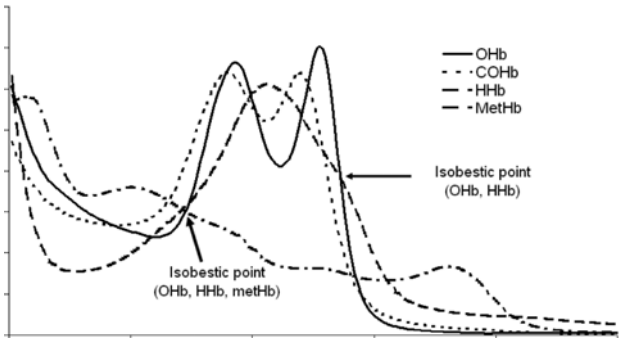
UNIVERSITY OF TROMSØ UIT

Faculty of Biosciences, Fisheries and Economics
Department of Norwegian College of Fishery Science

Quantification and characterisation of residual blood in fish muscle

– Impact of slaughtering methods



Stein Harris Olsen

A dissertation for the degree of Philosophiae Doctor
June 2011

Table of contents

Acknowledgments

The present work was carried out at the Norwegian College of Fishery Science, University of Tromsø. Funding of this work was provided by the Norwegian Research Council and the University of Tromsø (University grant).

I would like to express my sincere gratitude to my supervisor Nils-Kristan Sørensen who tragically passed away on September 30th in 2007. I would also like to express my gratitude to my supervisor Edel O. Elvevoll, who deserves thanks for her contribution, positive attitude, new ideas and guidance. I also wish to thank supervisor Kjell Ø. Midling for his guidance during the last three years. The supervisors' enthusiasm for new ideas and tasks has been inspiring, and their positive attitude and immense patience have been greatly appreciated.

I would also like to thank my co-authors at the Norwegian College of Fishery Science (Rune Larsen) and Nofima (Heidi Nilsen and Svein Kristian Stormo). Thanks to Karstein Heia, Heidi Nilsen and Agnar H. Siversen at Nofima, for helping me to study the possible application of using VIS/NIR imaging spectroscopy as a tool for visualising blood residuals in Atlantic salmon fillets. Thanks to Bjarne Østerud, at the Faculty of Medicine, for assistance with blood sampling. Thank you to Rune Larsen and Hanne Mæhre for critical reading of the manuscripts, which I am very grateful for.

Other persons deserving thanks are the office mates, co-workers, students and staff at Faculty of Biosciences, Fisheries and Economics for the good working atmosphere during these years.

To my dear beloved live-in partner Ingunn Bendiksen: Thank you for always being there for me with patience, encouragement and help to improve my written language.

Tromsø, 2010

Stein Harris Olsen

Summary

The quality of farmed fish is assessed according to multiple criteria including freshness, fat content, blood spots, flesh colour and gaping. Blood spots and residuals have become more frequent in fish fillets, after the reorganisation of the industry with a shift from small manually operated slaughterhouses to larger and more mechanized production lines. This has led to increased degree of rejection by consumers and financial losses. Today, blood residuals are mainly detected by manual visual inspection. However, this method is labour-intensive and involves subjective evaluation and thus a high degree of inaccuracy.

The main objectives of this work was to find and establish an appropriate method to measure residual blood and blood spots in fish muscle and to study factors contributing to the problem in order to help the industry improve slaughter procedures.

A chemical method used to quantify haem pigment in meat was adapted and established for quantifying blood in fish muscle. However, use of chemical methods involve toxic chemicals and are destructive to the product, they are therefore not suited in industrial food production. Nevertheless, the chemical method demonstrated that the amount of residual blood and blood spots were influenced both by pre-slaughter activity, killing procedures, chilling and storage conditions. In particular, percussive stunning prior to bleeding was proved better in terms of residual blood compared to carbon dioxide (CO_2) stunning. In terms of fish welfare, CO_2 anaesthesia is not recommended, as it creates vigorous activity among the fish before they were proper stunned. Pre-mortem activity also resulted in more blood in the fish muscle.

The primary chromophore that gives the blood its characteristic colour is haemoglobin (Hb). Visual and near infrared (VIS/NIR) spectroscopy is a rapid technique and may be a valuable tool for the assessment of the blood residuals in fish fillets. Thus, with the chemical method as a reference, it was anticipated that VIS/NIR spectroscopy could be used to quantify haem pigment in whole fish fillets. A correlation between the VIS/NIR and chemical measurements of Hb in fish muscle was found. Most of the information regarding Hb in the fillet was found in the visible range (400–700 nm), but the 400–1100 nm ranges resulted in the lowest error of prediction. Even so, the error of prediction was still too high to recommend this method for industrial application.

Detection limits and correlation of VIS/NIR measurements are influenced by the substance used for calibration. Mammalian Hb has been used as reference when measuring blood Hb. However, when comparing the optical spectra of mammalian and fish Hb, significant differences with respect to absorbance in the visible range (450-700 nm) was found. The fish Hb was also influenced by pH, resulting in deoxygenating and increased auto-oxidation and thus pH related differences in absorption spectres were evident.

The knowledge gained through this work, regarding blood residuals in fish muscle, suggests measures in the slaughterhouse to improve bleeding. Added knowledge to improve online or automated VIS/NIR inspection of blood residuals was also gained. With regard to fish welfare and muscle quality, the stunning methods used today are disputed. Further studies are needed to improve these methods and their goal should be to satisfy both the requests for fish welfare and to improve muscle quality. Further studies should also focus on possible industrial application of using non-contact imaging spectroscopy to assess residual blood in fish fillets, using fish Hb spectra as a reference. In addition, studies investigating possible differences in the Hb spectra between fish spices are both academically interesting and may also have relevance if VIS/NIR spectroscopy is to be applied industrially.

Sammendrag

Kvaliteten på fisk blir industrielt vurdert etter kriterier som ferskhet, fettinnhold, blodflekker, muskelfarge og filetspalting. En omorganisering og konsentrasjon i oppdrettnæringen har ført til en overgang fra små slakterier til større og mer mekaniserte eller automatiserte anlegg med høy slaktekapasitet. Samtidig har også uønskede blodflekker eller høyere innhold av blod i fiskemuskel blitt et økende problem. Dette har ført til reklamasjoner og økonomiske tap for produsentene. Fysisk aktivitet før slakting, levende kjøling, valg av bedøvelses- og bløggemetode antas å være faktorer som kan påvirke mengden av restblod i fiskemuskelen. I dag kontrolleres normalt rester av blod ved manuell visuell inspeksjon av fileten. Kontrollene er derfor både arbeidskrevende og de innebærer subjektive evalueringer med mulighet for høy grad av unøyaktighet. Dette gjør det vanskelig å vurdere forskjellig grad av utblødning mellom enkeltfisk.

Målet med dette arbeidet har derfor vært todelt, å bedre kunnskapen om påvirkningen de ulike enhetsoperasjonene ved slakting av fisk har på utblødning, samt framskaffe kunnskap som kan bidra til å etablere en mer objektiv kontroll av mengden blod i fiskemuskel etter slakting.

En kjemisk metode for måling av hem pigment ble adoptert fra kjøttindustrien og etablert som referansemetode i dette arbeidet. En kjemisk analyse av hem pigment involverer imidlertid giftige kjemikalier og er i likhet med manuelle visuelle metoder arbeidskrevende. I tillegg ødelegges produktet ved analyse og metoden er derfor ikke egnet ved industriell produksjon. Uansett, den kjemiske metoden viser at mengden hem pigment i muskel påvirkes både av stress før slakting, avlivningsmetoder, kjøling og lagring. Spesielt ble det funnet at bedøving ved bruk av slag før bløgging er optimalt med hensyn til innhold av restblod. Bruk av karbondioksid (CO_2) anbefales heller ikke til bedøving fra et produksjonsteknisk ståsted, da den fører til svært høyt aktivitetsnivå på fisken og derved større mengde blod i muskelen. Også ut fra et dyrevelferdsmessig aspekt er bruk av CO_2 som bedøvelse omstridt, da dette utvilsomt er stressende for fisken.

I blod er det hemoglobin (Hb) som er den primære fargebæreren og som bidrar til den karakteristiske rødfargen. Neste skritt var derfor uttesting av en ikke-destruktiv og objektiv metode basert på visuell og nær infrarød (VIS/NIR) spektroskopi for måling av restblod i fiskemuskelen, der den kjemiske metoden ble brukt som referanse. Vi fant en bedre sammenheng mellom kjemisk og VIS/NIR spektroskopisk måling av hem pigment i muskelen enn den ovenfor beskrevne manuelle metoden. I fiskemuskel er mesteparten av informasjonen rundt hem pigment funnet i det visuelle området (400-700 nm), men også informasjon fra NIR delen (700-1100 nm) av spekteret var av betydning. Hele VIS/NIR spekteret (400-1100 nm) ga derfor lavere prediksjonsfeil. Imidlertid ble sammenhengen mellom den kjemiske og VIS/NIR metoden vurdert til å ikke være god nok for å anbefale metoden brukt industrielt.

Ved slik bruk av VIS/NIR spektroskopi er forbindelsen som benyttes til kalibrering avgjørende for resultatet. Normalt benyttes Hb fra pattedyr som standard for bestemmelse av total Hb konsentrasjon i blod. En sammenligning av Hb spektra mellom pattedyr og fisk ble derfor gjennomført i det visuelle området (450-700 nm). Resultatene viste store forskjeller med hensyn til absorpsjon. Dette gjør Hb fra pattedyr er mindre egnet som referanse for kalibrering. Fiske-Hb er påvirket av pH, noe som resulterer i deoksygenering, samt økt autooksidasjon, og derved også endret absorpsjonsspekter. Automatisert VIS/NIR spektroskopisk metode for måling av restblod i fiskemuskel antas derfor å kunne utvikles og forbedres ved å bruke Hb fra samme fiskeart som referanse.

Kunnskap fra dette arbeidet kan benyttes til å bedre utblødning gjennom endringer i slakterutinene, samt fremover å utvikle bedre industrielle metoder for kontroll av rester av blod i produktet. Med hensyn til fiskevelferd og slaktekvalitet er bedøvelsesmetodene brukes i dag omstridt. Videre studier er nødvendig for å forbedre disse metodene slik at både velferd og kvalitetskrav blir tilfredsstilt. En bør også fokusere på mulig industriell anvendelse av avbildende spektroskopi for å vurdere restblod i fiskefilet, med spektra fra fisk Hb som referanse. Nye studier som ser på eventuelle forskjeller i Hb spektra mellom forskjellige fiskeslag er av faglig interesse og kan ha relevans dersom VIR/NIR spektroskopi skal anvendes industrielt.

List of papers

Paper I

Olsen, S.H., Sørensen, N.K., Stormo, S.K.,Elvevoll, E.O. 2006. Effect of slaughter methods on blood spotting and residual blood in fillets of Atlantic salmon (*Salmo salar*). Aquaculture 258, 462-469

Paper II

Olsen, S.H., Sørensen, N.K., Larsen, R., Elvevoll, E.O., Nilsen, H. 2008. Impact of pre-slaughter stress on residual blood in fillet portions of farmed Atlantic cod (*Gadus morhua*) - Measured chemically and by Visible and Near-infrared spectroscopy. Aquaculture 284, 90-97

Paper III

Olsen, S.H., Elvevoll, E.O. 2011. A pH induced shift in the haemoglobin spectra – A spectrophotometeric comparison of fish (*Gadus morhua*) and mammalian haemoglobin. J. Agric. Food Chem. 59 (4), pp 1415–1422

Additional work

In addition to papers included in this PhD thesis, I have co-authored papers in the same period. However, this are not regarded as part of this thesis.

