes and a molecular weight calculated to about 1400 g/mol, showed no significant differences in relative peak area during storage or between the chilling methods. The standard deviations were high, which indicates large individual differences between the samples.

Peak C, with a retention time of approximately 37 minutes and a molecular weight calculated to about 970 g/mol, showed a higher relative peak area for the chilled reference

samples before ten days of storage. After this there were no significant differences in relative peak areas between the chilling methods. The superchilled samples were low and stable during the entire storage time.

Peak D, with a retention time of approximately 40 minutes and a molecular weight of about 510 g/mol, showed a higher relative peak area for the samples superchilled using liquid carbon dioxide before 16 days of storage. There were no significant differences between the chilled reference samples and the samples superchilled using air, which stayed low and stable throughout the storage time.

Peak E, with a retention time of approximately 43 minutes and a molecular weight of about 250 g/mol, showed a high relative peak area for all the samples during the entire storage time. The samples superchilled using liquid carbon dioxide and air had high and stable peak areas with no significant differences through the length of the storage time. The peak areas of the chilled reference samples were significantly lower than the superchilled samples the entire storage time except for day 10. On day 10 and 14 the standard deviations of the chilled reference samples were large, indicating changes in the muscle at this time of storage.

Peak F, with a retention time of approximately 51 minutes and a molecular weight calculated to about 45 g/mol, showed a significant increase in relative peak area for the chilled reference samples during the entire storage period. The superchilled samples were stable, with no significant differences during storage time. The large increase in relative peak areas for the chilled reference samples may indicate that the content of free amino acids increase during storage, which is not unusual according to Bauchart (2007) and Moya et al. (2001). An increase in free amino acids could indicate proteolytic activity during storage. However, the content of free amino acids has not been measured during this work.

The results in this thesis indicate that the proteins from the superchilled samples are less exposed to deterioration and structural changes during storage, than the proteins from the

ice chilled samples. This indicates more proteolytic activity during storage of the chilled reference samples compared to the superchilled samples. This is not in accordance with the work of French (1988) and Rehbein and Caklis (2000) who stated that the enzymatic reactions in partially frozen salmon were faster than the reactions in frozen or ice chilled salmon. However the method used here will only detect exoproteases. Since the cathepsins are endoproteases, the activity of them might not be detected using these methods.

4.4 Mass spectrometry

The next step for identifying the compounds and the composition of the samples was mass spectrometry. Mass spectrometry was performed as described in chapter 2.8. The samples selected for analysis were extracts and four of the fractions collected during fractionated FPLC on extracts precipitated using 75 % ethanol. The fractions collected and sent for MS analysis corresponded to peak C, D, E and F.

The m/z values were sorted, and all abundances below four to five digits (5 000-10 000) were disregarded. The remaining m/z values were evaluated.

For the chilled reference samples the abundance for the different m/z values was plotted relative to the abundance at day zero (chapter 3.4.1). Most of the relative abundances of the m/z values decreased. There were however three relative abundances of the m/z values that increased. These values were compounds with a mass to charge ratio of 76.2, 151.2 and 152.2. The results indicates that these three m/z values are increasing the most during storage time compared to the total abundance. The standard deviations are large during the entire storage period, and especially at day fourteen. Large standard deviations may indicate some changes in the muscles at the end of the storage time.

Further, for the same samples, the abundances for the different m/z values was plotted relative to the abundance of the m/z value = 151.2. These results showed that most of the samples were stable during the entire storage period; however there were some increase in relative abundances at day fourteen. The three values increasing the most are 104.2, 132.2 and 241.1. This indicates that these compounds are increasing in quantity compared to 151.2. At day fourteen there are large standard deviations, compared to very low deviations earlier in the storage period which also indicates some changes in the composition and structure of the proteins at the end of storage.

The results reported in chapter 3.4.2 show that there are no significant differences in both the abundance relative to the total abundance and relative to day zero for the superchilled samples during storage. These results indicate that the proteins from the superchilled

samples are less exposed for deterioration and structural changes during storage than the proteins from the ice chilled samples.

There are some changes for the chilled reference samples. The results of the chilled reference samples show that the abundance of the m/z values relative to the total abundance is decreasing during storage for the m/z values of 76.2, 151.2 and 152.2. These three m/z values have increasing abundances relative to day zero for the chilled reference samples which is confirmed by the results in chapter 3.4.1. The decrease in content of these three compounds compared to the total abundance, indicate a decrease compared to the total content of detectable compounds. The increase in abundance relative to day zero indicates that the content of the three compounds increase during storage time.

For the chilled reference samples, the m/z values of 104.2, 132.2 and 241.1, the abundance relative to the total abundance is increasing, however not significantly for 132.2. When it comes to the abundance relative to day zero, the m/z values of 104.2, 132.2 and 241.1 are decreasing, however not significantly compared to the superchilled samples. This indicates that there is some increase of the compounds relative to the total content of detectable compounds. However, the abundance of the three compounds relative to the abundance at day zero is stable, indicating that the total content of detectable compounds must be decreasing.

The results from mass spectrometry of the peak fractions from FPLC, made it clear that the content of proteins in the peaks decreased with the retention time. As seen in chapter 3.4.3, the content of molecules in peak C and D is approximately 90 %, while in peak E it has decreased below 40 % and in peak F it has decreased below 30 %. The results showed that most of the remaining abundance could be accounted for as imidazole from the buffer.

The most important components in the peaks, exceeding 10 %, is for peak C the m/z values of 151.2 and 104.2. The third largest m/z value is 76.2. For peak D, 263.1 and

131.2 are characterized. Peak E have a lot of small abundances for several different m/z values, none of which constitutes more than 10 %. The three largest abundances are still found for the m/z values of 731.4, 307 and 282.1. For peak F 282.1 constitutes above 20 %, and 70.1 about 5 %.

The results from the mass spectrometry analysis can be compared to those of Bauchart et al. (2007) who found that MS analysis revealed the appearance of small non characterized peptides in trout muscle during the ice storage. However, they also, together with Skistad (2008), found it difficult to identify the compounds using this method. Work done on cheese (Piraino et al., 2007) and dry-cured ham (Mora et al., 2009) show promising results using mass spectrometry for the characterization of small compounds.

As no standards have been run, the work of identifying the compounds in the samples has been difficult. In addition the amount of work that has been done characterizing compounds in fish muscle has been limiting. By using MALDI-ToF analysis, the molecular weights could have been found to a higher certainty.

By assuming that there is only one charge in the mass/charge (m/z) ratios, some investigations have been done to identify some of the m/z values. The m/z value of 76.2 could be corresponding to glycine, with a molecular weight of appr. 75 g/mol. Taking this into consideration when looking at the results presented in chapter 3.4.2 for abundance relative to the total, this would correspond to the results of Bauchart et al. (2007) who found that glycine had a large decrease during ice storage.

The m/z value of 132.2 may correspond to creatine, with a molecular weight of appr. 131g/mol. This, taken into consideration when looking at the results of peak D presented in chapter 3.4.3, is plausible as the white muscle of salmon contain creatine up to 100 µmol/g dry weight (Erikson et al., 1999).

When looking at the results presented in chapter 3.4.2, the m/z value of 241.1 could correspond to anserine, with a molecular weight of appr. 240 g/mol. This is not in

accordance with the results of Moya et al. (2001) who found by working with pork meat that there was a general increase in all free amino acid concentrations except of, among others, anserine. However, there are differences in the degradation of proteins and peptides between species, so anserine could still be present and increase in salmon. Rustad et al. (2000) found that there were between 15 and 20 µmol anserine/g wet weight of salmon.

All results seen together with the standard deviations and changes in drip loss and liquid loss indicate that there are some changes in the proteins and peptides happening around 10-14 days of storage. This confirms the results by Hemmingsen (2002) who saw protein stability changes between 9 and 16 days of storage, and Benjakul (1997) who found that denaturations and degradation of proteins became obvious within 8 days of iced storage. These denaturations makes the proteins more susceptible to the action of cathepsin B as explained by Hultmann and Rustad (2004), and may lead to further degradation of the proteins and peptides in the muscle. The content of free amino acids has not been analyzed during this work, and should be included in further work.

5 Conclusions and further work

There were no significant differences in drip loss between the different chilling methods during storage. However, the significantly higher drip loss in samples superchilled by air indicate some formation of large ice crystals in the muscle compared to superchilling using liquid carbon dioxide. The liquid loss was significantly higher for samples superchilled using air before sixteen days of storage. After this there were no significant differences between the chilling methods.

There were no significant changes in the content of neither water nor ethanol soluble proteins on wet weight basis.

Gel filtration using FPLC was a good tool for fractionation and purification of the extracts. Seven peaks were compared during storage. Precipitating the extracts of water soluble proteins made the concentrations of large proteins and peptides > 80 000 g/mol decrease and mostly disappeared. The superchilled samples showed no significant changes during storage. A significant decrease in compounds with a molecular weight of approximately 970 g/mol and an increase in small compounds below 100 g/mol were observed for the chilled reference samples.

MS was used as a tool for isolation and identification of compounds in the extracts and FPLC fractions. Some changes were detected for certain compounds. However, the work of identifying these compounds was troublesome as the mass to charge ratio is not accurate enough to be certain of the compound. Further work in identifying compounds in salmon muscle should include the use of MALDI analysis, which is more accurate for determining correct molecular weights than MS.

Three of the compounds found by MS were identified as glycine, creatine and anserine.

The combined results from FPLC and MS show that the protein content and structure in the superchilled samples are generally more stable than the chilled reference samples. These results suggest that superchilled fish products have a more stable muscle quality than fish stored on ice, while the ice chilled reference samples indicate an increase of free amino acids during storage. This increase suggests increased proteolytic activity during storage on ice. The content of free amino acids should be included in further work.

Seen together, the results suggest that there are some changes in the composition of the proteins around 10 to 14 days of storage. Further work should investigate this period of storage, as it could give answers about important reactions concerning shelf life and product quality.

6 References

ALOMIRAH, H. F., ALLI, I., GIBBS, B. F. & KONISHI, Y. 1998. Identification of proteolytic products as indicators of quality in ground and whole meat. *Journal of Food Quality,* 21**,** 299-316.

ANDERSEN, E., JUL, M. & RIEMANN, H. 1965. *Industriel levnedsmiddelkonservering,* København, Teknisk Forlag.

ARIZONA, The University Of. 2010. *Mass Spectra Interpretation* [Online]. Available: http://www.chem.arizona.edu/quizplease/msintro/alcohol/images/0201spb.gif [Accessed].

BAHUAUD, D., UNIVERSITETET FOR MILJØ- OG BIOVITENSKAP, I. F. H.-O. A., MØRKØRE, T., AKVAFORSK, I. F. A., ØSTBYE, T.-K., AKVAFORSK, VEISETH-KENT, E., NOFIMA, M., THOMASSEN, M. S., OFSTAD, R. & MATFORSK 2010. Muscle structure responses and lysosomal cathepsins B and L in farmed Atlantic salmon (Salmo salar L.) pre- and post-rigor fillets exposed to short and long-term crowding stress
Food Chemistry. 118**,** 602-615.

BAHUAUD, D. S. O. 2009. *Atlantic salmon (Salmo salar L.) flesh quality role of lysosomes and cathepsins in muscle degradation,* Ås, Dept. of Animal and Aquacultural Sciences.

BARRETT, A. J. & KIRSCHKE, H. 1981. CATHEPSIN-B, CATHEPSIN-H, AND CATHEPSIN-L. *Methods in Enzymology,* 80**,** 535-561.

BAUCHART, C., CHAMBON, C., MIRAND, P. P., SAVARY-AUZELOUX, I., REMOND, D. & MORZEL, M. 2007. Peptides in rainbow trout (Oncorhynchus mykiss) muscle subjected to ice storage and cooking. *Food Chemistry,* 100**,** 1566-1572.

BENJAKUL, S., SEYMOUR, T. A., MORRISSEY, M. T. & AN, H. J. 1997. Physicochemical changes in Pacific whiting muscle proteins during iced storage. *Journal of Food Science,* 62**,** 729-733.

CORTESI, M. L., PANEBIANCO, A., GIUFFRIDA, A. & ANASTASIO, A. 2009. Innovations in seafood preservation and storage. *Veterinary Research Communications,* 33**,** S15-S23.

DAMODARAN, S. 2008. Amino Acids, Peptides and Proteins. *In:* DAMODARAN, S., PARKIN, K. L. & FENNEMA, O. R. (eds.) *Fennema's food chemistry.* 4th ed. / edited by Srinivasan Damodaran, Kirk Parkin, and Owen R. Fennema. ed. Boca Raton ; London: CRC Press.

DARBRE, A. 1986. Analytical Methods. *In:* DARBRE, A. (ed.) *Practical protein chemistry: a handbook.* Chichester: Wiley.

DOMON, B. & AEBERSOLD, R. 2006. Review - Mass spectrometry and protein analysis. *Science,* 312**,** 212-217.

DUUN, A. S. 2002. Superchilling - A review. Trondheim: Department of Biotechnology, Norwegian University of Science and Technology (NTNU).

DUUN, A. S. 2008. *Superchilling of muscle food storage stability and quality aspects of salmon (Salmo salar), cod (Gadus morhua) and pork,* Trondheim, Norwegian University of Science and Technology, Faculty of Natural Sciences and Technology, Institute of biotechnology.

DUUN, A. S. Unpublished Results.

DUUN, A. S. & RUSTAD, T. 2008. Quality of superchilled vacuum packed Atlantic salmon (Salmo salar) fillets stored at -1.4 and -3.6 degrees C. *Food Chemistry,* 106**,** 122-131.

EIDE, O., BORRESEN, T. & STROM, T. 1982. Minced Fish Production from Capelin (Mallotus-Villosus) - a New Method for Gutting, Skinning and Removal of Fat from Small Fatty Fish Species. *Journal of Food Science,* 47**,** 347-&.

ERIKSON, U., HULTMANN, L. & STEEN, J. E. 2006. Live chilling of Atlantic salmon (Salmo salar) combined with mild carbon dioxide anaesthesia I. Establishing a method for large-scale processing of farmed fish. *Aquaculture,* 252**,** 183-198.

ERIKSON, U., SIGHOLT, T., RUSTAD, T., EINARSDOTTIR, I. E. & JORGENSEN, L. 1999. Contribution of bleeding to total handling stress during slaughter of Atlantic salmon. *Aquaculture International,* 7**,** 101-115.

ERVIK, A., AURE, J. & HANSEN, P. K. 2006. Hvor mye havbruk tåler norskekysten? *In:* THOMASSEN, M., GUDDING, R., NORBERG, B. & JØRGENSEN, L. (eds.) *Havbruksforskning: Fra merd til mat.* Norges forskningsråd.

FERNANDEZ, K., ASPE, E. & ROECKEL, M. 2009. Shelf-life extension on fillets of Atlantic Salmon (Salmo salar) using natural additives, superchilling and modified atmosphere packaging. *Food Control,* 20**,** 1036-1042.

FRENCH, J. S., KRAMER, D. E. & KENNISH, J. M. 1988. PROTEIN HYDROLYSIS IN COHO AND SOCKEYE SALMON DURING PARTIALLY FROZEN STORAGE. *Journal of Food Science,* 53**,** 1014-&.

FYSON, D. 2008. United States Geological Survey. Available: http://pubs.usgs.gov/of/2001/ofr01-257/images/figure1.gif [Accessed].

GOFF, H. D. 1992. Low-Temperature Stability and the Glassy State in Frozen Foods. *Food Research International,* 25**,** 317-325.

GREASER, M. L. 2001. Postmortem Muscle Chemistry. *In:* HUI, Y. H. (ed.) *Meat science and applications.* New York: Marcel Dekker.

HANSEN, A. A., MORKORE, T., RUDI, K., LANGSRUD, O. & EIE, T. 2009. The combined effect of superchilling and modified atmosphere packaging using CO2 emitter on quality during chilled storage of pre-rigor salmon fillets (Salmo salar). *Journal of the Science of Food and Agriculture,* 89**,** 1625-1633.

HEALTHCARE, GE. 2006. Instructions 71-5017-97 AF High Performance Columns. GE Healthcare.

HEMMINGSEN, A. K. T. 2002. *Quality of fresh foods,* Trondheim, Norwegian University of Science and Technology, Faculty of Engineering Science and Technology.

HERRERA-MENDEZ, C. H., BECILA, S., BOUDJELLAL, A. & OUALI, A. 2006. Meat ageing: Reconsideration of the current concept. *Trends in Food Science & Technology,* 17**,** 394-405.

HORTIN, G. L. & MEILINGER, B. 2005. Cross-reactivity of amino acids and other compounds in the biuret reaction: Interference with urinary peptide measurements. *Clinical Chemistry,* 51**,** 1411-1419.

HULTMANN, L. 2003. *Endogenous proteolytic enzymes studies of their impact on fish muscle proteins and texture,* [Trondheim], Faculty of Natural Sciences and Technology, Department of Biotechnology, Norges teknisk-naturvitenskapelige universitet.

HULTMANN, L., RORA, A. M. B., STEINSLAND, I., SKARA, T. & RUSTAD, T. 2004. Proteolytic activity and properties of proteins in smoked salmon (Salmo salar) - effects of smoking temperature. *Food Chemistry,* 85**,** 377-387.

HULTMANN, L. & RUSTAD, T. 2004. Iced storage of Atlantic salmon (Salmo salar) - effects on endogenous enzymes and their impact on muscle proteins and texture. *Food Chemistry,* 87**,** 31-41.

HUSS, H. H. 1995. Quality and quality changes in fresh fish. *FAO Fisheries Technical Paper - 348.* Rome, Italy: Fisheries and Aquaculture Department

J. GRAHAM, JOHNSTON, W. A. & NICHOLSON, F. J. 1992. Ice in fisheries. *FAO Fisheries Technical Paper.* Rome, Italy: Fisheries and Aquaculture Department

KOOHMARAIE, M. 1996. Biochemical factors regulating the toughening and tenderization processes of meat. *Meat Science,* 43**,** S193-S201.

LARSEN, R. 2007. *Influence of weak brines on technological, biochemical and nutritional properties of cod (Gadus morhua L.) muscle,* [Tromsø], University of Tromsø, Norwegian College of Fishery Science.

LEISTNER, L. 1999. Combined Methods for Food Preservation. *In:* RAHMAN, S. (ed.) *Handbook of food preservation.* New York: Marcel Dekker.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. 1951. PROTEIN MEASUREMENT WITH THE FOLIN PHENOL REAGENT. *Journal of Biological Chemistry,* 193**,** 265-275.

LYNUM, L. 2005. *Fisk som råstoff,* Trondheim, Tapir akademisk forlag.

MACKIE, I. M. 1993. The Effects of Freezing on Flesh Proteins. *Food Reviews International,* 9**,** 575-610.

MAGNUSSEN, O. M., HAUGLAND, A., HEMMINGSEN, A. K. T., JOHANSEN, S. & NORDTVEDT, T. S. 2008. Advances in superchilling of food - Process characteristics and product quality. *Trends in Food Science & Technology,* 19**,** 418-424.

MARSHALL, R. C. A. I., ADAM S. 1986. Protein Oligomer Composition, Preparation of Monomers and Constituent Chains. *In:* DARBRE, A. (ed.) *Practical protein chemistry: a handbook.* Chichester: Wiley.

MICHALCZYK, M. & SUROWKA, K. 2007. Changes in protein fractions of rainbow trout (Oncorhynchus mykiss) gravads during production and storage. *Food Chemistry,* 104**,** 1006-1013.

MORA, L., SENTANDREU, M. A., KOISTINEN, K. M., FRASER, P. D., TOLDRA, F. & BRAMLEY, P. M. 2009. Naturally Generated Small Peptides Derived from Myofibrillar Proteins in Serrano Dry-Cured Ham. *Journal of Agricultural and Food Chemistry,* 57**,** 3228-3234.

MORKORE, T., HANSEN, A. A., UNANDER, E. & EINEN, O. 2002. Composition, liquid leakage, and mechanical properties of farmed rainbow trout: Variation between fillet sections and the impact of ice and frozen storage. *Journal of Food Science,* 67**,** 1933-1938.

MOYA, V. J., FLORES, M., ARISTOY, M. C. & TOLDRA, F. 2001. Pork meat quality affects peptide and amino acid profiles during the ageing process. *Meat Science,* 58**,** 197-206.

NAKAI, Y., NISHIMURA, T., SHIMIZU, M. & ARAI, S. 1995. Effects of freezing on the proteolysis of beef during storage at 4 degrees C. *Bioscience Biotechnology and Biochemistry,* 59**,** 2255-2258.

NILSSON, K. & EKSTRAND, B. 1993. THE EFFECT OF STORAGE ON ICE AND VARIOUS FREEZING TREATMENTS ON ENZYME LEAKAGE IN MUSCLE-TISSUE OF RAINBOW-TROUT (ONCORHYNCHUS-MYKISS). *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung,* 197**,** 3-7.

NORTVEDT, R., ESPE, M., GRIBBESTAD, I., JØRGENSEN, L., KARLSEN, Ø., OTTERÅ, H., RØRÅ, M. B., STIEN, L. H. & SØRENSEN, N. K. 2006. Fra kjemisk sammensetning til trygg og god sjømat basert på etisk og bærekraftig produksjon. *In:* THOMASSEN, M., GUDDING, R., NORBERG, B. & JØRGENSEN, L. (eds.) *Havbruksforskning: Fra merd til mat.* Norges forskningsråd.

NORWEGIAN, S. E. C. 2010. *Eksportrekord for norsk sjømat på 44,7 milliarder kroner* [Online]. Norwegian Seafood Export Council Available: http://www.seafood.no/Bransje/Nyheter/Vis+artikkel?key=59146 [Accessed 13.01.10 2010].

OLAFSDOTTIR, G., LAUZON, H. L., MARTINSDOTTIR, E., OEHLENSCHLAGER, J. & KRISTBERGSSON, K. 2006. Evaluation of shelf life of superchilled cod (Gadus morhua) fillets and the influence of temperature fluctuations during storage on microbial and chemical quality indicators. *Journal of Food Science,* 71**,** S97-S109.

OVERREIN, I., ELLINGSEN, T. E. & RUSTAD, T. 2010. Protein and lipid changes during storage and heating of *Calanus finmarchicus* harvests. *Submitted to: Process Biochemistry*.

PARKIN, K. L. 2008. Enzymes. *In:* DAMODARAN, S., PARKIN, K. L. & FENNEMA, O. R. (eds.) *Fennema's food chemistry.* 4th ed. / edited by Srinivasan Damodaran, Kirk Parkin, and Owen R. Fennema. ed. Boca Raton ; London: CRC Press.

PIRAINO, P., UPADHYAY, V. K., ROSSANO, R., RICCIO, P., PARENTE, E., KELLY, A. L. & MCSWEENEY, P. L. H. 2007. Use of mass spectrometry to characterize proteolysis in cheese. *Food Chemistry,* 101**,** 964-972.

REHBEIN, H. & CAKLI, S. 2000. The lysosomal enzyme activities of fresh, cooled, frozen and smoked salmon fish species (Onchorhyncus keta and Salmo salar). *Turkish Journal of Veterinary & Animal Sciences,* 24**,** 103-108.

RONSIVALLI, L. J. & BAKER, D. W. 1981. Low-Temperature Preservation of Seafoods - a Review. *Marine Fisheries Review,* 43**,** 1-15.

ROSENBERG, I. M. 1996. *Protein analysis and purification : benchtop techniques*, Boston, Birkhäuser.

RUSTAD, T. 2005. Muskelvevet i kjøtt og fisk. Trondheim: NTNU, Institutt for bioteknologi.

RUSTAD, T. 2010a. Peptides and proteins. *In:* NOLLET & TOLDRA, F. (eds.) *Seafood and Seafood Products Analysis.* CRC Press.

RUSTAD, T. 2010b. *RE: Personal communication.* Type to FOSSHAUG, G. M. E.

RUSTAD, T., SIGHOLT, T., AURSAND, M., SELAND, A. & BERG, A. J. 2000. Muscle chemistry and muscle quality of Atlantic salmon (Salmo salar). Effect of swimming activity. *In:* NICLASEN, A. G. A. O. (ed.) *Annales Societatis Scientarium Færoensis Supplementum XXVIII.* Thorshavn: Føroya Frodskaparfelag.

