UNIVERSITY OF TROMSØ UIT

FACULTY OF BIOSCIENCES, FISHERIES AND ECONOMICS
DEPARTMENT OF NORWEGIAN COLLEGE OF FISHERY SCIENCE

Spectral changes in fillet of Atlantic salmon as affected by freshness loss and spoilage during cold storage

Izumi Sone

A dissertation for the degree of Philosophiae Doctor

Fall 2012

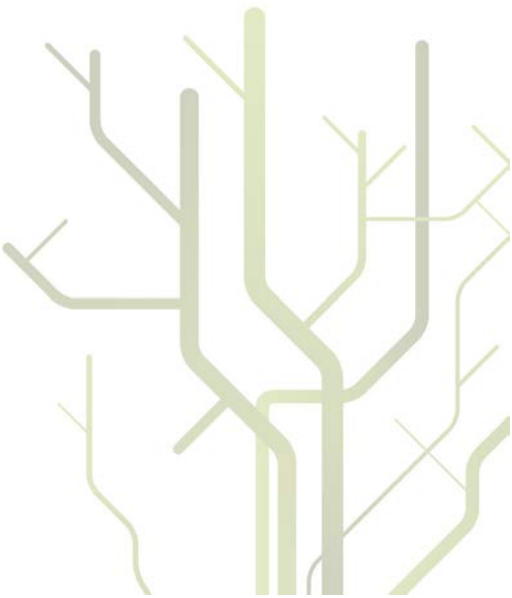

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Table of contents

List of publications

Paper I

Sone, I., Olsen, R.L., Dahl, R. & Heia, K., (2011). Visible/near-infrared spectroscopy detects autolytic changes during storage of Atlantic salmon (*Salmo salar* L.). *Journal of Food Science*, 76(3), S203-S209.

Paper II

Sone, I., Olsen, R.L., Sivertsen, A.H., Eilertsen, G. & Heia, K., (2011). Classification of fresh Atlantic salmon (*Salmo salar* L.) fillets stored under different atmospheres by hyperspectral imaging. *Journal of Food Engineering*, 109(3), 482-489.

Paper III

Sone, I., Olsen, R.L., & Heia, K., (2012). Spectral changes of Atlantic salmon (*Salmo salar* L.) muscle during cold storage as affected by the oxidation state of haem. Revised and resubmitted to *Journal of Agricultural and Food Chemistry.*

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Tromsø, September 2012

Izumi Sone

Abstract

The spectral properties of fish muscle change during storage. These spectral changes have previously been applied to predicting storage time and the quality parameters of fresh, frozen and thawed-chilled samples of different fish species. However, which post-mortem processes were causing the changes in the spectral properties of fish muscle during storage remains undetermined. The main objective of this thesis was to identify the source of spectral variations occurring in fresh salmon during storage. The specific aims were to investigate the spectral effects of microbial growth, packaging atmospheres and haem oxidation.

The storage experiment using fresh salmon fillets with and without microbial growth revealed that the presence of microorganisms and their growth had little effect on the development of spectral features prior to extensive spoilage. The spectral changes in the fillets originated from non-microbial processes such as biochemical and physical changes in fish muscle. The spectral effects of bacterial growth could only be verified at late storage when microorganisms had grown to a large number and utilised a wide range of substrates in the muscle for growth and metabolic activities. Degradation of the physical properties and microbial metabolites accumulating in the muscle may have caused spectral shifts in the spoiled fillets and contributed to separation between the spectra of fillets with and without microbial growth.

Spectra of fresh salmon fillets stored under different packaging atmospheres (air, 60% CO_2/40% N_2 and 90% vacuum) exhibited their main spectral variations in the visible region of the spectrum, especially at 606 and 636 nm. The spectral variations at 606 and 636 nm were dependent on both storage time and packaging atmosphere and allowed successful classification (>88 %) of the fillets by packaging type. Oxidised haem (met-haemoglobin and myoglobin) are known to exhibit an absorption peak around at 630 nm. A shoulder peak of unknown origin has been described at 606 nm in the spectra of cod, mackerel and salmon. The results indicated possible spectral effects of haem oxidation in fresh salmon. This was further investigated by incorporating spectra of isolated salmon haemoglobin in different oxidation states for the PCA spectral projection of the salmon fillets. A gradual increase in similarity between the spectra of air-stored salmon fillets and those of methaemoglobin was observed during storage. This was attributed to the changes in the spectral features at 636 nm. The results demonstrated that haem oxidation was the primary source of spectral variations at 636 nm in salmon fillets during air storage.

The origin of the shoulder peak at 606 nm was investigated with mince of fresh salmon with different water content during storage in air and under 100% N_2. The mince with a higher water content showed a significantly greater peak area centred at 606 nm than the mince containing less water on all storage days independently of storage atmospheres. The spectra of the two minces showed varied absorption at the water peak of 970 nm. Accordingly, the results demonstrated that the origin of the shoulder peak at 606 nm was also absorption due to water in the muscle. Furthermore, the area around the shoulder peak changed distinctively under the different storage conditions. Air storage led to a decrease in the shoulder peak area while storage under modified atmosphere resulted in an increase. The results illustrated that the visibility of the water shoulder peak in the spectrum depended on the dominant oxidation state of haem in the muscle. The predominance of haem in the deoxy form under 100% N_2 allowed the water shoulder peak to appear more distinctly while haem oxidation in air storage resulted in a decline in the visibility of the water shoulder in prior extensive spoilage.

Introduction

Atlantic salmon (*Salmo salar* L.) is the most important commercial fish species in Norway. In 2011, the export value of farmed Atlantic salmon was 29.3 billion Norwegian kroner and accounted for 55% of the total export value of fish and fishery products. Most Atlantic salmon produced in Norway are exported as gutted, whole fresh fish or fillets and a smaller amount is exported frozen (Norwegian Seafood Council, 2011). Accurate monitoring and control of product quality are important for the salmon industry to maintain global competitiveness and achieve cost reduction. Quality is a term frequently used to describe a product, yet it has no single consistent definition. Its definition depends on each individual and their expectations, which involve a multitude of features and characteristics of the particular product such as availability, safety, freshness, convenience and price. Fish and fishery products are highly perishable. Freshness is therefore an important attribute of quality. Method of catch/slaughter, post-mortem treatment and storage time and temperature determine fish freshness. Fresh fish refers to the absence of processing, for example canning, freezing, curing and cooking. Changes in the sensory characteristics such as appearance, odour/flavour and texture of fresh fish define the degree of freshness during storage (Botta, 1995; Olafsdóttir et al., 1997).

In the industry, challenges remain with conventional analytical methods of freshness assessment as they are often time-consuming and destructive. Tedious chemical/biochemical methods and microbiological analysis do not satisfy industrial requirements for on-line and at-line routine control throughout production and processing. During recent decades, spectroscopic methods, including visible/near-infrared spectroscopy and hyperspectral imaging, have become feasible as rapid and non-destructive alternatives for freshness and quality determination of various agricultural and food products including fish (Mathiassen et al., 2011; Osborne and Fearn, 1986). The spectroscopic techniques offer the possibility of

measuring several biochemical and physical properties simultaneously. In addition, these techniques will permit measurement through plastic food packaging whilst operating at speeds suitable for implementation in the production line (ElMasry and Sun, 2010; Lin et al., 2004; Nilsen and Heia, 2009). Within the field of seafood quality, correlation between storage time and spectroscopic parameters has been established in fresh, frozen and thawed-chilled samples of different origins (Bøknæs et al., 2002; Heia et al., 2003; Nilsen et al., 2002; Sigernes et al., 1998). Visible/near-infrared spectroscopy and hyperspectral imaging have been applied for determination of proximate composition (Downey, 1996; ElMasry and Wold, 2008; Wold et al., 1996) and for evaluation of freshness parameters including Quality Index Method scores and total bacterial count (Lin et al., 2006; Nilsen and Esaiassen, 2005).

Previous studies have established that the spectral properties of fish muscle change during storage. These spectral changes can be used to predict storage time and determine quality attributes related to fish freshness. However, which post-mortem processes are causing the changes in the spectral properties of the fish muscle during storage has yet to be determined. The main objective of this thesis was to identify the source of spectral variations occurring in fish muscle during storage. For this purpose, Atlantic salmon was selected due to its economic significance. The specific aims were to investigate how

1. Microbial growth (Paper I).
2. Packaging atmospheres (Paper II).
3. Haem oxidation states (Paper III).

affect the spectral properties of fresh salmon muscle during storage.

General background

1. Storage of fresh fish

Fish is one of the most perishable foods. The low content of connective tissue, high water activity in the muscle and the high post-mortem pH make the fish susceptible to microbial spoilage and fish muscle rapidly undergoes quality changes. Fresh fish degrades post-mortem due to autolysis by endogenous enzymes and bacterial growth and metabolites will finally render the fish inedible (spoilage). Lipid oxidation may also occur particularly if the fish is not well bled (Maqsood et al., 2012; Richards and Hultin, 2002). The endogenous enzymes in fish muscle are responsible for the initial freshness loss where autolysis of nucleotides reduces the desirable odours and flavours of freshly harvested fish. The structural and physical properties of fresh fish are altered by autolytic degradation of muscle proteins. Fish spoilage during cold storage is microbial and characterised by the strong off-odours and flavours of volatile metabolites from spoilage microorganisms. Species, initial microflora, handling stress, storage temperature as well as type of processing and packaging atmosphere are among factors affecting the rate and nature of quality changes in the fish muscle during storage (Ashie et al., 1996; Olafsdottir et al., 2004; Sivertsvik et al., 2002).