Larsen, R., Olsen, S.H., Kristoffersen, S., Elvevoll, E.O. 2008. Low salt brining of pre-rigor filleted farmed cod (*Gadus morhua* L.) and the effects on different quality parameters. LWT - Food Science and Technology 41, 7, 1167-1172.

Midling, K.Ø., Mejdell, C., Olsen, S.H., Tobiassen, T., Aas-Hansen, Ø., Aas, K., Harris, S., Oppedal, K., Femsteinevik, Å. 2008. Slakting av oppdrettslaks på båt, direkte fra oppdrettsmerd, NOFIMA Rapport 6/2008, ISBN: 978-82-7251-637-5. NOFIMA AS, Tromso, Norway.

Abbreviations

Carbon dioxide (CO_2)

Carbon monoxide (CO)

Carboxyhaemoglobin (COHb)

Deoxyhaemoglobin (HHb)

Haemoglobin (Hb)

Methaemoglobin (metHb)

Millimolar Absorptivities (L x $mmol^{-1}$x cm^{-1})

Nanometre (nm)

Nitrogen (N_2)

Oxygen (O_2)

Oxyhaemoglobin (OHb)

Packed cells volume (PCV)

Partial least squares (PLS) regression

Principal component analysis (PCA)

Principal component (PC)

Refrigerated sea water (RSW)

Rigor mortis (rigor)

Standard deviation (SD)

Standard error of mean (SEM)

Visible and Near-infrared (VIS/NIR)

1 Introduction

In Norway, Atlantic salmon, rainbow trout and Atlantic cod are the most important commercial farmed fish species. In 2009 859 056 metric ton salmon, 76 091 metric ton rainbow trout and 20 683 metric ton cod were produced (SSB, 2010). Successful farming has enabled a continuous supply of high quality fresh fish to the market, giving fish farming an advantage over traditional fisheries. In salmon and trout industry, control of the biological production process, the post-mortem biochemistry and various quality parameters has enabled a number of productivity enhancing innovations. These have contributed to reduce the production cost and price to the consumer during the last two decades. Altogether this has made farmed salmon products competitive compared to meat, chicken and wild-caught salmon (Asche *et al.*, 2008; Borderías and Sánchez-Alonso, 2011). Opposite of this, Atlantic cod farming industry is struggling due to a high production costs. The cost of capture and inherent marked price of wild cod is substantially lower than the production cost of farmed cod (Engelsen *et al.*, 2004; Fauske, 2008). Due to high production costs and market price, it is important that loss of quality and value due to common defects such as poor bleeding, fillet gaping and muscle softening is avoided. Thus farmed cod may have the same potential, in terms of production efficiency and cost reduction, as farmed salmon (Standal and Utne, 2007; Fauske, 2008).

The colour of fish muscle is an important criterion when potential consumers evaluate fish fillets. Customers expect white fish to be white and it may hence be rejected if the muscle is only slightly darkened or discoloured. The main factor responsible for colour change in white fish is residual blood, i.e. the colour intensity is depending on the content and chemical state of haemoglobin (Hb) (Love, 1978; Mancini and Hunt, 2005; Saenz *et al.*, 2008). In the fish farming industry, blood spots have lately become more frequent, leading to unacceptable appearance and financial loss (Michie, 2001; Robb and Whittington, 2004). Residual blood in fish fillet is mainly an aesthetic problem, but can also contribute to and alter several other

quality parameters, including reduced shelf life, lipid oxidation, rancid odour, muscle softening and inherent loss of important nutrients (Tretsven and Patten, 1981; Ando *et al.*, 1999; Undeland *et al.*, 2004; Pazos *et al.*, 2005; Sakai *et al.*, 2006; Larsson *et al.*, 2007; Richards *et al.*, 2007; Vareltzis *et al.*, 2008; Pazos *et al.*, 2009; Richards *et al.*, 2009; Maqsood and Benjakul, 2010). Despite the economic importance for the industry, the problem of residual blood has not been extensively studied. Several production parameters, such as pre-slaughter stress due to crowding, chilling, anaesthetisation and exsanguination techniques, may influence the amount of residual blood in the fillet (Skjervold *et al.*, 1999; Robb *et al.*, 2003; Roth *et al.*, 2005a; Olsen *et al.*, 2006; Olsen *et al*., 2008; Roth *et al.*, 2009a; Roth *et al.*, 2009b). A more strict control of these factors may improve the total quality of the product.

2 Fish slaughtering

Traditionally, fish slaughtering methods were chosen based on their simplicity and operating costs. For captured fish, suffocation in air or live chilling on ice (hypothermia) are the oldest methods and are characterised by a prolonged suffering period before death. Other methods used for killing fish are gill- or throat cutting and direct gutting in combination with the former to ease the slaughter process (Tretsven and Patten, 1981; Botta *et al.*, 1986; Robb and Kestin, 2002; Poli *et al.*, 2005). In the fish farming industry, the most common processing line include live chilling, carbon dioxide (CO_2) stunning in seawater, cutting of gill arches, bleeding, gutting, washing, chilling, sorting/grading and packing on ice (Erikson *et al.*, 1999). During slaughtering, the chances of exposing fish to considerable acute stressors and handling are evident and this has a detrimental effect on bleeding and overall muscle quality (Erikson *et al.*, 1999; Roth *et al.*, 2005a; Olsen *et al.*, 2006; Erikson and Misimi, 2008; Olsen *et al.*, 2008). Thus, gentle handling, stunning and bleeding are important factors in improving flesh quality of fish (Ando *et al.*, 1999; Richards and Hultin, 2002; van de Vis *et al.*, 2003; Undeland *et al.*, 2004; Pazos *et al.*, 2005; Sakai *et al.*, 2006; Roth *et al.*, 2009a). Therefore, chilling of live farmed fish prior to anaesthetisation and slaughter was introduced in Norway in order to reduce pre-slaughter stress and delay onset of rigor mortis (rigor), and accordingly to enable pre-rigor filleting (Skjervold *et al.*, 1999; Skjervold *et al.*, 2001).

However, in the last decade there has been an increased attention to welfare of fish during slaughter procedures. In Norway, it is decreed by law that all farmed fish must be stunned prior to bleeding to assure fish welfare (Lovdata, 2009). Therefore, rapid loss of consciousness and death is important (Robb and Roth, 2003; Robb and Whittington, 2004; Sorensen *et al.*, 2004). Carbon dioxide is the only allowed chemical stunning agent used in fish slaughtering intended for human consumption. Thermal cold shock, combined with CO_2, is the most common method to stun farmed fish (Burka *et al.*, 1997; Erikson *et al.*, 1999; van de Vis *et al.*, 2003; Roth *et al.*,

2006; Erikson, 2008). Nevertheless, the use of CO_2 as a stunning agent is disputed with regard to fish welfare (van de Vis *et al.*, 2003; Hastein *et al.*, 2005; Poli *et al.*, 2005; Olsen *et al.*, 2006; Roth *et al.*, 2006; Digre *et al.*, 2009). It has also been demonstrated, that killing with reduction of acute stressors such as percussive stunning and specific electrocution techniques can improve muscle quality of slaughtered fish (Robb and Kestin, 2002; van de Vis *et al.*, 2003; Robb and Whittington, 2004; Hastein *et al.*, 2005; Poli *et al.*, 2005; Olsen *et al.*, 2006; Ashley, 2007; Terlouw *et al.*, 2008; Digre *et al.*, 2009). In 2003 the Norwegian government authorised both percussive stunning and electrocution as new methods for the stunning of fish prior to slaughter.

2.1 Pre-slaughter handling

Farmed fish are in general fasted for 7-14 days depending on the season and the seawater temperature. The starvation, prior to slaughter, enables the gut to empty and slows down the metabolic rate. This contributes to improved quality and hygiene of the final product (Einen and Thomassen, 1998; Morkore *et al.*, 2008).

Live fish are transported by well boats to the processing plant where they are either processed immediately or kept in holding cages in order to recover after transportation. Harvest of fish from the cage and transport to the slaughter facility involve handling and thus stressors (Erikson, 2008). Today, pressure-vacuum pumps are frequently used to transport fish from the vessel or cage. Before pumping, the fish is crowded by reduction of the water volume in the cage. Crowding may also take place when the fish is unloaded from the vessels. Crowding may results in physical damage and suffocation of the fish (Hastein *et al.*, 2005; Poli *et al.*, 2005; Midling *et al.*, 2008).

2.2 Stunning

2.2.1 Carbon dioxide

Traditionally, hard carbon dioxide (CO_2) stunning is an easy method to stun large amounts of fish and loss of consciousness is obtained within minutes. Gaseous CO_2 is highly soluble in cold seawater, and the pH of the water decreases as it becomes saturated with CO_2. The fish are netted or pumped into CO_2 saturated water and left until movement stops (Bernier and Randall, 1998; van de Vis *et al.*, 2003; EFSA, 2004). The CO_2 narcosis occurs as a result of severe acidosis which reduces the brain pH and inhibits the cerebral activity (Yoshikawa *et al.*, 1991; Yoshikawa *et al.*, 1994). An alternative method to the hard stunning is mild CO_2 anaesthesia in combination with sufficient supply of oxygen. A one step process, by means of CO_2/O_2 in a refrigerated seawater (RSW) tank, has been introduced. This method has gradually grown to be the most frequently used stunning method for farmed fish in Norway. However, the fish tend to show vigorous activity due to the sensations of strangulation when they enter the anoxic water (Robb and Kestin, 2002; Erikson *et al.*, 2006; Olsen *et al.*, 2006; Roth *et al.*, 2006; Erikson, 2008) and CO_2 stunning is thus considered to be inhumane (EFSA, 2009). A ban on CO_2 stunning of fish has therefore been adopted but has not yet been implemented. The industrial transition from CO_2 to electrocution or percussive stunning has led to an increase in quality defects such as increased blood spot formation and bruises in the product (Digre *et al.*, 2009; Lambooij *et al.*, 2010). Dispensation from the ban is still granted if the welfare of the fish can be documented throughout the process. However, a reconsideration of the different stunning methods, and combinations of these, are under evaluation by the Norwegian Food authorities (Hjeltnes *et al.*, 2010).

2.2.2 Electrocution

Electrical stunning methods are used in the fish farming industry today, both in the UK and in Norway, but these methods are still under development as technologies. This is an efficient method to stun large amounts of fish. The principle is to pass sufficient current through the brain to ensure proper stunning. The electrocution is achieved by directly applying electrodes to the head of the fish (dry stunning) or by exposing the fish to an electrified water bath (Lines *et al.*, 2003; Robb and Roth, 2003; Lambooij *et al.*, 2004; Roth *et al.*, 2009a; Lambooij *et al.*, 2010). The challenge with electrocution is to avoid carcass damage such as muscle haemorrhages caused by bursting blood vessels and broken spinal columns. Electrocution can also influence muscle pH, onset of rigor, muscle texture and fillet gaping (Robb and Kestin, 2002; Roth *et al.*, 2003; van de Vis *et al.*, 2003; Gregory, 2005; Roth *et al.*, 2009a; Lambooij *et al.*, 2010).