SIEBERT, G. 1958. Aktivitat Eiweiss Spaltender Enzyme in Fischen. *Experientia,* 14**,** 65-66.

SIVERTSVIK, M., ROSNES, J. T. & KLEIBERG, G. H. 2003. Effect of modified atmosphere packaging and superchilled storage on the microbial and sensory quality of Atlantic salmon (Salmo salar) fillets. *Journal of Food Science,* 68**,** 1467-1472.

SKISTAD, I. E. 2008. *Superchilling of meat and fish.* Master's Master's thesis, Norwegian University of Science and Technology.

STOKNES, I. 1994. *Proteases in fish : purification and characterization of alkaline muscle proteases.* Norwegian Institute of Technology. Department of Biotechnolog.

STRASBURG, G., XIONG, Y. L. & CHIANG, W. 2008. Physiology and Chemistry of Edible Muscle Tissues. *In:* DAMODARAN, S., PARKIN, K. L. & FENNEMA, O. R. (eds.) *Fennema's food chemistry.* 4th ed. / edited by Srinivasan Damodaran, Kirk Parkin, and Owen R. Fennema. ed. Boca Raton ; London: CRC Press.

SØVIK, S. L. 2005. *Characterisation of enzymatic activities in by-products from cod species effect of species, season and fishing ground elektronisk ressurs.* PhD Doctoral Thesis, Norwegian University of Science and Technology, Fakultet for naturvitenskap og teknologi.

TIRONI, V. A., TOMAS, M. C. & ANON, M. C. 2007. Lipid and protein deterioration during the chilled storage of minced sea salmon (Pseudopercis semifasciata). *Journal of the Science of Food and Agriculture,* 87**,** 2239-2246.

VIRGINIA BIOINFORMATICS INSTITUTE. 2010. *Liquid Chromatography / Mass Spectrometry* [Online]. Blacksburg: Virginia Bioinformatics Institute. Available: https://www.vbi.vt.edu/core_laboratory_facility/proteomics/lc-ms_technology_and_faq [Accessed 25.03.10 2010].

WINTHER, U., ZIEGLER, F., SKONTORP HOGNES, E., EMANUELSON, A., SUND, V., ELLINGSEN, H. 2009. Carbon footprint and energy use of Norwegian seafood products. Trondheim: SINTEF Fisheries and Aquaculture.

YAMASHITA, M. & KONAGAYA, S. 1991. HYDROLYTIC ACTION OF SALMON CATHEPSIN-B AND CATHEPSIN-L TO MUSCLE STRUCTURAL PROTEINS IN RESPECT OF MUSCLE SOFTENING. *Nippon Suisan Gakkaishi,* 57**,** 1917-1922.

Appendix

A. Labelling samples A:3
B. Composition of mix protein standard A:5
C. Numerical examples A:7
C.1. Error analysis A:7
C.1.1. Mean values and standard deviations A:7
C.1.2. Rejection of data A:8
C.2. Drip loss A:10
C.3. Calculations of water content A:11
C.4. Calculations of liquid loss A:12
C.5. Calculation of protein concentration by Lowry's method A:13
C.6. Fast protein liquid chromatography A:15
C.6.1. Calculation of peaks A:15
C.6.2. Calculation of protein size distribution, found by FPLC A:16
C.7. Mass spectrometry A:18
C.7.1. Changes in composition during storage relative to day zero A:18
C.7.2. Changes in composition during storage relative to 151,2 ..... A:19
C.7.3. Changes in composition during storage relative to total abundance A:20
C.7.4. Mass spectrometry of FPLC fractions A:20
D. Drip loss and water holding capacity A:21
D.1. Drip loss – measured data A:21
D.2. Water content – measured data A:22
D.3. Water holding capacity – measured and calculated data A:24
E. Protein content A:27
E.1. Protein concentrations of salmon A:27
F. Fast protein liquid chromatography A:31
F.1. Standards A:31
F.2. Water soluble proteins A:32
F.2.1. Chromatograms A:32
F.2.2. Assembled table A:38
F.3. Ethanol soluble proteins A:39
F.3.1. Chromatograms A:39
F.3.2. Assembled table A:45

F.4. Tables for changes in peaks A:46
G. Mass spectrometry A:49
G.1. Changes relative to day zero A:49
G.2. Changes relative to 151,2 ..... A:51
G.3. Changes relative to total abundance A:52
G.4. Composition of peaks found using FPLC A:55

A. Labelling samples

The samples were labeled as shown in figure A.1.

Samples			
Method	Code	Withdrawal (date)	Storage time (days)
REF	LK-01 LK-02 LK-03 LK-04 LK-05 LK-06	20.may	0
LIC 20 %	LY 20-01 LY 20-02 LY 20-03 LY 20-04 LY 20-05 LY 20-06	26.may	6
	LY 20-07 LY 20-08 LY 20-11 LY 20-12	05.jun	16
	LY 20-28 LY 20-29 LY 20-30	19.jun	30
LUFT 10 %	LL 10-8 LL 10-9 LL 10-10 LL 10-11 LL 10-12	28.may	8
	LL 10-25 LL 10-26 LL 10-27 LL 10-28 LL 10-29	19.jun	30
KR	KR 5H 01 KR 5H 02 KR 5V 01 KR 5V 02	12.feb	0
	KR 1H 01 KR 1H 02 KR 1V 01 KR 3V 03	17.feb	5
	KR 1H 03 KR 2H 01 KR 4H 02 KR 4V 02	22.feb	10
	KR 2H 03 KR 2V 03 KR 3V 01 KR 4H 02	26.feb	14
	KR 2V 01 KR 3H 02 KR 3H 03 KR 4H 03	4.mar	20

LABELLING SAMPLES

REF: Samples collected at day 0 (2 days after slaughter). No superchilling.

LIC: Samples superchilled using Liquid CO_2 and cabinets from Yara. Ice fraction; 20 %.

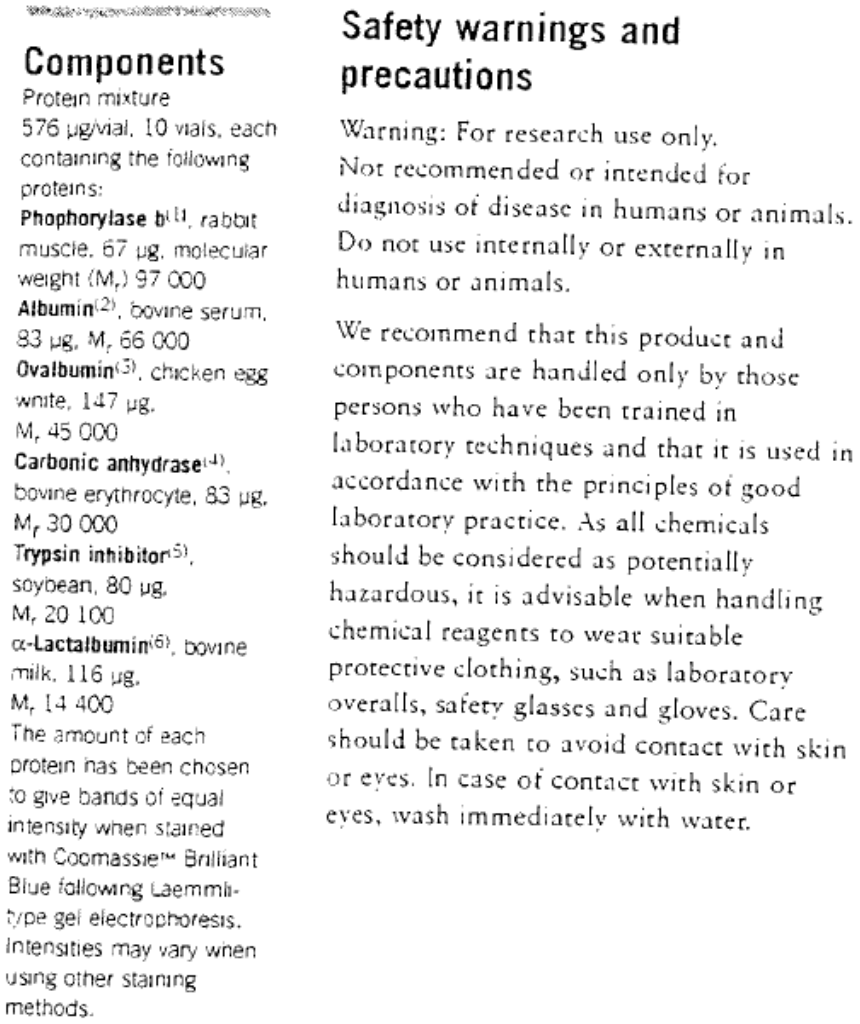
LUFT: Samples superchilled using air tunnel. Ice fraction; 10 %.

KR: Cooling reference samples stored on ice. Storage started 2 days after slaughter (i.e. day 0 = 2 days after slaughter).

Figure A.1: The labelling of the samples including storage method, code, day of withdrawal and days of storage. The methods are explained in the text to the left.

B. Composition of mix protein standard

One of the standards used were a mixture of proteins as shown in figure B.1. For chromatograms of this standard, see appendix F.1.



Components

Protein mixture
576 µg/vial, 10 vials, each containing the following proteins:
Phophorylase b[1], rabbit muscle, 67 µg, molecular weight (M_r) 97 000
Albumin[2], bovine serum, 83 µg, M_r 66 000
Ovalbumin[3], chicken egg white, 147 µg, M_r 45 000
Carbonic anhydrase[4], bovine erythrocyte, 83 µg, M_r 30 000
Trypsin inhibitor[5], soybean, 80 µg, M_r 20 100
α-Lactalbumin[6], bovine milk, 116 µg, M_r 14 400
The amount of each protein has been chosen to give bands of equal intensity when stained with Coomassie™ Brilliant Blue following Laemmli-type gel electrophoresis. Intensities may vary when using other staining methods.

Safety warnings and precautions

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

We recommend that this product and components are handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. As all chemicals should be considered as potentially hazardous, it is advisable when handling chemical reagents to wear suitable protective clothing, such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In case of contact with skin or eyes, wash immediately with water.

Figure B.1: The composition of mix protein standard, and safety warnings and precautions.

C. Numerical examples

C.1. Error analysis

C.1.1. Mean values and standard deviations

Errors of the methods in this thesis originate from weighing samples and measuring optical density (OD). As one can expect the samples to be distributed around the mean value ($\bar{x}$) as a Gauss curve, there will be errors between the mean value and the actual measurement of the samples.

The mean values of each sample withdrawal are calculated as

$$\bar{x} = \frac{\sum_i x_i}{N} \tag{C.1}$$

where *x* is the measured value and N is the number of samples.

Standard deviations were found as

$$\sigma_x = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(\delta x_i)^2} \tag{C.2}$$

where δx is the difference between the sample value and the mean value, and N is the number of samples.

When calculating relative numbers, the mean value is calculated as

$$\bar{x}_{rel} = \frac{\bar{x}_y}{\bar{x}_0} \tag{C.3}$$

where $\bar{x}_y$ is the mean value at time y, and $\bar{x}_0$ is the mean value at time zero (0).

Standard deviations of relative values ($\sigma_{\bar{x}rel}$) were found as

$$\sigma_{\bar{x}rel} = \left(\frac{\bar{x}_y}{\sigma_{\bar{x}y}} + \frac{\bar{x}_0}{\sigma_{\bar{x}0}}\right) \times \bar{x}_{rel} \tag{C.4}$$

where $\bar{x}_y$ and $\bar{x}_0$ is the mean values of time y and 0, respectively, and $\bar{x}_{rel}$ is the relative mean value.

C.1.2. Rejection of data

When rejecting data, there is always a problem of setting a boundary. The application of Chauvenet's criterion may provide a solution for this.

With N measurements of a single quantity (x), we have calculated $\bar{x}$ and σ_x as shown in appendix C.1.1. The value of rejection (x_{sus}) should differ from $\bar{x}$ so much that it looks suspicious. Then we use the equation as follows

$$t_{sus} = \frac{|x_{sus} - \bar{x}|}{\sigma_x} \tag{C.5}$$

where t_{sus} is the number of standard deviations by which x_{sus} differs from $\bar{x}$.

From the table C.1 below, you can find the probability

$$Prob(\text{outside } t_{sus}\,\sigma) \tag{C.6}$$

that a legitimate measurement would differ from $\bar{x}$ by t_{sus} or more standard deviations.

Finally, multiplying by N, the total number of measurements gives

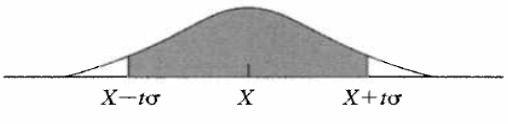
$$\begin{aligned} n &= (\text{expected number as deviant as } x_{sus}) \\ &= N \times Prob(\text{outside } t_{sus}\,\sigma) \end{aligned} \tag{C.7}$$

If this expected number n is less than one-half, then, according to Chauvenet's criterion, x_{sus} can be rejected. If the value is rejected, $\bar{x}$ and σ_x is recalculated for the remaining values. (Taylor, 1997)[1]

[1] TAYLOR, J. R. 1997. *An introduction to error analysis the study of uncertainties in physical measurements,* (2nd edition) Sausalito, Calif., University Science Books.

Table C.1: The percentage probability as a function of t. (Taylor, 1997)

Table A. The percentage probability, $Prob(\text{within } t\sigma) = \int_{X-t\sigma}^{X+t\sigma} G_{X,\sigma}(x)\,dx$, as a function of t.



t	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
0.0	0.00	0.80	1.60	2.39	3.19	3.99	4.78	5.58	6.38	7.17
0.1	7.97	8.76	9.55	10.34	11.13	11.92	12.71	13.50	14.28	15.07
0.2	15.85	16.63	17.41	18.19	18.97	19.74	20.51	21.28	22.05	22.82
0.3	23.58	24.34	25.10	25.86	26.61	27.37	28.12	28.86	29.61	30.35
0.4	31.08	31.82	32.55	33.28	34.01	34.73	35.45	36.16	36.88	37.59
0.5	38.29	38.99	39.69	40.39	41.08	41.77	42.45	43.13	43.81	44.48
0.6	45.15	45.81	46.47	47.13	47.78	48.43	49.07	49.71	50.35	50.98
0.7	51.61	52.23	52.85	53.46	54.07	54.67	55.27	55.87	56.46	57.05
0.8	57.63	58.21	58.78	59.35	59.91	60.47	61.02	61.57	62.11	62.65
0.9	63.19	63.72	64.24	64.76	65.28	65.79	66.29	66.80	67.29	67.78
1.0	68.27	68.75	69.23	69.70	70.17	70.63	71.09	71.54	71.99	72.43
1.1	72.87	73.30	73.73	74.15	74.57	74.99	75.40	75.80	76.20	76.60
1.2	76.99	77.37	77.75	78.13	78.50	78.87	79.23	79.59	79.95	80.29
1.3	80.64	80.98	81.32	81.65	81.98	82.30	82.62	82.93	83.24	83.55
1.4	83.85	84.15	84.44	84.73	85.01	85.29	85.57	85.84	86.11	86.38
1.5	86.64	86.90	87.15	87.40	87.64	87.89	88.12	88.36	88.59	88.82
1.6	89.04	89.26	89.48	89.69	89.90	90.11	90.31	90.51	90.70	90.90
1.7	91.09	91.27	91.46	91.64	91.81	91.99	92.16	92.33	92.49	92.65
1.8	92.81	92.97	93.12	93.28	93.42	93.57	93.71	93.85	93.99	94.12
1.9	94.26	94.39	94.51	94.64	94.76	94.88	95.00	95.12	95.23	95.34
2.0	95.45	95.56	95.66	95.76	95.86	95.96	96.06	96.15	96.25	96.34
2.1	96.43	96.51	96.60	96.68	96.76	96.84	96.92	97.00	97.07	97.15
2.2	97.22	97.29	97.36	97.43	97.49	97.56	97.62	97.68	97.74	97.80
2.3	97.86	97.91	97.97	98.02	98.07	98.12	98.17	98.22	98.27	98.32
2.4	98.36	98.40	98.45	98.49	98.53	98.57	98.61	98.65	98.69	98.72
2.5	98.76	98.79	98.83	98.86	98.89	98.92	98.95	98.98	99.01	99.04
2.6	99.07	99.09	99.12	99.15	99.17	99.20	99.22	99.24	99.26	99.29
2.7	99.31	99.33	99.35	99.37	99.39	99.40	99.42	99.44	99.46	99.47
2.8	99.49	99.50	99.52	99.53	99.55	99.56	99.58	99.59	99.60	99.61
2.9	99.63	99.64	99.65	99.66	99.67	99.68	99.69	99.70	99.71	99.72
3.0	99.73									
3.5	99.95									
4.0	99.994									
4.5	99.9993									
5.0	99.99994									

C.2. Drip loss

The drip loss was measured as explained in chapter 2.2. The error analysis were done as described in appendix C.1, including rejected data.

Table C.2 shows the measured weights and the calculations done for the chilled reference samples (KR). The value marked in yellow has been rejected as explained in appendix C.1.2. For an assembled table of all mean values and standard deviations see appendix D.1.

Table C.2: The measured weights and the calculated drip, means and standard deviation of the chilled reference samples (KR). The value marked in yellow has been rejected. The boxes shows the equations used.

	A	B	C	D	E	F	G	H	I	J	K
58	Sample	Sample +bag (g)	Sample + bag a. drying (g)	Sample (g)	Dried bag (g)	Start-weight sample (g)	Drip (g)	Drip (%)	Mean	St. Dev	N
59	KR 1H 02	164,40	160,90	152,90	8,00	156,40	3,50	2,24 %			
60	KR 3V 03	127,50	125,30	117,30	8,00	119,50	2,20	1,84 %	2,04 %	0,28 %	2
61	KR 4V 01	145,00	141,50	133,50	8,00	137,00	3,50	2,55 %			
62	KR 2H 01	219,70	212,90	204,90	8,00	211,70	6,80	3,21 %			
63	KR 3V 02	118,30	115,00	107,00	8,00	110,30	3,30	2,99 %			
64	KR 1H 03	143,20	139,40	131,40	8,00	135,20	3,80	2,81 %	2,89 %	0,28 %	4
65	KR 1V 03	148,40	143,00	135,00	8,00	140,40	5,40	3,85 %			
66	KR 2H 03	205,40	198,60	190,60	8,00	197,40	6,80	3,44 %			
67	KR 3V 01	146,20	143,00	135,00	8,00	138,20	3,20	2,32 %			
68	KR 2V 03	143,90	137,20	129,20	8,00	135,90	6,70	4,93 %	3,63 %	1,08 %	4
69	KR 2H 01	185,50	172,10	164,10	8,00	177,50	13,40	7,55 %			
70	KR 3H 02	153,80	150,20	142,20	8,00	145,80	3,60	2,47 %			
71	KR 3H 03	195,00	188,00	180,00	8,00	187,00	7,00	3,74 %			
72	KR 4H 03	122,30	118,40	110,40	8,00	114,30	3,90	3,41 %	3,21 %	0,66 %	4

F-D

G/F

Expr. C.1 (page A:7)

Expr. C.2 (page A:7)

C.3. Calculations of water content

The water content was measured as explained in chapter 2.3. The error analysis was done as explained in appendix C.1.

Table C.3 gives the values and calculations of the water content for the reference samples (REF). For an assembled table of means and standard deviations see appendix D.2.

Table C.3: The measured weights and the calculated water content (%), means and standard deviations of the reference samples (REF). The boxes shows the equations used.

	A	B	C	D	E	F	G	H	I	J	K
3		Duplicate	Empty glas (g)	glas w/sample (g)	glas w/sample a. drying (g)	Water content (%)	Mean	St. dev.	Mean	St. dev.	N
4	LK01	A	8,1721	10,1927	8,8715	65,4 %	64,8 %	0,8 %			
5	LK01	B	8,2956	10,3056	9,0140	64,3 %					
6	LK02	A	8,5033	10,5378	9,2436	63,6 %	64,2 %	0,8 %			
7	LK02	B	8,2767	10,2709	8,9788	64,8 %					
8	LK03	A	8,4414	10,5037	9,1423	66,0 %	65,8 %	0,3 %			
9	LK03	B	8,2536	10,2780	8,9499	65,6 %			63,7 %	1,6 %	6
10	LK04	A	8,2915	10,3936	9,0458	64,1 %	63,5 %	0,9 %			
11	LK04	B	8,4054	10,3552	9,1298	62,8 %					
12	LK05	A	8,3911	10,4470	9,1799	61,6 %	61,9 %	0,4 %			
13	LK05	B	8,3459	10,3797	9,1157	62,1 %					
14	LK06	A	8,4226	10,4023	9,1717	62,2 %	61,9 %	0,4 %			
15	LK06	B	8,4075	10,4050	9,1749	61,6 %					

(D-E)/(D-C)

Expr.C.1 (page A:7)

Expr.C.2 (page A:7)

C.4. Calculations of liquid loss

Liquid loss was measured as explained in chapter 2.3. Error analysis was done as explained in appendix C.1.

Table C.4 shows the measured values and calculations of liquid loss for the chilled reference samples (KR). For an assembled table of calculated means and standard deviations see appendix D.3.

Table C.4: Measured values and calculated liquid loss (Δr), means and standard deviations of the chilled reference samples (KR). The boxes shows the equations used.

	A	B	C	D	E	F	G	H	I	J	K	L
139		Duplicate	Empty test tube (g)	Test tube containing sample (g)	Test tube + sample a. drying (g)	Δr	Mean	St. dev.	N	Mean	St. dev.	N
140	KR 5V 03	a	15,9777	17,9845	17,9132	3,6 %						
141		b	15,0114	16,9543	16,8722	4,2 %	3,9 %	0,5 %	2	3,1 %	0,9 %	2
142	KR 5H 03	a	14,9516	16,9853	16,9434	2,1 %						
143		b	15,9381	17,9245	17,8700	2,7 %	2,4 %	0,5 %	2			
144	KR 1H 02	a	15,0561	17,0473	17,0314	0,8 %						
145		b	16,2343	18,3326	18,3155	0,8 %	0,8 %	0,0 %	2	2,7 %	2,2 %	2
146	KR 3V 03	a	13,6013	15,4520	15,3730	4,3 %						
147		b	14,9583	17,0230	16,9210	4,9 %	4,6 %	0,5 %	2			
148	KR 2H 01	a	15,0256	17,2868	17,2000	3,8 %						
149		b	14,9671	17,0522	16,9687	4,0 %	3,9 %	0,1 %	2	3,2 %	0,9 %	2
150	KR 1H 03	a	15,0670	17,2750	17,2300	2,0 %						
151		b	15,9600	18,0721	18,0130	2,8 %	2,4 %	0,5 %	2			
152	KR 1V 03	a	15,9149	17,9148	17,8339	4,0 %						
153		b	14,9012	16,9201	16,8545	3,2 %	3,6 %	0,6 %	2	3,1 %	0,7 %	2
154	KR 2H 03	a	14,9185	16,9082	16,8557	2,6 %						
155		b	14,7983	16,7221	16,6715	2,6 %	2,6 %	0,0 %	2			
156	KR 2V 01	a	16,2262	18,3365	18,2279	5,1 %						
157		b	14,9174	16,9115	16,8073	5,2 %	5,2 %	0,1 %	2	4,7 %	0,6 %	2
158	KR 3H 03	a	14,9468	17,0353	16,9435	4,4 %						
159		b	15,9830	18,0094	17,9278	4,0 %	4,2 %	0,3 %	2			

(D-E)/(D-C)

Expr. C.1 (page A:7)

Expr. C.2 (page A:7)

C.5. Calculation of protein concentration by Lowry's method

The protein concentration of the samples was determined creating a standard curve using known concentrations of the protein standard bovine serum albumin (BSA). The standard and samples were treated as described in chapter 2.6.

Table C.5: Measured optical density and mean values used to create a standard curve.