1.1. Autolysis

The word autolysis means “self-digestion” (Huss, 1995). Following death, the supply of oxygen to the tissues stops and energy production in the form of adenosine triphosphate (ATP) is no longer possible through normal (aerobic) respiration. The reservoir of creatine phosphate in the cells regenerates ATP only temporarily and becomes rapidly depleted. Alternatively, ATP is produced anaerobically by conversion of adenosine diphosphate (ADP) by the enzyme adenylate kinase. The third pathway by which ATP is produced is the anaerobic degradation of glycogen (Fig. 1). The end product of anaerobic glycolysis is lactic

acid which will accumulate in the muscle. The formation of lactate correlates well with the drop in post-mortem pH resulting from the hydrolysis of ATP (Foegedin et al., 1996).

Reduction of pH can interfere with the structural properties of proteins and enzyme activities in fish muscle. As the post-mortem pH approaches the isoelectric point of muscle proteins, the water binding capacity of the muscle will be reduced affecting the fish's organoleptic quality (Lawrie and Ledward, 2006). The pH drop leads to inactivation or reduced activity of enzymes adapted to near-neutral pH (e.g. glycolytic enzymes) while other enzymes with an acidic pH optimum will become more active. Well known examples of acidic active enzymes are the lysosomal proteases the cathepsins. The softening of fish flesh, colour change and increased incidence of fillet gaping may occur as a result of the pH-induced decrease in protein stability and increased activity of acidic proteases in fish muscle (Godiksen et al., 2009; Kristoffersen et al., 2006; Wang et al., 2011).

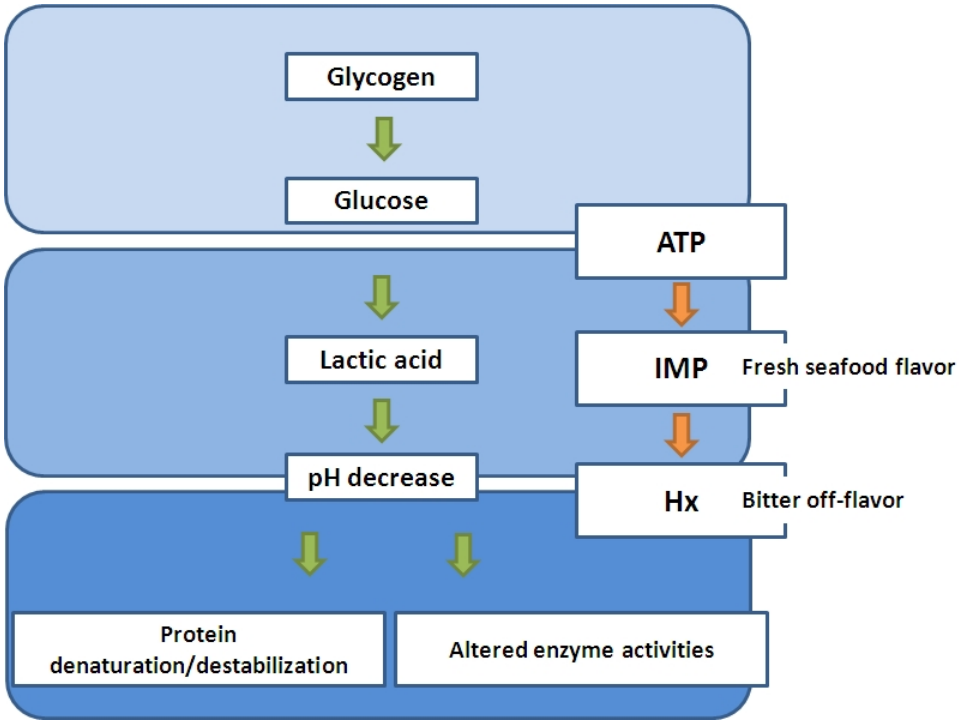


Fig. 1: Post-mortem changes in fish muscle due to autolytic degradation. Adenosine triphosphate (ATP), inosine monophosphate (IMP) and hypoxanthine (Hx). Adapted from Green (2011).

With the cessation of ATP regeneration, the ATP concentration in the muscle continues to decrease after death and the nucleotides are catabolized to yield degradation products (Fig. 1). The ADP formed from ATP is first degraded to adenosine monophosphate (AMP) by dephosphorylation. Deamination further degrades AMP to inosine monophosphate (IMP). The degradation of ATP to IMP via AMP is catalysed entirely by endogenous enzymes. In the following degradation processes, IMP is decomposed to inosine and inosine is broken down to hypoxanthine by endogenous as well as bacterial enzymes. The organoleptic importance of nucleotide degradation is associated with the initial freshness loss, where the flavour of fresh seafood is lost as the flavour enhancer IMP is decomposed to the flavourless inosine. Later in storage, bitter off-flavour may develop at increased concentrations of hypoxanthine.

1.2. Microbial spoilage

The flesh of live and newly caught fish is sterile. Microorganisms are only found on the outer surface of skin and gills and in the intestines. The initial bacterial population reflects the microbial communities of the surrounding environment. For marine temperate (cold) water fish such as salmon, most microbes present on the fish at the time of catch/slaughter are aerobic or facultative anaerobes, psychrotolerant Gram-negative bacteria belonging to the genera *Pseudomonas*, *Acinetobacter*, *Shewanella*, *Aeromonas* and *Vibrio* (Huss, 1995). During the subsequent storage after gutting, the number of microorganisms increases on the surface of skin and gills. The bacteria eventually penetrate into the fish flesh and grow exponentially following an initial lag phase. Only a fraction of the total microflora has the ability to produce volatile metabolites responsible for off-odour and flavour in spoiled fish. These microorganisms are known as specific spoilage bacteria. The *Pseudomonas* spp. and *Shewanella putrefaciens* are the specific spoilage bacteria of fresh fish stored aerobically at a chilled temperature, regardless of the origin of the fish (Gram and Dalgaard, 2002).

Microorganisms utilise non-protein nitrogen (NPN) components in fish muscle as substrates for continuous growth and metabolism. The development of fish spoilage is associated with this microbial utilisation of NPNs and subsequent metabolite production, resulting in offensive fishy, rotten off-odours and flavours. Trimethylamine oxide (TMAO), nucleotides and free amino acids are the main NPN fractions giving rise to spoilage metabolites during storage. The characteristic fishy odour of spoiled fish is attributed to bacterial reduction of TMAO to TMA by the facultative anaerobe *S. putrefaciens*. This specific spoilage bacterium is capable of using TMAO as a terminal electron acceptor in a microaerophilic and anaerobic respiration and has a growth advantage over the strict aerobe *Pseudomonas* spp. when oxygen supply becomes limited. Many of the off-odours and flavours associated with fish spoilage arise from bacterial degradation of free amino acids. Ammonia is an odour-active compound with a characteristic pungent smell and is produced when the amino acids are deaminated by bacteria to attain a carbon source for growth. The specific spoilage bacterium responsible for the intense sulphurous off-odour in spoiled fish is *S. putrefaciens* producing H_2S from the sulphur-containing amino acid cysteine. Other volatile sulphides such as methyl mercaptan and dimethyl sulphide are generated from the amino acid methionine by *S. putrefaciens* as well as *Pseudomonas* spp.. In contrast to the potent fishy, H_2S off-odours dominating spoilage by *S. putrefaciens*, little TMA and H_2S is produced by *Pseudomonas* spp.. Fruity, musty, soapy and sulfhydryl off-odours characterise spoilage by *Pseudomonas* spp., mainly resulting from production of volatile compounds such as fatty acids, carbonyls, alcohols and esters.

1.3. Modified atmosphere and vacuum packaging

Modified atmosphere (MA) and vacuum packaging have become increasingly common as the food industry attempts to meet the market's demand for fresh fish with extended shelf-life. Both MA and vacuum packaging involve removal of air from the package

to inhibit deteriorative effects of air storage on freshness and to extend the shelf-life of fresh fish. In MA packaging, air removal is followed by the addition of a single or mixed gas into an enclosed gas-barrier material. Carbon dioxide (CO_2) is often used in the gas mixture for packaging of fresh fish due to its bacteriostatic properties. Water and fat content of the fish muscle, storage temperature and the CO_2 partial pressure in the package headspace influence the solubility of CO_2 into the product and determine the bacteriostatic effect of CO_2 (McMillin, 2008). In addition to CO_2, nitrogen (N_2) can be included as a filler gas since the low solubility of N_2 in water and fat prevents packaging collapse during storage. High levels of oxygen may be used in the gas packaging of red meat and red flesh fish such as tuna to maintain the red colour of the muscle. A typical gas mixture used for packaging of fatty fish such as salmon is 40-60% CO_2 balanced with N_2 (Sivertsvik et al., 2002). For cod, which has a relatively high level of TMAO in the muscle, the optimal MA should include sufficient oxygen (e.g. 63% oxygen and 37% CO_2) to prevent the production of TMA by microaerophilic and anaerobic bacteria (Sivertsvik, 2007).

Under the oxygen limited conditions of MA and vacuum packaging, a shift in the population of specific spoilage bacteria occurs. The growth of strict aerobic, Gram-negative *Pseudomonas* spp. is strongly inhibited while the absence of CO_2 in vacuum-packaged fish may allow limited growth of the facultative anaerobe *S. putrefaciens* (Hansen et al., 2009; Rosnes et al., 2003). Gram-positive, CO_2 resistant microorganisms such as *Carnobacterium* spp., lactic acid bacteria and *Photobacterium phosphoreum* dominate the microbial flora of spoiled fish under MA and vacuum packaging (Emborg et al., 2002; Macé et al., 2012; Rudi et al., 2004; Schirmer et al., 2009). The specific spoilage bacterium of fresh cod stored under CO_2-enriched MA and vacuum has been identified as *P. phosphoreum* (Dalgaard, 1995; Dalgaard et al., 1993). The TMAO reducing ability of *P. phosphoreum* is 10-100 times the amount per cell of that of *S. putrefaciens* due to the greater diameter of the former while little

H_2S is produced during growth of *P. phosphoreum* (Dalgaard et al., 1996). Use of MA and vacuum packaging does not fully eliminate microbial spoilage. The bacteriostatic effect of CO_2 and air removal can however delay the onset of exponential microbial growth and retard the formation of spoilage volatile compounds (Wierda et al., 2006), resulting in a longer shelf life for fresh fish. In addition, packaging under MA and vacuum may be beneficial for fatty fish like salmon as the oxygen exclusion limits the occurrence of lipid oxidation. The inhibitory effect of MA on lipid oxidation is not attributed to the presence of CO_2, but to air replacement in the form of the gas mixture. Lipid oxidation may still occur even at high CO_2 partial pressures unless oxygen is fully removed from the packaging atmosphere (Giménez et al., 2002).