2.2.3 Percussion

Traditionally, percussive stunning has been achieved by delivering a blow to the head of the fish with a wooden priest. This disrupts the normal brain functions. Provided that the blow is accurate, percussive stunning renders the fish unconscious immediately (Robb *et al.*, 2000; Robb and Kestin, 2002; van de Vis *et al.*, 2003; Sorensen *et al.*, 2004). Today mechanical percussive stunners, which also enhance bleeding immediately after stunning, are used (Roth *et al.*, 2007a; Midling *et al.*, 2008). However, large variation in fish size is a challenge when applying this technique. This can result in broken jaws, burst eyes, haemorrhages and insufficient stunning (Roth *et al.*, 2007a; Lambooij *et al.*, 2010).

2.3 Bleeding fish

Bleeding is normally applicable to medium or large fish species, but not small pelagic species like herring, sardines, sprat and mackerel (Borderías and Sánchez-Alonso, 2011). Bleeding fish to improve flesh quality and accelerate death has a long tradition in the Nordic countries. A process known from the Old Norse (blóðga) is to cut the throat of fish in such a way that the fish are exsanguinated. However, in Norway bleeding fish was first decreed by law in fisheries in 1933 (Anderssen, 1934; Tretsven and Patten, 1981). Bleeding is also regulated by law in fish farming. Several methods have been practised to accelerate death and remove the majority of blood volume from the fish. Bleeding techniques such as cutting the gill arches, the throat, or the arteries in the neck (Figure 1), and even direct evisceration are frequently used methods. However, the method used has minimal influence on the residual blood in the muscle. Vasodilatation in peripheral tissues due to pre-slaughter stress or crowding, the time elapsed between death and bleeding, chilling, anaesthetisation, muscle contraction, the blood coagulation time and gravity are more important factors (Warriss and Wilkins, 1987; Skjervold *et al.*, 1999; Richards and Hultin, 2002; Robb and Kestin, 2002; Robb *et al.*, 2003; Lambooij *et al.*, 2004; Roth *et al.*, 2005a; Olsen *et al.*, 2006; Olsen *et al.*, 2008; Roth *et al.*, 2009a; Roth *et al.*, 2009b).

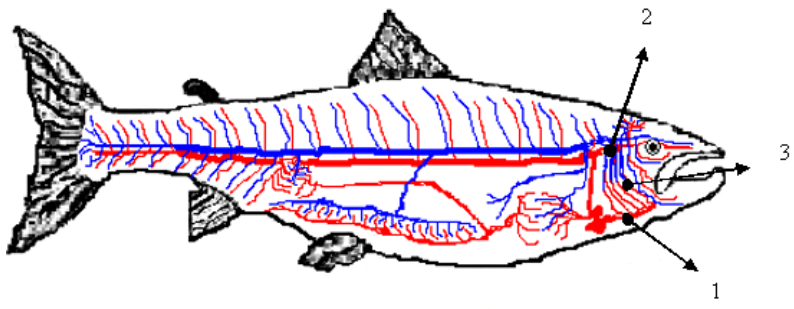


Figure 1. Three common methods used for bleeding fish. 1. Cutting the throat and ventral aorta. 2. Cutting the dorsal aorta in the neck. 3. Cutting the gill arches.

3 Fish muscle, cardiovascular system and blood

3.1 Muscle

Fish typically have two distinct muscle types and these are classified according to colour; a large mass of white muscle and a smaller band of red muscle running the length of the animal just under the skin (Johnston, 1980; Summers, 2004; Saguer *et al.*, 2006). Generally in whitefleshed fish species, 90% of the fish skeletal muscle is composed of anaerobic white fibres, which gives the flesh its characteristic light colour. The haem pigment found in white muscle originates mainly from Hb in blood (Johnston, 1980; 1981; 1983). In the skeletal muscle of tuna and other darkfleshed fish species the level of haem proteins (myoglobin and haemoglobin) is high, which cause the attractive red muscle colour (Brown, 1962; Schubring, 2008). In contrast, the salmonid species have the ability to deposit dietary carotenoids in their muscle tissues, which gives the distinctive red flesh coloration (Shahidi *et al.*, 1998).

Myoglobin is the predominant haem pigment present in cardiac and aerobic red skeletal muscles of all vertebrates. This is a single chain globular protein with an iron containing haem group in the centre. The primary role of myoglobin is as an oxygen storage unit, providing oxygen from the capillary to the mitochondria in aerobic red muscles (Marcinek *et al.*, 2001; Hankeln *et al.*, 2005). Dark muscle fibres generally have higher levels of haem pigment (myoglobin and haemoglobin), mitochondria, fat and glycogen compared to white muscle fibres (Haard, 1992). The capillary vascularisation differs between dark and white muscle tissues. The capillaries cover 18-25% of the dark aerobic muscle fibre surface, whereas only 0.2 – 9.0% in white anaerobic muscle (Soldatov, 2006).

3.2 Cardiovascular system

The teleost circulation system is of considerable interest when studying exsanguination during slaughter. The cardiovascular systems of fish differ from the system found in mammals (Figure 2). A fish heart is constructed for single circulation, containing a two chambered structure. The fish heart consists of a sinus venosus, atrium, ventricle and bulbus arteriosus that ends in the ventral aorta. A mammalian heart is constructed for double circulation (pulmonary and systemic), and consists of a four chambered structure. The pulmonary circulation is related to the lung aeration of blood. Deoxygenated blood from the body is pumped to the lungs where it is oxygenated and then returns back to the heart to increase the blood pressure. The systemic circulation is the mayor circulation of oxygen-rich blood pumped from the heart to the arteries and capillaries in the body (Randall, 1970; Farrell and Jones, 1992; Huss, 1995; Moorman and Christoffels, 2003). In fish, venous blood is entering the heart via sinus venosus with a pressure below atmospheric and up to 10 mmHg. The fish heart then forces the venous blood towards the gills via the ventral aorta, with a blood pressure between 10-40 mmHg (species variation). After being aerated in the gills, the blood pressure decreases by 50%. However, in fish, the aerated blood is not returned back to the heart (Figure 2). Instead, the arterial blood is forced into the dorsal aorta that runs just beneath the vertebral column and is dispersed further into the different tissues and organs via arteries, arterioles and the capillaries (Figure 3).

Fish also have a secondary circulation system, which is an arterial-capillary-venous system derived from the primary circulation system. This system supplies the fins, tail and skin with blood. The capillaries are drained into a large collecting vein, which flows towards the head. In salmonids, a “caudal heart” forces blood from the secondary capillaries system in the tail into the caudal vein, driven by skeletal muscle contractions. The venous blood then returns to the heart, by the posterior cardinal vein, which is also located beneath the vertebral column. All the veins gather into one blood vessel (sinus venosus) before entering the heart, with a venous

blood pressure close to zero. Thus, skeletal muscle contractions, vasodilators and capillary attraction are believed to play important roles in the transportation of venous blood, which is helped by a system of paired valves inside the veins, preventing counter flow (Randall, 1970; Farrell, 1991; Bushnell *et al.*, 1992; Satchell, 1992; Huss, 1995; Olson, 1996; Farrell *et al.*, 2001; Sandblom *et al.*, 2005; Sandblom and Axelsson, 2007). This indicates that a beating heart is not necessary for efficient exsanguination during slaughter, which furthermore has been confirmed in slaughter experiments where the impact of different killing procedures has been studied (Robb *et al.*, 2003; Roth *et al.*, 2005a; Olsen *et al.*, 2006; Roth *et al.*, 2006; Olsen *et al.*, 2008; Roth *et al.*, 2009a; Roth *et al.*, 2009b).

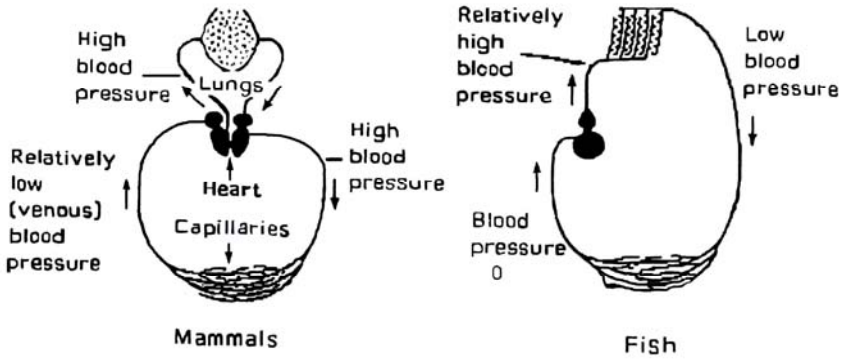


Figure 2. Blood circulation in fish and mammals (Eriksson and Johnson, 1979; Huss, 1995)

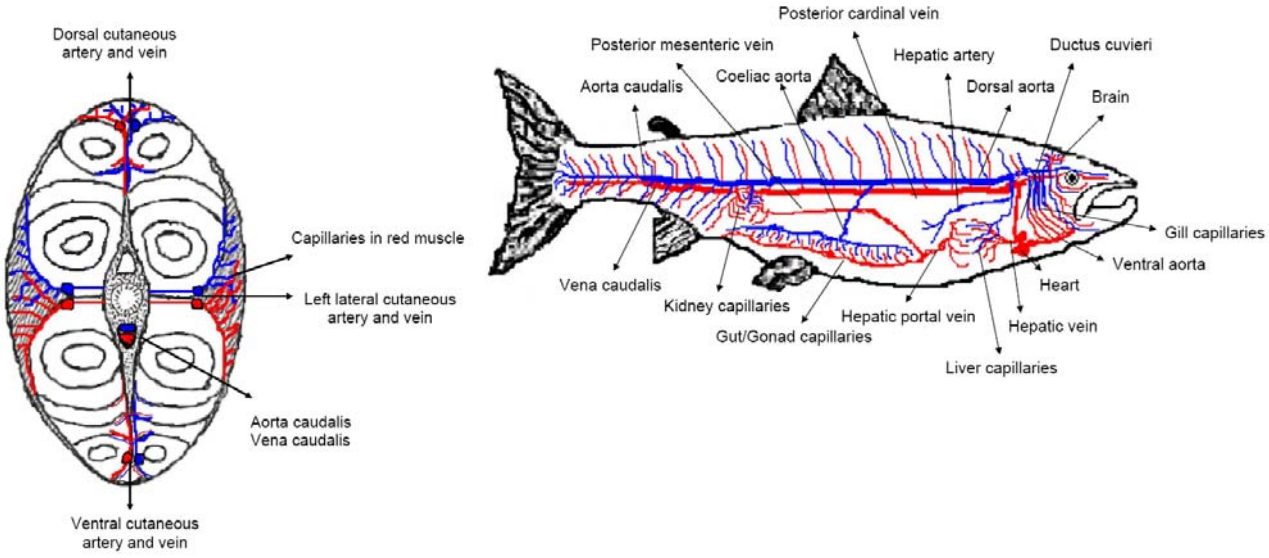


Figure 3. A general sketching of the blood circulation in fish. Drawn after Bushnell and Satchell (Bushnell *et al.*, 1992; Satchell, 1992).