Standard curve							
ug/ml	12,5	25	50	100	150	200	300
OD 750	0,032	0,044	0,073	0,238	0,326	0,417	0,544
	0,032	0,043	0,071	0,23	0,307	0,411	0,543
	0,033	0,038	0,112	0,225	0,322	0,485	0,564
Average	0,032	0,042	0,085	0,231	0,318	0,438	0,550

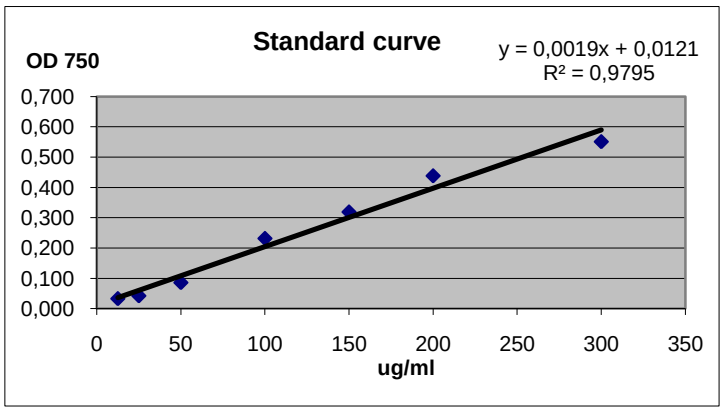


Figure C.1: Optical densities (OD) relative to protein concentration (µg/ml) make up the standard curve.

The mean values of the measured values of optical density (OD) on the different standard concentrations (table C.5) were plotted relative to protein concentration (figure C.1) and used to create a standard curve with the equation:

$$y = ax + b \tag{C.8}$$

where y is the average value of the measured OD and x is the concentration of protein in the sample. By rewriting this expression, the protein concentration of a sample with measured OD can be found.

Protein µg/ml diluted sample:

$$x = \frac{y-b}{a} \tag{C.9}$$

The dilution factor must be considered to find the protein concentration in the initial sample. In our case the sample is diluted 1:20 with distilled water prior to analyses. This gives us the following expression for the protein concentration (µg/ml extract) in the sample:

$$x = \frac{(y-b)*20}{a} \tag{C.10}$$

When precipitating the extract in ethanol as described in chapter 2.5, the expression has to be modified once more:

$$x = \frac{(y-b)*20*2{,}8}{a*11{,}2} \tag{C.11}$$

As shown in table C.6, the formulas above have been used to calculate protein concentrations in a sample precipitated in water (A) and a sample precipitated in 75 % ethanol (B).

Table C.6: Calculations of sample A (precipitated in water) and B (precipitated in ethanol). a=0,0019 and b=0,0121. The boxes shows the equations used.

		Samples	
		A	B
Weigh in (g)		10,6817	10,7786
Volume extract (ml)		100	100
Dilution		20	20
OD 750	A1	0,346	0,061
	A2	0,346	0,066
	A3	0,344	0,063
prot.ug/ml (diluted)	1	175,74	25,74
	2	175,74	28,37
	3	174,68	26,79
Average:		175,39	26,96
ug/ml extract		3507,72	134,82
protein % wet weight		3,284	0,125

Expression C.9

Expression C.10

Expression C.11

C.6. Fast protein liquid chromatography

Fast protein liquid chromatography (FPLC) was measured as described in chapter 2.7.

C.6.1. Calculation of peaks

The chromatograms of the samples (appendix F.2.1 and F.3.1) were studied and the retention time, peak area and total area were noted for the seven largest peaks. These values were used together with values of protein content (appendix E.1.) to calculate the relative area in each of the seven selected peaks (A-G).

Table C.7 shows an example of noted and calculated data for the chilled reference samples (KR) stored for 5 days.

Table C.7: Noted values from chromatograms and calculated data for peaks. The samples calculated are chilled reference samples stored for 5 days and solubilised in water. The boxes shows the expressions used.

	A	B	C	D	E	F	G	H	I	J	K	L
1	File		Peak name	Retention min	Area mAU*min	Height mAU	Total area	Protein µg/ml	Protein put on	Total protein	Protein peaks	Rel. Protein
2	Peak A:											
3												
4	KR 5V 01		A	15,96	796,53	486,51	1235,49	4471,43	894,29	1,38	0,89	64 %
5	KR 5V 02		A	16,21	240,22	159,41	406,89	3189,68	637,94	0,64	0,38	60 %
6	KR 5H 01		A	15,91	791,99	513,377	1217,62	4348,02	869,60	1,40	0,90	64 %
7	KR 5H 02		A	15,97	755,54	458,61	1213,59	3749,206349	749,84	1,62	1,01	62 %
8	Mean		A	16,01	644,82	365,51	1018,40	3939,58	787,92	1,26	0,80	0,63
9	Std. Dev.		A	0,13	266,94	179,39	407,78	591,12	118,22	0,43	0,28	0,02
10	Rel. Std. Dev.			1 %	41 %	49 %	40 %	15 %	15 %	34 %	35 %	3 %
11	Peak B:											
12												
13	KR 5V 01		B	35,33	42,54	19,18	1235,49	4471,43	894,29	1,38	0,05	3 %
14	KR 5V 02		B	35,44	14,64	8,87	406,89	3189,68	637,94	0,64	0,02	4 %
15	KR 5H 01		B	35,36	41,25	18,95	1217,62	4348,02	869,60	1,40	0,05	3 %
16	KR 5H 02		B	35,45	46,16	21,97	1213,59	3749,206349	749,84	1,62	0,06	4 %
17	Mean		B	35,41	36,15	17,24	1018,40	3939,58	787,92	1,26	0,04	0,04
18	Std. Dev.		B	0,07	14,49	5,74	407,78	591,12	118,22	0,43	0,02	0,00
19	Rel. Std. Dev.			0 %	40 %	33 %	40 %	15 %	15 %	34 %	36 %	5 %

From chromatogram (Appendix F.2)

From protein content (Appendix E.1)

H X 0,2

G/I

E/I

K/J

C.6.2. Calculation of protein size distribution, found by FPLC

Protein and peptide standards were analyzed by FPLC as described in chapter 2.7. The chromatograms of the standards were studied and the retention time (t_e) was noted. The flow rate (V_{flow}) and elution volume (V_e) for the protein is given by the product of the retention time and the flow rate. The partition coefficient (K_{av}) for the main peak in the chromatogram is given by

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad \text{(C.12)}$$

Where V_t is the total bed volume given as approximately 24 mL by the supplier. V_0 is the void volume of the column given as 8 ml in the connected software.
Table C.8 shows the molecular weight (M_w), the retention time and elution volume, plus the calculated partition coefficient for the standards used.

Table C.8: shows calculated data for standards.

Standard	Molecular weight [g/mol]	Retention time [min]	V_e	K_{av}	log10(Mw)
Cys	121,20	47,49	23,75	0,98	2,08
Lys	146,19	42,79	21,40	0,84	2,16
B12	1355,37	33,27	16,64	0,54	3,13
Aprotinin	6512,42	29,84	14,92	0,43	3,81
Cyt C	12384,00	29,14	14,57	0,41	4,09
Carbonic Anhydrase	30000,00	19,04	9,52	0,10	4,48
Ovalbumin	45000,00	17,77	8,89	0,06	4,65
Albumin	66000,00	15,93	7,97	0,00	4,82

Plotting the logarithm of the molecular weight for each standard as a function of the corresponding retention time gives a standard curve (figure C.1). Figure C.2 displays the distribution of the different protein standards.

The curve is described by the equation

$$y = -0.0912x + 6{,}3368 \quad \text{(C.13)}$$

The squared correlation coefficient is 0,9584, which gives a linear correlation coefficient of 0,979.

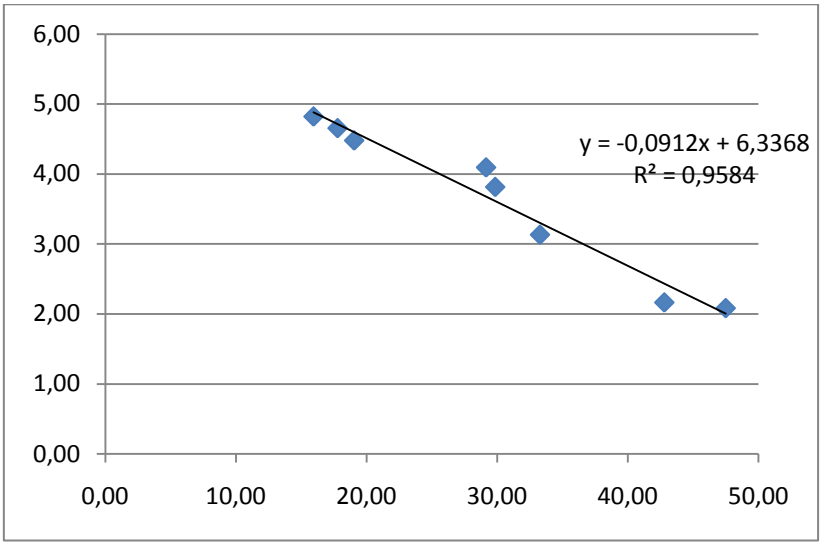


Figure C.2: Standard curve for estimating molecular weight using FPLC.

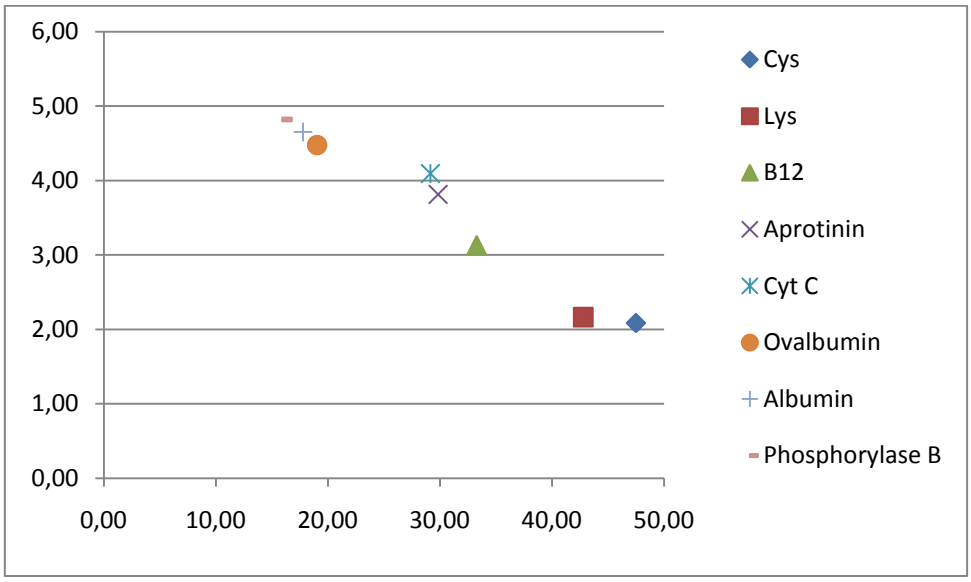


Figure C.3: Distribution of the different protein standards used for estimating a standard curve with FPLC.

To find the molecular weight of a peak in a chromatogram, the retention time is noted and inserted into the rewritten expression describing the standard curve

$$Mw = 10^{((-0{,}0912 \times \text{retention time}) + 6{,}3368)} \quad \text{(C.14)}$$

C.7. Mass spectrometry

Mass spectrometry (MS) was analysed as described in chapter 2.8. The results from the mass spectrometry analysis were sorted out according to the m/z values. The values with no abundances were zero filled, and then decimal reduced. After this, the m/z values with no abundances were deleted from the file. These calculations were carried out by SINTEF prior to releasing them.

C.7.1. Changes in composition during storage relative to day zero

Table C.9 shows an example of the calculated mass spectrometry data for chilled reference samples relative to day zero. An assembled table of the mean values and standard deviations calculated are found in appendix G.1.

Table C.9: Results from mass spectrometry and calculated data for mean and relative mean values and standard deviations for abundances. The samples calculated are chilled references samples stored for 0 and 5 days relative to day zero. The boxes shows the expressions used.

	A	B	C	D	E	F	G	H	I
1	Day zero (0)								
2	m/z	KR 5H 01	KR 5H 02	KR 5V 01	KR 5V 02	Mean value	Standard deviation	Relative mean value	Relative Standard deviation
3	76,2			157602,20	167106,70	149158,05	37829,06	1,00	0,51
4	104,2			33259,80	32907,00	31239,93	6235,81	1,00	0,40
5	118,2			2902,20	2614,90	2539,60	362,74	1,00	0,29
6	132,15			53979,90	99215,00	56051,58	41765,01	1,00	1,49
7	133,15	27737,10	0,00	2215,30	5318,80	8817,80	12800,12	1,00	2,90
8	151,2			1174038,30	1067467,90	1001120,55	261200,34	1,00	0,52
9	152,2			90691,10	85553,00	78569,18	24629,58	1,00	0,63
10	154,1			2753,30	7133,10	3851,33	2321,46	1,00	1,21
11	170			12539,70	44363,60	26113,63	17861,11	1,00	1,37
12	193,1			2859,80	1394,90	1656,98	880,36	1,00	1,06
13	207,1			30283,10	60741,20	39652,53	19893,00	1,00	1,03
14	208			5145,90	8925,00	6148,28	2562,72	1,00	0,83
15	241,1			1485,80	42026,50	24308,85	27634,07	1,00	2,27
16	279	0,00	10862,30	122,20	1677,90	3165,60	5187,67	1,00	3,28
17	296			903,90	81,00	3275,08	5907,68	1,00	3,61
18	307			12081,70	5274,20	9300,55	7964,42	1,00	1,71
19									
20	Day five (5)								
21	m/z	KR 1H 01	KR 1H 02	KR 1V 01	KR 3H 03	Mean value	Standard deviation	Relative mean value	Relative Standard deviation
22	76,2			196602,20	216437,30	201900,93	18773,98	0,74	0,26
23	104,2			41212,60	55592,50	54571,25	10835,52	0,57	0,23
24	118,2			1919,00	1195,80	2879,48	2406,71	0,88	0,86
25	132,15			63579,80	43226,00	32952,28	27034,75	1,70	2,66
26	133,15			2860,70	1402,50	14885,05	26245,22	0,59	1,90
27	151,2			1319653,10	1442502,60	1315426,05	175155,99	0,76	0,30
28	152,2	85108,30	109233,70	99234,00	106418,00	99998,73	10782,70	0,79	0,33
29	154,1			11118,20	11037,70	10533,05	1571,29	0,37	0,27
30	170			21774,30	21189,50	33718,28	15296,75	0,77	0,88
31	193,1			4446,30	0,00	4050,73	4143,77	0,41	0,64
32	207,1			42608,30	33636,30	38771,08	6614,45	1,00	0,68
33	208	0,00	9899,20	8242,90	5937,30	6019,85	4329,62	1,02	1,16
34	241,1	14972,00	37922,10	36946,10	34515,70	31088,98	10839,69	0,78	1,16
35	279	61,90	5052,70	2958,50	9484,40	4389,38	3965,42	0,72	1,83
36	296	23863,50	15830,50	12005,70	33,20	12933,23	9918,60	0,25	0,65
37	307	11846,30	1927,40	6579,50	21,80	5093,75	5277,46	1,83	3,46

Expression C.3 (F/F) (page A:7)

Expression C.4 (page A:7)

Expression C.1 (page A:7)

Expression C.2 (page A:7)

Expression C.3 (F_{5days}/F_{0days}) (page A:7)

Expression C.4 (page A:7)

C.7.2. Changes in composition during storage relative to 151.2

Table C.10 shows an example of the calculated mass spectrometry data for chilled reference samples relative to the m/z value = 151.2. An assembled table of mean values and standard deviations calculated are found in appendix G.2.

Table C.10: Results from mass spectrometry and calculated data for mean and relative mean values and standard deviations for abundances. The samples calculated are chilled references samples stored for 0 and 5 days relative to m/z value = 151.2. The boxes shows the expressions used.

	A	B	C	D	E	F	G	H	I	J	K
1						Day zero (0)					
2	m/z	KR 5H 01	Rel. val.	KR 5H 02	Rel. val.	KR 5V 01	Rel. val.	KR 5V 02	Rel. val.	Mean values	Sta. Deviations
3	76,2	93736,5	15 %	177826,8	15 %	157962,2	13 %	167106,7	16 %	15 %	1 %
4	104,2	22220,1	4 %	36572,8	3 %	33259,8	3 %	32907,0	3 %	3 %	0 %
5	118,2	2036,3	0 %	2605,0	0 %	2902,2	0 %	2614,9	0 %	0 %	0 %
6	132,15	27174,7	4 %	71011,4	6 %	53979,9	5 %	99215,0	9 %	6 %	2 %
7	133,15	562,4	0 %	3990,6	0 %	2215,3	0 %	5318,8	0 %	0 %	0 %
8	151,2	615264,0	100 %	1147712,0	100 %	1174038,3	100 %	1067467,9	100 %	100 %	0 %
9	152,2	38573,8	6 %	89000,3	8 %	84990,1	7 %	79291,7	7 %	7 %	1 %
10	154,1	1808,8	0 %	3710,1	0 %	2753,3	0 %	7133,1	1 %	0 %	0 %
11	170	9133,0	1 %	38418,2	3 %	12539,7	1 %	44363,6	4 %	3 %	1 %
12	193,1	1225,7	0 %	1043,1	0 %	2959,8	0 %	1394,9	0 %	0 %	0 %
13	207,1	15435,5	3 %	48150,3	4 %	30283,1	3 %	60741,2	6 %	4 %	2 %
14	208	3091,8	1 %	7429,8	1 %	5145,9	0 %	8925,6	1 %	1 %	0 %
15	241,1	0,0	0 %	47011,2	4 %	1485,8	0 %	36550,6	3 %	2 %	2 %
16	279	0,0	0 %	10862,3	1 %	84,7	0 %	6165,2	1 %	0 %	0 %
17	296	0,0	0 %	12115,4	1 %	903,9	0 %	81,0	0 %	0 %	1 %
18	307	16995,8	3 %	824,4	0 %	12081,7	1 %	5274,2	0 %	1 %	1 %
19											
20						Day ten (10)					
21	m/z	KR 1H 03	Rel. val.	KR 2H 01	Rel. val.	KR			Rel. val.	Mean values	Sta. Deviations
22	76,2	178454,9	15 %	89068,3	17 %			16478,9	16 %	16 %	1 %
23	104,2	67023,2	6 %	98084,8	19 %			52174,8	7 %	10 %	6 %
24	118,2	6436,1	1 %	5579,5	1 %			5310,8	1 %	1 %	0 %
25	132,15	55829,8	5 %	91701,1	18 %			59221,1	8 %	10 %	6 %
26	133,15	2241,8	0 %	4623,7	1 %	4305,5	0 %	2385,6	0 %	0 %	0 %
27	151,2	1214885,4	100 %	521173,8	100 %	1002379,9	100 %	723985,0	100 %	100 %	0 %
28	152,2	92342,2	8 %	40224,9	8 %	73121,8	7 %		7 %	7 %	0 %
29	154,1	3457,0	0 %	11930,1	2 %	6663,9			%	1 %	1 %
30	170	37030,0	3 %	47668,8	9 %	47432,1			%	5 %	3 %
31	193,1	8881,6	1 %	5440,5	1 %	5923,9			%	1 %	0 %
32	207,1	40580,4	3 %	4734,8	1 %	42068,4			%	3 %	2 %
33	208	8589,0	1 %	33522,9	6 %	7133,8			%	2 %	3 %
34	241,1	44913,2	4 %	4986,1	1 %	52458,1	5 %	51532,6	7 %	4 %	3 %
35	279	8994,1	1 %	71513,0	14 %	12918,3	1 %	12596,5	2 %	4 %	6 %
36	296	18406,5	2 %	13215,9	3 %	0,0	0 %	0,0	0 %	1 %	1 %
37	307	12601,0	1 %	41845,1	8 %	0,0	0 %	6529,0	1 %	2 %	4 %

C/$B$8

C/$B$27

Expression C.1 (page A:7)

Expression C.2 (page A:7)

C.7.3. Changes in composition during storage relative to total abundance

Table C.11 shows an example of the calculated data for analyzing changes during storage relative to total abundance. A complete table of the calculated data is found in appendix G.3.

Table C.11: Results from mass spectrometry and calculated data for relative mean values and standard deviations for abundances. The samples calculated are chilled reference samples stored for 0 and 5 days relative to the total abundance. The boxes shows the expressions used.

	A	B	C	D	E	F	G	H	I	J	K
1	Day zero (0)										
2	m/z	KR 5H 01	Rel. val.	KR 5H 02	Rel. val.	KR 5V 01	Rel. val.	KR 5V 02	Rel. val.	Mean values	Sta. deviations
3	76,2	93736,5	11 %	177826,8	10 %	157962,2	10 %	167106,7	10 %	10 %	0 %
4	104,2	22220,1	3 %	3		33259,8	2 %	32907	2 %	2 %	0 %
5	118,2	2036,3	0 %			2		9	0 %	0 %	0 %
6	132,15	0	0 %	71011,4	4 %	53		5	6 %	3 %	3 %
7	133,15	27737,1	3 %	0	0 %	2		8	0 %	1 %	2 %
8	151,2	615264	72 %	1147712	67 %	1174		9	65 %	70 %	4 %
9	152,2	42168,4	5 %	95864,2	6 %	90		3	5 %	5 %	0 %
10	154,1	1808,8	0 %	3710,1	0 %	2753,3	0 %	7133,1	0 %	0 %	0 %
11	170	9133	1 %	38418,2	2 %	12		6	3 %	2 %	1 %
12	193,1	1230,1	0 %	1043,1	0 %	2		9	0 %	0 %	0 %
13	207,1	15435,5	2 %	48150,3	3 %	30		2	4 %	3 %	1 %
14	208	3091,8	0 %	7429,8	0 %	5		6	1 %	0 %	0 %
15	241,1	0	0 %	53723,1	3 %	1		5	3 %	1 %	2 %
16	279	0	0 %	10862,3	1 %	722,2	0 %	1077,9	0 %	0 %	0 %
17	296	0	0 %	12115,4	1 %	903,9	0 %	81	0 %	0 %	0 %
18	307	19021,9	2 %	824,4	0 %	12081,7	1 %	5274,2	0 %	1 %	1 %
19	Total abundance	852883,5		1707868,9		1583324,2		1631801,3			
20											
21	Day five (5)										
22	m/z	KR 1H 01	Rel. val.	KR 1H 02	Rel. val.	KR 1V 01	Rel. val.	KR 3H 03	Rel. val.	Mean values	Sta. deviations
23	76,2	177797,3	11 %	213766,9	10 %	196602,2	11 %	219437,3	11 %	11 %	0 %
24	104,2	53789,3	3 %	67690,6	3 %	41212,6	2 %	55592,5	3 %	3 %	1 %
25	118,2	1951,7	0 %	6451,4	0 %	1919	0 %	1195,8	0 %	0 %	0 %
26	132,15	25003,3	2 %	0	0 %	63579,8	3 %	43226	2 %	2 %	1 %
27	133,15	1041,8	0 %	54235,2	3 %	2860,7	0 %	1402,5	0 %	1 %	1 %
28	151,2	1066400	68 %	1433148,5	70 %	1319653,1	71 %	1442502,6	73 %	70 %	2 %
29	152,2	85108,3	5 %	109233,7	5 %	99234,9	5 %	106418	5 %	5 %	0 %
30	154,1	8225,2	1 %	11751,1	1 %	11118,2	1 %	11037,7	1 %	1 %	0 %
31	170	53127	3 %	38782,3	2 %	21774,3	1 %	21189,5	1 %	2 %	1 %
32	193,1	2118,8	0 %	9637,8	0 %	4446,3	0 %	0	0 %	0 %	0 %
33	207,1	32737	2 %	46102,7	2 %	42608,3	2 %	33636,3	2 %	2 %	0 %
34	208	0	0 %	9899,2	0 %	8242,9	0 %	5937,3	0 %	0 %	0 %
35	241,1	14972	1 %	37922,1	2 %	36946,1	2 %	34515,7	2 %	2 %	0 %
36	279	61,9	0 %	5052,7	0 %	2958,5	0 %	9484,4	0 %	0 %	0 %
37	296	23863,5	2 %	15830,5	1 %	12005,7	1 %	33,2	0 %	1 %	1 %
38	307	11846,3	1 %	1927,4	0 %	6579,5	0 %	21,8	0 %	0 %	0 %
39	Total abundance	1558043,4		2061432,1		1871742,1		1985630,6			

C.7.4. Mass spectrometry of FPLC fractions

The FPLC fractions were from peak C-F. The calculations of the composition of the four peaks were identical to those shown in appendix C.7.3. An assembled table of mean values and standard deviations calculated is found in appendix G.4.