1.4. Haem oxidation state, colour and lipid oxidation

Colour affects consumers' purchasing decisions and product acceptance (Francis, 1995). Muscle colour may depend on the oxidation state of haem proteins and the partial oxygen pressure in the food package determines which oxidation state will be favoured during storage. Myoglobin and haemoglobin are the most abundant haem compounds in muscle. A high level of myoglobin is found in beef and poultry and the dark muscles of broiler chickens (Fox, 1966; Kranen et al., 1999). The effect of myoglobin on meat colour and colour changes during storage is well established in the literature (Mancini and Hunt, 2005). For most fish species, the amount of haem proteins in the fillet is small due to the anaerobic nature of light muscle. The concentration of haemoglobin in the fish muscle may be more substantial than that of myoglobin (Richards and Hultin, 2002). The fibre diameter of fish muscle allows better diffusion of oxygen from capillaries into the muscle cells and this makes the intracellular oxygen storage of myoglobin less necessary (Richards et al., 2005). In addition to haemoglobin, carotenoids such as astaxanthin are important components influencing muscle colour in farmed salmon.

A haemoglobin molecule consists of four polypeptide globin chains, each having an attached haem group composed of an iron atom and a protoporphyrin molecule (Fig. 2). At the centre of each haem group is the iron and four of the six coordination sites around this atom are occupied by protoporphyrin nitrogen atoms. Nitrogen from a histidine side chain in the protein structure binds to the fifth coordination site of the iron. Depending on the redox state of the haem iron, different molecules bind at the sixth coordinate site. Haemoglobin with an oxygen molecule attached at the ferrous (+2 redox state) haem iron is called oxyhaemoglobin and imparts a bright red colour. When no ligand is bound at the ferrous haem iron, haemoglobin exists in its deoxygenated form (deoxyhaemoglobin) and appears in a purple-blue colour. Brown methaemoglobin is formed when the iron atom in the haem group takes an extra positive charge upon oxidation and converts from the ferrous to the ferric state (+3). The sixth coordinate site of the ferric iron in methaemoglobin is occupied by a molecule of water.

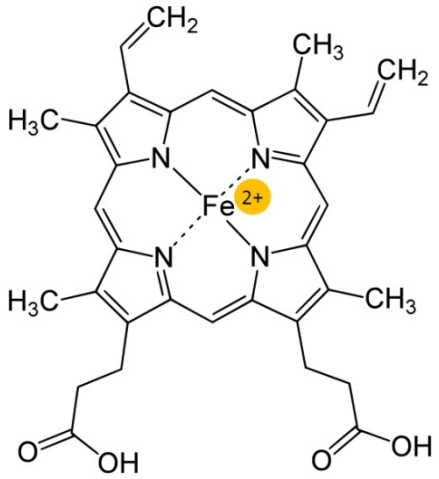


Fig. 2: A haem group of deoxyhaemoglobin carrying the ferrous iron (Fe^{2+}) at the centre and no ligand attached at the sixth coordinate site.

The surface of fish muscle immediately after cutting is characterised by the predominance of deoxyhaemoglobin which upon exposure to oxygen turns to oxyhaemoglobin. This ability of haemoglobin to bind to oxygen and convert to

oxyhaemoglobin is dependent on both intrinsic (e.g. species) and extrinsic factors (e.g. temperature, pH and oxygen partial pressure). Fish haemoglobin exhibits a low oxygen affinity compared to mammalian haemoglobin, especially at the low pH found in post-mortem fish muscle (Bohr effect) (Olsen and Elvevoll, 2011). At the start of air storage, deoxy- and oxyhaemoglobin constitute the majority of haemoglobin in the muscle. Unless high oxygen partial pressure is provided to favour oxygenation, the ferrous iron reacts with oxygen in air and becomes oxidised to the ferric iron to form methaemoglobin (Fig. 3A). The enzymatic reduction of methaemoglobin to the deoxy form is prevented in post-mortem fish muscle as the necessary cofactor (NADPH/NADH) for methaemoglobin reductase is oxidised. The drop in pH post-mortem can also inactivate the reducing enzyme systems.

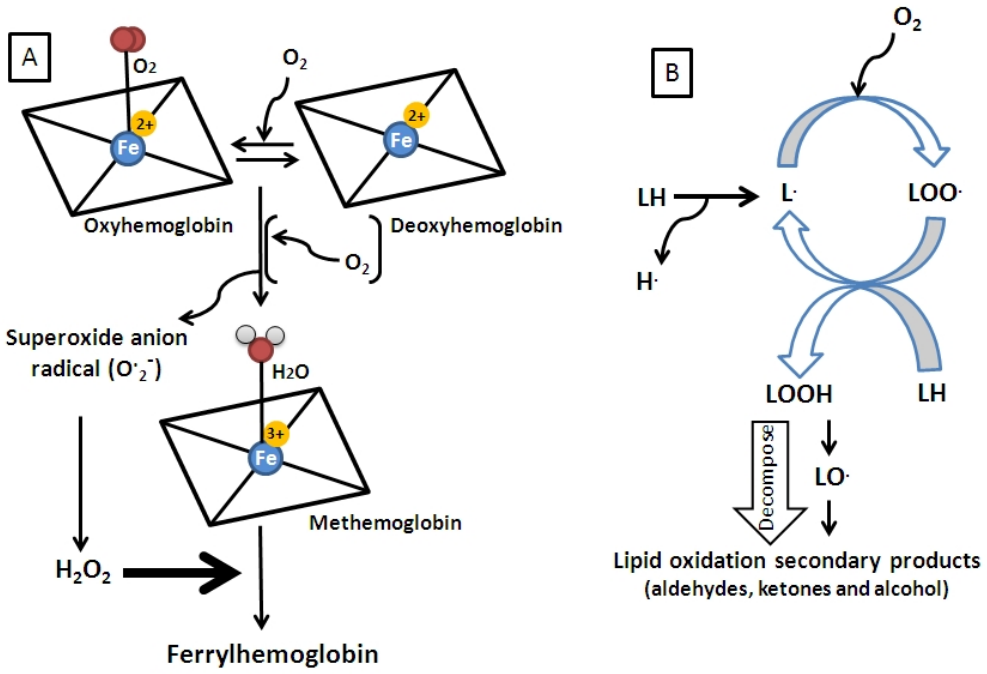


Fig. 3. (A) The schematic diagram of haemoglobin oxidation and prooxidative role of haemoglobin in lipid oxidation. (B) Lipid oxidation. A polyunsaturated fatty acid (LH), hydrogen ($H^{\cdot}$), a lipid free radical ($L^{\cdot}$), a lipid peroxy radical ($LOO^{\cdot}$), lipid peroxides (LOOH) and alkoxy radical ($LO^{\cdot}$).

Haemoglobin is a major contributor to lipid oxidation in the fish muscle (Maqsood et al., 2012). Fatty fish containing a high proportion of polyunsaturated fatty acids are especially prone to lipid oxidation. Lipid oxidation (Fig. 3B) is an autocatalytic chain reaction initiated

when a polyunsaturated fatty acid (LH) is abstracted hydrogen ($H^{\cdot}$) and a lipid free radical ($L^{\cdot}$) is released. The unstable lipid radical interacts readily with an oxygen molecule to form a lipid peroxy radical ($LOO^{\cdot}$), which then reacts with another polyunsaturated fatty acid to produce the lipid oxidation primary product lipid peroxide (LOOH). Lipid peroxides are tasteless and odourless, with no direct effect on sensory quality. However, they are further decomposed to free radicals (e.g. alkoxy radicals; $LO^{\cdot}$) and degraded to the secondary lipid oxidation products such as carbonyls and alcohols, which contribute to the off-odour/flavours of stored fish (e.g. rancidity).

Haem oxidation is an important step in the ability of haemoglobin to stimulate lipid oxidation. This occurs when the ferrous iron is oxidised to the ferric iron and the superoxide anion radical ($O^{\cdot}_2^{-}$) is liberated in this process (Fig. 3A). The dismutation of $O^{\cdot}_2^{-}$ to hydrogen peroxide (H_2O_2) and the following reaction of H_2O_2 with methaemoglobin lead to the formation of ferrylhaemoglobin, previously shown to initiate and propagate lipid oxidation (Baron et al., 1997; Everse and Hsia, 1997). The presence of haemoglobin can also catalyse the decomposition of lipid peroxides, thereby promoting the production of free radicals (e.g. alkoxy radicals) and secondary lipid oxidation products. The prooxidative activity of haemoglobin is enhanced at low pH. The post-mortem pH favours deoxygenation of haemoglobin in the fish muscle due to the Bohr effect. Increase in deoxyhaemoglobin content has been linked to enhanced lipid oxidation in fish although the exact mechanism is not fully understood (Maqsood et al., 2012; Richards and Hultin, 2000; Richards et al., 2002a). Deoxyhaemoglobin is highly susceptible to haem oxidation because of the high spin state of the iron atom inside the haem ring. It has been suggested that accelerated formation of methaemoglobin from deoxygenated haemoglobin and the subsequent increase in ferrylhaemoglobin may explain the elevated level of lipid oxidation in fish muscle (Richards et al., 2002b).