3.2.1 Regulation of the vascular system

Like all other vertebrates, fish cannot fully perfuse the vascular system, because there is insufficient capacity to simultaneously support the blood flow demands for exercise, digestion and maintenance functions. During digestion and exercise a redistribution of blood flow occurs. In resting unfed fish, the blood flow to the gastrointestinal tract accounts for approximately 30%–40% of the cardiac output, but during periods of aerobic exercise the gastrointestinal blood flow decreases. However, after feeding the gastrointestinal blood flow can increase up to 85% via an increased cardiac output and redistribution of blood to the digestive system. Under physical stress fish are also able to redistribute the blood flow and thus increase the blood content in the muscle, prioritising swimming over digestion (Randall, 1970; Axelsson and Fritsche, 1991; Thorarensen *et al.*, 1993; Farrell *et al.*, 2001; Soldatov, 2006; Altimiras *et al.*, 2008). Pre-slaughter activity and stress can therefore increase the blood volume in the muscle. Thus, only the blood present in the gills, or in the vessels close to them, may be purged when the gills are cut (Olsen *et al.*, 2006).

3.3 Blood

Blood volume in birds and mammals is normally 7-10% of the body weight (7-22% in marine mammals), 3-8% in reptiles and amphibians, and 3-7% in most elasmobranchs and teleost fish (Brill *et al.*, 1998; Williams and Wortby, 2002; Saguer *et al.*, 2006). It is widely accepted that in fish blood, like in most other vertebrate blood, erythrocytes (red blood cells) are the dominant cell type and the numbers range from 0.8-3.5 million μL^{-1}. Fish erythrocytes are different from mammalian erythrocytes. Fish erythrocytes have an oval shape, they may be nucleated or not, and are larger than mammalian erythrocytes (de Souza and Bonilla-Rodriguez, 2007; Vazquez and Guerrero, 2007). Teloeosts generally have a volume of packed red blood cells (PCV) greater than 20%. In spite of that, the volume of PCV is depending on temperature,

salinity, stress, disease etc. Stress among other factors can result in spleen red cell release and erythrocytic swelling. The PCV range from 15-30% in Atlantic cod whole blood and for salmonids as an group the PCV range from 20-50% (Gallaugher and Farrell, 1998).

3.3.1 Blood coagulation

Fish are aquatic vertebrates and are members of the largest and most diverse vertebrate taxon. They have adapted numerous mechanisms for clotting wounds rapidly in water. Stress affects the coagulation rate of the blood, as the rate of coagulation increases as stress increases (Ruis and Bayne, 1997; Tavares-Dias and Oliveira, 2009). It is well known that chilling delays blood coagulation. Rapid blood clotting can prevent proper exsanguination. Therefore, both chilling and rinsing blood from the wound can play an important role during exsanguination (Connell, 1995; Olsen *et al.*, 2006; Roth *et al.*, 2009b).

3.3.2 Haemoglobin (Hb)

Haemoglobin is a globular protein that is present in red blood cells of all vertebrates. Generally, the Hb molecule consists of four globin chains, each chain having an iron containing haem group that is responsible for the reversible binding of O_2. The main function of Hb is O_2-transport from the gas-exchange organs to peripheral tissues (Marcinek *et al.*, 2001; Hankeln *et al.*, 2005; de Souza and Bonilla-Rodriguez, 2007). There is a chemical similarity between myoglobin in muscle and Hb in red blood cells (erythrocytes). The different chemical states of Hb in the erythrocytes are called Hb derivatives and the most common derivatives are oxyhaemoglobin (OHb), deoxyhaemoglobin (HHb), carboxyhaemoglobin (COHb) and methaemoglobin (metHB). Hence, when recording optical absorbance spectrum of blood, these derivatives can be identified (Figure 4) by visible spectroscopy (450–700 nm) (Snell and Marini, 1988; Steinke and Shepherd, 1992; Zijlstra and Buursma, 1997; Volkel and Berenbrink, 2000; Zijlstra *et al.*, 2000; Jensen, 2007). In freshly drawn blood, the OHb and HHb forms are

the primary chromophores that give the blood its characteristic cherry-red colour (McMurdy *et al*., 2008). Nevertheless, the Hb in a fish blood sample will rapidly start to oxidise when exposed to air and the colour will then change from bright red to brownish. Thus it is the state of haem-iron that dictates the blood colour (Mancini and Hunt, 2005; Olsen *et al.*, 2006; Olsen *et al.*, 2008). Among vertebrates, fish display the most extensive presence of multiple Hb components, which show considerable differences in amino acid sequence and functional properties (Pellegrini *et al*., 2003). This is probably due to the enormous plasticity in the selection of environments and the capacity of some species to cope with different conditions of temperature, pressure, salinity, pH and oxygen availability (Pelster and Decker, 2004; Brittain, 2005; de Souza and Bonilla-Rodriguez, 2007; Verde *et al.*, 2007). In some fish species, a part of the Hb is extremely pH sensitive – this is known as the Root effect. The Root effect decreases the oxygen carrying capacity rapidly when pH is reduced, and parts of the Hb remain deoxygenated (HHb) even at a high partial pressure of oxygen (Pelster and Randall, 1998; Pelster and Decker, 2004; Brittain, 2005; Verde *et al.*, 2007). The Root effect does not occur in mammalian Hb. However, mammalian and fish have another effect in common, the Bohr effect, which generally alter the oxygen binding properties of Hbs in response to changes in both partial pressure of oxygen and pH (Giardina *et al.*, 2004; Brittain, 2005).

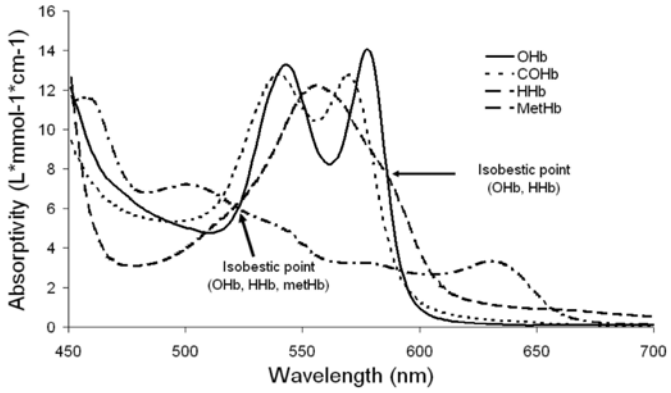


Figure 4. Absorption curves of the four common derivatives of mammalian haemoglobin at pH 7.4. Millimolar absorptivities (L x $mmol^{-1}$ x cm^{-1}) spectra of oxyhaemoglobin (OHb), deoxyhaemoglobin (HHb), carboxyhaemoglobin (COHb) and methaemoglobin (metHb) in the spectral region of 450-700 nm (Olsen and Elvevoll, 2011)

4 Detecting blood in fish

4.1 Traditional methods

Poor exsanguination is visualised as blood effusion and blood spots, both on the surface and within fish fillets (Robb *et al.*, 2003; Roth *et al.*, 2005a; Olsen *et al.*, 2006; Roth *et al.*, 2009b; Erikson *et al.*, 2010). In the Norwegian white fish industry, poor bleeding is detected on whole gutted fish by visual inspection of the cavity canal. If the fish is poorly bled, the blood vessels in the belly flaps are not sufficiently drained. After filleting, residual blood is registered as blood spots or blood effusions in the fillets by candling. This involves manual inspection over an illuminated table where removal of defects is done manually (Stormo *et al.,* 2007). If residual blood is considered unacceptable in a particular product, it can either be trimmed from the fillet with a knife or sorted out and used in some frozen-coated products. Coatings may be enriched with antioxidants which will protect the product against oxidation during frozen storage (Love, 1978). Manual inspections are also practised on slaughtered farmed fish; such as blood spot counting on salted and smoked salmon fillet (Robb *et al.*, 2003; Olsen *et al.*, 2006) or manual inspection of the cavity canal of gutted fish (Erikson *et al.*, 2010). Both candling and manual inspections are labour-intensive procedures that involve subjective evaluation with a high level of inaccuracy. The efficiency of candling depends on the thickness of the fillet and the amount of muscle pigments, because both may conceal the embedded bloodspots in the fillet (Olsen *et al.*, 2006; Heia *et al.*, 2007; Stormo *et al*., 2007; Roth *et al.*, 2009b). In addition, due to the high scattering and absorption of visible light in fish muscle, the illumination of the fillet is reduced. However, many factors such as rigor mortis, water influx, drip loss, gaping and muscle degradation can affect the light scattering and thus the colour of fish muscle (Stormo *et al*., 2007).

4.2 Chemical methods

For more accurate measurement of blood residuals or haem pigments in muscle, chemical methods have been established. Most of the reported procedures for determination of total Hb in muscle are based on chemicals which convert all Hb derivatives into a stabile haem pigment that can be quantified spectrophotometrically. Today there is no standard procedure for quantification of Hb in the muscle of mammals, birds or fish. However, the most widely used chemical method for the determination of total haem pigment in meat is the Hornsey acid haematin method (Hornsey, 1956; Warriss and Wilkins, 1987; Karlsson and Lundstrom, 1991; Carpenter and Clark, 1995; Olsen *et al.*, 2006; Olsen *et al.*, 2008). This method converts all forms of Hb into a stabile acid haematin (hemin) compound formed by the oxidation of hemin-iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state, with a peak transmission at 512 and 640 mn (Hornsey, 1956). A alternative to the acid haematin method is the alkaline haematin method (Karlsson and Lundstrom, 1991).

A common colorimetric method used for Hb determination in both blood and muscle, is the Drabkins cyanomethaemoglobin method (Drabkin, 1950). This method involves highly toxic cyanide compounds and the principle of this method is to convert Hb to cyanomethaemoglobin in a solution containing potassium ferricyanide and potassium cyanide. Cyanomethaemoglobin is a stable colour pigment with peak transmission at 540 nm (Drabkin, 1950; Rodkey, 1967; Warriss, 1976; Warriss and Rhodes, 1977; Balasubramaniam and Malathi, 1992; Synowiecki *et al.*, 1992; Kranen *et al.*, 1999). Chemical methods based on carbon monoxide or sodium dithionite have also been used. These convert Hb either into COHb or HHb and both have a peak transmission at 432 nm (Brown, 1961; Richards, 2000; Richards and Hultin, 2000; 2002; Pazos *et al.*, 2005; Pazos *et al.*, 2009). Chemical methods are labour-intensive, involve some highly toxic chemicals and are destructive to the product. Thus they are not useful for industrial online production (Olsen *et al.*, 2008).