D. Drip loss and water holding capacity

D.1. Drip loss – measured data

Table D.1 shows measured data of drip loss as described in chapter 2.2 and calculated as described in appendix C.2. Values marked in yellow were rejected as explained in appendix C.1.

Table D.1: Measured and calculated data of drip loss. LK (REF), LL (LUFT) and LY (LIC) were measured in 2008 by Anne Sissel Duun (unpublished). The values marked in yellow were kept out of the calculations.

	A	B	C	D	E	F	G	H	I	J
1	Drypptap				Pakket	R = RSW	L = LUFT	Y = LIC (YARA)		DAG 0
2				DATO	19. mai.	20. mai.	20. mai.	23. mai.		20. mai.
3	Prøve	Prøve +pose (g)	prøve +pose e. tørking (g)	prøve (g)	tørket pose (g)	startvekt prøve (g)	drypp (g)	drypp (%)	snitt	stdev
4	LK-01	259,74		248,74	8,11	251,63	2,89	1,15 %		
5	LK-02	293,78		282,10	8,07	285,71	3,61	1,26 %		
6	LK-03	288,44		276,51	8,01	280,43	3,92	1,40 %	1,34 %	0,23 %
7	LK-04	315,43		304,15	8,05	307,38	3,23	1,05 %		
8	LK-05	271,88		259,47	8,09	263,79	4,32	1,64 %		
9	LK-06	315,36		302,49	8,10	307,26	4,77	1,55 %		
10	LL10-01	248,82		236,40	8,04	240,78	4,38	1,82 %		
11	LL10-02	273,81		262,41	8,13	265,68	3,27	1,23 %		
12	LL10-03	250,83		238,88	8,10	242,73	3,85	1,59 %	1,50 %	0,24 %
13	LL10-04	324,80		312,41	7,96	316,84	4,43	1,40 %		
14	LL10-05	313,67		301,51	8,26	305,41	3,90	1,28 %		
15	LL10-06	249,60		237,26	8,21	241,39	4,13	1,71 %		
16	LL10-07	258,34		245,01	8,12	250,22	5,21	2,08 %		
17	LL10-08	257,79		244,41	8,16	249,63	5,22	2,09 %		
18	LL10-09	279,93		265,25	8,09	271,84	6,59	2,42 %	2,23 %	0,36 %
19	LL10-10	220,42		206,35	8,15	212,27	5,92	2,79 %		
20	LL10-11	301,09		288,70	7,28	293,81	5,11	1,74 %		
21	LL10-12	270,02		255,87	8,19	261,83	5,96	2,28 %		
22	LL10-13	334,05		305,32	7,54	326,51	21,19	6,49 %		
23	LL10-14	264,78		244,95	7,04	257,74	12,79	4,96 %		
24	LL10-15	309,36		295,75	7,21	302,15	6,40	2,12 %	3,42 %	1,02 %
25	LL10-16	285,63		269,54	6,23	279,4	9,86	3,53 %		
26	LL10-17	246,78		231,91	7,13	239,65	7,74	3,23 %		
27	LL10-18	312,42		296,01	6,37	306,05	10,04	3,28 %		
28	LL10-19	248,74		232,55	8,54	240,2	7,65	3,18 %		
29	LL10-20	292,12		275,42	8,48	283,64	8,22	2,90 %		
30	LL10-21	237,83		220,99	8,66	229,17	8,18	3,57 %	3,48 %	0,54 %
31	LL10-22	220,53		203,65	7,62	212,91	9,26	4,35 %		
32	LL10-23	285,38		266,55	8,27	277,11	10,56	3,81 %		
33	LL10-24	306,54		288,85	8,50	298,04	9,19	3,08 %		
34	LL10-25	346,68		324,78	8,25	338,43	13,65	4,03 %		
35	LL10-26	238,71		220,40	8,16	230,55	10,15	4,40 %		
36	LL10-27	294,61		276,71	8,16	286,45	9,74	3,40 %	4,04 %	0,38 %
37	LL10-28	300,78		281,41	8,14	292,64	11,23	3,84 %		
38	LL10-29	258,17		239,65	8,20	249,97	10,32	4,13 %		
39	LL10-30	270,76		251,03	8,14	262,62	11,59	4,41 %		
40	LY20-01	293,10		279,24	8,49	284,61	5,37	1,89 %		
41	LY20-02	285,13		265,80	8,88	276,25	10,45	3,78 %		
42	LY20-03	288,20		273,72	8,28	279,92	6,20	2,21 %	2,70 %	0,73 %
43	LY20-04	340,40		320,88	8,66	331,74	10,86	3,27 %		
44	LY20-05	332,43		314,88	8,46	323,97	9,09	2,81 %		
45	LY20-06	290,42		275,75	8,38	282,04	6,29	2,23 %		
46	LY20-07	306,73		291,69	7,26	299,47	7,78	2,60 %		
47	LY20-08	256,98		245,30	6,88	250,1	4,80	1,92 %		
48	LY20-09	281,67		269,77	7,37	274,3	4,53	1,65 %	2,22 %	0,38 %
49	LY20-10	268,64		254,44	7,98	260,66	6,22	2,39 %		
50	LY20-11	335,75		320,58	8,02	327,73	7,15	2,18 %		
51	LY20-12	270,52		256,45	7,29	263,23	6,78	2,58 %		
52	LY20-25	280,88		265,84	8,20	272,68	6,84	2,51 %		
53	LY20-26	275,02		259,63	8,09	266,93	7,30	2,73 %		
54	LY20-27	300,87		285,50	8,19	292,68	7,18	2,45 %	2,85 %	0,35 %
55	LY20-28	350,99		332,61	8,18	342,81	10,20	2,98 %		
56	LY20-29	285,21		267,75	8,18	277,03	9,28	3,35 %		
57	LY20-30	277,02		260,58	8,20	268,82	8,24	3,07 %		
58	KR 1H 02	164,40	160,90	152,90	8,00	156,40	3,50	2,24 %		
59	KR 3V 03	127,50	125,30	117,30	8,00	119,50	2,20	1,84 %	2,04 %	0,28 %
60	KR 4V 01	145,00	141,50	133,50	8,00	137,00	3,50	2,55 %		
61	KR 2H 01	219,70	212,90	204,90	8,00	211,70	6,80	3,21 %		
62	KR 3V 02	118,30	115,00	107,00	8,00	110,30	3,30	2,99 %		
63	KR 1H 03	143,20	139,40	131,40	8,00	135,20	3,80	2,81 %	2,89 %	0,28 %
64	KR 1V 03	148,40	143,00	135,00	8,00	140,40	5,40	3,85 %		
65	KR 2H 03	205,40	198,60	190,60	8,00	197,40	6,80	3,44 %		
66	KR 3V 01	146,20	143,00	135,00	8,00	138,20	3,20	2,32 %		
67	KR 2V 03	143,90	137,20	129,20	8,00	135,90	6,70	4,93 %	3,63 %	1,08 %
68	KR 2H 01	185,50	172,10	164,10	8,00	177,50	13,40	7,55 %		
69	KR 3H 02	153,80	150,20	142,20	8,00	145,80	3,60	2,47 %		
70	KR 3H 03	195,00	188,00	180,00	8,00	187,00	7,00	3,74 %		
71	KR 4H 03	122,30	118,40	110,40	8,00	114,30	3,90	3,41 %	3,21 %	0,66 %

D.2. Water content – measured data

The water content of a sample was determined as described in chapter 2.3 and displayed in table D.2. The samples were weighed before and after drying and water content (%) and calculated as described in appendix C.3. The values marked in yellow were rejected as explained in appendix C.1.

Table D.2: Measured and calculated data of water content. Samples marked LK (REF), LL (LUFT) and LY (LIC) were measured in 2008 by Anne Sissel Duun (unpublished). Values marked in yellow were rejected. (continues next page)

	A	B	C	D	E	F	G	H	I	J	K
1											
2						Vanninnhold (%)			Vanninnhold (%)		
3		Parallell	tomt veieglass (g)	veieglass m/prøve (g)	veieglass m/prøve e. tørk (g)	singel	snitt	stdev	snitt	stdev	N
4	LK01	A	8,1721	10,1927	8,8715	65,4 %	64,8 %	0,8 %	63,7 %	1,6 %	6
5	LK01	B	8,2956	10,3056	9,0140	64,3 %					
6	LK02	A	8,5033	10,5378	9,2436	63,6 %	64,2 %	0,8 %			
7	LK02	B	8,2767	10,2709	8,9788	64,8 %					
8	LK03	A	8,4414	10,5037	9,1423	66,0 %	65,8 %	0,3 %			
9	LK03	B	8,2536	10,2780	8,9499	65,6 %					
10	LK04	A	8,2915	10,3936	9,0458	64,1 %	63,5 %	0,9 %			
11	LK04	B	8,4054	10,3552	9,1298	62,8 %					
12	LK05	A	8,3911	10,4470	9,1799	61,6 %	61,9 %	0,4 %			
13	LK05	B	8,3459	10,3797	9,1157	62,1 %					
14	LK06	A	8,4226	10,4023	9,1717	62,2 %	61,9 %	0,4 %			
15	LK06	B	8,4075	10,4050	9,1749	61,6 %					
16	LL10-01	A	11,0663	12,9830	11,7874	62,4 %	62,4 %	0,0 %	62,6 %	1,8 %	6
17	LL10-01	B	11,0261	13,2716	11,8709	62,4 %					
18	LL10-02	A	10,8267	12,9948	11,6338	62,8 %	62,7 %	0,0 %			
19	LL10-02	B	11,0672	13,1968	11,8612	62,7 %					
20	LL10-03	A	11,8643	13,9319	12,6833	60,4 %	60,9 %	0,7 %			
21	LL10-03	B	11,0670	13,0660	11,8395	61,4 %					
22	LL10-04	A	13,0208	15,1744	13,8092	63,4 %	62,9 %	0,7 %			
23	LL10-04	B	11,3337	13,3929	12,1073	62,4 %					
24	LL10-05	A	7,3492	9,3321	8,1330	60,5 %	60,8 %	0,5 %			
25	LL10-05	B	8,3119	10,3368	9,0980	61,2 %					
26	LL10-06	A	7,2268	9,4171	7,9648	66,3 %	65,8 %	0,7 %			
27	LL10-06	B	7,9631	9,9536	8,6545	65,3 %					
28	LL10-08	A	8,4611	10,4920	9,2673	60,3 %	60,4 %	0,1 %	61,7 %	1,9 %	3
29	LL10-08	B	8,2329	10,2110	9,0155	60,4 %					
30	LL10-10	A	8,4413	10,4723	9,2380	60,8 %	60,9 %	0,2 %			
31	LL10-10	B	8,2991	10,2322	9,0520	61,1 %					
32	LL10-12	A	8,3447	10,4175	9,0916	64,0 %	63,8 %	0,2 %			
33	LL10-12	B	8,3753	10,5896	9,1794	63,7 %					
34	LL10-13	A	8,3371	10,4358	9,2229	57,8 %	57,8 %	0,0 %	60,4 %	2,6 %	3
35	LL10-13	B	8,3438	10,3552	9,1930	57,8 %					
36	LL10-16	A	8,4409	10,5845	9,2829	60,7 %	60,5 %	0,4 %			
37	LL10-16	B	8,3460	10,3379	9,1386	60,2 %					
38	LL10-17	A	8,2621	10,3271	9,0282	62,9 %	63,0 %	0,1 %			
39	LL10-17	B	8,3881	10,5284	9,1791	63,0 %					
40	LL10-25	A	8,5075	10,6819	9,0498		57,1 %	#DIV/0!	59,9 %	2,5 %	3
41	LL10-25	B	8,3436	10,4058	9,2282	57,1 %					
42	LL10-28	A	8,1769	10,2840	9,0066	60,6 %	60,5 %	0,2 %			
43	LL10-28	B	7,3159	9,2814	8,0951	60,4 %					
44	LL10-29	A	8,4226	10,5099	9,2387	60,9 %	62,1 %	1,6 %			
45	LL10-29	B	8,2808	10,2835	9,0173	63,2 %					

	A	B	C	D	E	F	G	H	I	J	K
2						Vanninnhold (%)			Vanninnhold (%)		
3		Parallell	tomt veieglass (g)	veieglass m/prøve (g)	veieglass m/prøve e. tørk (g)	singel	snitt	stdev	snitt	stdev	N
46	LY20-01	A	8,3266	10,4218	9,0948	63,3 %	63,3 %	0,0 %	63,7 %	0,8 %	3
47	LY20-01	B	8,3849	10,4152	9,1306	63,3 %					
48	LY20-03	A	8,5238	10,6810	9,2843	64,7 %	64,7 %	0,0 %			
49	LY20-03	B	8,3929	10,4399	9,1159	64,7 %					
50	LY20-06	A	8,2410	10,2220	8,9627	63,6 %	63,2 %	0,5 %			
51	LY20-06	B	8,2587	10,3761	9,0448	62,9 %					
52	LY20-15	A	7,6157	9,6653	8,4094	61,3 %	61,2 %	0,1 %	61,6 %	0,5 %	3
53	LY20-15	B	8,3045	10,4815	9,1515	61,1 %					
54	LY20-17	A	8,3065	10,2489	9,0540	61,5 %	61,4 %	0,1 %			
55	LY20-17	B	8,4097	10,3137	9,1463	61,3 %					
56	LY20-18	A	8,3975	10,5276	9,2058	62,1 %	62,2 %	0,2 %			
57	LY20-18	B	8,3119	10,3482	9,0800	62,3 %					
58	LY20-28	A	8,5477	10,6234	9,3482	61,4 %	61,0 %	0,6 %	61,6 %	0,6 %	3
59	LY20-28	B	7,2859	9,2414	8,0578	60,5 %					
60	LY20-29	A	8,4062	10,4131	9,1734	61,8 %	61,8 %	#DIV/0!			
61	LY20-29	B	8,4588	10,4164	11,5991						
62	LY20-30	A	8,3509	10,4210	9,1584	61,0 %	62,1 %	1,6 %			
63	LY20-30	B	8,3777	10,3775	9,1117	63,3 %					
64	KR 5V 03	a	8,5234	10,5445	8,9033	81,2 %	64,5 %	23,6 %	64,8 %	13,6 %	2
65		b	8,2203	10,2272	9,2663	47,9 %					
66	KR 5H 03	a	8,2799	10,2367	8,9680	64,8 %	65,1 %	0,3 %			
67		b	8,6144	10,6031	9,3044	65,3 %					
68	KR 1H 02	a	8,3126	10,2483	8,9517	67,0 %	66,8 %	0,3 %	67,4 %	0,7 %	2
69		b	10,7853	12,6884	11,421	66,6 %					
70	KR 3V 03	a	11,0494	13,0553	11,6913	68,0 %	68,0 %	0,0 %			
71		b	8,4349	10,5842	9,124	67,9 %					
72	KR 2H 01	a	8,2946	10,3130	8,9376	68,1 %	68,1 %	0,1 %	67,8 %	0,3 %	2
73		b	8,3568	10,2298	8,9557	68,0 %					
74	KR 1H 03	a	8,4960	10,4984	9,1451	67,6 %	67,5 %	0,1 %			
75		b	8,4845	10,7332	9,2163	67,5 %					
76	KR 1V 03	a	8,2735	10,2535	8,9301	66,8 %	66,8 %	0,0 %	67,5 %	0,7 %	2
77		b	8,6142	10,4995	9,2392	66,8 %					
78	KR 2H 03	a	8,2303	10,2986	8,8904	68,1 %	68,1 %	0,0 %			
79		b	8,2476	10,2015	8,8717	68,1 %					
80	KR 2V 01	a	8,3970	10,3916	9,0550	67,0 %	66,9 %	0,2 %	65,5 %	1,6 %	2
81		b	8,2809	10,2802	8,9452	66,8 %					
82	KR 3H 03	a	8,3862	10,4589	9,1250	64,4 %	64,2 %	0,3 %			
83		b	8,3289	10,4142	9,0806	64,0 %					

D.3. Water holding capacity – measured and calculated data

The water holding capacity was measured as described in chapter 2,3 and shown in table D.3. The values were calculated as explained in appendix C.4.

Table D.3: Measured and calculated data of the muscles water holding capacity. The samples marked LK (REF), LL (LUFT) and LY (LIC) were measured in 2008 by Anne Sissel Duun (unpublished). (continuing next page)

	A	B	C	D	E	F	G	H	I	J	K	L
1	Liquid loss beregnet v sentrifugering						Pakket	R = RSW	Y = LIC (YARA)		DAG 0	
2	SUP 2008MAI LAKS					DATO	19. mai.	20. mai.	23. mai.		20. mai.	
3	MAI/ JUNI 2008											
4												
5							Liquid loss (%)			Liquid loss (%)		
6		Parallell	tom prøveholder (g)	prøveholder m/prøve (g)	prøveholder m/prøve e. sentr. (g)	Δr	snitt	stdev	N	snitt	stdev	N
7	LK01	A	15,0396	17,1780	17,0140	7,7 %						
8	LK01	B	15,0146	17,2067	17,0396	7,6 %	7,5 %	0,2 %	4			
9	LK01	C	16,3064	18,5115	18,3433	7,6 %						
10	LK01	D	16,0048	18,4851	18,3054	7,2 %						
11	LK02	A	14,8515	16,9928	16,7699	10,4 %						
12	LK02	B	14,9480	17,0626	16,9185	6,8 %	8,1 %	2,0 %	4			
13	LK02	C	14,6726	17,0132	16,8456	7,2 %						
14	LK02	D	16,3618	18,7059	17,1852							
15	LK03	A	15,0611	17,1283	16,9645	7,9 %						
16	LK03	B	15,9874	18,2853	18,1395	6,3 %	6,8 %	0,8 %	4			
17	LK03	C	16,3373	18,4086	18,2759	6,4 %						
18	LK03	D	15,9753	18,0297	17,8990	6,4 %				6,9 %	0,8 %	6
19	LK04	A	15,1579	17,2070	17,0755	6,4 %						
20	LK04	B	16,1085	18,4664	18,3194	6,2 %	6,1 %	0,3 %	4			
21	LK04	C	16,0360	18,1757	18,0511	5,8 %						
22	LK04	D	15,1640	17,2934	17,1658	6,0 %						
23	LK05	A	14,8733	16,8271	16,6912	7,0 %						
24	LK05	B	15,8626	18,5000	18,3631	5,2 %	6,0 %	1,0 %	4			
25	LK05	C	15,0778	17,0948	16,9580	6,8 %						
26	LK05	D	15,0560	17,2397	17,1287	5,1 %						
27	LK06	A	16,2899	18,2876	17,5468							
28	LK06	B	15,9027	17,9488	17,7860	8,0 %	6,7 %	1,1 %	4			
29	LK06	C	15,0327	17,2673	17,1297	6,2 %						
30	LK06	D	15,1254	17,2362	17,1090	6,0 %						
31	LL10-01	A	14,8992	16,8460	16,7520	4,8 %						
32	LL10-01	B	14,9410	17,0530	16,9128	6,6 %	5,3 %	1,7 %	4			
33	LL10-01	C	15,0807	17,1016	17,0405	3,0 %						
34	LL10-01	D	15,0611	17,1169	16,9790	6,7 %						
35	LL10-02	A	15,9048	18,5450	17,9142							
36	LL10-02	B	15,9141	18,1479	18,0326	5,2 %	6,2 %	2,2 %	4			
37	LL10-02	C	15,9337	18,0644	17,9630	4,8 %						
38	LL10-02	D	16,3046	18,4179	18,2325	8,8 %						
39	LL10-03	A	15,9629	18,0462	17,8745	8,2 %						
40	LL10-03	B	15,1448	17,3362	17,1494	8,5 %	8,6 %	0,9 %	4			
41	LL10-03	C	15,9187	18,0556	17,8933	7,6 %						
42	LL10-03	D	16,3542	18,3467	18,1505	9,8 %				7,8 %	1,8 %	6
43	LL10-04	A	16,1154	18,2937	18,1910	4,7 %						
44	LL10-04	B	15,8928	18,0958	18,0046	4,1 %	7,5 %	3,6 %	4			
45	LL10-04	C	15,9936	17,9627	17,7565	10,5 %						
46	LL10-04	D	15,1800	17,1072	16,8997	10,8 %						
47	LL10-05	A	15,2660	17,3604	17,1430	10,4 %						
48	LL10-05	B	16,0541	18,1158	17,9206	9,5 %	10,1 %	0,5 %	4			
49	LL10-05	C	16,0234	18,0415	17,8312	10,4 %						
50	LL10-05	D	16,1236	18,0834	17,1870							
51	LL10-06	A	15,9898	18,1355	17,9461	8,8 %						
52	LL10-06	B	15,1830	17,1293	16,9660	8,4 %	9,1 %	0,6 %	4			
53	LL10-06	C	16,3608	18,4742	18,2759	9,4 %						
54	LL10-06	D	16,3267	18,4607	18,2535	9,7 %						
55	LL10-08	A	16,1146	18,2479	18,0682	8,4 %						
56	LL10-08	B	14,7983	16,7991	16,5559	12,2 %	8,4 %	2,8 %	4			
57	LL10-08	C	15,9613	18,0613	17,9482	5,4 %						
58	LL10-08	D	16,0447	18,0224	17,8715	7,6 %						
59	LL10-10	A	16,2840	17,2277	17,0974	13,8 %						
60	LL10-10	B	14,9353	17,8199	16,7077		8,3 %	4,8 %	4	7,1 %	2,2 %	3
61	LL10-10	C	16,1227	18,1732	18,0763	4,7 %						
62	LL10-10	D	14,9310	16,9741	16,8450	6,3 %						
63	LL10-12	A	15,8679	17,9324	17,8394	4,5 %						
64	LL10-12	B	16,0141	18,0417	17,9319	5,4 %	4,7 %	0,5 %	4			
65	LL10-12	C	16,0551	18,1542	18,0665	4,2 %						
66	LL10-12	D	15,4070	17,3499	17,2610	4,6 %						
67	LL10-13	A	15,1738	17,2502	17,0758	8,4 %						
68	LL10-13	B	15,0289	17,0821	16,9835	4,8 %	7,2 %	1,7 %	4			
69	LL10-13	C	15,2826	17,1947	17,0563	7,2 %						
70	LL10-13	D	16,0716	18,0040	17,8438	8,3 %						
71	LL10-16	A	15,9336	17,9247	17,8059	6,0 %						
72	LL10-16	B	15,2629	17,2839	17,1668	5,8 %	5,4 %	0,6 %	4	4,9 %	0,7 %	3
73	LL10-16	C	16,1110	18,2313	18,1301	4,8 %						
74	LL10-16	D	15,0002	17,1010	16,9944	5,1 %						
75	LL10-17	A	15,0680	17,0196	16,9690	2,6 %						
76	LL10-17	B	15,8081	17,9448	17,8993	2,1 %	2,2 %	0,3 %	4			
77	LL10-17	C	16,1244	18,1128	18,0751	1,9 %						
78	LL10-17	D	14,8226	16,8062	16,7615	2,3 %						