2. Methods for freshness evaluation

2.1. Traditional methods

Freshness assurance is one of the most important goals for the fish industry as freshness is closely related to quality. Sensory, chemical/biochemical and microbiological methods are conventionally used to measure the degree of fish freshness. Texture and colour analysis as well as determination of post-mortem pH also provide indications of physical changes occurring in fish muscle during storage. Sensory methods such as the Quality Index Method (QIM) may be applied in the industry due to their non-invasive approach. Appearance, smell and meat flexibility of whole, gutted fresh fish are evaluated by trained graders according to a standardised scheme. Chemical/biochemical methods involve determining the concentration of a specific compound in the fish muscle and relating the observed concentration to a level of a sensory attribute (e.g. off-odour/flavour) and freshness. The various nucleotide degradation products, spoilage volatile compounds (e.g. ammonia and TMA) and primary and secondary products of lipid oxidation are examples of compounds chemically analysed to give indications of freshness. Many standard guidelines for acceptable levels of fish freshness are based on microbiological methods such as total viable counts. A microbial load of 10^6-10^8 cfu/g is usually considered as an acceptable limit for freshness. Sensory deterioration and remaining shelf-life of fresh fish correlate well with the increasing number of SSO during storage (Alasalvar et al., 2011; Olafsdóttir et al., 1997).

Challenges remain in applying the traditional analytical methods for routine control and assessment of freshness along the production and processing lines in the industry. Labour costs and the time required for sample preparation and conducting measurement are the main factors limiting implementation. Microbiological and chemical/biochemical methods depend on destructive sampling while inconsistency and variability introduced by observer-based evaluation and the surrounding environment may be high in sensory methods. In addition,

several chemical/biochemical methods target spoilage compounds increasing at the time of sensory rejection and are only applicable to evaluate medium to poor freshness and to detect spoilage (Botta, 1995) (Fig. 4). The lag phase of bacterial growth may also limit microbiological methods in identifying the early stages of deterioration in fish freshness.

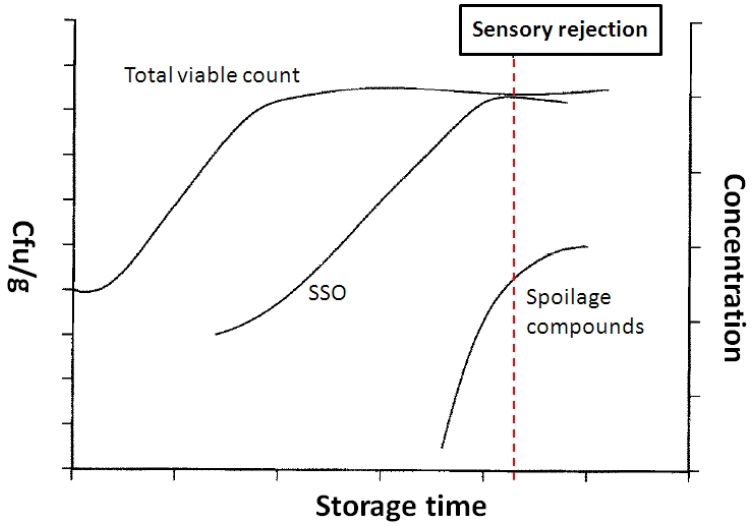


Fig. 4. Changes in total viable count, the number of specific spoilage organisms (SSO) and concentration of spoilage compounds during cold storage of fresh fish. Adapted from Gram and Huss (1996).

2.2. Rapid spectroscopic methods

2.2.1. Visible/near-infrared spectroscopy

Online control and evaluation of fish freshness in the industry require rapid and non-destructive instrumentation. Light spectroscopy such as visible/near-infrared (VIS/NIR) spectroscopy has gained attention during recent decades as an alternative to the conventional methods of freshness determination. Measurements based on VIS/NIR spectroscopy require little or no sample preparation and results can be made available for analysis within minutes or seconds. The ability of NIR light to penetrate through plastic food packaging may also facilitate online and at-line implementation of VIS/NIR spectroscopy in the industry (Lin et al., 2004; Osborne and Fearn, 1986). Another attractive feature of VIS/NIR spectroscopy is that it allows simultaneous determination of biochemical as well as physical parameters of

fish muscle related to freshness. Freshness is not a single attribute, but rather consists of many properties and characteristics. The VIS/NIR spectra may therefore provide a more detailed and refined representation of freshness in fish samples than the traditional methods.

Light spectroscopy is based on the interaction between light and matter. Light is a form of electromagnetic radiation with specific wavelengths and propagated as waves and as particles (photons). Light in VIS/NIR spectroscopy constitutes the visible (400-750 nm) and near-infrared (750-2500 nm) regions of the electromagnetic spectrum. All organic matter consists mainly of atoms, of carbon, oxygen, hydrogen and nitrogen combined by chemical bonds to form molecules. The nature of bonds and electrostatic charges on the atoms and molecules keep the molecules in constant motion, called the ground energy state. Different molecules have characteristic energy vibrations such as stretching and bending and each vibration has its specific frequency depending on the structure and composition of the molecule.

The energies of the molecules are quantized. This means that the molecules only exist within certain energy levels and can transit between these levels through interactions with light. The energy of photons is related to the wavelength at which the light is emitted. Photons emitted in the visible region have energy to excite and raise the energy level of bound electrons in the molecules. The photons in the NIR light carry energy corresponding to the frequencies of molecular vibration. The molecules absorb photons at specific wavelengths in the NIR region where the frequency of the photons is coincident with the vibrational frequency of the specific molecule. When a molecule absorbs photons, the ground state of the molecule is elevated to a higher energy level. Energy transition from the ground state to the first excited vibrational state is called the fundamental vibration and occurs in the mid-infrared region. Depending on the amount of energy in the photon, the molecule can reach the second or third energy levels and create the first, second and higher overtones of fundamental

vibrations in the NIR region. Two or more molecular vibrations can be excited simultaneously and appear as combination bands. Physical interactions between different molecules may also alter states of vibrational energy and result in a shift in existing absorption bands or give rise to new bands in the spectrum. A NIR absorbance spectrum is therefore a direct consequence of the chemical composition and physiochemical states in a biological sample and often referred to as a fingerprint spectrum.

Modes of operation of VIS/NIR spectroscopy include reflectance, transmission and interactance (Fig. 5). A reflectance mode consists of a light source and detector unit which are placed on the same side of the sample. The reflected light is measured at a 45^{o} angle to the incident beam. In transmission mode, the light source and detector unit are positioned on the opposite sides of the sample which is placed between the two components. Light is transmitted from beneath and passes through the sample before it reaches the detector unit. The disadvantage with transmission is that it is prone to variation due to sample thickness. In interactance, the light source and detector unit are positioned on the same side, as in reflectance, but light is transmitted into the sample then back-scattered to the surface. Interactance mode is suitable for analysis of biological samples of uneven thickness such as fish fillet. The reduced intensity variation due to sample thickness in interactance is attributed to a constant optical path length of the light interacting with the samples (Sivertsen, 2011).

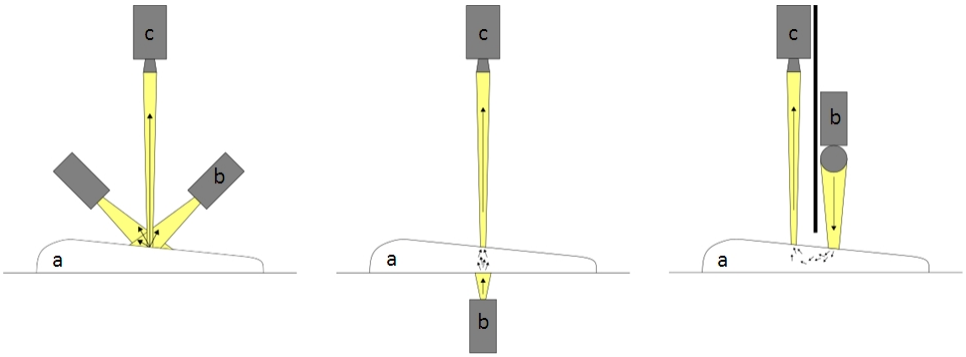


Fig. 5: Three modes of operation. Left: Refection. Middle: Transmission. Right: Interactance. The sample (a) is illuminated by the light source (b) and the detector unit (c) collects the reflected, transmitted and interacted light, respectively. Borrowed from Agnar Sivertsen, Nofima.

2.2.2. VIS/NIR hyperspectral imaging

In addition to traditional VIS/NIR spectroscopy, a new technique referred to as hyperspectral imaging (HSI) has been developed. This technique integrates conventional imaging and VIS/NIR spectroscopy. This allows simultaneous acquisition of spatial and spectral information from an object. A hyperspectral image is known as a hypercube consisting of a data block with three dimensions, two spatial (x, y) and one spectral (λ) (Fig. 6). Each spatial position (x_i, y_i) on a hyperspectral image contains a spectrum. Each value in the spectrum of the pixel (x_i, y_i) corresponds to a light intensity obtained at a given wavelength (λ_i). While traditional spectroscopy relies on relatively small point-source measurements, the advantage of HSI lies in its ability to characterise biochemical and physical properties of a sample at any specific region of interest, based on the spectra obtained at the particular position of the image of the sample. As with conventional spectroscopy, operation modes such as reflectance, transmittance and interactance can be applied in HSI. Hyperspectral imaging for food analysis is usually carried out in the VIS-NIR (400-1000 nm) or the NIR (1000-2500nm) region (Gowen et al., 2007).