4.3 Instrumental methods

The focus on instrumental analysis and evaluation of fish quality is increasing. The main principle being used is based on the optical properties of intact muscle, which may enable rapid at-line or on-line determination of fish quality (Scotter, 1997; Wold and Isaksson, 1997; Nilsen *et al.*, 2002; Wold *et al.*, 2006; Heia *et al.*, 2007; Roth *et al.*, 2007b; Stormo *et al.*, 2007; Folkestad *et al.*, 2008; Olsen *et al.*, 2008; Sivertsen *et al.*, 2009; Erikson *et al.*, 2010; Sanchez-Zapata *et al.*, 2010; Sivertsen *et al.*, 2011). Due to the evolution in computer technology and multivariate statistical software during the last decades, spectrophotometric multicomponent analysis has become a powerful analytical tool. This is because only small differences in the absorbance spectra of the individual compounds are sufficient for quantitative analysis. Advantage of using spectrophotometric multicomponent analysis is the ability to measure components in materials with little or no sample preparation, thus not destructive to the product (Ni and Gong, 1997; Workman, 1999; Blanco and Villarroya, 2002; Prevolnik *et al.*, 2004; Mlcek *et al.*, 2006; Nicolai *et al.*, 2007).

4.3.1 VIS/NIR-spectroscopy

Visible (VIS, 400-780 nm) and near-infrared (NIR, 780-2500 nm) spectroscopy is a rapidly developing scientific field and has been used to assess chemical and physical properties of food products. For example, the colour changes of fish muscle during storage have recently been studied in order to predict the fish freshness spectrophotometrically (Nilsen *et al.*, 2002; Nilsen and Esaiassen, 2005; Chau *et al.*, 2009; Sivertsen *et al.*, 2011). VIS/NIR-spectroscopy is a fast and non-destructive instrumental technique based on light absorption and reflection in samples at different wavelengths. Molecules and particles in a sample interact with light energy in the 400–2500 nm spectral region through absorption, reflection and scattering processes. Generally, there are three models of measurements (Figure 5) based on transmission, reflection or transflection of light (Osborne, 2001; Reich, 2005; Nilsen and Heia, 2009). Reflectance

measurement is based on reflection of light, and a detector normally measures the reflected light at 45° to the incident beam (Figure 5A). The illumination and detector units are located on the same side of the sample. Transmittance measurements are like the classical spectroscopy, and when light radiation is sent through the sample, the proportion of radiation subdued by the sample is measured as transmittance (Figure 5B). A typical VIS/NIR (400-2500 nm) transmittance, or reflectance measurements, is used as an online by-pass sampler and is well suited for measuring homogenous samples. A sample is normally measured either in a standard quartz sample cell or in a flow-through quartz cell. In transflectance (diffuse reflectance) measurements (Figure 5C), the illumination and detector unit are also located on the same side of the sample. However, the illumination and detector units are not focused on the same location, and are thus a hybrid of both transmittance and reflectance. Transflectance measurements are normally accomplished by using a fiberoptic probe in which one set of fiberoptic bundles carry the incident radiation and another carry the reflected radiation. Fiberoptic probes probably have the widest range of applications in online food analysis. However, fiberoptic probes require direct contact with the product and can cause problems regarding both food safety and the mechanics (Wilson and Tapp, 1999; Osborne, 2001; Wold *et al.*, 2006; Benito *et al.*, 2008; Huang *et al.*, 2008; Nilsen and Heia, 2009).

The main differences in the absorption spectra of blood in fish muscle are found in the visible region (Olsen *et al*., 2008). However, visible light scatters greatly in fish muscle and scattering increases with decreasing wavelength, thus obstructing the illumination of the fillet (Stormo *et al*., 2007; Stormo, 2009). It is well known that muscle density and degradation in fish is affected by large seasonal and individual variations (Love, 1975; Bjørnevik, 2003; Roth *et al*., 2005b). These factors can also influence light scatter in fish fillets, and can therefore limit the detection when using transmittance measurements. The preferred method used for fish fillet is thus transflectance measurements. This method allows both the absorbed and the scattered light to

be calculated, and the sample measurement results in a spectrum in the VIS/NIR region (Figure 6). However, the existence of compounds with specific absorption characteristics is normally separated and identified through chemicall analysis. Specific information about components, either in blood or fish muscle, can be used as optical signatures. Eventually, a model between the recorded spectra and a chemical reference measurement can be built (Stormo *et al*., 2007; Stormo, 2009). The recorded spectral information of the chemical composition of the muscle can then be selectively highlighted and used in algorithms for blood detection. Thus, several chemical components in the muscle can be estimated from the same measurement (Lee *et al.*, 1992; Chen *et al.*, 1996; Scotter, 1997; Wold and Isaksson, 1997; Blanco and Villarroya, 2002; Nilsen *et al.*, 2002; Uddin *et al.*, 2005; Folkestad *et al.*, 2008; Huang *et al.*, 2008; Olsen *et al.*, 2008).

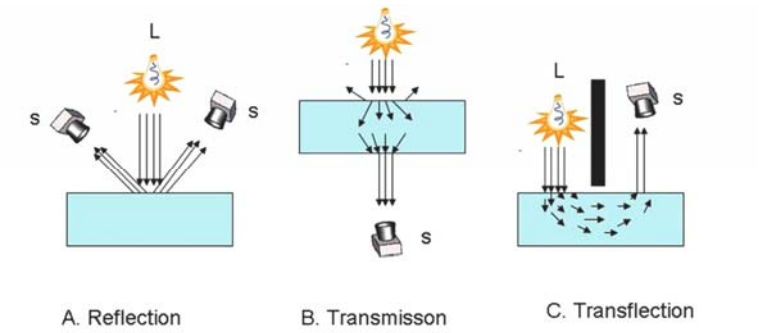


Figure 5. NIR measuring models; (A) reflectance, (B) transmittance and (C) transflectance. Light source (L), sensor (S) (Nilsen and Heia, 2009)

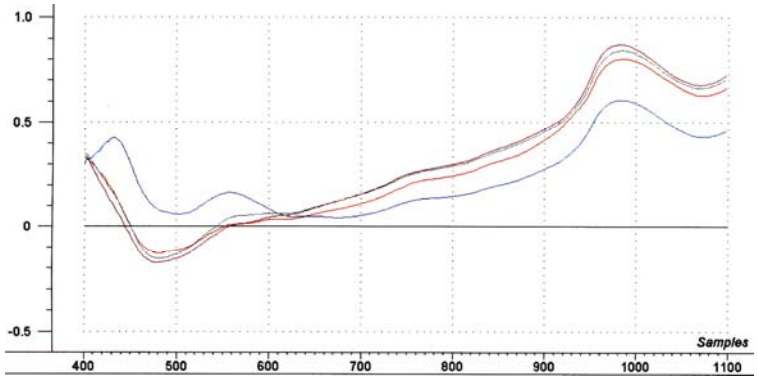


Figure 6. Example spectra of Atlantic cod light muscle in the 400-1100 nm region. The spectrum differing from the others in the visible range (blue line), with distinct peaks at about 430 and 550 nm, contained visible blood speckles. In general the spectra showed the same peaks of absorption, with a broad band of absorption at around 970 nm, the water peak (Olsen *et al.*, 2008).

4.3.2 Multivariate data analysis

When using VIS/NIR spectroscopy, multivariate statistical analysis is required for quantitative analysis. Algorithms for principal component analysis (PCA) and partial least squares (PLS) regression are commonly used methods to build linear models (Gomez *et al.*, 2006; He *et al.*, 2006b; He *et al.*, 2006a; Nilsen and Heia, 2009; Sivertsen *et al.*, 2011). In order to evaluate if the data may be considered similar or dissimilar, a principal component analysis (PCA) can be used to observe clustering or to separate the main principal components (PCs) from the noise within sample sets. The result from a PCA is typically displayed in the form of a scores plot (Figure 7). The most commonly used scores plot is for the scores vectors from principal component 1 (PC1) and principal component 2 (PC2). These represent the two largest variations in magnitude within the data. A partial least squares (PLS) analysis is applied to evaluate any correlation between the measured spectra and the reference method used (Figure 8). A complete description of a PCA analysis is described earlier by others (Tzeng and Berns, 2005).

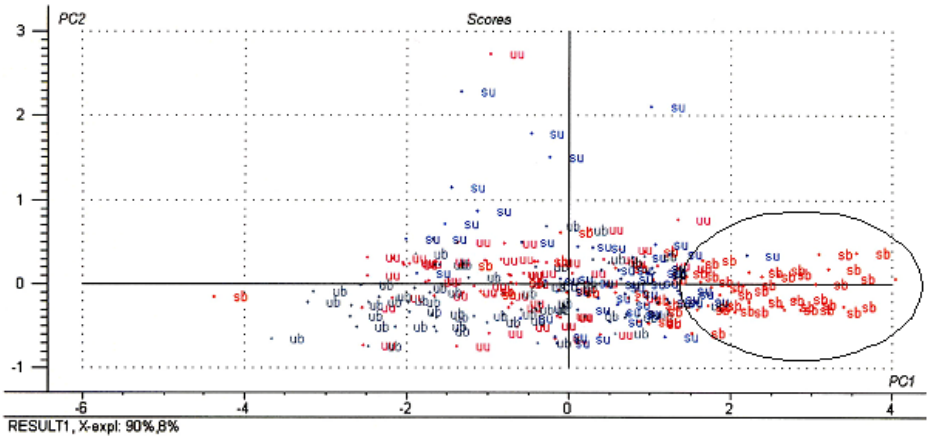


Figure 7. Result from a principal component analysis (PCA) and score plot of spectral data (Olsen *et al.*, 2008). The data is assigned according to: sb – stressed bled, n= 50; su – stressed unbled, n=50; ub – unstressed bled, n=50; uu – unstressed unbled, n=50. Most of the spectra from the stressed bled fish are encircled on the right side of the PC1.

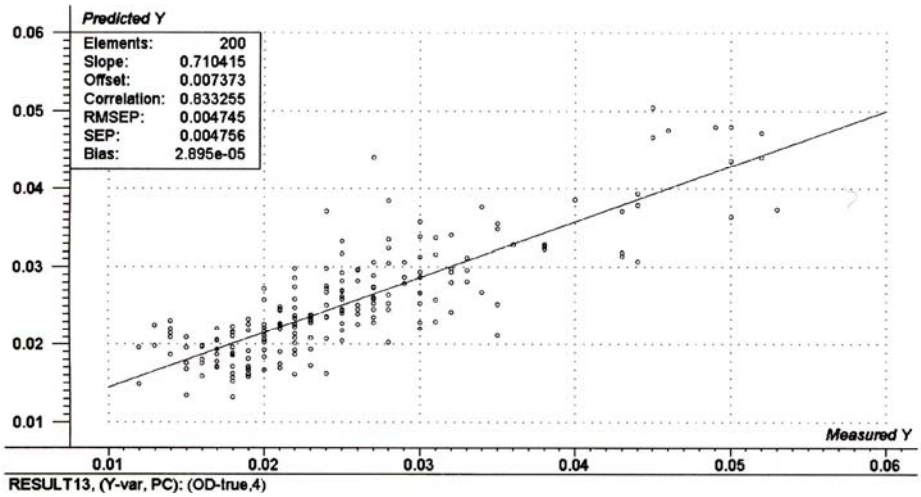


Figure 8. The result of the Partial least squares 1 (PLS1) analysis of the correlation between chemically measured haem pigment values (optical density measured) and spectral measurements (optical density predicted, based on 4 principal components) are shown (Olsen *et al.*, 2008).