	A	B	C	D	E	F	G	H	I	J	K	L
5							Liquid loss (%)			Liquid loss (%)		
6		Parallell	tom prøveholder (g)	prøveholder m/prøve (g)	prøveholder m/prøve e. sentr. (g)	Δr	snitt	stdev	N	snitt	stdev	N
79	LL10-22	A	15,1494	17,2054	17,1346	3,4 %						
80	LL10-22	B	14,7844	16,7283	16,6634	3,3 %	3,3 %	0,2 %	4			
81	LL10-22	C	14,9064	16,9816	16,9152	3,2 %						
82	LL10-22	D	15,3544	17,2982	17,2394	3,0 %						
83	LL10-23	A	16,0217	18,0521	17,9266	6,2 %						
84	LL10-23	B	15,2543	17,3081	17,1813	6,2 %	6,1 %	0,2 %	3	5,4 %	0,7 %	3
85	LL10-23	C										
86	LL10-23	D	15,9325	17,9472	17,8292	5,9 %						
87	LL10-24	A	15,2554	17,2621	17,1251	6,8 %						
88	LL10-24	B	16,0369	18,0231	17,9124	5,6 %	7,0 %	1,4 %	4			
89	LL10-24	C	16,1049	18,1124	17,9792	6,6 %						
90	LL10-24	D	15,0674	17,0247	16,8516	8,8 %						
91	LL10-25	A	16,3863	18,3163	18,1376	9,3 %						
92	LL10-25	B	16,0823	18,1130	17,9831	6,4 %	8,4 %	1,4 %	4			
93	LL10-25	C	16,0682	18,0833	17,8946	9,4 %						
94	LL10-25	D	14,8739	16,8504	16,6807	8,6 %						
95	LL10-28	A	15,0494	17,1199	17,0239	4,6 %						
96	LL10-28	B	15,9507	17,9097	17,8132	4,9 %	4,5 %	0,3 %	4	6,1 %	0,5 %	3
97	LL10-28	C	15,1135	17,1708	17,0851	4,2 %						
98	LL10-28	D	15,9609	17,9399	17,8554	4,3 %						
99	LL10-29	A	15,9465	18,0131	17,8901	6,0 %						
100	LL10-29	B	15,8786	17,9016	17,7805	6,0 %	5,3 %	0,9 %	4			
101	LL10-29	C	15,1631	17,1698	17,0632	5,3 %						
102	LL10-29	D	14,9761	16,8997	16,8227	4,0 %						
103	LY20-01	A	16,3726	18,4015	18,3261	3,7 %						
104	LY20-01	B	15,1617	17,1249	17,0489	3,9 %	3,3 %	0,6 %	4			
105	LY20-01	C	16,3408	18,3422	18,2911	2,6 %						
106	LY20-01	D	15,8944	17,9587	17,8980	2,9 %						
107	LY20-03	A	14,9137	16,8846	16,8259	3,0 %						
108	LY20-03	B	16,0417	18,1303	18,0846	2,2 %	2,9 %	0,5 %	4	3,6 %	0,2 %	3
109	LY20-03	C	16,0674	18,0597	17,9982	3,1 %						
110	LY20-03	D	15,1911	17,1724	17,1059	3,4 %						
111	LY20-06	A	15,0742	17,1197	17,0058	5,6 %						
112	LY20-06	B	14,8211	16,7477	16,6592	4,6 %	4,5 %	0,8 %	4			
113	LY20-06	C	16,0696	18,0311	17,9625	3,5 %						
114	LY20-06	D	15,0448	17,0612	16,9716	4,4 %						
115	LY20-15	A	16,2517	18,2531	18,1218	6,6 %						
116	LY20-15	B	15,0769	16,9154	16,8147	5,5 %	5,7 %	0,8 %	4			
117	LY20-15	C	14,7095	16,7791	16,6540	6,0 %						
118	LY20-15	D	16,0568	18,0948	17,9969	4,8 %						
119	LY20-17	A	14,8011	16,9630	16,8110	7,0 %						
120	LY20-17	B	15,0255	16,9357	16,8340	5,3 %	6,1 %	0,7 %	4	5,7 %	0,1 %	3
121	LY20-17	C	16,0092	18,0678	17,9437	6,0 %						
122	LY20-17	D	16,0301	18,0469	17,9226	6,2 %						
123	LY20-18	A	16,1307	18,1532	18,0512	5,0 %						
124	LY20-18	B	15,0215	16,9324	16,8441	4,6 %	5,3 %	0,6 %	4			
125	LY20-18	C	15,9815	18,0456	17,9300	5,6 %						
126	LY20-18	D	15,9343	18,0582	17,9341	5,8 %						
127	LY20-28	A	15,3070	17,3313	17,2139	5,8 %						
128	LY20-28	B	15,9458	17,9775	17,8898	4,3 %	4,8 %	0,7 %	4			
129	LY20-28	C	15,0676	17,0384	16,9502	4,5 %						
130	LY20-28	D	14,8217	16,8820	16,7855	4,7 %						
131	LY20-29	A	15,1984	17,2259	17,0801	7,2 %						
132	LY20-29	B	15,9954	17,9593	17,7869	8,8 %	8,0 %	0,9 %	4	6,5 %	0,1 %	3
133	LY20-29	C	15,2842	17,2654	17,0927	8,7 %						
134	LY20-29	D	15,0831	16,9985	16,8590	7,3 %						
135	LY20-30	A	16,0793	18,0250	17,8885	7,0 %						
136	LY20-30	B	14,9994	17,0612	16,9413	5,8 %	6,7 %	0,9 %	4			
137	LY20-30	C	15,9321	18,0542	17,9267	6,0 %						
138	LY20-30	D	15,2035	17,2014	17,0455	7,8 %						
139	KR 5V 03	a	15,9777	17,9845	17,9132	3,6 %						
140		b	15,0114	16,9543	16,8722	4,2 %	3,9 %	0,5 %	2	3,1 %	0,9 %	2
141	KR 5H 03	a	14,9516	16,9853	16,9434	2,1 %						
142		b	15,9381	17,9245	17,8700	2,7 %	2,4 %	0,5 %	2			
143	KR 1H 02	a	15,0561	17,0473	17,0314	0,8 %						
144		b	16,2343	18,3326	18,3155	0,8 %	0,8 %	0,0 %	2	2,7 %	2,2 %	2
145	KR 3V 03	a	13,6013	15,4520	15,3730	4,3 %						
146		b	14,9583	17,0230	16,9210	4,9 %	4,6 %	0,5 %	2			
147	KR 2H 01	a	15,0256	17,2868	17,2000	3,8 %						
148		b	14,9671	17,0522	16,9687	4,0 %	3,9 %	0,1 %	2	3,2 %	0,9 %	2
149	KR 1H 03	a	15,0670	17,2750	17,2300	2,0 %						
150		b	15,9600	18,0721	18,0130	2,8 %	2,4 %	0,5 %	2			
151	KR 1V 03	a	15,9149	17,9148	17,8339	4,0 %						
152		b	14,9012	16,9201	16,8545	3,2 %	3,6 %	0,6 %	2	3,1 %	0,7 %	2
153	KR 2H 03	a	14,9185	16,9082	16,8557	2,6 %						
154		b	14,7983	16,7221	16,6715	2,6 %	2,6 %	0,0 %	2			
155	KR 2V 01	a	16,2262	18,3365	18,2279	5,1 %						
156		b	14,9174	16,9115	16,8073	5,2 %	5,2 %	0,1 %	2	4,7 %	0,6 %	2
157	KR 3H 03	a	14,9468	17,0353	16,9435	4,4 %						
158		b	15,9830	18,0094	17,9278	4,0 %	4,2 %	0,3 %	2			

E. Protein content

E.1. Protein concentrations of salmon

The protein concentrations of salmon stored at different conditions were measured as explained in chapter 2.6 and calculated as shown in appendix C.5.

Table E.1(rotated): Protein concentrations [µg/ml] and protein % wetweight of reference samples (REF), samples superchilled using liquid carbon dioxide (LIC 20 %) and air (LUFT 10 %) and chilled reference samples. (continuing next page)

	A	B	C	D	E	F	G	H	I	J	K
1	Superchilling of salmon										
2											
3		Samples									
4				Storage time	Weight of muscle	Water soluble protein	Ethanol soluble protein	Water soluble protein	Ethanol soluble protein	Water soluble protein	Ethanol soluble protein
5	Method	Code	Withdrawal (date)	(days)	made to extract (g)	protein µg/ml	protein µg/ml	protein % wet weight	protein % wet weight	protein % dry matter	protein % dry matter
6	REF	LK-01	20 May 2009	0	10,103	2736,111	321,000	2,708	0,079	7,699	0,226
7		LK-02			10,3509	2819,444	157,667	2,724	0,038	7,609	0,106
8		LK-03			10,3601	2934,524		2,833		8,284	
9		LK-04			10,6803	2400,351	263,158	2,247		6,154	0,675
10		LK-05			10,2829	2990,333	304,444	2,908	0,074	7,631	0,194
11		LK-06			10,4097	2179,298		2,094		5,491	
12		Average value			10,3645	2676,877	261,567	2,586	0,064	7,145	0,300
13		Standard deviation			0,1892	320,163	73,413	0,333	0,022	1,075	0,285
14											
15	REF	LY 20-01	26 May 2009	6	10,0902	2040,333	460,000	2,022	0,115	5,510	0,115
16		LY 20-02			10,0107	3458,333		3,455			
17		LY 20-03			10,0485	3152,778	160,000	3,138	0,040	8,892	0,113
18		LY 20-04			10,671	2853,667	289,630	2,674	0,068		
19		LY 20-05			10,017	3783,730	245,614	3,777			
20		LY 20-06			10,0434	2664,912		2,653		7,213	
21		Average value			10,1471	2892,292	288,811	2,953	0,074	7,205	0,114
22		Standard deviation			0,2582	616,757	126,181	0,632	0,038	1,691	0,002
23											
24	LIC 20%	LY 20-07	05 June 2009	16	10,1543	2007,368	452,593	1,977	0,111		
25		LY 20-08			10,5879	2183,704	583,704	2,062	0,138		
26		LY 20-11			10,4632	2670,526	319,259	2,552	0,218		
27		LY 20-12			9,9916	2198,519	520,741	2,200	0,130		
28		Average value			10,2993	2265,029	469,074	2,198	0,149	#DIV/0!	#DIV/0!
29		Standard deviation			0,2744	283,933	113,322	0,253	0,047	#DIV/0!	#DIV/0!
30											
31	LIC 20%	LY 20-28	19 June 2009	30	10,1643	3712,698	531,852	3,653	0,131	9,361	0,335
32		LY 20-29			10,0201	2843,651	413,333	2,838	0,103	7,424	0,270
33		LY 20-30			11,0677	2069,841	-5,185	1,870	-0,003	4,940	0,013
34		Average value			10,4174	2875,397	472,593	2,787	0,117	7,242	0,303
35		Standard deviation			0,5678	821,889	83,805	0,893	0,020	2,216	0,046
36											

	A	B	C	D	E	F	G	H	I	J	K
4				Storage time	Weight of muscle	Water soluble protein	Ethanol soluble protein	Water soluble protein	Ethanol soluble protein	Water soluble protein	Ethanol soluble protein
5	Method	Code	Withdrawal (date)	(days)	made to extract (g)	protein µg/ml	protein µg/ml	protein % wet weight	protein % wet weight	protein % dry matter	protein % dry matter
37		LL 10-8			9,8036	3613,000	372,157	3,685	0,095	9,299	0,239
38		LL 10-9			10,8422	5176,333	701,667	4,774	0,179		0,469
39		LL 10-10	28 May 2009	8	10,861	4823,000	352,549	4,441	0,081	11,361	0,208
40		LL 10-11			10,6268	2513,000	301,369	2,365	0,118		
41		LL 10-12			9,902	4549,667	441,667	4,595	0,102	12,702	
42		**Average value**			**10,4071**	**4135,000**	**473,922**	**3,972**	**0,115**	**11,121**	**0,305**
43		**Standard deviation**			0,5155	1076,224	140,321	**0,989**	**0,038**	**1,714**	**0,142**
44	Air 10%										
45		LL 10-25			10,0015	5913,000	389,123	5,912	0,092		0,233
46		LL 10-26			10,1373	3338,000	476,842	3,293	0,112	7,525	0,283
47		LL 10-27	19 June 2009	30	10,0698	3626,333	551,667	3,601	0,130	10,566	0,344
48		LL 10-28			10,4029	3093,000	485,882	2,973	0,114		
49		LL 10-29			10,8597	4353,000	728,333	4,008	0,171		
50		**Average value**			**10,2542**	**4064,667**	**526,369**	**3,958**	**0,124**	**9,046**	**0,286**
51		**Standard deviation**			0,3508	1136,199	126,845	1,158	0,030	2,150	0,056
52											
53		KR 5V 01			10,65	4471,429	483,158	4,195	0,120		
54		KR 5V 02			9,9624	3189,683	166,316	3,202	0,042		
55		KR 5V 03									
56		KR 5H 01	12 February 2010	0	10,6817	4348,016	349,825	4,071	0,082		
57		KR 5H 02			10,0772	3749,206	264,561	3,720	0,066		
58		KR 5H 03									
59		**Average value**			**10,3428**	**3939,583**	**315,965**	**3,797**	**0,078**	**#DIV/0!**	**#DIV/0!**
60		**Standard deviation**			0,3762	591,121	134,334	0,445	0,033	#DIV/0!	#DIV/0!
61											
62		KR 1H 01			10,6905	4550,794	511,228	4,257	0,120		
63		KR 1H 02			10,5297	4332,540	397,895	4,115	0,094	12,390	0,284
64		KR 1V 01	17 February 2010	5	9,9913	3900,000	518,246	3,903	0,130		
65		KR 2H 02									
66		KR 3V 03			10,0653	4003,175	317,193	3,977	0,079	12,417	0,246
67		KR 4V 03									
68		**Average value**			**10,3192**	**4196,627**	**436,140**	**4,063**	**0,106**	**12,403**	**0,265**
69		**Standard deviation**			0,3436	299,621	96,593	0,156	0,023	0,019	0,027

	A	B	C	D	E	F	G	H	I	J	K
4				Storage time	Weight of muscle	Water soluble protein	Ethanol soluble protein	Water soluble protein	Ethanol soluble protein	Water soluble protein	Ethanol soluble protein
5	Method	Code	Withdrawal (date)	(days)	made to extract (g)	protein µg/ml	protein µg/ml	protein % wet weight	protein % wet weight	protein % dry matter	protein % dry matter
70											
71		KR 1H 03			10,8796	3459,905	353,333	3,180	0,081	9,791	0,250
72		KR 2H 01			10,3234	4169,841	503,158	4,039	0,122	12,656	0,382
73		KR 3V 02	22 February 2010	10	10,329	4356,349	331,228	4,218	0,080		
74	Ice storage	KR 4V 01			10,7786	3717,460	539,298	3,449	0,125		
75		KR 1V 02									
76		KR 4H 01									
77		**Average value**			**10,5777**	**3925,889**	**431,754**	**3,722**	**0,102**	**11,223**	**0,316**
78		**Standard deviation**			0,2933	410,444	104,753	0,488	0,025	2,025	0,093
79											
80		KR 1V 03									
81		KR 2H 03			10,4779	2439,216	497,778	2,328	0,119	7,291	0,372
82		KR 2V 03	26 February 2010	14	10,9025	3839,216	616,296	3,521	0,141		
83		KR 3V 01									
84		KR 4H 02			10,1939	3400,000	429,259	3,335	0,105		
85		KR 4V 02			10,0021	3619,608	405,185	3,619	0,101		
86		**Average value**			**10,3941**	**3324,510**	**487,130**	**3,201**	**0,117**	**7,291**	**0,372**
87		**Standard deviation**			0,3913	616,833	94,624	0,594	0,018	#DIV/0!	#DIV/0!
88											
89		KR 2V 01			10,1565	3388,235	640,370	3,336	0,158	10,076	0,476
90		KR 2V 02									
91		KR 3H 01									
92		KR 3H 02	04 March 2010	20	10,3773	3737,255	562,593	3,601	0,136		
93		KR 3H 03			10,0528	4415,686	347,778	4,392	0,086	12,254	0,241
94		KR 4H 03			10,2642	3819,608	571,852	3,721	0,139		
95		**Average value**			**10,2127**	**3840,196**	**530,648**	**3,763**	**0,130**	**11,165**	**0,359**
96		**Standard deviation**			0,1396	426,801	126,753	0,449	0,031	1,540	0,166

F. Fast protein liquid chromatography

F.1. Standards

The standards were run as described in chapter 2.7. by Diana Herbon and Mahmoudreza Ouissipour. An example of a chromatogram is given in figure F.1.

Figure F.1 shows the standard run by Mahmoudreza Ouissipour. The composition of this protein mixture standard is shown in appendix B.

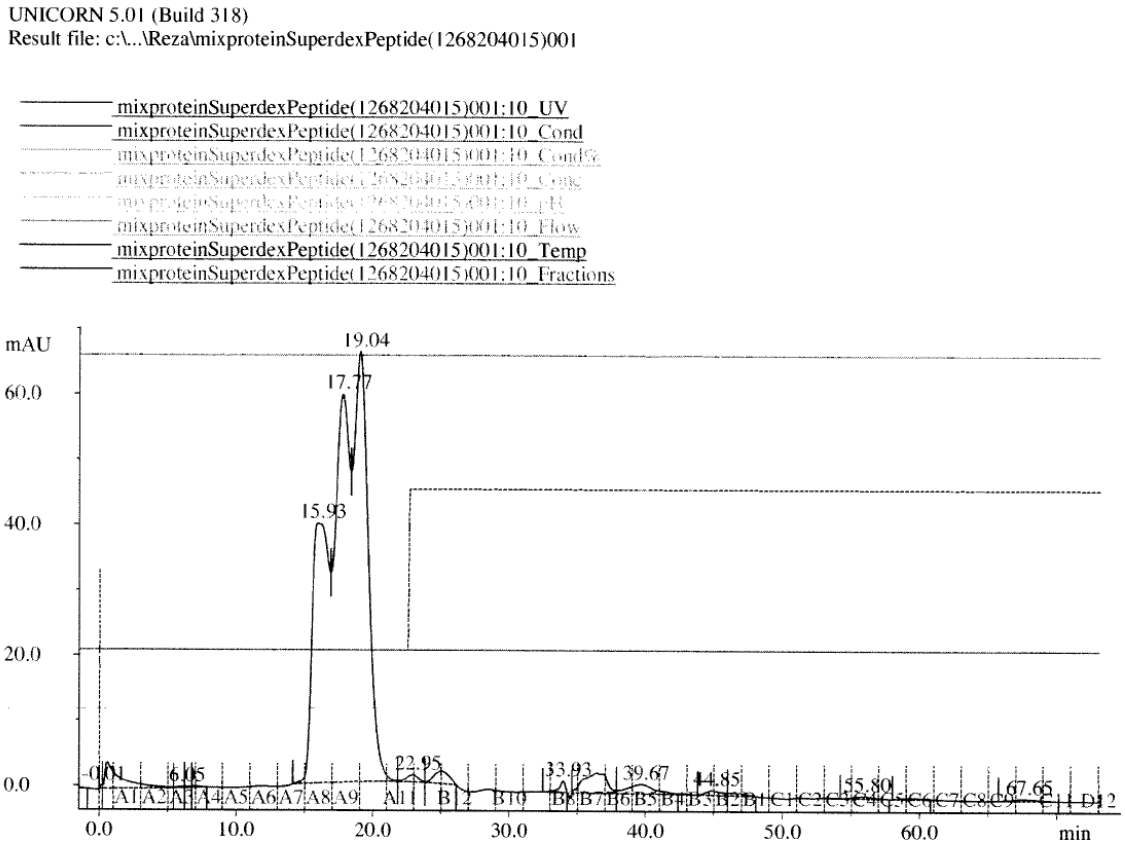


Figure F.1: Chromatogram of the mix protein standard, run by Mahmoudreza Ouissipour.

The protein size distribution was calculated as explained in appendix C.6.2.

F.2. Water soluble proteins

FPLC of water soluble proteins were run as described in chapter 2.7. Values from chromatograms (appendix F.2.1) were used to calculate the relative peak area of the samples (appendix F.2.2) as explained in appendix C.6.1.

F.2.1. Chromatograms

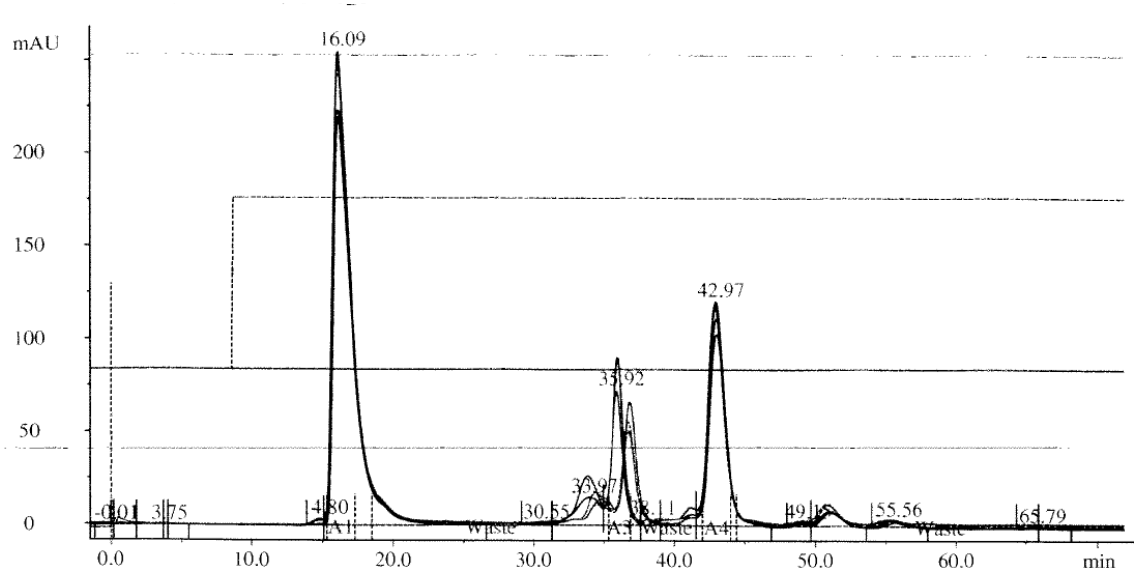


Figure F.2: Chromatogram of the reference samples (REF) withdrawn before superchilling.

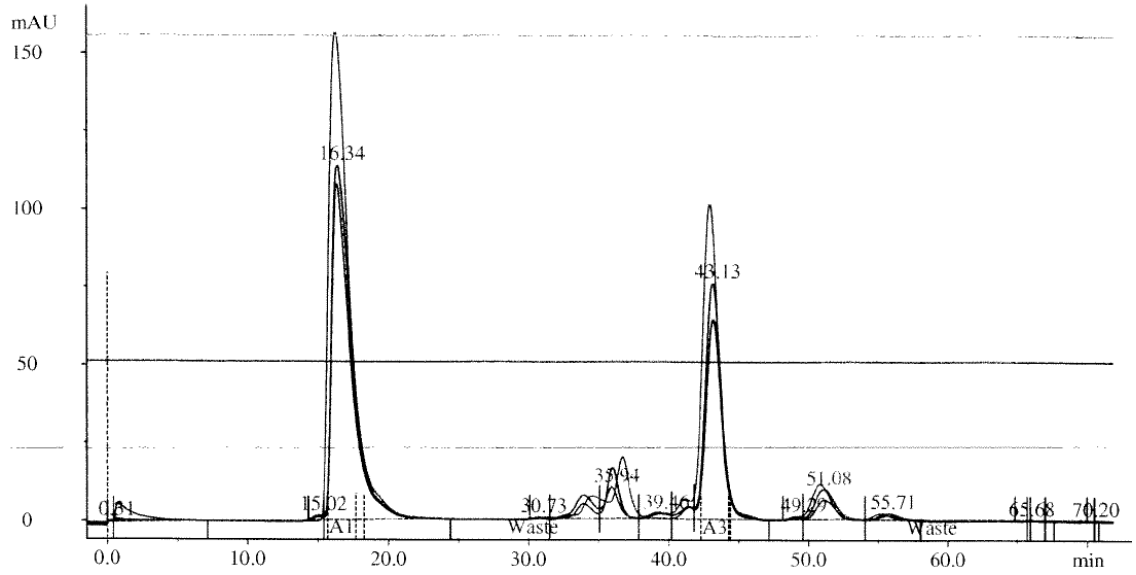


Figure F.3: Chromatogram of the samples superchilled using liquid carbon dioxide (LIC 20 %) and stored for 6 days.