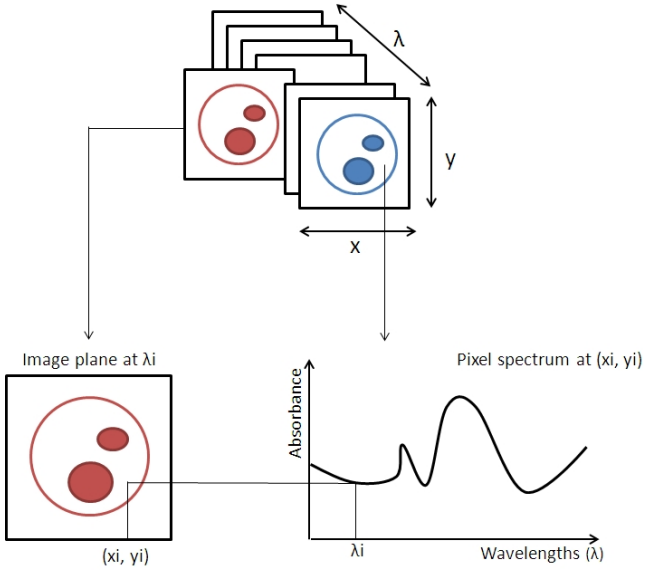


Fig. 6: A hyperspectral image (hypercube). Adapted from Gowen et al. (2007).

2.3. Spectral Analysis

2.3.1. Pre-treatment

One major prerequisite for using VIS/NIR spectroscopy and HSI for food analysis is that spectra reflect biochemical composition and physical properties of a sample of interest. Considerable variations can be introduced into the spectra due to experimental factors and the complex and heterogeneous nature of biological samples. These effects can influence spectral signals and obscure acquisition of relevant information in the spectral data. Different mathematical correction methods can be applied to pre-treat raw spectra and remove redundant background noise and unwanted variability. Examples of pre-treatment can be normalization (Griffiths, 1995), derivation (McLure, 1993, 1994), multiplicative scatter correction (Geladi et al., 1985), standard normal variate and de-trending (Barnes et al., 1989) or a combination of these treatments.

2.3.2. Multivariate analysis

Measurements by VIS/NIR spectroscopy and HSI produce an extensive dataset containing a large number of spectral variables. Studying each variable independently of the others, referred to as univariate analysis is not sufficient for complex spectral datasets where important information may be found in the covariance of the variables. Multivariate analysis such as principal component analysis (PCA) and partial least squares regression (PLS) are common methodologies employed in combination with the spectral data.

Principal component analysis

The main objective of principal component analysis (PCA) is to identify the main variation in a spectral data matrix X by reducing the high dimensionality of the spectral data into a smaller set of new variables called principal components (PC). Assume spectra collected for N number of samples, where each spectrum is composed of *J* number of features

(wavelengths). Each feature defines a coordinate axis in the *J* dimensional descriptor space. The sample *i* is described by a point, with the coordinates corresponding to the obtained values at each of the *J* wavelengths. This creates a swarm of points representing N samples in the *J* dimensional descriptor space (Fig. 7). The next step is to find a direction through the points that accounts for the largest variation within the distribution of the N samples in the descriptor space. This vector is called the first principal component (PC1). The PC1 vector is projected so that it is anchored in the average point of the samples. All the points *i* in the descriptor space can be redefined by the distance of orthogonal projections *i*' to this average point on PC1 and expressed as a new set of coordinates called scores t_{1i}, i = 1, 2, 3, …, N. After extraction of the variation in PC1, the largest variation left in the data matrix can be described in the second principal component (PC2). The direction of the PC2 vector is orthogonal to PC1 and projected through the average point. As in PC1, the data points in the descriptor space are redefined as the corresponding scores t_{2i} on PC2. This may be repeated until enough variation in the spectral data is described (e.g. elbow point in a plot showing explained variance in X).

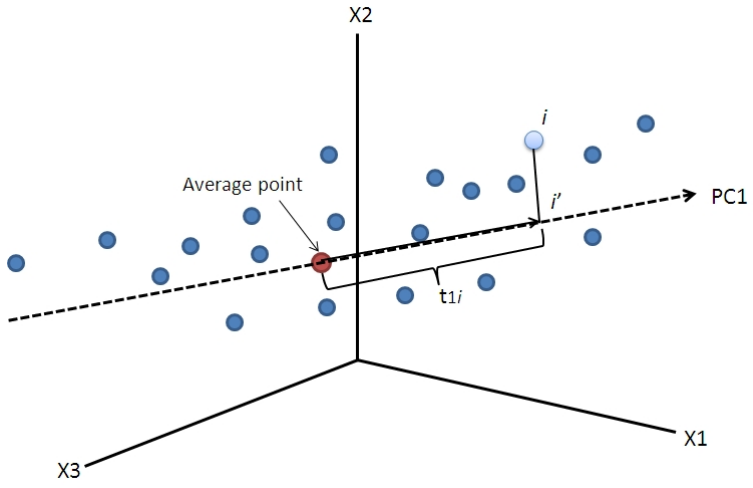


Fig. 7: Projection of data points (blue dots) along the first principal component vector PC1 in the descriptor space constructed of three variables X_1, X_2 and X_3. The score of the sample *i* in PC1 is denoted as t_{1i}. Adapted from Carlson and Carlson (2005).

The corresponding scores of samples can be plotted onto a new co-ordinate system called a score plot where the PC1 is the first axis, PC2 the second axis and so on. In this way, the higher dimensionality of the spectroscopic data can be reduced down to a lower dimensional plane spanned by the PCs while describing as much as possible of the systematic variations in the original data. The score projection in the new co-ordinate system can portray similarities and dissimilarities between samples. Samples with similar spectral properties will have similar score values and may be visualized as a cluster in the score plot while those with different spectral properties will be projected apart from each other. The angle at which the PC vector is tilted towards each of the *J* feature axes in the descriptor space is called a loading. The loading describes the extent to which each feature (wavelength) contributes to determining the direction of the PC vector. The larger the absolute value of a loading is at a given wavelength, the greater the influence of spectral variations occurring at the respective wavelength is on the corresponding PC. In cases where samples are grouped in a separate cluster along one of the PCs in the score plot, inspection of the loading for this PC will indicate at which wavelength the largest spectral variation occurred in the samples.

Partial least squares regression

The aim of partial least squares regression (PLS) is to establish a relationship between the spectroscopic data (X) and one or more measured reference values (Y). Determined by the quantitative relation between the two matrices, it is possible to predict a Y value of an unknown sample based on its spectral values. The X and Y matrices are modelled simultaneously by linear models, where the two matrices are factorized into a score matrix T and U and a loading matrix P and C in addition to some fitting errors. Because of the high dimensionality of the X and Y matrices, PLS is designed to find a relationship between T and U in a lower dimensional space. This is called inner relation and describes correlation between the spectral data (X) and the measured reference value(s) (Y). The projection

directions (PLS components) are obtained so that correlation between X and Y is maximized. After fitting the first PLS component, systematic variation left in the matrices is described by a second PLS component and so on. The score matrix T and U can be displayed in a plot constructed on the PLS components to examine the variation in each matrix. The loading plot based on the P matrix illustrates the degree of contribution each X variable (wavelength) makes to a PLS component as in PCA loadings. The C loading plot is constructed as the projection of the Y variables onto the T score plot. It can be used to examine the relationship between the X and Y variables based on the location of the projected Y points in the plot.

The quality of the model (i.e. how well the model would perform when applied to predict new, unknown samples) is evaluated through validation. Model validation can be performed by setting aside a portion of the dataset for testing (test set). On the remaining dataset, PLS is applied to estimate the parameters of the underlying model and X values of the test set are fed into the model to predict their Y values. The predicted Y values of the test set are then compared to the measured Y values to compute the prediction error. Alternatively, full cross validation can be used when the number of samples is small. In full cross validation, a sample is left out of the dataset and PLS is applied to the remaining samples. The Y value of the omitted sample is predicted by the model and the prediction error between the predicted and measured values is calculated. This procedure is repeated until each sample has been omitted.

Validation yields the mean square of prediction errors (residual variance). Complementary to residual variance is explained variance, expressed as a percentage of the original variations in the dataset which are explained by the model. The residual and explained variances can be used to determine the optimal number of PLS components. The fraction of total residual and explained variances decrease and increases with additional model components respectively, but this stagnates when the optimal number of PLS

components has been reached. The model with too many PLS components may bear the risk of overfitting where the model includes components describing noise and uninformative variations that are irrelevant for Y. The overfitted model may perform poorly if it is applied to predict a new set of samples. In addition to the validation variances, R^2 may be used to evaluate the predictive ability of the model. Also known as the coefficient of determination, R^2 is computed as 1-(residual variance) and normally ranges between 0 and 1. The R^2 value equal to 1 implies that the model provides perfect predictions of samples in the test set. Root mean square error of prediction (RMSEP) is a measure of uncertainty on future predictions by the model. Expressed in the same units as in original Y, RMSEP is interpreted as the expected mean of prediction error if the model is to be applied to predict Y values of new samples.

In PLS, the Y values are approximated by a linear combination of the values of the X variables and the coefficients of that combination are called regression coefficients. Regression coefficients can be used to check the importance of each X variable (wavelength) in modelling Y. In prior PLS modelling, X and Y variables can be standardized independently to give all variables an equal chance to influence the model regardless of their original variance. A common example of standardization is to divide each variable by its standard deviation. Large absolute coefficient values indicate the importance of variations occurring at the respective wavelengths for modelling Y while values close to zero indicate little contribution from the particular wavelengths. The significance of each X variable can be determined by a modified Jack-knifing method based on cross validation (Martens and Martens, 2000). During full cross-validation, a set of regression coefficients β_i is estimated at the respective wavelengths each time PLS is applied by leaving a single sample *i* out of the dataset $i = 1, 2, 3\ldots N$. The sum of the squares of the difference between the regression coefficients β_i and the regression coefficient β for the total model expresses the variance of β

at each wavelength. A t-test can be performed relative to the square root of variance, giving the significance level of the regression coefficient.