4.3.3 Digital imaging

It is often necessary to analyse the texture, colour and shape of food samples both qualitatively and quantitatively. Qualitative analysis involves visual inspection and comparison of samples, while quantitative analysis involves distribution and averages. Normally, a labour intensive visual inspection has been used to assess quality changes, hence alternative objective methods have been strongly warranted. The advances in computer technologies and digital colour image processing techniques have increased the effectiveness of imaging analysis. Therefore, focus on digital imaging as an objective tool in food research is increasing. Digital imaging analysis is based on pixels and colours obtained from the red, green and blue colour distribution and greyscale outputs from digital camera pictures. However, a proper light source is important since the colour of the sample depends on the part of spectrum it reflects (Yam and Papadakis, 2004; Faucitano *et al.*, 2005; Yang *et al.*, 2006; Roth *et al.*, 2007b; Erikson and Misimi, 2008; Erikson *et al.*, 2010).

Due to this, a new field of digital imaging analysis (imaging spectroscopy) has been developed during the last decade. A digital imaging spectroscopic analysis is based on non-contact VIS/NIR transflectance measurements. The technique is based on irradiation of the sample

surface with a broadband continuous light source, followed by detection of the radiation backscattered by the sample surface with a suitable camera device (Figure 9). The reflected light from the object is captured in a great number of narrow spectral bands through the ultraviolet, visible and infrared part of the electromagnetic spectrum. Typical parameters for multispectral imaging include spectral range, spatial and spectral resolution and acquisition rate/time. The multispectral data obtained from the sample surface can be viewed as an entire image (Figure 10) at any wavelength or as a full spectrum of any pixel in the image. Digital imaging techniques are promising tools to detect or visualise objects and discoloration in fish muscle, such as loose fish scales, black membranes and parasitic round worms (nematodes) which are not easily recognised upon visual inspections (Sigernes *et al.*, 2000; Wold *et al.*, 2006; Stormo *et al.*, 2007; Qiao *et al.*, 2007; Nilsen *et al.*, 2009; Nilsen and Heia, 2009; Stormo, 2009). The techniques also enable the detection of blood spots embedded and concealed within a fish fillet (Midling *et al.*, 2008; Sivertsen *et al.*, 2011).

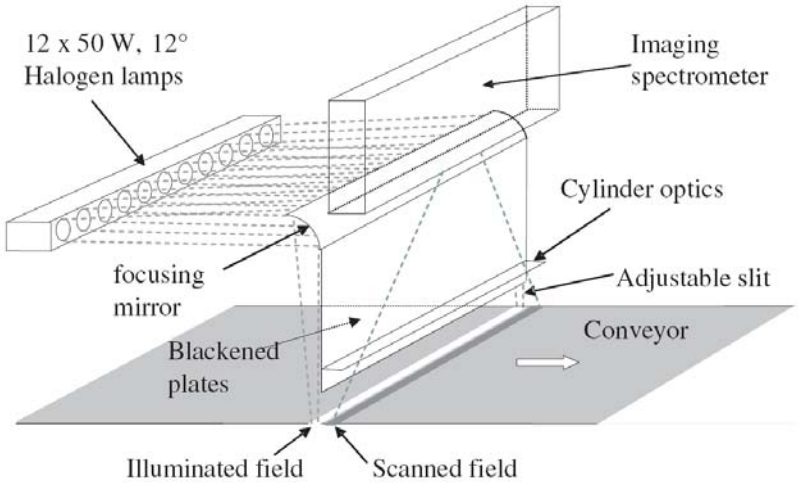


Figure 9. Principle drawing of on-line non-contact NIR transflection imaging system (Wold *et al.*, 2006)

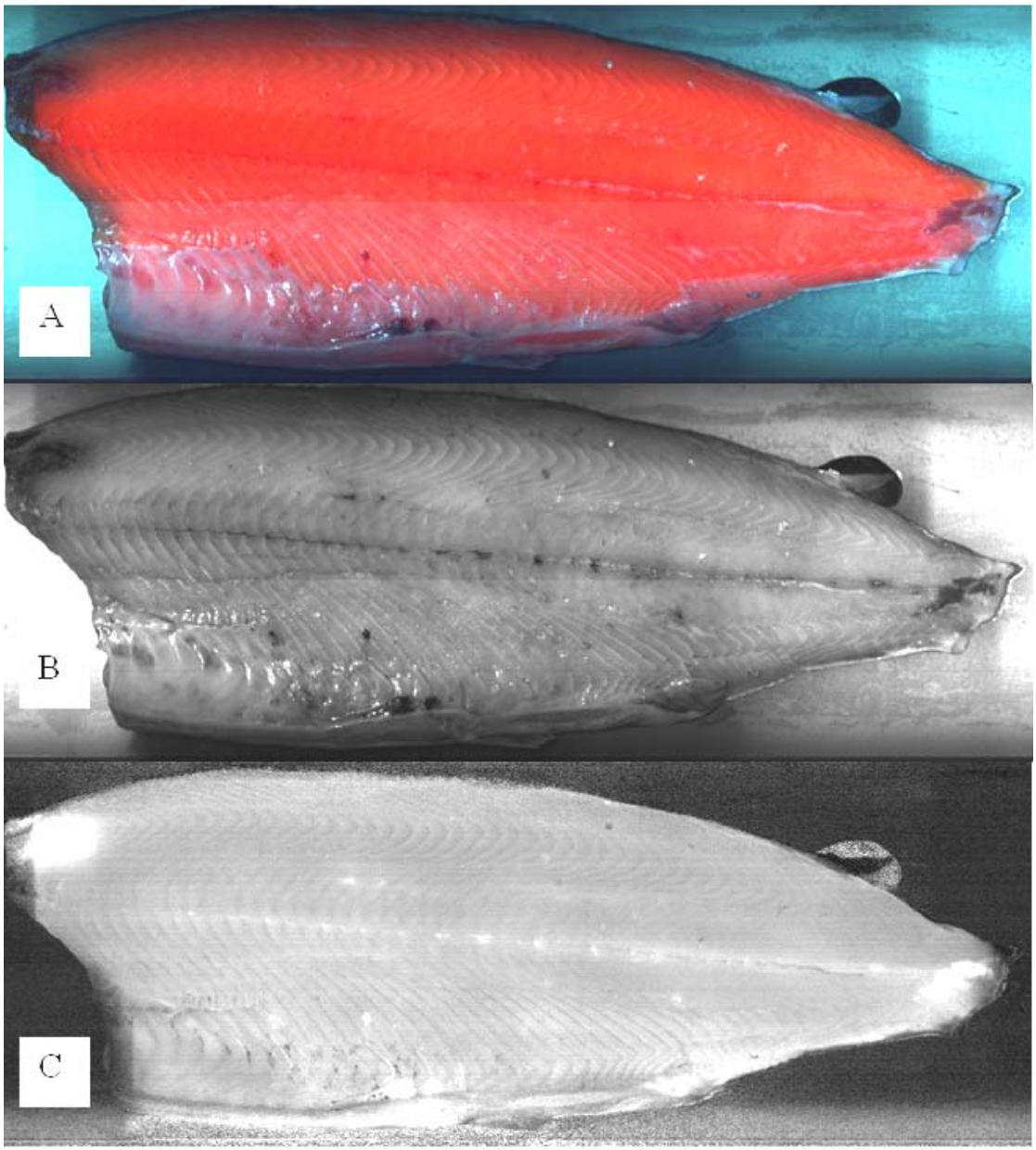


Figure 10. Hyperspectral images of an Atlantic salmon fillet, with a spectral resolution of 5 nm and pixel resolution of 0.5 mm^2. The multispectral data were obtained from imaging spectroscopy with transflection setup to record data from the VIS/NIR (400-1000 nm) region, with a conveyer speed of 80 cm/second. The measurements were carried out in February 2007 at Fiskeriforskning (NOFIMA), in cooperation with Karstein Heia, Heidi Nilsen and Agnar Siversen. Figure 10A is showing a colour picture of the fillet composed of three colours - red (641 nm), green (552 nm) and blue (458 nm). This is a colour composition that is close to what we actually can see with our eyes. Figure 10B, is focusing on oxy- and deoxyhaemoglobin (572 nm) and blood is visualised as dark spots. Figure 10C, is focusing on methaemoglobin (630 nm) and blood is visualised as luminous spots.

5 Aim of study

Residual blood in fish fillet is mainly an aesthetic problem for the fish industry as it leads to an unacceptable appearance for the customer and hence, financial loss. Despite the economic importance the problem has not been extensively studied. The main purpose of this project has therefore been to add knowledge to this field, helping to understand which factors contributing to the blood spot formation in fish fillets. Then evaluating the application of using Visible and Near-infrared (VIS/NIR) spectroscopy as a method for assessing residual blood in intact fish fillets, with a chemical haem pigment measurement as a reference.

The specific aims were:

1. Establishing a method to measure residual blood (paper I & II).
2. Evaluating the influence of slaughter procedures on residual blood (paper I & II).
3. Evaluating the applicability of a non-destructive method (paper II).
4. Selecting reference material in VIS/NIR spectroscopy (paper III)

6 General results and discussion

6.1 Establishing a method to measure residual blood

In order to quantify residual blood, a modified chemical method (Hornsey, 1956) for measuring haemoglobin in fish muscle was adopted, evaluated and further developed (Paper I and II). An established method (Robb *et al*., 2003) involving salting, slicing and blood spot counting was used for comparison (Paper I), but this method lacked the sensitivity to differentiate between residual blood in fillets among the different slaughtering procedures.

When applying the latter method (Robb *et al*., 2003), frequent blood spots were observed only in unbled fish. No differences resulting from slaughter procedures, such as percussive killing and gill cutting, percussive killing and direct gutting, or CO_2 stunning and gill cutting could be observed. The variation in bloodspot count was high within each group, and thus large sample sizes are generally required to find statistical differences between treatments groups. When applying this method. In addition, the chemical method produces continuous data instead of integers, thus increasing the chance of using the more powerful parametric methods instead of non-parametric statistical methods to evaluate differences between groups.

Total counts of bloodspots in the fillet and the haemoglobin content measured by the chemical method correlated only between bled and unbled fish. However, the results obtained through the established method lacked precision to recommend a slaughter procedure that led to the lowest content of residual blood in the fillets. The chemical method was more sensitive and considered a better method to quantitatively evaluate residual blood in fish muscle. Nevertheless, it is important to note that detecting blood spots closely resembles the consumer's evaluation of the final product. Salmon farmers and buyers base their evaluations of residual blood on inspection of the cavity canal of gutted fish or on the surface of the fillets.

Although such evaluations probably involve a higher level of inaccuracy compared to the chemical method, it is convenient and practical, and it is the method currently in use.

Haemoglobin in white muscle of fish originates mainly from haemoglobin in residual blood. The Hornsey's (1956) haem iron method was adapted since it is the most widely used chemical procedure for determining the total haem pigment in meat. Haemoglobin is converted into a stable haem pigment (acid haematin) that can be quantified spectrophotometrically with absorption peaks at 512 and 640 nm. The accuracy of the chemical method was tested and calculated as percent recovery (95.4 ± 3.2%) of Hb in spiked muscle after acid acetone extraction (paper II). The accuracy of the method showed that it was well suited for quantifying even low levels of haem pigments in fish muscle. However, the naturally present of astaxanthin in salmonid muscle is influencing the absorption at 512 nm but not at 640 nm (Shahidi et al., 1998). Thus the chemical method was therefore chosen and applied for evaluating residual blood in both salmon (640 nm) and cod (512 nm) muscle. In addition, this metod was faster to carry out, compared to the established visual method. When compared to other chemical methods for measurement of Hb in meat, it is safe, fast and repeatable and thus, the chemical method was preferred as a reference method for further work.