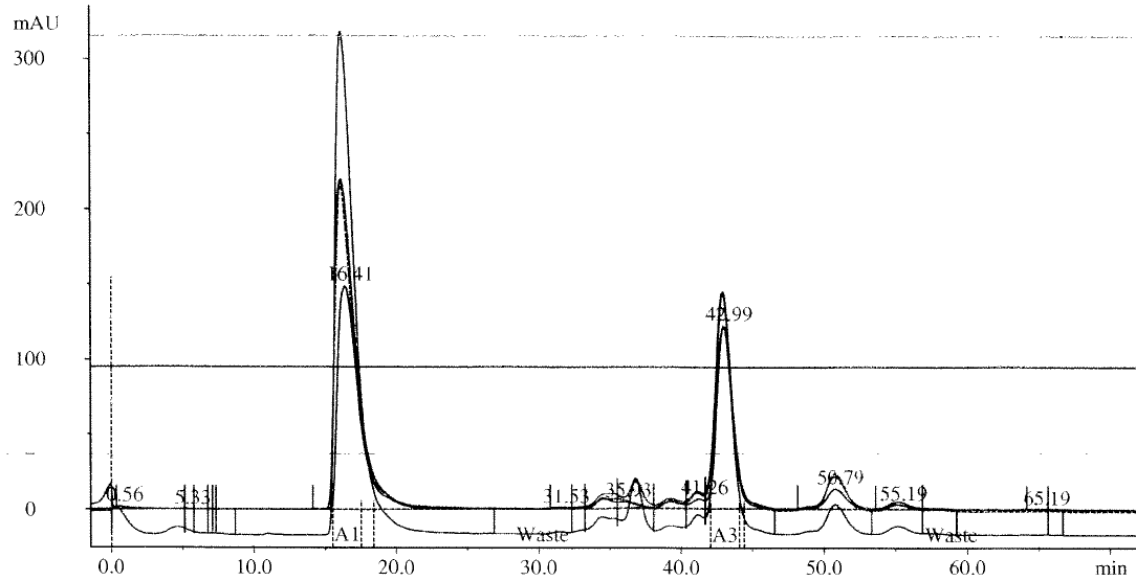


Figure F.4: Chromatogram of the samples superchilled by liquid carbon dioxide (LIC 20 %) and stored for 16 days.

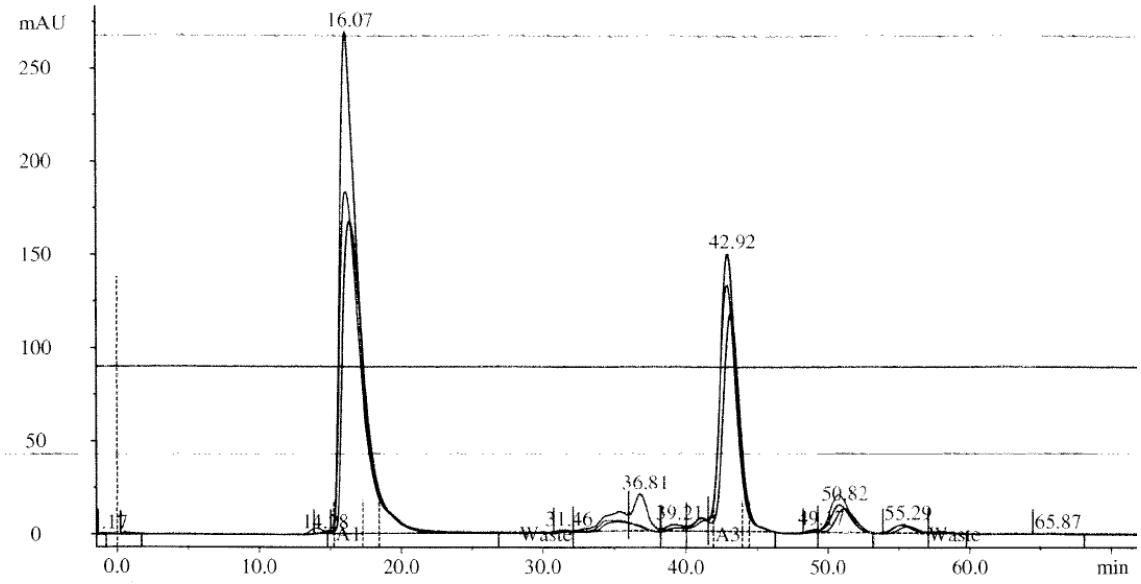


Figure F.5: Chromatogram of the samples superchilled by liquid carbon dioxide (LIC 20 %) and stored for 28 days.

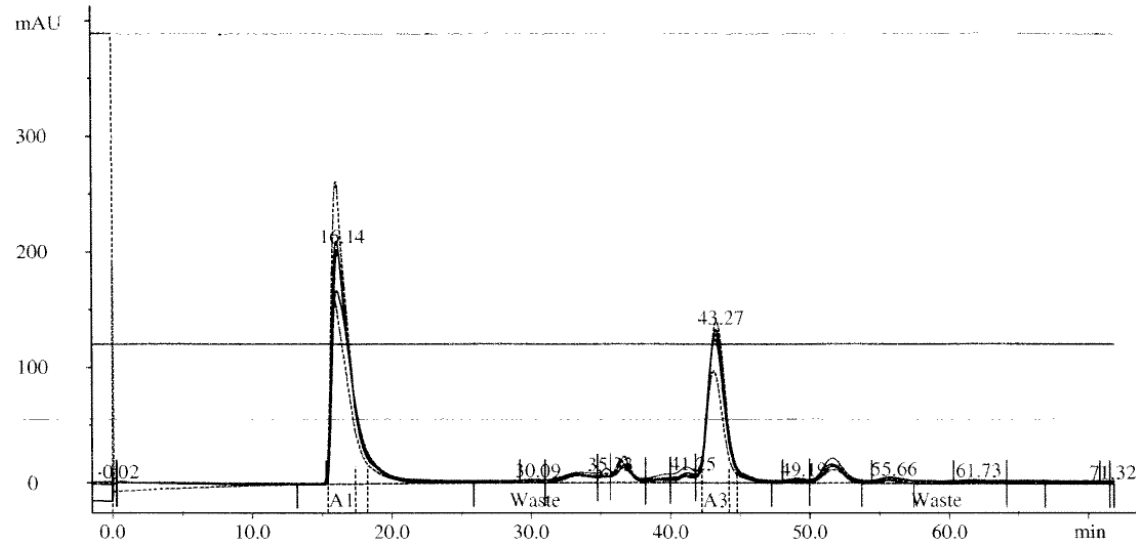


Figure F.6: Chromatogram for the samples superchilled by air (LUFT 10 %) and stored for 8 days.

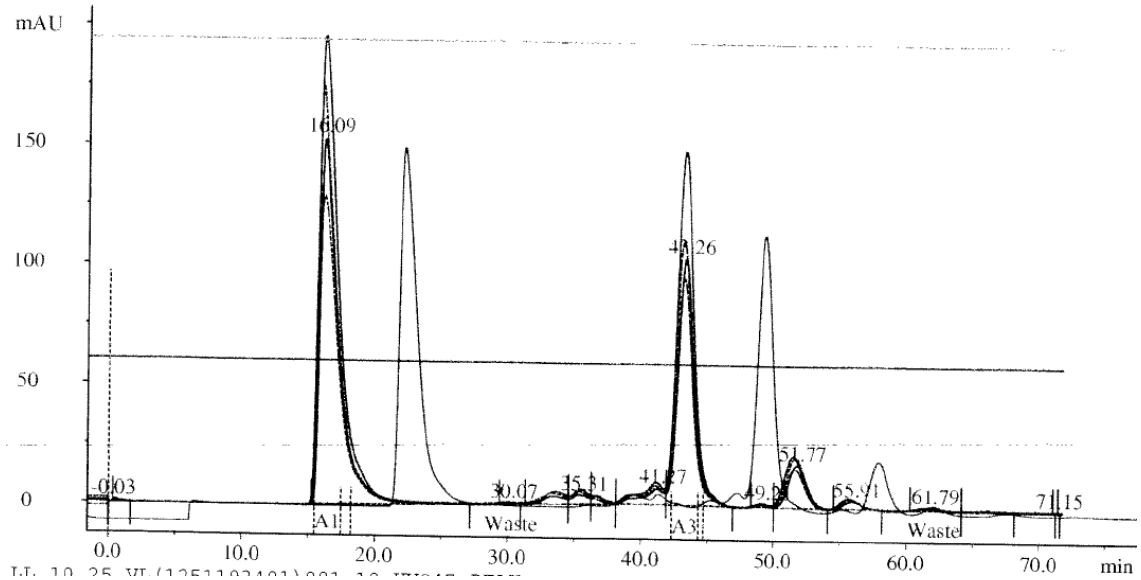


Figure F.7: Chromatogram for the samples superchilled by air (LUFT 10 %) and stored for 28 days.

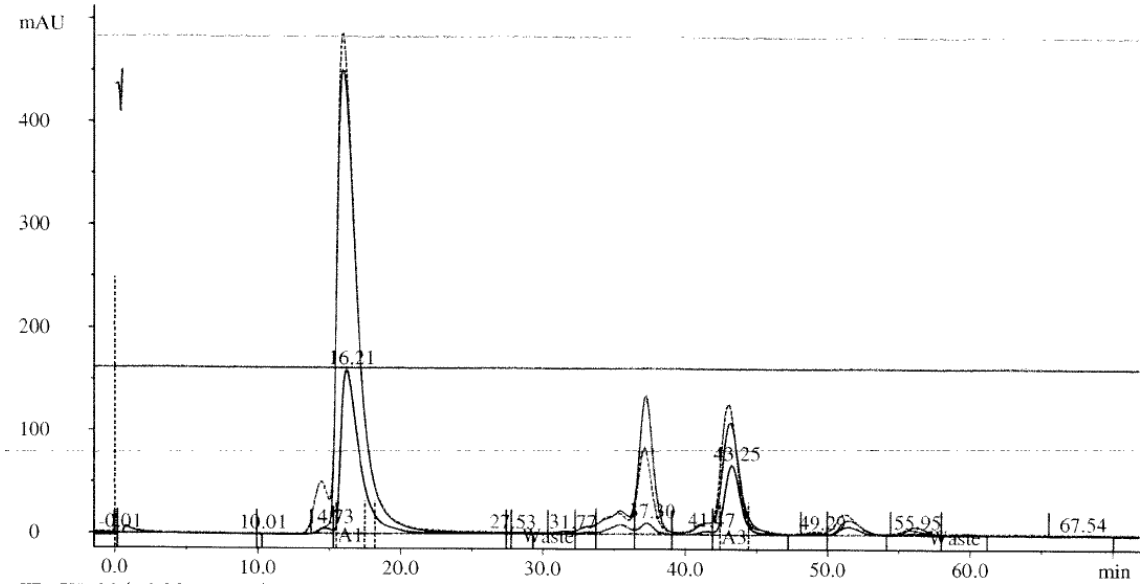


Figure F.8: Chromatogram of the chilled references (KR) stored on ice for 0 days.

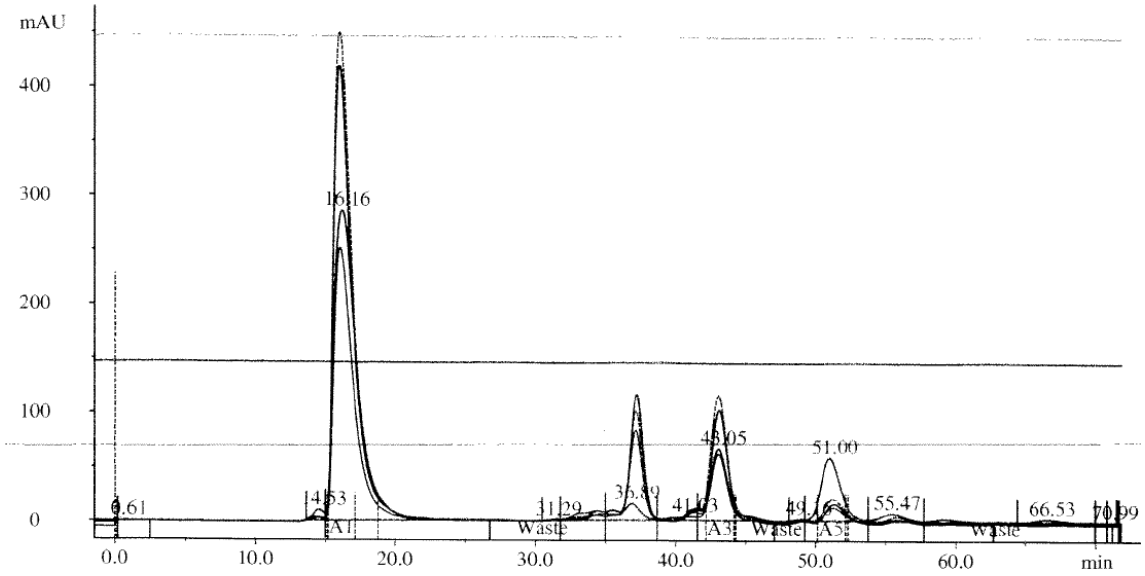


Figure F.9: Chromatogram of the chilled references (KR) stored on ice for 5 days.

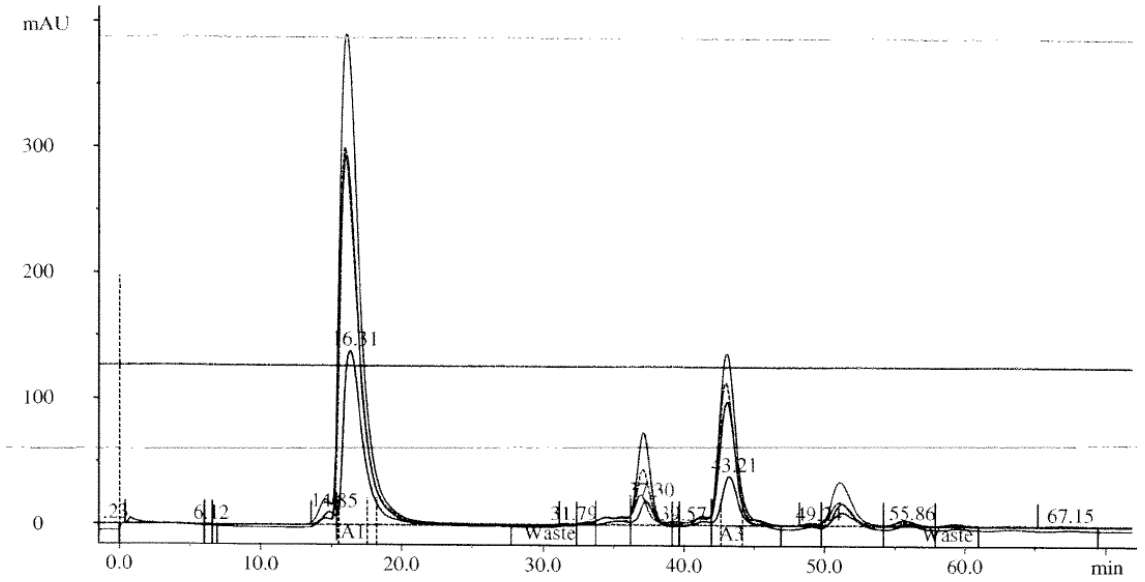


Figure F.10: Chromatogram of the chilled references (KR) stored on ice for 10 days.

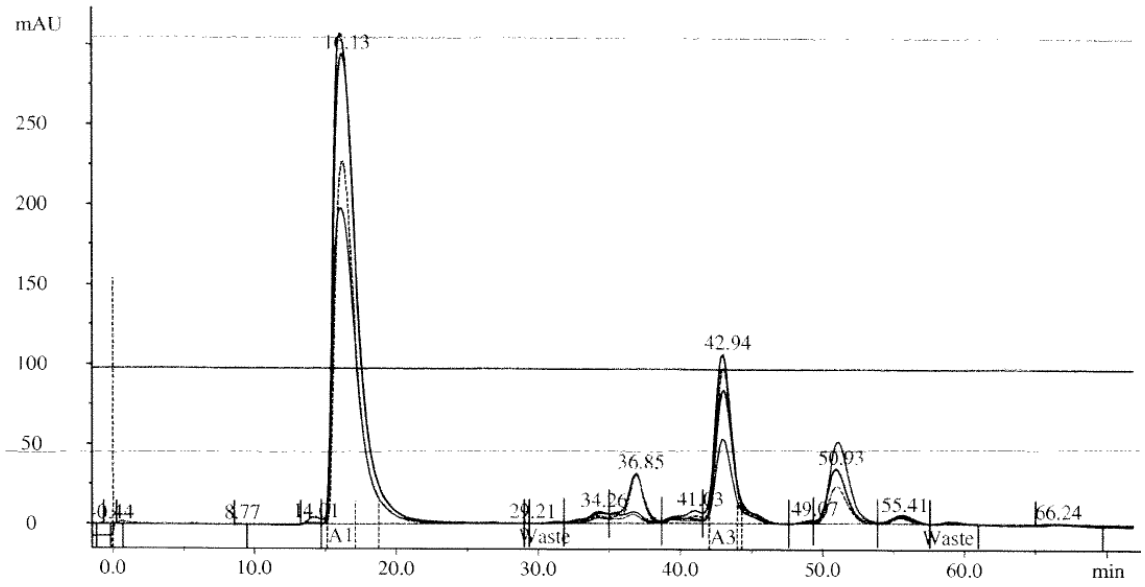


Figure F.11: Chromatogram of the chilled references (KR) stored on ice for 14 days.

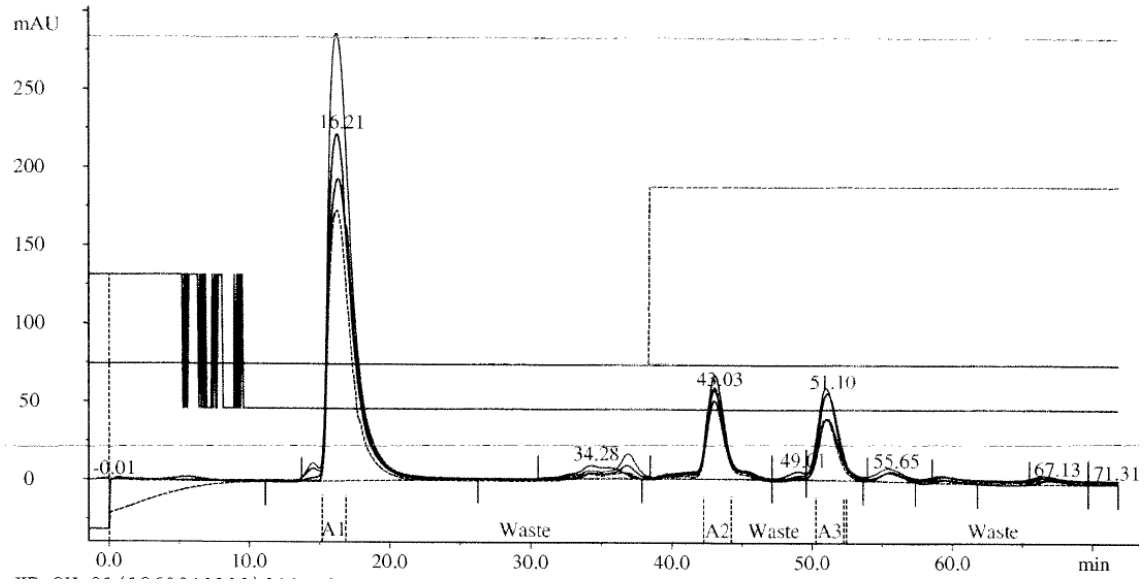


Figure F.12: Chromatogram of the chilled references (KR) stored on ice for 20 days.

F.2.2. Assembled table

The values from the chromatograms (appendix F.2.1) were used to find the relative peak areas, as described in appendix C.6.1., and combine them to get a better view of the occurring changes during storage at different conditions (chapter 3.3.1). Table F.1 shows the assembled table of the relative peak areas and standard deviations of these.

Table F.1: Assembled table of the relative peak areas and standard deviations of samples solubilised in water.

Mean values

	REF	LY 20 - 6 days	LY 20 - 16 days	LY 20 - 28 days
Peak A	55 %	54 %	52 %	57 %
Peak B	5 %	3 %	2 %	3 %
Peak C	11 %	4 %	3 %	1 %
Peak D	1 %	1 %	2 %	1 %
Peak E	22 %	27 %	27 %	30 %
Peak F	2 %	4 %	5 %	5 %
Peak G	1 %	1 %	1 %	1 %
Sum	97 %	95 %	92 %	99 %
Other	3 %	5,42 %	8,40 %	1 %

	REF	LL 10 - 8 days	LL 10 - 28 days	
Peak A	55 %	53 %	52 %	
Peak B	5 %	2 %	2 %	
Peak C	11 %	3 %	2 %	
Peak D	1 %	2 %	3 %	
Peak E	22 %	30 %	32 %	
Peak F	2 %	4 %	6 %	
Peak G	1 %	1 %	1 %	
Sum	97 %	95 %	97 %	
Other	3 %	5,40 %	2,75 %	

Standard deviations

	REF	LY 20 - 6 days	LY 20 - 16 days	LY 20 - 28 days
Peak A	1 %	3 %	4 %	2 %
Peak B	1 %	1 %	1 %	0 %
Peak C	1 %	1 %	1 %	2 %
Peak D	0 %	0 %	0 %	0 %
Peak E	1 %	2 %	6 %	2 %
Peak F	0 %	1 %	1 %	1 %
Peak G	0 %	0 %	0 %	0 %
Sum	1 %	5 %	11 %	0 %
Other	1 %	5,43 %	11 %	0 %

	REF	LL 10 - 8 days	LL 10 - 28 days	
Peak A	1 %	4 %	1 %	
Peak B	1 %	0 %	0 %	
Peak C	1 %	1 %	0 %	
Peak D	0 %	1 %	0 %	
Peak E	1 %	2 %	2 %	
Peak F	0 %	1 %	0 %	
Peak G	0 %	0 %	0 %	
Sum	1 %	5 %	1 %	
Other	1 %	5,19 %	1,16 %	

Mean values - Chilled references (KR)

Days	0	5	10	14	20
Peak A	63 %	64 %	63 %	65 %	61 %
Peak B	4 %	1 %	1 %	1 %	2 %
Peak C	8 %	12 %	6 %	4 %	2 %
Peak D	1 %	1 %	1 %	1 %	1 %
Peak E	17 %	14 %	18 %	16 %	13 %
Peak F	3 %	3 %	5 %	8 %	11 %
Peak G	1 %	1 %	1 %	1 %	2 %
Sum	94 %	95 %	94 %	95 %	90 %
Other	6 %	5 %	6 %	5 %	10 %

Standard deviations - Chilled references (KR)

Days	0	5	10	14	20
Peak A	2 %	2 %	3 %	3 %	5 %
Peak B	0 %	1 %	0 %	0 %	1 %
Peak C	4 %	2 %	3 %	3 %	1 %
Peak D	0 %	0 %	0 %	1 %	0 %
Peak E	3 %	1 %	3 %	3 %	2 %
Peak F	0 %	0 %	1 %	2 %	1 %
Peak G	0 %	0 %	0 %	0 %	0 %
Sum	1 %	1 %	2 %	2 %	6 %
Other	1 %	1 %	2 %	2 %	6 %

F.3. Ethanol soluble proteins

FPLC of ethanol soluble proteins were run as described in chapter 2.7. Values from chromatograms (appendix F.3.1) were used to calculate the relative peak area of the samples (appendix F.3.2) as described in appendix C.6.1.

F.3.1. Chromatograms

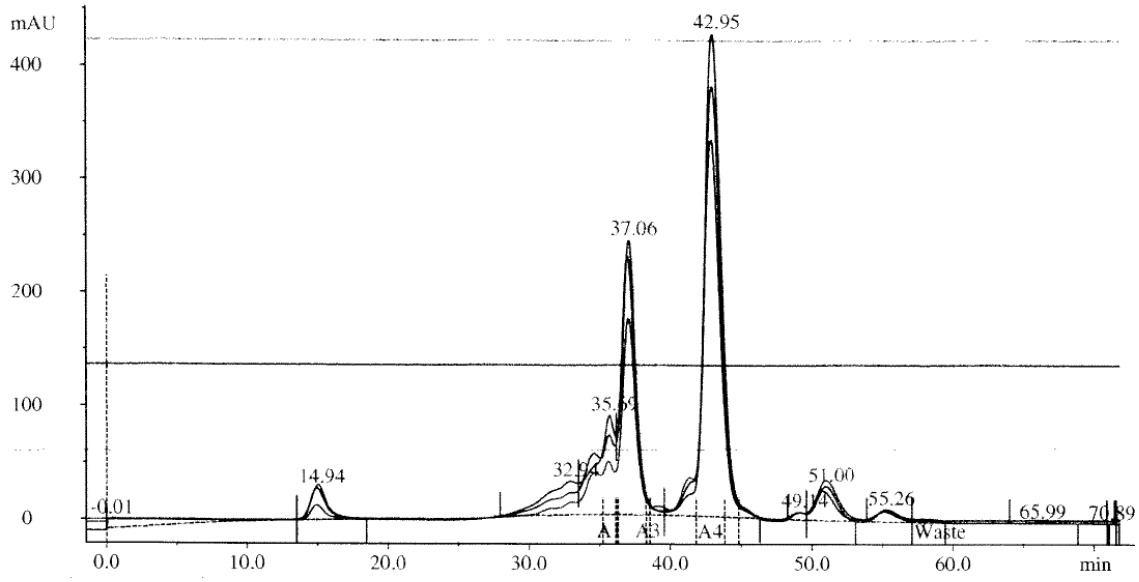


Figure F.13: Chromatogram of the reference samples (REF) withdrawn before superchilling.