2.4. Applications of spectroscopy for evaluation of freshness and other attributes

Light spectroscopy was suggested to be a rapid method for evaluation of fish freshness for the first time by Ólafsdóttir et al. in 1997. A year later, the potential of VIS/NIR spectroscopy was demonstrated in fresh cod fillets on ice whose storage time was predicted with an error of less than 30 hours (Sigernes et al., 1998). The VIS/NIR technique was later also shown to be feasible for storage prediction in iced salmon fillets (Nilsen et al., 2002). This study identified the upper loin of the fillet as the area for extracting a best prediction model for storage time. Thawing and packaging with modified atmosphere (40% CO_2/40% N_2/20% oxygen) did not hinder NIR spectroscopy in estimating the duration of storage in minced cod fillets (Bøknæs et al., 2002). The viability of VIS spectroscopy was also proven for predicting the storage time of chilled and frozen hake fillets with an error of 23 hours and 1.6 month, respectively (Heia et al., 2003).

In these early years of research with VIS/NIR spectroscopy, correlation between storage time and spectroscopic parameters was established in fresh, frozen as well as thawed-chilled whole fish and fillets of several fish species. Nilsen and Essaiassen (2005) extended the potential application of VIS/NIR spectroscopy to predict changes in the QIM scores during iced storage of whole gutted cod. The study demonstrated that VIS/NIR spectroscopy was applicable for the determination of freshness attributes other than storage time and robust enough to deal with possible sample variations due to different capture methods. Physical attributes such as water holding capacity and muscle texture have also shown reasonable correlation with VIS/NIR spectroscopy (Bechmann and Jørgensen, 1998; Isaksson et al., 2001), illustrating the ability of the spectroscopic technique to perform rapid and non-

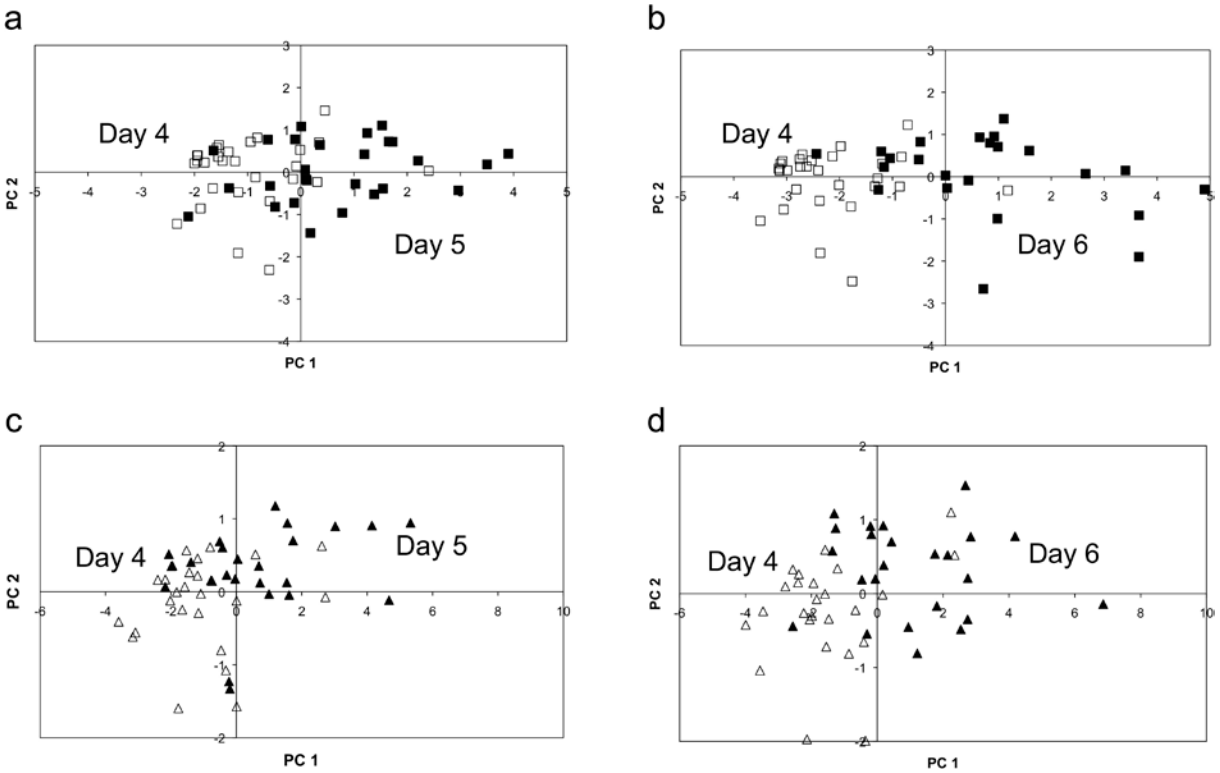
destructive characterisation of fish samples. In addition, spectra-based assessment of freshness parameters was attempted by VIS and shortwave NIR spectroscopy to predict microbial load (total viable count) in minced rainbow trout (Lin et al., 2006). Colour changes in fresh salmon and mackerel fillets were also investigated by VIS/NIR spectroscopy during storage under different atmosphere conditions (Ottestad et al., 2012; Ottestad et al., 2011).

Originally developed for remote sensing, HSI has emerged in recent years as a powerful analytical tool for non-invasive food analysis. Referring to the use of HSI within the field of seafood, fewer studies have been reported in the literature for determination of storage time and freshness parameters. Previous examples of HSI application on fish and fishery products include detection of parasites (Heia et al., 2007; Sivertsen et al., 2011a; Stormo et al., 2007) and determination of proximate composition in fish fillet (ElMasry and Wold, 2008; Segtnan et al., 2009; Wold et al., 2006). Recently, the feasibility of HSI to differentiate between fresh and thawed cod fillets and to predict storage time has been demonstrated (Sivertsen et al., 2011b).

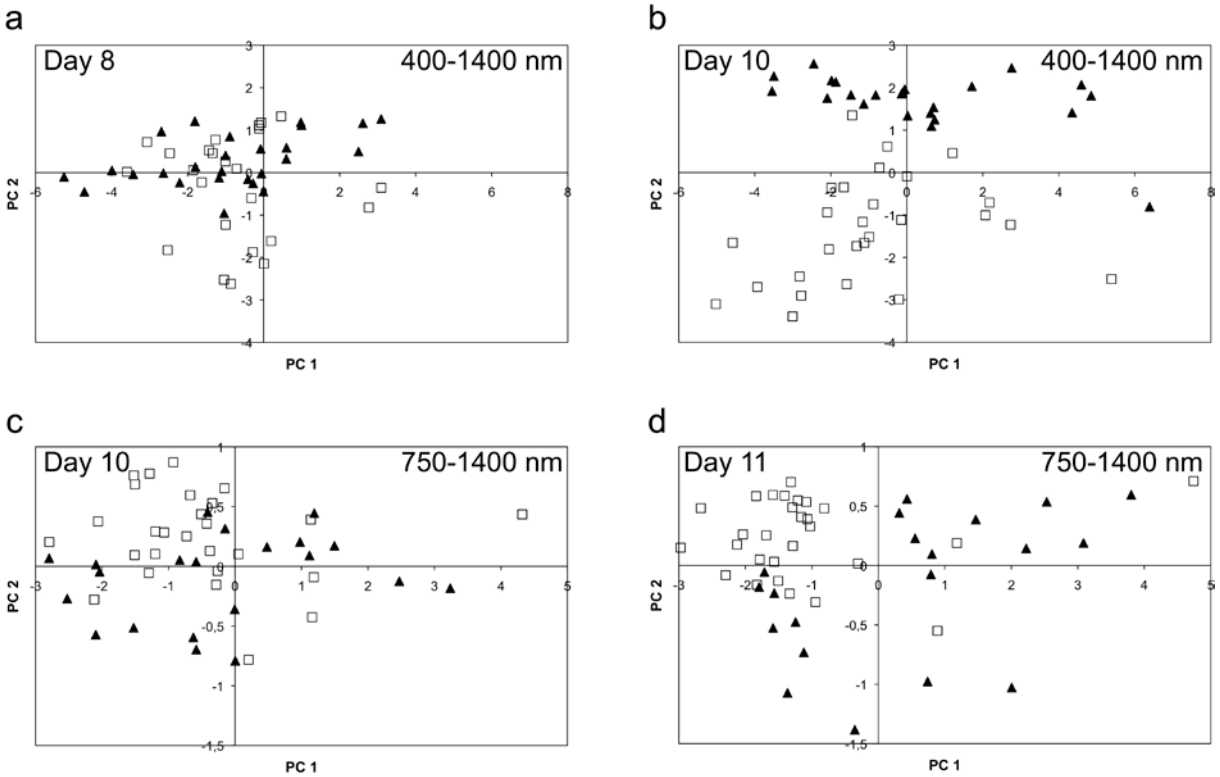
Main results and discussion

Based on the aims presented in the Introduction, the experiments were carried out to investigate the spectral effects of bacterial growth (Paper I), packaging atmospheres (Paper II) and haem oxidation states (Paper III) in fresh salmon during storage. In Paper I, a storage experiment was conducted with post-rigor fillets of fresh salmon after treating half of the fillets with the antimicrobial agent NaN_3 (treated). The other half retained its normal microbial growth (control). Thereafter, VIS/NIR spectra of treated and control fillets were obtained during storage under conditions accelerating spoilage. In Paper II, hyperspectral interactance images of pre-rigor salmon fillets were collected during storage under three different atmospheres (air, 60% CO_2/40% N_2 and 90% vacuum) at 4 ^oC. In Paper III, absorption spectra of isolated salmon haemoglobin in different oxidation states were obtained at post-mortem pH of salmon fillets from Paper II (mean pH 6.1) and incorporated into PCA for the spectral projection of the same fillets. In addition, minces with different water content were prepared from post-rigor salmon fillets and analysed by HSI during storage in air and under modified atmosphere (100% N_2).