6.2 Evaluating the influence of slaughter procedures on residual blood

Previous studies have indicated that the amount of residual blood in fish is influenced both by pre-slaughter activity, anaesthetisation and killing procedures (Robb *et al*., 2003; Roth *et al*., 2005a; 2006). The effect of ante mortem stress and slaughtering procedures on residual blood was evaluated in both cod and salmon (Paper I and II).

When the large arteries or veins in the throat are cut during bleeding, the blood pressure drops rapidly and there is no driving force from the heart to empty the capillaries embedded in the muscle. It has been argued that efficient exsanguination is obtained only if the fish is alive (a

beating heart) during bleeding. Results from Paper I showed that both CO_2 stunned and percussive killed fish were almost equally exsanguinated. In contrast to what has been reported by others, a beating heart is not necessary for effective exsanguination (Robb *et al.*, 2003; Roth *et al.*, 2005a; Roth *et al.*, 2006; Roth *et al.*, 2009a; Roth *et al.*, 2009b). In addition, it was found that a blow to the head followed by evisceration was the best procedure in terms of reducing levels of residual blood in fish muscle. The quick removal of blood from the muscles by cutting the larger vessels in the abdominal cavity can explain these results.

Physical activity and stress is reported to increase the amount of residual blood in the muscle (Randall, 1970; Axelsson and Fritsche, 1991; Thorarensen *et al.*, 1993; Farrell *et al.*, 2001; Soldatov, 2006; Altimiras *et al.*, 2008), and results from paper I and II corroborated with these findings. This can be explained by the ability of the fish to redistribute and thus increase blood volume in its muscles, which slows down the exchange of blood between parts of the vascular system. Thus, only blood present in the gills, or in the vessels close to them, is purged when the gills are cut. Increased blood viscosity and ability to clot is another well-known physiological response to stress (Ruis and Bayne, 1997; Tavares-Dias and Oliveira, 2009).

Therefore, use of CO_2 stunning was not optimal, because it created vigorous activity and stress for several minutes before the fish was properly stunned (Paper I). It is known that Atlantic salmon have CO_2/pH-sensitive chemoreceptors on the gill arches, which respond to external changes in CO_2 and pH (Evans *et al*., 2005). The vigorous activity may be linked with fish feeling a sensation of strangulation when it enters anoxic water. Thus use of CO_2 to stun fish is accordingly disputed with regard to fish welfare. Carbon dioxide stunning also resulted in a rapid drop (pH 7.3 to 6.8) in the muscle pH ante mortem (Paper I). This is unfavourable if the fish is to be filleted pre-rigor. For newly percussive killed fish, not exposed to CO_2, the muscle pH was measured to pH ~ 7.4. However, the pH declined within hours after death, reaching the ultimate muscle pH at 6.2-6.4 from one tills four days post-mortem (Paper I, II and III). This is

within the normal range of both farmed salmon and cod (Olsen *et al.*, 2006; Kristoffersen *et al.*, 2007; Olsson *et al.*, 2007)

In the Norwegian fish industry, the fish is eviscerated within an hour after killing. The gutted fish can then either be filleted pre-rigor, or kept on ice for some days and then filleted post-rigor. However, independent of the slaughter methods used initially, it is known that gravity can result in more blood in fish muscle facing downward during ice storage (Roth *et al.*, 2007a; Roth *et al.*, 2009b). Furthermore, chilling prolongs the blood coagulation time (Paper I). Improved exsanguination can therefore be obtained if gutted fish is properly chilled, cleaned and then stored with the belly down, allowing the residual blood to drain out through the open belly as a result of gravitation during storage.

6.3 Evaluating the applicability of a non-destructive method

Both visual and chemical methods used to predict residual blood in fish muscle are labour-intensive. Chemical tests also involve toxic chemicals and are destructive to the product. Therefore, an investigation was carried out to examine whether already industrialized, visible and near-infrared (VIS/NIR) spectroscopy could be used to assess residual blood in intact fish (farmed cod) muscle (Paper II).

The VIS/NIR system was operated in fibre optic transflection mode, enabling spectral recordings in the range 400 to 1100 nm. The probe was located on the surface of the fillet and ten spectra from each sample were averaged in order to reduce noise. Multivariate statistical analysis of the VIS/NIR spectra was performed, including a partial least square (PLS) regression analysis to evaluate the correlation between the measured spectra and the chemically measured Hb content. Then a principal component analysis (PCA) was performed on the data in order to observe any clustering and to separate the main principle components (PCs) from the noise within the sample sets.

A correlation between the VIS/NIR and the chemical measurements was found. However, the correlations in the VIS- (400–700 nm) or the NIR region (700–1100 nm) alone were not as good as the complete VIS/NIR region (400–1100 nm, $R^2 = 0.83$). Information on Hb in muscle, both in the visible and NIR range of the spectrum is of importance. However, upon PCA cluster examination it was clear that the visible range of 400-700 nm contained most of the variations with regard to the presence of Hb in fish muscle, and the results seem to be related to the treatment of fish. Thus, the VIS/NIR measurement managed to demonstrate differences in whole fillet. These findings indicate that it may be possible to develop a spectroscopic method to measure residual blood in intact muscle. However, in this experiment the correlation was not sufficient to apply VIS/NIR spectroscopy as a feasible method for accessing residual blood in intact muscle. The correlation between the chemical haem pigment measured and the VIS/NIR spectral recording indicate how well this method applies to assess the chemical value. The weakness of the reference method will influence VIS/NIR measurements, meaning that the detection limits, robustness, variability and correlation will never be better than the reference method used initially.

With this in mind, the accuracy of the spectroscopic measurements was reduced when measuring Hb on whole muscle samples. This because whole muscle samples both contain different myoglobin derivatives in dark muscle (Marcinek *et al*., 2001; Hankeln *et al*., 2005), and different Hb derivatives (OHb, HHb, metHb) in blood filled vessels traversing the muscle (Zijlstra *et al*., 2000; Soldatov, 2006), which increases the heterogeneity. From the viewpoint of the fish processing industry, measurements on minced fish muscle are not an option for the evaluation of residual blood content in fish. The fiberoptic VIS/NIR probe as operated in this work also requires contact with the product that can cause problems regarding both food safety and the mechanics. Therefore, it does not satisfy the industrial requirements for online processing regarding speed, accuracy and food safety. For industrial application the preferred

instrumentation should operate at an online basis. An option could be non-contact imaging VIS/NIR spectroscopy. However, this method is still under development (Wold *et al.*, 2006; Heia *et al.*, 2007; Stormo *et al.*, 2007; Sivertsen *et al.*, 2009; Sivertsen *et al.*, 2011). By the using imaging spectroscopy it is possible to simultaneously record spectral and spatial data and obtain information about blood spots, as well as their location in the fillet (Figure 10). An optical imaging method will probably comply with the requirements for online fillet inspection. Although, it should be kept in mind that when using VIS/NIR spectroscopy to predict components in fish muscle, the reference method for calibration is decisive.

6.4 Selecting reference material in VIS/NIR spectroscopy

Bovine Hb is normally used as a reference for the determination of total Hb concentration in meat or blood, because there are insignificant variations in the optical Hb spectra among mammalian species (Zijlstra *et al.*, 2000). In mammalian Hb spectra, a pH independent triplicate isobestic point at ~ 523 nm is found. This point has been used to estimate the total concentration of Hb in solutions containing OHb, HHb and metHb in any combination (Snell and Marini, 1988; McMurdy *et al.*, 2008; Bender *et al.*, 2009). Due to this, it has been assumed that the isobestic point at ~ 523 nm also could be used to estimate the total concentration of Hb in fish muscle.

The suitability of using mammalian Hb as reference material when determining Hb in fish muscle spectrophotometrically was evaluated in Paper III. The aim was to examine the optical spectra of mammalian (human) and fish (farmed Atlantic cod) Hb subjected to pH 7.4 and 6.5. These two pH values were chosen in order to reflect both muscle pH at slaughter and the ultimate pH, which is relevant for post-rigor-processed fish.

The fish hemolysate was more affected by pH which induced alterations in mixtures of Hb derivatives, compared the mammalian hemolysate (Paper III). At pH 7.4, both the human and

the cod Hb were nearly fully oxygenated when exposed to oxygen during tonometry. However, at pH 6.5, it was mainly the cod Hb that was deoxygenated, and the concentration of HHb and metHb increased. Thus, the oxyHb, COHb, and HHb spectra obtained after tonometry was not pure, but rather a mixture of derivatives. Therefore, a spectral deconvolution was included to predict the exact mixture of each individual Hb (OHb, COHb, HHb, and metHb) spectrum. A pure spectrum of metHb was obtained by adding potassium ferricyanide to the hemolysate, thus assuming that all the relevant Hb derivatives in the solution were converted to metHb.

In the mammalian Hb spectra, a pH-independent isobestic point between OHb, HHb, and metHb at 523 nm was found (Figure 4). This was not found in the absorption spectra of fish Hb, and this clearly demonstrated the species differences in Hb spectra. The most significant difference between fish and mammalian Hb were found in the spectra of OHb and metHb.

The main pH-induced change was observed in the metHb spectrum. Compared to mammalian metHb, fish metHb was less influenced by changes in pH. No pH related differences were observed in the fish COHb and HHb spectra, or in the mammalian OHb, COHb, and HHb spectra. In the fish Hb spectra, a pH independent isobestic point was observed between OHb and HHb at 583 nm. In contrast to the isobestic point at 523 in the mammalian Hb spectra, the isobestic point at 583 nm was influenced by Hb auto-oxidation. In blood of newly killed fish, the OHb and HHb are the primary haemoglobin pigments (McMurdy *et al*., 2008). Thus an isobestic point between these two Hb derivatives could be used to highlight Hb in muscle. However, it is known that the auto-oxidation of Hb is dependent on the concentration of HHb and the presence of O_2 (Richards and Hultin, 2002; Undeland *et al*., 2004; Larsson *et al*., 2007; Richards *et al*., 2007; Maqsood and Benjakul, 2010; 2011). It is also known that both the rate of Hb oxidation and losses of hemin from fish Hb depend on changes in pH. This is partly explained by the differences found in the amino acid sequence, which reduce the oxygen affinity and cause structural changes in fish Hb when pH decline (Maestre *et al*., 2009;

Richards *et al*., 2009). A drop in pH will deoxygenate the Hb and in presence of O_2 this will initiate a spontaneous oxidation of Hb, thus changing the Hb spectra.