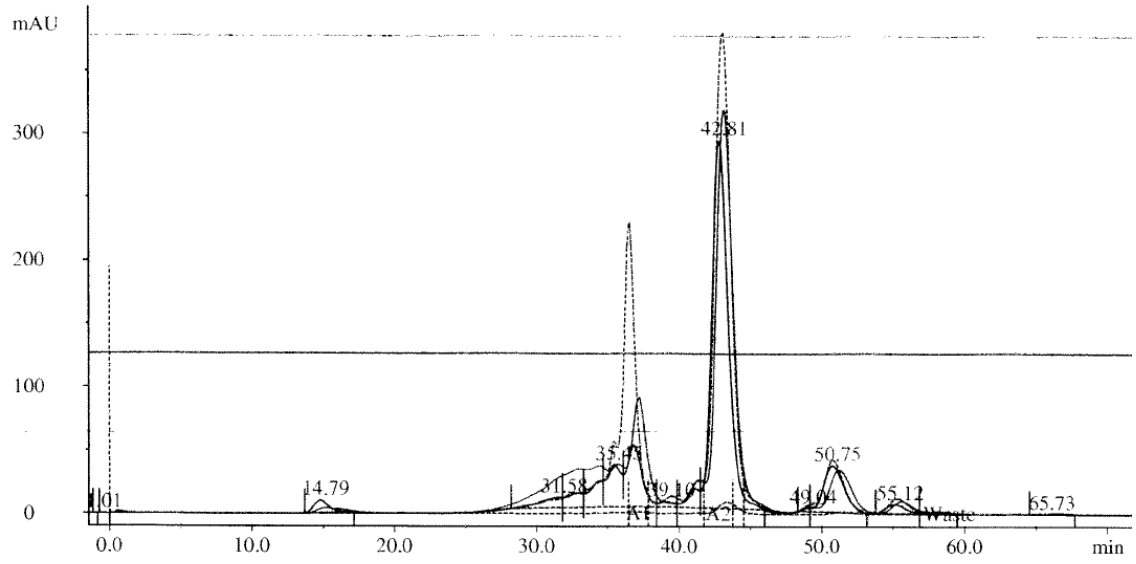


Figure F.14: Chromatogram of the samples superchilled by liquid carbon dioxide (LIC 20 %) stored for 6 days.

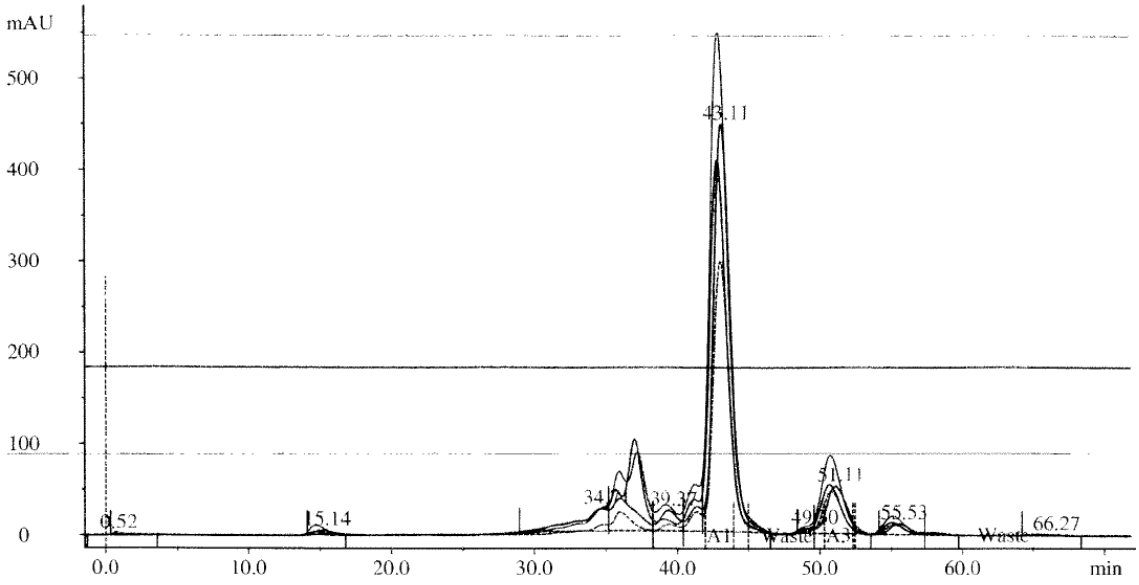


Figure F.15: Chromatogram of the samples superchilled by liquid carbon dioxide (LIC 20 %) and stored for 16 days.

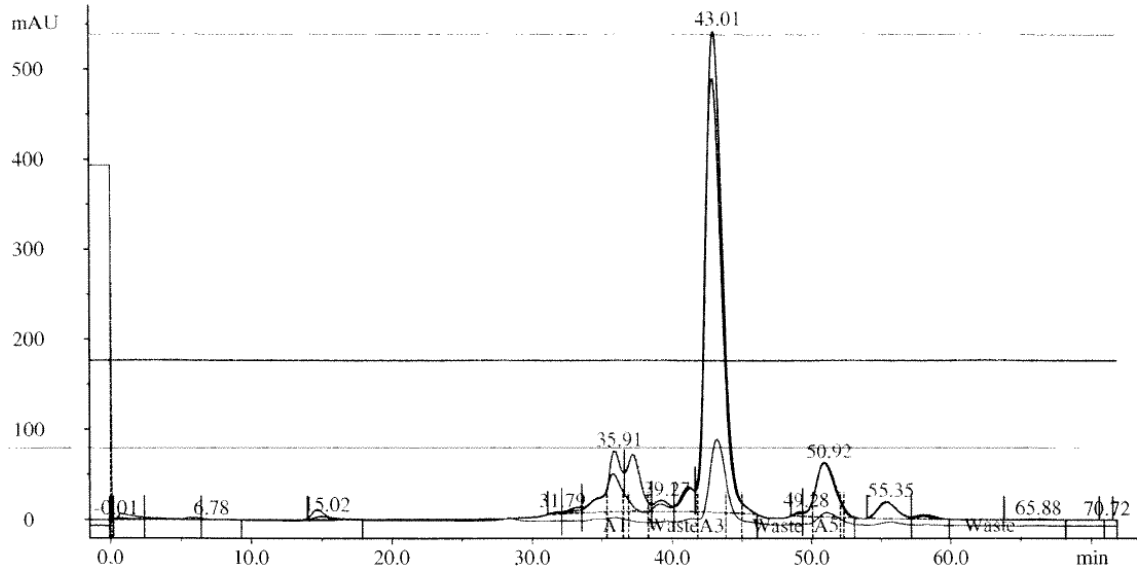


Figure F.16: Chromatogram of the samples superchilled by liquid carbon dioxide (LIC 20 %) and stored for 28 days.

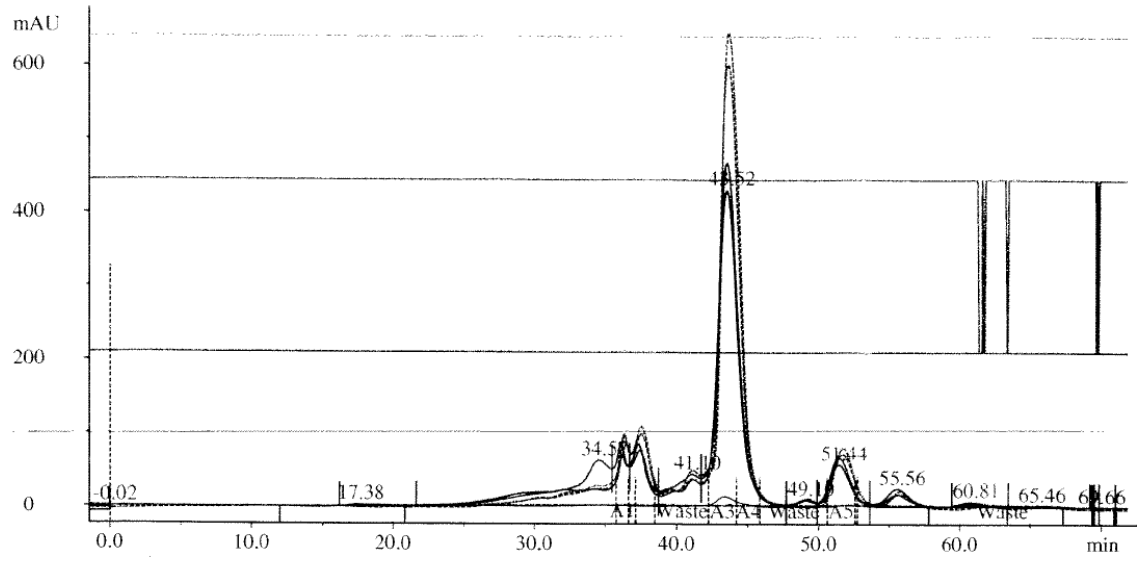


Figure F.17: Chromatogram of the samples superchilled by air (LUFT 10 %) and stored for 8 days.

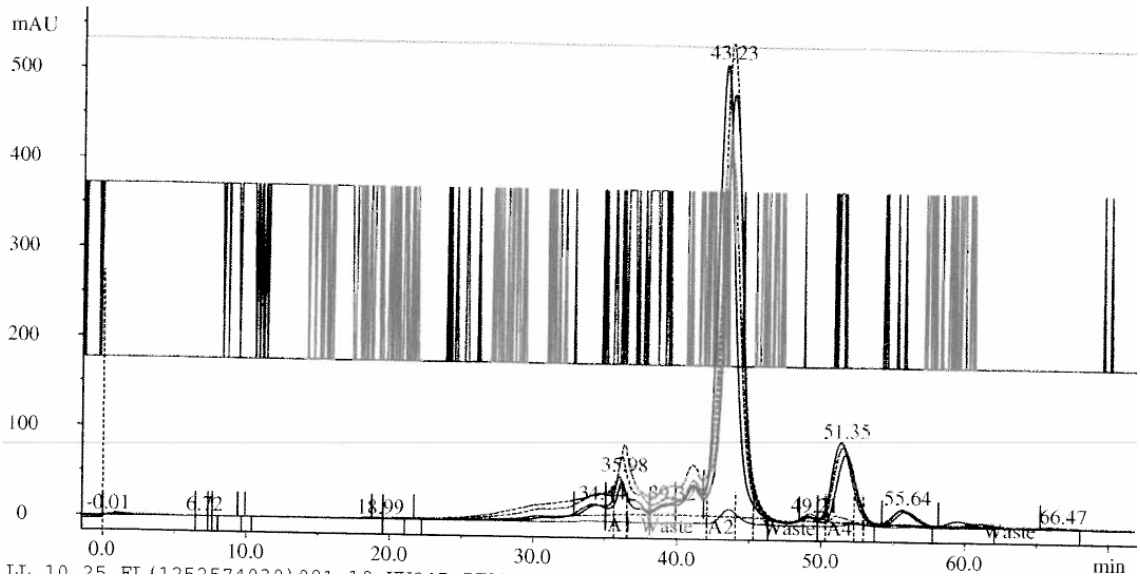


Figure F.18: Chromatogram of the samples superchilled by air (LUFT 10 %) and stored for 28 days.

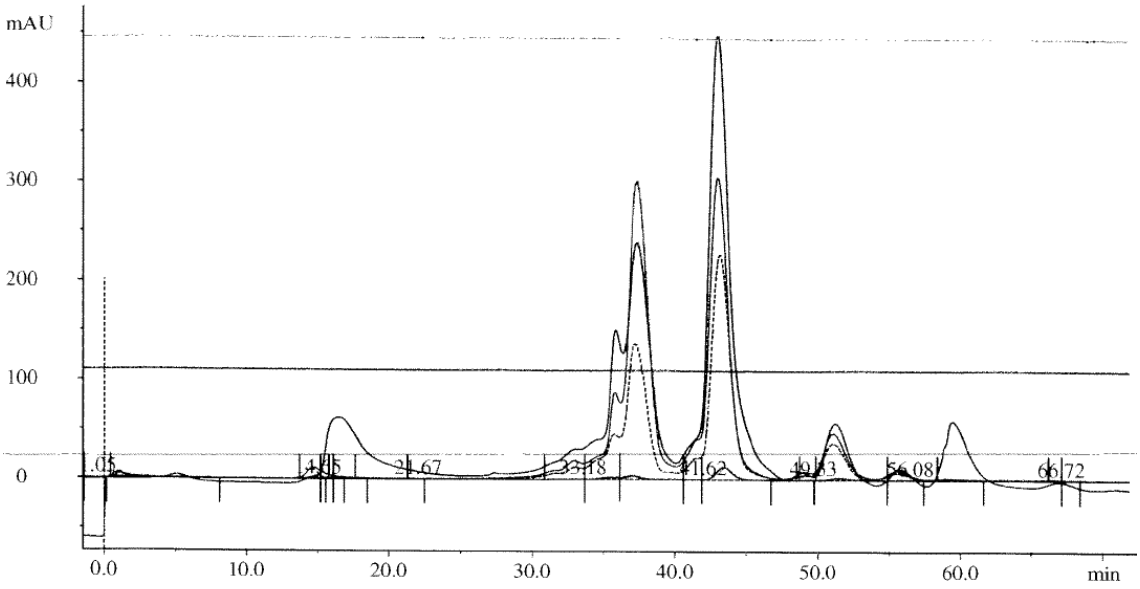


Figure F.19: Chromatogram of the chilled reference samples (KR) stored on ice for 0 days.

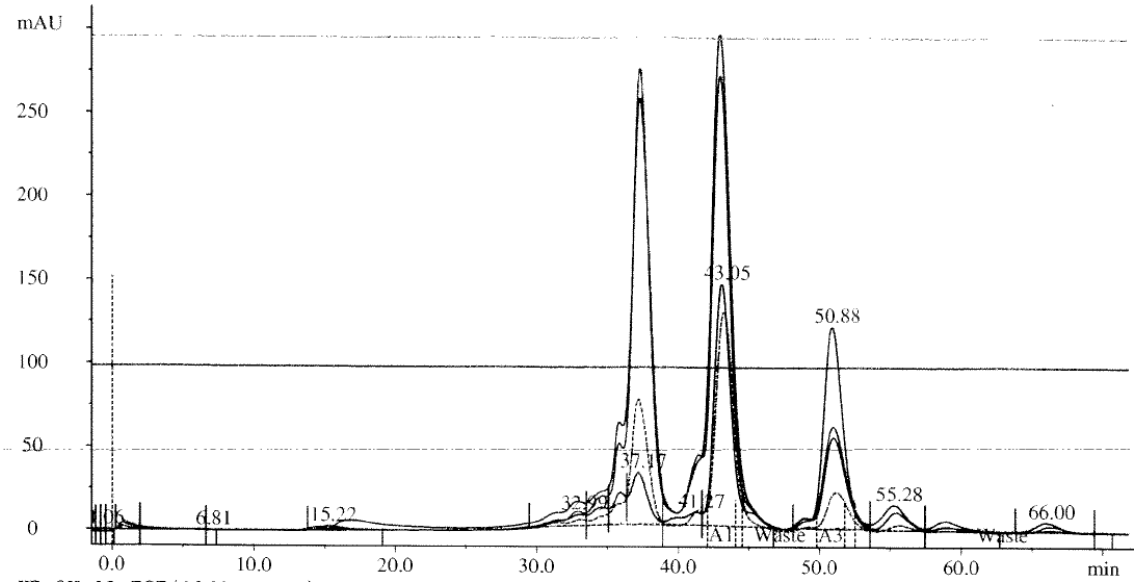


Figure F.20: Chromatogram of the chilled reference samples (KR) stored on ice for 5 days.

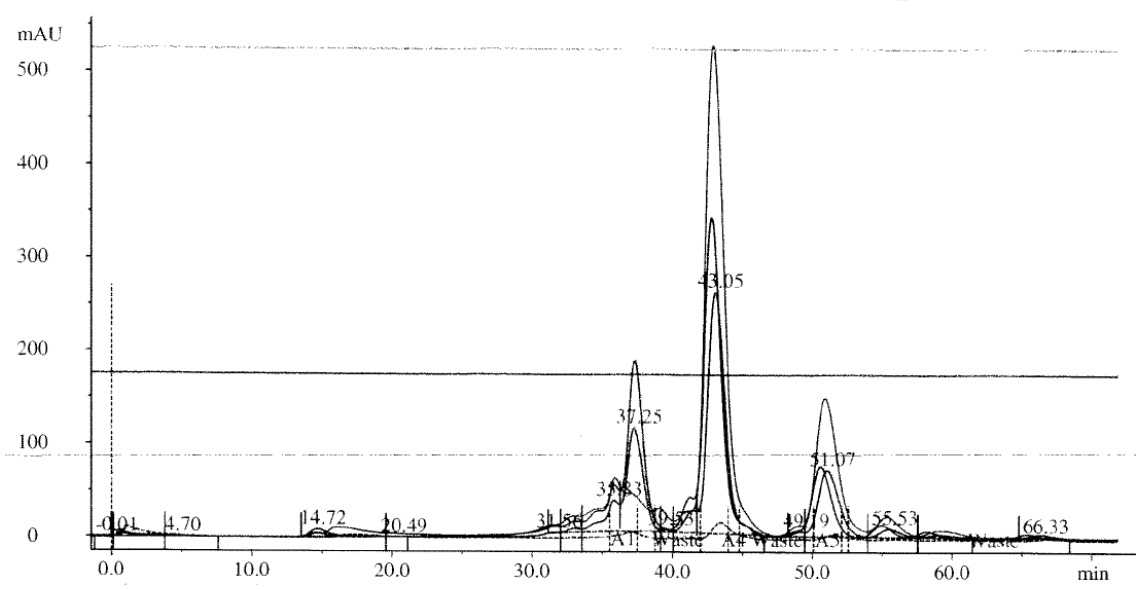


Figure F.21: Chromatogram of the chilled reference samples (KR) stored on ice for 10 days.

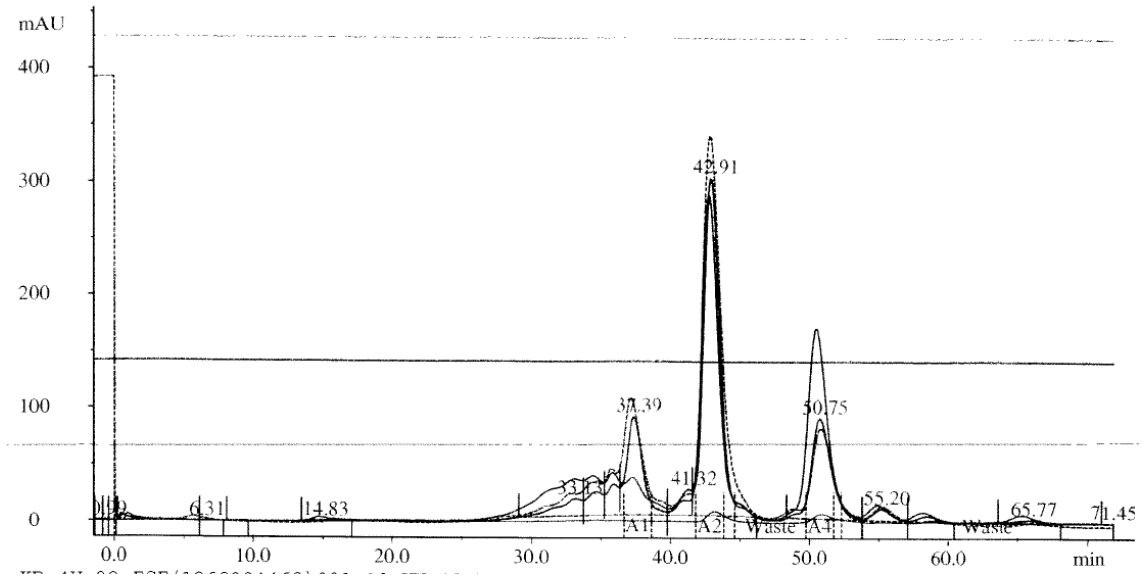


Figure F.22: Chromatogram of the chilled reference samples (KR) stored on ice for 14 days.

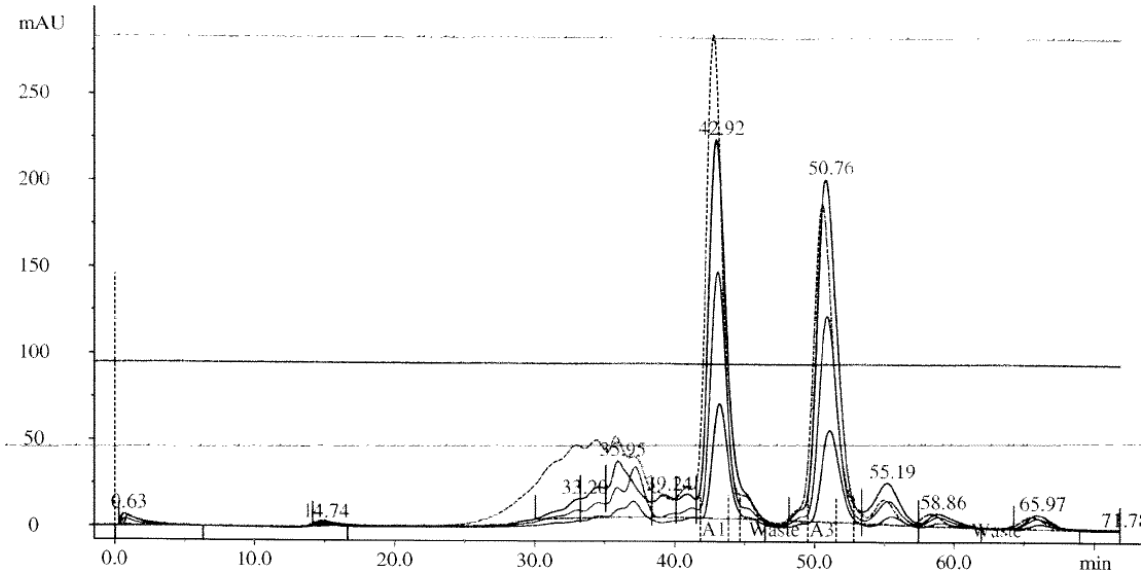


Figure F.23: Chromatogram of the chilled reference samples (KR) stored on ice for 20 days.

F.3.2. Assembled table

The values from the chromatograms (appendix F.3.1) were used to find the relative peak areas, as described in appendix C.6.1., and combine them to get a better view of the occurring changes during storage at different conditions (chapter 3.3.2). Table F.2 shows the assembled table of the relative peak areas and standard deviations of these.

Table F.2: Assembled table of the relative peak areas and standard deviations of protein samples solubilised in ethanol.