Spectral changes occurred in the salmon fillets regardless of the presence of microbial growth (Paper I, Figure 3). The changes were detectable in the spectra of the control and treated fillets within a single day after filleting (day 4 post-mortem). At this time of storage (day 5), microorganisms still remained in the lag phase of growth in the control fillets and no microbial growth was detected in the treated fillets. The fillets with and without microbial spoilage were spectroscopically distinguishable only after the control fillets suffered extensive microbial spoilage (10^8-10^9 cfu/g, i.e. day 10 post-mortem) (Paper I, Figure 4/5).



Paper I, Figure 3: Score plots in PCA for the control fillets at day 4 (□) compared to day 5 (a) and to day 6 (b) (■) post-mortem in the wavelength range 400 to 1400 nm. Score plots in PCA for the treated fillets at day 4 (Δ) compared to day 5 (c) and to day 6 (d) (▲) post-mortem in the wavelength range 400 to 1400 nm.



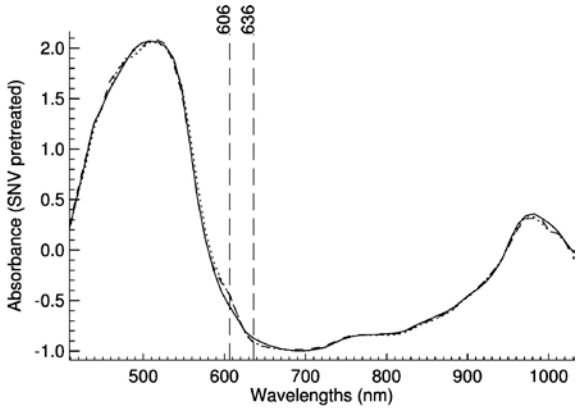
Paper I, Figure 4/5: Comparison of the control (□) and treated fillets (▲) in PCA at day 8 (a) and 10 (b) post-mortem in the wavelength range 400 to 1400 nm. Comparison of the control (□) and treated fillets (▲) in PCA at day 10 (c) and day 11 (d) post-mortem in the wavelength range 750 to 1400 nm excluding the visible region 400 to 750 nm.

Perhaps because microorganisms constitute a major limiting factor for the shelf-life of fresh fish, spectral changes occurring in the fish muscle have been related to the growth of microorganisms during storage. Lin et al. (2006) suggested that VIS and short-wavelength NIR spectra could distinguish between fresh and stored rainbow trout when the difference in the microbial load exceeded 1 log cycle after 5 days of storage at 4 ^{o}C. Similar results had been obtained earlier with fresh chicken meat (Lin et al., 2004). However, Lin et al. (2004; 2006) did not study the spectral changes in samples where microbial growth had been inhibited. The log difference in the microbial load between the control and treated fillets in Paper I exceeded 5 before their spectra could be separated (Paper I, Figure 4/5). Our results were similar to those reported recently by Alexandrakis et al. (2012) who showed that the spectra of fresh and stored chicken breasts could only be differentiated after the aerobic count in the stored samples reached over 10^9 cfu/g. The source of the spectral changes in control as well as treated samples must have been non-microbial, biochemical and physical processes in the muscle prior to the extensive spoilage in the control fillets.

Paper I demonstrated that the main spectral changes in salmon fillets were not related to the growth of microorganisms during storage. The spectral effects of bacterial growth could only be verified at late storage when microorganisms had grown to a large number and utilised a wide range of substrates in the muscle for growth and metabolic activities. Degradation of the physical properties and microbial metabolites accumulating in the muscle may have caused spectral shifts in the spoiled fillets and contributed to separation between the spectra of fillets with and without microbial growth.

Important spectral variations that occurred in fresh salmon originated in the visible region of the spectrum, particularly at 606 and 636 nm during storage (Paper I and II). The main spectral differences between the fillets with and without microbial spoilage appeared in the visible region (400-750 nm), reflecting microbial degradation in the spoiled fillets at late

storage (Paper I). Changes in the visible region in the spoiled samples were also observed earlier than the changes in the NIR region (Paper I, Figure 4/5). Moreover, it was mainly in the visible region of the spectrum that fresh salmon fillets developed distinctive absorption features during storage in air, under modified atmosphere (60% CO_2/40% N_2) and 90% vacuum (Paper II, Fig. 2b). Especially, changes occurring at 606 and 636 nm accounted for substantial spectral differences between the samples and allowed successful classification (>88 %) of fillets by packaging type. The spectral variations at 606 and 636 nm were dependent on both storage time and packaging atmosphere (Paper II).

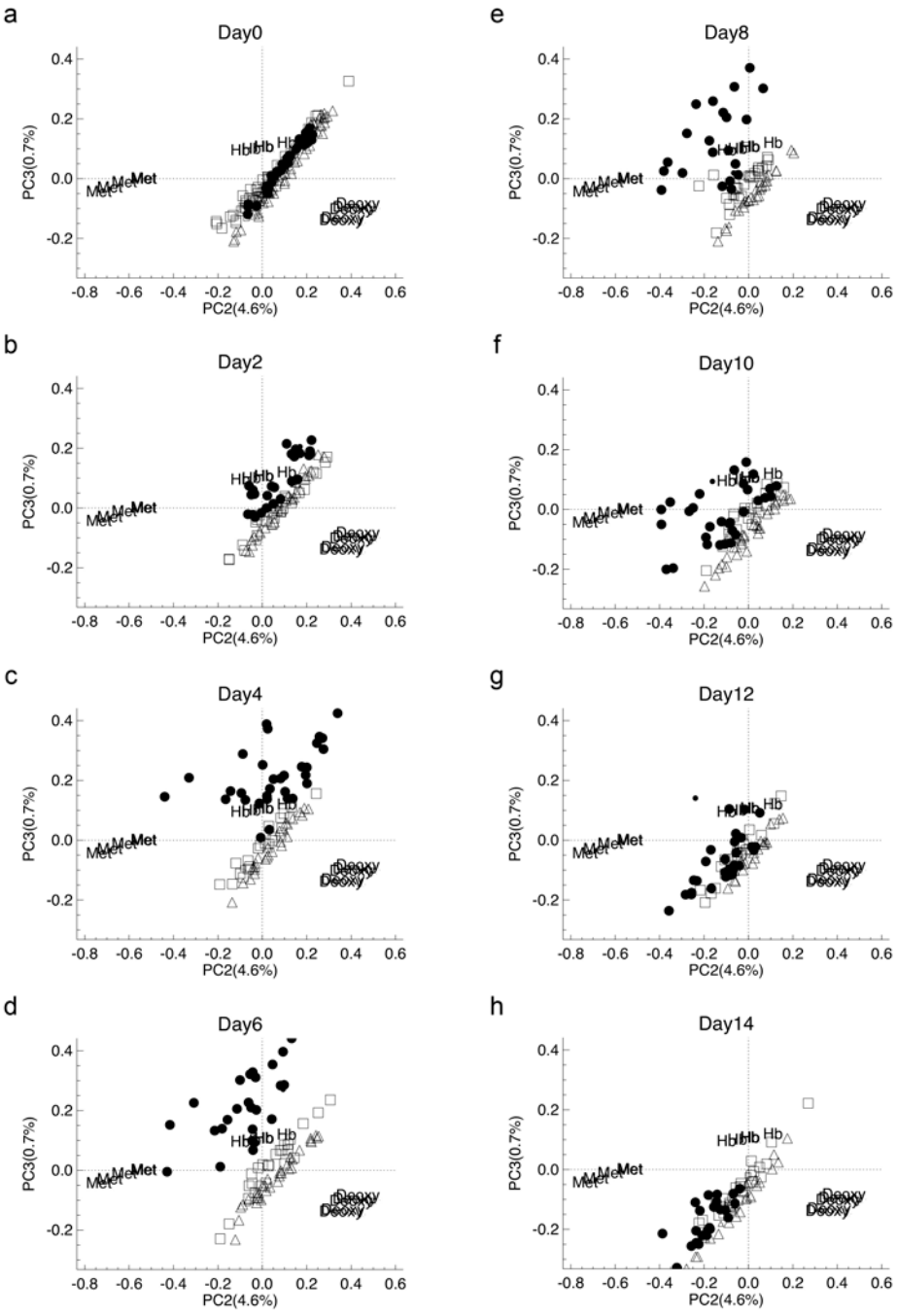


Paper II, Fig. 2b: Mean spectra of fresh salmon fillets stored in air (solid), under modified atmosphere (60% CO_2/40% N_2) (dotted) and vacuum (dashed) after 4 days of storage. The interactance spectra were natural log transformed to the absorbance $A(\lambda,x,y) = -\ln I(\lambda,x,y)$ and pre-treated by SNV.

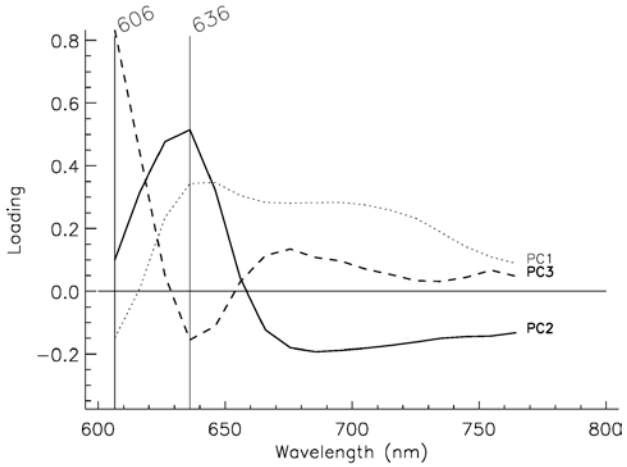
In agreement with these findings, the best prediction model for storage time of fresh cod has been obtained using the visible region of the spectrum (Nilsen and Esaiassen, 2005; Nilsen et al., 2002). A large part of spectral variations in cod fillets occurred in the spectral features at 606 and 646 nm during cold storage (Sivertsen et al., 2011b). The results in Paper II corresponded well with the spectral changes at 606 and 632 nm observed by Ottestad et al. (2011) in fresh salmon and mackerel fillets after 6 days of storage in air, carbon monoxide and under vacuum.