Mean values

	REF 0 day	LY 6 days	LY 16 days	LY 28 days
Peak A	2 %	1 %	1 %	1 %
Peak B	4 %	5 %	6 %	8 %
Peak C	6 %	6 %	8 %	3 %
Peak D	23 %	15 %	2 %	1 %
Peak E	50 %	55 %	64 %	66 %
Peak F	3 %	8 %	11 %	9 %
Peak G	1 %	2 %	2 %	3 %
Sum	87 %	92 %	95 %	92 %
Other	12,64 %	8,38 %	5,41 %	8 %

	REF 0 day	LL 8 days	LL 28 days
Peak A	2 %	0 %	0 %
Peak B	4 %	5 %	7 %
Peak C	6 %	8 %	0 %
Peak D	23 %	0 %	2 %
Peak E	50 %	63 %	66 %
Peak F	3 %	8 %	10 %
Peak G	1 %	2 %	2 %
Sum	87 %	87 %	88 %
Other	12,64 %	13,20 %	12,45 %

Standard deviations

	REF 0 day	LY 6 days	LY 16 days	LY 28 days
Peak A	1 %	1 %	0 %	1 %
Peak B	3 %	6 %	3 %	1 %
Peak C	5 %	1 %	3 %	5 %
Peak D	3 %	7 %	1 %	0 %
Peak E	4 %	4 %	4 %	1 %
Peak F	2 %	1 %	2 %	1 %
Peak G	1 %	0 %	0 %	0 %
Sum	10 %	3 %	1 %	3 %
Other	10,04 %	3,02 %	1 %	3 %

	REF 0 day	LL 8 days	LL 28 days
Peak A	1 %	0 %	0 %
Peak B	3 %	1 %	3 %
Peak C	5 %	0 %	0 %
Peak D	3 %	0 %	2 %
Peak E	4 %	8 %	6 %
Peak F	2 %	1 %	6 %
Peak G	1 %	1 %	1 %
Sum	10 %	9 %	3 %
Other	10,04 %	9,24 %	3,29 %

Chilled references - mean values

Days	0	5	10	14	20
Peak A	4 %	0 %	1 %	0 %	0 %
Peak B	8 %	5 %	6 %	4 %	4 %
Peak C	29 %	33 %	14 %	7 %	6 %
Peak D	1 %	2 %	2 %	1 %	2 %
Peak E	41 %	43 %	51 %	39 %	36 %
Peak F	6 %	10 %	16 %	24 %	32 %
Peak G	1 %	2 %	2 %	2 %	4 %
Sum	90 %	93 %	91 %	76 %	80 %
Other	10,16 %	7,29 %	8,93 %	24 %	20 %

Chilled references - standard deviations

Days	0	5	10	14	20
Peak A	5 %	0 %	1 %	0 %	0 %
Peak B	1 %	1 %	2 %	1 %	5 %
Peak C	10 %	6 %	12 %	6 %	5 %
Peak D	1 %	2 %	1 %	1 %	1 %
Peak E	4 %	3 %	8 %	12 %	1 %
Peak F	2 %	0 %	4 %	5 %	2 %
Peak G	0 %	0 %	0 %	0 %	2 %
Sum	8 %	2 %	4 %	15 %	3 %
Other	8,37 %	2,47 %	4,49 %	15 %	3 %

F.4. Tables for changes in peaks

The changes in peaks were calculated as described in appendix C.6.1. Table F.3 shows the mean values (%) and table F.4 shows the standard deviations (%) relative to storage time of the different storage methods. Figures of the peak changes are to be found in chapter 3.3.3.

Table F.3: Mean values (%) of peak areas from ethanol soluble proteins relative to storage time of the different storage methods.

Mean values

Peak A									
	0	5	6	8	10	14	16	20	28
REF	2								
LUFT 10%				0					0
LIC 20%			1				1		1
KR	4	0			1	0		0	

Peak B									
	0	5	6	8	10	14	16	20	28
REF	4								
LUFT 10%				5					7
LIC 20%			5				6		8
KR	8	5			6	4		4	

Peak C									
	0	5	6	8	10	14	16	20	28
REF	6								
LUFT 10%				8					0
LIC 20%			6				8		3
KR	29	33			14	7		6	

Peak D									
	0	5	6	8	10	14	16	20	28
REF	23								
LUFT 10%				0					2
LIC 20%			15				2		1
KR	1	2			2	1		2	

Peak E									
	0	5	6	8	10	14	16	20	28
REF	50								
LUFT 10%				63					66
LIC 20%			55				64		66
KR	41	43			51	39		36	

Peak F									
	0	5	6	8	10	14	16	20	28
REF	3								
LUFT 10%				8					10
LIC 20%			8				11		9
KR	6	10			16	24		32	

Peak G									
	0	5	6	8	10	14	16	20	28
REF	1								
LUFT 10%				2					2
LIC 20%			2				2		3
KR	1	2			2	2		4	

Table F.4: Standard deviations (%) of peak areas from ethanol soluble proteins relative to storage time of the different storage methods.

Standard deviations

Peak A									
	0	5	6	8	10	14	16	20	28
REF	1								
LUFT 10%				1					0
LIC 20%			1				0		1
KR	5	0			1	0		0	

Peak B									
	0	5	6	8	10	14	16	20	28
REF	3								
LUFT 10%				6					3
LIC 20%			6				3		1
KR	1	1			2	1		5	

Peak C									
	0	5	6	8	10	14	16	20	28
REF	5								
LUFT 10%				1					0
LIC 20%			1				3		5
KR	10	6			12	6		5	

Peak D									
	0	5	6	8	10	14	16	20	28
REF	3								
LUFT 10%				7					2
LIC 20%			7				1		0
KR	1	2			1	1		1	

Peak E									
	0	5	6	8	10	14	16	20	28
REF	4								
LUFT 10%				4					6
LIC 20%			4				4		1
KR	4	3			8	12		1	

Peak F									
	0	5	6	8	10	14	16	20	28
REF	2								
LUFT 10%				1					6
LIC 20%			1				2		1
KR	2	0			4	5		2	

Peak G									
	0	5	6	8	10	14	16	20	28
REF	1								
LUFT 10%				0					1
LIC 20%			0				0		0
KR	0	0			0	0		2	

G. Mass spectrometry

Mass spectrometry was run as described in chapter 2.8. Results from the analysis were used to calculate the changes during storage as described in appendix C.7.

G.1. Changes relative to day zero

Table G.1 shows the assembled table of mean values and standard deviations calculated relative to day zero as explained in appendix C.7.1. Table G.2 shows the assembled table for the data shown graphical in chapter 3.4.2.

Table G.1: The mean values and standard deviations calculated relative to day zero (0) for the chilled reference samples.

	A	B	C	D	E	F	G	H	I
1									
2	Mean values relative to day zero					Standard deviations rel. to day zero			
3	m/z	day 0	day 5	day 10	day 14	day 0	day 5	day 10	day 14
4	76,2	1,00	0,74	1,10	2,40	0,51	0,26	0,61	2,19
5	104,2	1,00	0,57	0,43	0,22	0,40	0,23	0,20	0,09
6	118,2	1,00	0,88	0,47	0,23	0,29	0,86	0,15	0,05
7	132,2	1,00	1,70	m	1,04	1,49	2,66	m	2,04
8	133,2	1,00	0,59	0,12	0,15	2,90	1,90	0,20	0,41
9	151,2	1,00	0,76	1,16	2,71	0,52	0,30	0,71	2,65
10	152,2	1,00	0,79	1,20	2,56	0,63	0,33	0,77	2,46
11	154,1	1,00	0,37	0,38	0,47	1,21	0,27	0,32	0,59
12	170	1,00	0,77	0,63	0,50	1,37	0,88	0,53	0,51
13	193,1	1,00	0,41	0,29	0,17	1,06	0,64	0,36	0,14
14	207,1	1,00	1,00	1,02	1,30	1,03	0,68	0,66	1,35
15	208	1,00	1,02	1,02	1,07	0,83	1,16	0,81	0,83
16	241,1	1,00	0,78	0,43	0,38	2,27	1,16	0,57	0,49
17	279	1,00	0,72	0,26	0,19	3,28	1,83	0,47	0,43
18	296	1,00	0,25	0,22	0,12	3,61	0,65	0,67	0,35
19	307	1,00	1,83	0,92	0,78	1,71	3,46	1,58	0,97

Table G.2: Changes relative to total sorted by m/z-values for the chilled reference samples (KR), the reference samples (REF) and the samples superchilled using air (LL) and liquid carbon dioxide (LY).

	A	B	C	D	E	F	G	H	I	J	K
1	Mean values 76,2						Standard deviations 76,2				
2		REF	LL	LY	KR			REF	LL	LY	KR
3	0	1			1		0	0,4368102			0,50723453
4	5				0,73876853		5				0,25605966
5	6			1,07762573			6			0,20654687	
6	8		1,20592101				8		0,83512962		
7	10				1,10		10				0,60687361
8	14				2,40169502		14				2,1898836
9	16			1,21384265			16			0,30075802	
10	28		0,99350878	1,15259634			28		0,45860336	0,14860623	
11											
12	Mean values 104,2						Standard deviations 104,2				
13		REF	LL	LY	KR			REF	LL	LY	KR
14	0	1			1		0	0,42387284			0,3992203
15	5				0,57246123		5				0,22793539
16	6			0,65543455			6			0,20142883	
17	8		1,02458572				8		1,10588822		
18	10				0,43		10				0,19636296
19	14				0,22253441		14				0,09187921
20	16			0,50680763			16			0,26486372	
21	28		0,77767992	0,84931284			28		0,74981918	0,3709967	
22											
23	Mean values 132,2						Standard deviations 132,2				
24		REF	LL	LY	KR			REF	LL	LY	KR
25	0	1			1		0	0,15603196			1,49023507
26	5				1,70099257		5				2,66297009
27	6			1,22155738			6			0,50036617	
28	8		0,75732351				8		0,38421519		
29	10						10				
30	14				1,04044005		14				2,03770377
31	16			0,983067			16			0,31568633	
32	28		1,38775323	0,77396939			28		0,27206895	0,32610956	
33											
34	Mean values 151,2						Standard deviations 151,2				
35		REF	LL	LY	KR			REF	LL	LY	KR
36	0	1			1		0	0,36851736			0,52181595
37	5				0,76106182		5				0,33101994
38	6			1,07754222			6			0,20656743	
39	8		1,14246558				8		0,85431281		
40	10				1,16		10				0,7736516
41	14				2,71109258		14				2,4623013
42	16			1,16166593			16			0,25962437	
43	28		1,03145131	1,14272859			28		0,46061829	0,20901505	
44											
45	Mean values 152,2						Standard deviations 152,2				
46		REF	LL	LY	KR			REF	LL	LY	KR
47	0	1			1		0	0,40189131			0,62695284
48	5				0,78570177		5				0,27494343
49	6			1,06324031			6			0,22254891	
50	8		1,16969117				8		0,89863846		
51	10				1,20		10				0,32258855
52	14				2,56410074		14				0,58696678
53	16			1,17679995			16			0,24330958	
54	28		1,04279635	1,14380516			28		0,51003737	0,15689783	
55											
56	Mean values 241,1						Standard deviations 241,1				
57		REF	LL	LY	KR			REF	LL	LY	KR
58	0	1			1		0	0,28367368			2,2735808
59	5				0,78191224		5				1,16149715
60	6			1,03755161			6			0,18234691	
61	8		0,98721572				8		0,62002041		
62	10				0,43		10				0,57232461
63	14				0,37618531		14				0,48968506
64	16			1,07945925			16			0,19825848	
65	28		1,13224341	1,06519984			28		0,44204498	0,26785775	

G.2. Changes relative to 151.2

Table G.3 shows the assembled table of mean values and standard deviations calculated relative to the abundance of 151.2 (m/z) as shown in appendix C.7.2.

Table G.3: The mean values and standard deviations calculated relative to 151.2(m/z) for the chilled reference samples.

	A	B	C	D	E	F	G	H	I
1		Mean values relative to 151,2				Standard deviations relative to 151,2			
2	m/z	day 0	day 5	day 10	day 14	day 0	day 5	day 10	day 14
3	76,2	15 %	15 %	16 %	18 %	1 %	1 %	1 %	2 %
4	104,2	3 %	4 %	10 %	75 %	0 %	1 %	6 %	75 %
5	118,2	0 %	0 %	1 %	6 %	0 %	0 %	0 %	5 %
6	132,2	6 %	4 %	10 %	57 %	2 %	1 %	6 %	64 %
7	133,2	0 %	0 %	0 %	3 %	0 %	0 %	0 %	4 %
8	151,2	100 %	100 %	100 %	100 %	0 %	0 %	0 %	0 %
9	152,2	7 %	8 %	7 %	9 %	1 %	0 %	0 %	2 %
10	154,1	0 %	0 %	1 %	5 %	0 %	0 %	1 %	6 %
11	170	3 %	3 %	5 %	29 %	1 %	2 %	3 %	34 %
12	193,1	0 %	0 %	1 %	4 %	0 %	0 %	0 %	3 %
13	207,1	4 %	3 %	3 %	11 %	2 %	0 %	2 %	9 %
14	208	1 %	1 %	2 %	3 %	0 %	0 %	3 %	3 %
15	241,1	2 %	2 %	4 %	32 %	2 %	1 %	3 %	34 %
16	279	0 %	0 %	4 %	7 %	0 %	0 %	6 %	7 %
17	296	0 %	1 %	1 %	19 %	1 %	0 %	1 %	23 %

G.3. Changes relative to total abundance

Table G.4 shows the assembled table of mean values and table G.5 shows the assembled table of standard deviations calculated relative to total abundance as shown in appendix C.7.3. Table G.6 shows the assembled table for the total abundance relative to the total abundance sorted by the m/z values as shown graphically in chapter 3.4.2.

Table G.4: Mean values calculated relative to total abundance for reference samples (REF) and samples superchilled using air (LUFT) and liquid carbon dioxide (LIC).

	A	B	C	D	E	F	G
1	Mean values						
2			LUFT		LIC		
3	m/z	REF	18 days	28 days	8 days	16 days	28 days
4	76,25	5 %	5 %	5 %	4 %	5 %	5 %
5	90,05	0 %	0 %	0 %	1 %	0 %	0 %
6	104,25	2 %	3 %	2 %	1 %	1 %	2 %
7	114,15	2 %	2 %	2 %	1 %	1 %	1 %
8	118,15	1 %	1 %	1 %	0 %	0 %	0 %
9	132,15	14 %	11 %	12 %	15 %	12 %	11 %
10	133,05	1 %	1 %	1 %	1 %	1 %	1 %
11	137,05	1 %	1 %	1 %	1 %	1 %	1 %
12	151,15	27 %	29 %	27 %	25 %	27 %	28 %
13	152,15	2 %	3 %	2 %	2 %	2 %	2 %
14	154,05	1 %	1 %	1 %	1 %	1 %	1 %
15	156,05	1 %	1 %	1 %	1 %	1 %	1 %
16	167,05	1 %	1 %	0 %	0 %	1 %	1 %
17	170,05	9 %	7 %	8 %	9 %	8 %	8 %
18	170,95	0 %	0 %	0 %	1 %	0 %	0 %
19	172,05	1 %	0 %	1 %	1 %	1 %	1 %
20	175,05	1 %	1 %	1 %	1 %	1 %	1 %
21	189,05	0 %	1 %	1 %	0 %	0 %	0 %
22	207,05	2 %	2 %	2 %	2 %	2 %	2 %
23	241,05	10 %	10 %	10 %	9 %	10 %	9 %
24	242,05	1 %	1 %	1 %	1 %	1 %	1 %
25	251,95	2 %	2 %	2 %	2 %	2 %	2 %
26	258,15	0 %	1 %	2 %	1 %	2 %	1 %
27	263,05	5 %	4 %	4 %	6 %	4 %	4 %
28	264,05	1 %	0 %	0 %	1 %	0 %	0 %
29	269,05	0 %	1 %	1 %	1 %	1 %	0 %
30	279,01	1 %	1 %	1 %	0 %	1 %	1 %
31	296,05	1 %	2 %	3 %	2 %	4 %	3 %
32	307,05	3 %	4 %	4 %	4 %	5 %	5 %
33	308,05	1 %	1 %	1 %	1 %	1 %	1 %
34	309,05	0 %	0 %	0 %	0 %	0 %	0 %
35	316,15	0 %	1 %	1 %	1 %	1 %	1 %
36	317,05	1 %	1 %	1 %	1 %	1 %	1 %
37	372,15	0 %	0 %	0 %	0 %	0 %	0 %
38	400,15	0 %	0 %	1 %	1 %	1 %	0 %
39	403,05	1 %	1 %	0 %	1 %	0 %	0 %
40	414,85	0 %	0 %	0 %	0 %	0 %	0 %
41	441,15	0 %	0 %	0 %	0 %	0 %	0 %
42	452,95	0 %	0 %	0 %	0 %	0 %	1 %

Table G.5: Standard deviations calculated relative to total abundance for reference samples (REF) and samples superchilled using air (LUFT) and liquid carbon dioxide (LIC).

	I	J	K	L	M	N	O
1	Standard deviations						
2			LUFT		LIC		
3	m/z	REF	18 days	28 days	8 days	16 days	28 days
4	76,25	0 %	1 %	0 %	0 %	1 %	0 %
5	90,05	0 %	0 %	0 %	0 %	0 %	0 %
6	104,25	1 %	1 %	1 %	0 %	0 %	1 %
7	114,15	0 %	0 %	0 %	0 %	0 %	0 %
8	118,15	0 %	0 %	0 %	0 %	0 %	0 %
9	132,15	1 %	3 %	1 %	3 %	2 %	2 %
10	133,05	0 %	0 %	0 %	0 %	0 %	0 %
11	137,05	0 %	1 %	0 %	0 %	0 %	0 %
12	151,15	2 %	4 %	2 %	2 %	3 %	3 %
13	152,15	0 %	0 %	0 %	0 %	0 %	0 %
14	154,05	0 %	0 %	0 %	0 %	0 %	0 %
15	156,05	0 %	0 %	0 %	0 %	0 %	0 %
16	167,05	0 %	0 %	0 %	0 %	0 %	0 %
17	170,05	1 %	2 %	0 %	0 %	1 %	1 %
18	170,95	0 %	0 %	0 %	0 %	0 %	0 %
19	172,05	0 %	0 %	0 %	0 %	0 %	0 %
20	175,05	0 %	0 %	0 %	0 %	0 %	0 %
21	189,05	0 %	0 %	0 %	0 %	0 %	0 %
22	207,05	0 %	0 %	0 %	0 %	0 %	0 %
23	241,05	0 %	0 %	1 %	1 %	1 %	1 %
24	242,05	0 %	0 %	0 %	0 %	0 %	0 %
25	251,95	0 %	0 %	0 %	0 %	0 %	0 %
26	258,15	0 %	0 %	1 %	0 %	0 %	1 %
27	263,05	0 %	1 %	0 %	2 %	1 %	1 %
28	264,05	0 %	0 %	0 %	0 %	0 %	0 %
29	269,05	0 %	0 %	0 %	0 %	0 %	0 %
30	279,01	0 %	0 %	0 %	0 %	0 %	1 %
31	296,05	0 %	1 %	1 %	0 %	1 %	2 %
32	307,05	1 %	2 %	0 %	1 %	1 %	1 %
33	308,05	0 %	0 %	0 %	0 %	0 %	0 %
34	309,05	0 %	0 %	0 %	0 %	0 %	0 %
35	316,15	0 %	0 %	0 %	0 %	0 %	0 %
36	317,05	0 %	0 %	0 %	0 %	0 %	0 %
37	372,15	0 %	0 %	0 %	0 %	0 %	0 %
38	400,15	0 %	0 %	0 %	0 %	0 %	0 %
39	403,05	0 %	0 %	0 %	0 %	0 %	0 %
40	414,85	0 %	0 %	0 %	0 %	0 %	0 %
41	441,15	0 %	0 %	0 %	0 %	0 %	0 %
42	452,95	0 %	0 %	0 %	0 %	0 %	0 %

Table G.6: Changes relative to total sorted by m/z-values for the chilled reference samples (KR), the reference samples (REF) and the samples superchilled using air (LL) and liquid carbon dioxide (LY).

	A	B	C	D	E	F	G	H	I	J	K
1	Mean - 76,25						Standard deviations - 76,25				
2		KR	REF	LY	LL			KR	REF	LY	LL
3	0	10 %	5 %				0	0 %	0 %		
4	5	11 %					5	0 %			
5	6			4 %			6			0 %	
6	8				5 %		8				1 %
7	10	9 %					10	1 %			
8	14	6 %					14	3 %			
9	16			5 %			16			1 %	
10	28			5 %	5 %		28			0 %	0 %
11											
12	Mean - 104,2						Standard deviations - 104,2				
13		KR 0	REF	LY 6	LL 8			KR 0	REF	LY 6	LL 8
14	0	2 %	2 %				0	0 %	1 %		
15	5	3 %					5	2 %			
16	6			1 %			6			7 %	
17	8				3 %		8				1 %
18	10	5 %					10	1 %			
19	14	16 %					14	1 %			
20	16			1 %			16			0 %	
21	28			2 %	2 %		28			1 %	0 %
22											
23	Mean - 151,2						Standard deviations - 151,2				
24		KR	REF	LY	LL			KR	REF	LY	LL
25	0	70 %	27 %				0	4 %	2 %		
26	5	70 %					5	2 %			
27	6			25 %			6			2 %	
28	8				29 %		8				4 %
29	10	60 %					10	9 %			
30	14	35 %					14	19 %			
31	16			27 %			16			3 %	
32	28			28 %	27 %		28			3 %	2 %
33											
34	Mean - Histidin (152,15)						Standard deviation - Histidin (152,15)				
35		KR	REF	LY	LL			KR	REF	LY	LL
36	0	5 %	2 %				0	0 %	0 %		
37	5	5 %					5	0 %			
38	6			2 %			6			0 %	
39	8				3 %		8				0 %
40	10	5 %					10	0 %			
41	14	3 %					14	1 %			
42	16			2 %			16			0 %	
43	28			2 %	2 %		28			0 %	0 %
44											
45	Mean - 132,15						Standard deviation - 132,15				
46		KR	REF	LY	LL			KR	REF	LY	LL
47	0	3 %	14 %				0	3 %	1 %		
48	5	2 %					5	1 %			
49	6			15 %			6			3 %	
50	8				11 %		8				3 %
51	10	0 %					10	0 %			
52	14	6 %					14	6 %			
53	16			12 %			16			2 %	
54	28			11 %	12 %		28			2 %	1 %
55											
56	Mean - 241,05						Standard deviation - 241,05				
57		KR	REF	LY	LL			KR	REF	LY	LL
58	0	1 %	10 %				0	2 %	0 %		
59	5	2 %					5	0 %			
60	6			9 %			6			1 %	
61	8				10 %		8				0 %
62	10	4 %					10	2 %			
63	14	7 %					14	2 %			
64	16			10 %			16			1 %	
65	28			10 %	9 %		28			1 %	1 %

G.4. Composition of peaks found using FPLC

Table G.7 shows the assembled table of mean values and standard deviations for the composition of peaks relative to the total abundance as explained in appendix C.7.4. The m/z value = 69.2 corresponds to imidazole from the buffer, and was not part of the graphical presentation in chapter 3.4.3.

Table G.7: Mean values and standard deviations for the composition of peaks (C-F) relative to the total abundance.

	A	B	C	D	E	F
1	m/z	C	D	E	F	Comments
2	69,2	38 %	40 %	51 %	80 %	Imidazole
3	70,1	0 %	1 %	1 %	1 %	
4	76,2	5 %	0 %	0 %	0 %	
5	90	0 %	0 %	1 %	0 %	
6	104,2	9 %	2 %	0 %	0 %	
7	118,2	1 %	0 %	0 %	0 %	
8	132,2	0 %	25 %	0 %	0 %	
9	133,2	0 %	1 %	0 %	0 %	
10	151,2	30 %	0 %	1 %	0 %	
11	156	2 %	0 %	0 %	0 %	
12	193,1	2 %	0 %	0 %	0 %	
13	207,1	0 %	2 %	0 %	0 %	
14	240	0 %	1 %	0 %	0 %	
15	263,1	0 %	18 %	0 %	0 %	
16	264,1	0 %	2 %	0 %	0 %	
17	268	0 %	0 %	1 %	0 %	
18	282,1	2 %	2 %	3 %	4 %	
19	307	0 %	0 %	3 %	0 %	
20	337,1	0 %	0 %	1 %	0 %	
21	731,4	4 %	0 %	5 %	0 %	
22	732,4	2 %	0 %	2 %	0 %	
23	733,4	1 %	0 %	1 %	0 %	
24	Sum	95 %	94 %	70 %	85 %	
25	Other	5 %	6 %	30 %	15 %	