The absorption at 636 nm was greater in the mean spectrum of air-stored fillets than the spectra of fillets under modified atmosphere and vacuum after 4 days of storage (Paper II, Fig. 2b). An increased absorption has been observed at around 630 nm in the spectra of tuna (Viriyarattanasak et al., 2008), mackerel (Ottestad et al., 2011) and cod fillets (Sivertsen et al., 2011b) during air storage. Oxidised haem (met-haemoglobin and -myoglobin) is known to exhibit an absorption peak at wavelengths around 630 nm (Millar et al., 1996; Olsen and Elvevoll, 2011; Zijlstra and Buursma, 1997). Sivertsen et al. (2011b) proposed that oxidation of haem proteins may explain most spectral variations in cod fillets appearing in the visible region of the spectrum during storage. For salmon, the concentration of haem proteins in the muscle has been assumed to be small and their spectral contribution supposedly minimal compared to the dominant influence of astaxanthin. However, several authors have recently presented findings suggesting a contribution of haem proteins particularly that of haemoglobin to the colour change of salmon muscle during storage (Bjørlykke et al., 2011; Heia et al., 2012). Indications of a possible spectral effect of haem oxidation in salmon were first presented by Ottestad et al. (2011) based on a PCA model constructed with the spectra of salmon fillets and bovine myoglobin in different oxidation states.

Paper III established the spectral effects of haem oxidation in fresh salmon (Paper III, Figure 2a-h). A gradual increase in similarity between the spectra of air-stored salmon fillets and those of salmon methaemoglobin was observed in PCA, resulting from changes in the spectral features at 636 nm (Paper III, Figure 3).



Paper III, Figure 2a-h: The spectral projection of salmon fillets stored in air (filled circle), under modified atmosphere (square) and vacuum (triangle) during storage from day 0 to day 14 (a-h) post-mortem. The spectra of isolated salmon haemoglobin and the two derivatives (methaemoglobin and deoxyhaemoglobin) are labelled as Hb, Met and Deoxy, respectively. The PC2 and PC3 separated the spectra of haemoglobin in the three oxidation states.



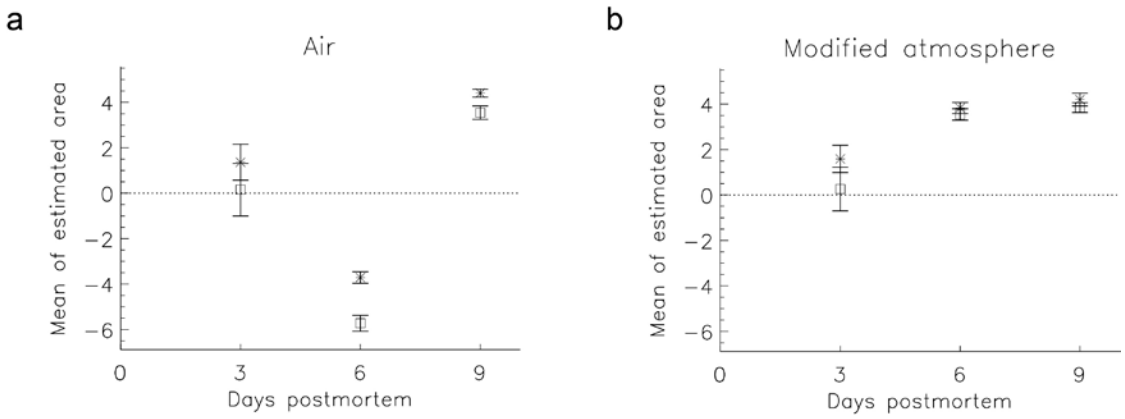
Paper III, Figure 3: Loading plot of PC 1 (dotted), PC2 (solid) and PC3 (dashed) obtained from PCA analysis of spectra of salmon fillets and isolated salmon haemoglobin in the different oxidation states.

Fillets stored under modified atmosphere and vacuum did not show spectral shift in the direction of methaemoglobin in PCA (Paper III, Figure 2a-h). Since modified atmosphere and vacuum packaging must have allowed little haem oxidation in the fillets (McMillin, 2008; Shikama, 1998), spectral changes at 636 nm contributed to separating the spectra of the air-stored salmon from those under the oxygen limited conditions (Paper II and III, Figure 2a-h). The results in Paper II and III established that haem oxidation was the primary source of spectral variations occurring at 636 nm in the air-stored salmon fillets. The greater absorption at 636 nm in the air-stored fillets compared to the fillets under modified atmosphere and vacuum originated from the formation of oxidised haem in haemoglobin (methaemoglobin) during air storage.

Storage resulted in a lower absorption at 606 nm in the spectrum of air-stored fillets than that of fillets under modified atmosphere and vacuum after 4 days (Paper II, Fig. 2b). A distinct shoulder peak has been described at 606 nm in the spectra of cod, mackerel and salmon (Ottestad et al., 2012; Ottestad et al., 2011; Sivertsen et al., 2011b). This illustrates that the shoulder peak is not related to astaxanthin in salmon muscle. The origin of the peak is not of haem as it does not correspond with the absorption characteristics of haem in any

oxidation states. Sivertsen et al. (2011b) suggested that the peak could arise from water in the fish muscle as water exhibits a shoulder at similar wavelengths (Pope and Fry, 1997).

The results in Paper III demonstrated that the origin of the shoulder peak at 606 nm was absorption due to water in the salmon muscle. Salmon mince with higher water content (66.5%) showed a significantly greater peak area centred at 606 nm than the mince containing less water (59.0%) on all storage days independently of packaging type (air or 100% N_2) (Paper III, Figure 5).



Paper III, Figure 5: Mean and standard deviation of the peak area centred at 606 nm in the mince with high (asterisk) and low (square) water content on day 3, 6 and 9 of storage in air (a) and under 100% N_2 (b).

The difference of 7.5% in water content was large enough to generate a spectral variation between the two minces at approximately 970 nm, where water is known to show absorption in the spectrum (Osborne and Fearn, 1986). Pope and Fry (1997) demonstrated that water exhibits a shoulder peak at approximately 600 nm. Accordingly, the greater area around the shoulder peak at 606 nm could also be explained by the higher water content in the mince.

Furthermore, the area around the shoulder peak changed distinctively under the different storage conditions (Paper III, Figure 5). Air storage from day 3 and 6 post-mortem led to a decrease in the shoulder peak area while storage under modified atmosphere resulted

in an increase. Absorption at the shoulder peak at 606 nm has previously been reported to decrease over time during air storage (Ottestad et al., 2012). The opposite correlations between 606 and 636 nm to the overall spectral variations were observed in the salmon fillets during storage in air, under modified atmosphere and vacuum (Paper II). A similar pattern of reverse correlation occurred between 606 and 646 nm in PLS developed to predict storage time of cod fillets on ice (Sivertsen et al., 2011b). The results indicated a relationship of the spectral variation occurring at 606 nm to haem oxidation. With the increase in oxidised haem during air storage, absorption at 636 nm consequently increases while the shoulder absorption at 606 nm may decrease or disappear.

When extensive spoilage was expected in the air-stored mince (day 9), the peak area increased to a value comparable to that under modified atmosphere. These findings were similar to the pattern of spectral development observed in the PCA projection of salmon fillets after day 8 post-mortem (Paper III, Figure 2a-h). The spectra of spoiled air-stored fillets ($>10^8$ cfu/g) moved away from the spectra of methaemoglobin and towards the spectra of fillets under modified atmosphere and vacuum. This spectral development at late storage was largely attributed to the variations occurring at 606 nm (Paper III, Figure 3). Both results illustrated that the absorption features at 606 nm in the air-stored samples changed at this time of storage and resembled more closely those under the oxygen limited condition.

The shoulder peak area increased under 100% N_2 where most haem in the mince probably existed in its deoxy form. The similar increase observed in the air-stored mince on day 9 may have resulted from the formation of deoxygenated haem by the activity of microorganisms. With a high microbial load expected in the air-stored mince at late storage, microorganisms such as *Pseudomonas* spp. are capable of converting oxidised haem to the deoxy form (Motoyama et al., 2010). The bacterial haem conversion may have induced

changes in the spectrum, particularly in the absorption features at 606 nm and contributed to the increase in the shoulder peak area of the air-stored mince on day 9.

The underlying water absorption at 606 nm most likely remained unchanged in the spectrum as storage and packaging did not affect the water content of the mince. The change in the haem oxidation state due to storage and packaging led to the change in the absorption features at 606 nm, causing the underlying water shoulder peak to appear differently in the spectrum. The results in Paper III illustrated that the visibility of the water shoulder peak in the spectrum depended on the dominant oxidation state of haem in the muscle. The predominance of deoxyhaem under modified atmosphere allowed the water shoulder peak to appear more distinctly in the spectrum while generating little change in absorption at 636 nm. The formation of oxidised haem (methaemoglobin) in air storage resulted in an increased absorption at 636 nm and a decline in absorption at the water shoulder peak prior to extensive spoilage.

Conclusions

The main conclusions of this thesis are;

- The presence of microorganisms and their growth have little effect on the development of spectral features in fresh salmon during storage prior to extensive spoilage.
- Important spectral changes occur at 606 and 636 nm in the spectra of fresh salmon during storage.
- The formation of oxidised haem (methaemoglobin) is the primary source of spectral changes occurring at 636 nm in fresh salmon fillets during air storage.
- The origin of the shoulder peak appearing at 606 nm is absorption due to water in the salmon muscle.
- The visibility of the water shoulder peak in the spectrum depends on the dominant oxidation state of haem in the muscle.

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Paper I

Paper II

Paper III

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