As previously mentioned astaxanthin is important when measuring Hb in salmonids because it influences the absorption spectra between 300-600 nm (Shahidi *et al*., 1998). The luminosity in the visible range (450–700 nm) will therefore depend on the quantity of both haem pigments and astaxanthin (Sanchez-Zapata *et al.*, 2010). However, the influence of the astaxanthin at 583 nm is minimal. Thus, an isobestic point at 583 nm between OHb and HHb can also be used to highlight blood spots in fillets of newly killed salmon and trout (Figure 10). Alternatively, a spectral deconvolution can be included; a method which has been used earlier to measure haem pigments in meat (Krzywicki, 1982). Then all changes in the Hb spectra, due to changes in pH and auto-oxidation, can be calculated. Furthermore, when using spectral deconvolution, the influence of astaxanthin can probably be calculated by including a spectrum of astaxanthin in the analysis. However, due to the differences found in the fish Hb spectra and the speed of the Hb auto-oxidation compared to mammalian Hb, a pure spectrum of all relevant Hb derivatives obtained for each species should be used as a standard for the determination of total Hb content in a sample.

7 Concluding remarks

Results obtained in this work showed that improved slaughter procedures might be implemented in order to reduce residual blood in fish muscle. Percussive stunning prior to bleeding was the best method in terms of minimising residual blood, but also to maintain fish welfare. Keeping control of the ante mortem activity is important for the fish welfare, as well as for the exsanguination and overall muscle quality. By using VIS/NIR spectroscopy it is possible to predict or highlight blood constituents in intact fish muscle, but further development is needed to make it applicable for the fish industry. However, results from this

study have highlighted the requirement of choosing proper reference material when implementing VIS/NIR spectroscopy to measure blood residuals in fish. Fish Hb differs from the mammalian and is in particular sensitive to pH changes. In addition, fish Hb is more reactive and has a faster rate of auto-oxidation. Such differences may lead to analytical errors if mammalian Hb is used as a reference.

8 Further work

With regard to fish welfare and muscle quality, the stunning methods used today are disputed. Further studies are needed to improve these methods and their goal should be to satisfy both the requests for fish welfare and to improve muscle quality. Further studies should also focus on possible industrial application of using non-contact imaging spectroscopy to assess residual blood in fish fillets, using fish Hb spectra as a reference. In addition, studies investigating possible differences in the Hb spectra between fish spices are both academically interesting and may also have relevance if VIS/NIR spectroscopy is to be applied industrially.

9 References:

Altimiras, J., Claireaux, G., Sandblom, E., Farrell, A.P., McKenzie, D.J., Axelsson, M., 2008. Gastrointestinal blood flow and postprandial metabolism in swimming sea bass Dicentrarchus labrax. Physiol. Biochem. Zool. 81, 663-672.

Anderssen, S., 1934. Årsberetning vedkommende Norges fiskerier 1934 - Nr. 2, Lofotfisket 1934. in: Fiskeridirektøren (Ed.). John Griegs Boktrykkeri AS, Bergen, Norway, pp. 96.

Ando, M., Nishiyabu, A., Tsukamasa, Y., Makinodan, Y., 1999. Post-mortem softening of fish muscle during chilled storage as affected by bleeding. J. Food Sci. 64, 423-428.

Asche, F., Roll, K.H., Tveteras, S., 2008. Future trends in aquaculture: productivity growth and increased production. in: Holmer, M., Black, K., Duarte, C.M., Marbà, N., Karakassis, I. (Eds.), Aquaculture in the ecosystem. Springer, New York, USA, pp. 271–292.

Ashley, P.J., 2007. Fish welfare: Current issues in aquaculture. Appl. Anim. Behav. Sci. 104, 199-235.

Axelsson, M., Fritsche, R., 1991. Effects of exercise, hypoxia and feeding on the gastrointestinal blood-flow in the Atlantic cod (*Gadus morhua*). J. Exp. Biol. 158, 181-198.

Balasubramaniam, P., Malathi, A., 1992. Comparative study of hemoglobin estimated by Drabkin's and Sahli's methods. J. Postgrad. Med. 38, 8-9.

Bender, J.E., Shang, A.B., Moretti, E.W., Yu, B., Richards, L.M., Ramanujam, N., 2009. Noninvasive monitoring of tissue hemoglobin using UV-VIS diffuse reflectance spectroscopy: a pilot study. Opt. Express. 17, 23396-23409.

Benito, M.T.J., Ojeda, C.B., Rojas, F.S., 2008. Process analytical chemistry: Applications of near infrared spectrometry in environmental and food analysis: An overview. Appl. Spectrosc. Rev. 43, 452-484.

Bernier, N.J., Randall, D.J., 1998. Carbon dioxide anaesthesia in rainbow trout: effects of hypercapnic level and stress on induction and recovery from anaesthetic treatment. J. Fish Biol. 52, 621-637.

Bjørnevik, M., 2003. White muscle distribution in Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) as affected by external factors, and relation to flesh quality. PhD thesis. University of Bergen, Bergen, Norway.

Blanco, M., Villarroya, I., 2002. NIR spectroscopy: a rapid-response analytical tool. Trac-Trend Anal. Chem. 21, 240-250.

Borderías, A.J., Sánchez-Alonso, I., 2011. First Processing Steps and the Quality of Wild and Farmed Fish. J. Food Sci. 76, R1-R5.

Botta, J.R., Squires, B.E., Johnson, J., 1986. Effect of bleeding gutting procedures on the sensory quality of fresh raw Atlantic cod (*Gadus morhua*). Can. Inst. Food Sci. Technol. J. 19, 186-190.

Brill, R.W., Cousins, K.L., Jones, D.R., Bushnell, P.G., Steffensen, J.F., 1998. Blood volume, plasma volume and circulation time in a high-energy-demand teleost, the yellowfin tuna (*Thunnus albacares*). J. Exp. Biol. 201, 647-654.

Brittain, T., 2005. Root effect hemoglobins. J. Inorg. Biochem. 99, 120-129.

Brown, W.D., 1961. Chromatography of myoglobin on diethylaminoethyl cellulose columns. J. Biol. Chem. 236, 2238-&.

Brown, W.D., 1962. The concentration of myoglobin and hemoglobin in tuna flesh. J. Food Sci., 27, 26–28.

Burka, J.F., Hammell, K.L., Horsberg, T.E., Johnson, G.R., Rainnie, D.J., Speare, D.J., 1997. Drugs in salmonid aquaculture - A review. J. Vet. Pharmacol. Therapeut. 20, 333-349.

References:

Bushnell, P.G., Jones, D.R., Farrell, A.P., 1992. The arterial system. in: Hoar, W.W., Randall, D.J., Farrell, A.P. (Eds.), The cardiovascular system, Fish physiology. Vol. 12A. Academic Press, San Diego, California, USA, pp. 89–139,.

Carpenter, C.E., Clark, E., 1995. Evaluation of methods used in meat iron analysis and iron content of raw and cooked meats. J. Agric. Food Chem. 43, 1824-1827.

Chau, A., Whitworth, M., Leadley, C., Millar, S., 2009. Innovative sensors to rapidly and non-destructively determine fish freshness. Report Nr. CMS/REP/110284/1. Campden BRI, Gloucestershire, UK., pp. 40.

Chen, Y.R., Huffman, R.W., Park, B., 1996. Changes in the visible/near-infrared spectra of chicken carcasses in storage. J. Food Process Eng. 19, 121-134.

Connell, J.J., 1995. in: Connell, J.J. (Ed.), Control of Fish Quality, 4th ed. Fishing News Books. The University Press, Cambridge, UK, pp. 61

de Souza, P.C., Bonilla-Rodriguez, G.O., 2007. Fish hemoglobins. Braz. J. Med. Biol. Res. 40, 769-778.

Digre, H., Erikson, U., Misimi, E., Lambooij, B., deVis, H.v., 2009. Electrical stunning of farmed Atlantic cod (*Gadus morhua* L): a comparison of an industrial and experimental method. Aquac. Res, 1-13.

Drabkin, D.L., 1950. The distribution of the chromoproteins, hemoglobin, myoglobin, and cytochrome-C, in the tissues of different species, and the relationship of the total content of each chromoprotein to body mass. J. Biol. Chem. 182, 317-333.

EFSA, 2004. Stunning and stun/killing methods for farmed fish, In: Welfare aspects of animal stunning and killing methods. EU Food Safety Authority, AHAW/04-027, pp. 155-185.

EFSA, 2009. Scientific opinion of the panel on animal health and welfare on a request from the European Commission on species-specific welfare aspects of the main systems of stunning and killing of farmed Atlantic salmon. in: EU Food Safety Authority (Ed.). The EFSA Journal 2012, pp. 1-77.

Einen, O., Thomassen, M.S., 1998. Starvation prior to slaughter in Atlantic salmon (*Salmo salar*) II. White muscle composition and evaluation of freshness, texture and colour characteristics in raw and cooked fillets. Aquaculture. 169, 37-53.

Engelsen, R., Asche, F., Skjennum, F., Adoff, G., 2004. New species in aquaculture: some basic economic aspects. in: Moksness, E., Kjørsvik, E., Olsen, Y. (Eds.), Culture of Cold-water Marine Fish. Blaxwell Publishing, Oxford, UK, pp. 487–515.

Erikson, U., 2008. Live chilling and carbon dioxide sedation at slaughter of farmed Atlantic salmon: a description of a number of commercial case studies. J. Appl. Aquac. 20, 38-61.

Erikson, U., Misimi, E., 2008. Atlantic salmon skin and fillet color changes effected by perimortem handling stress, rigor mortis, and ice storage. J. Food Sci. 73, C50-C59.

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., Misimi, E., Fismen, B., 2010. Bleeding of anaesthetized and exhausted Atlantic salmon: body cavity inspection and residual blood in pre-rigor and smoked fillets as determined by various analytical methods. Aquac. Res. 41, 496-510.

Erikson, U., Sigholt, T., Rustad, T., Einarsdottir, I.E., Jorgensen, L., 1999. Contribution of bleeding to total handling stress during slaughter of Atlantic salmon. Aquac. Int. 7, 101-115.

Eriksson, N.E., Johnson, G., 1979. Fisken. Landbruksforlaget, Oslo, Norway.

References:

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The nultifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97-177

Farrell, A.P., 1991. From hagfish to tuna - a perspective on cardiac-function in fish. Physiol. Zool. 64, 1137-1164.

Farrell, A.P., Jones, D.R., 1992. The heart. in: Hoar, W.W., Randall, D.J., A. P. Farrell (Eds.), The Cardiovascular System, Fish Physiology. Vol. 12a. Academic Press San Diego, California, USA, pp. 1-73.

Farrell, A.P., Thorarensen, H., Axelsson, M., Crocker, C.E., Gamperl, A.K., Cech, J.J., 2001. Gut blood flow in fish during exercise and severe hypercapnia. Comp. Biochem. Physiol. A. 128, 551-563.

Faucitano, L., Huff, P., Teuscher, F., Gariepy, C., Wegner, J., 2005. Application of computer image analysis to measure pork marbling characteristics. Meat Sci. 69, 537-543.

Fauske, M., 2008. Lønnsomhet i produksjon av oppdrettstorsk. Directorate of Fisheries, Bergen, Norway, http://www.fiskeridir.no/akvakultur/aktuelt/2008/loennsomhet-i-produksjon-av-oppdrettstorsk.

Folkestad, A., Wold, J.P., Rorvik, K.A., Tschudi, J., Haugholt, K.H., Kolstad, K., Morkore, T., 2008. Rapid and non-invasive measurements of fat and pigment concentrations in live and slaughtered Atlantic salmon (*Salmo salar* L.). Aquaculture. 280, 129-135.

Gallaugher, P., Farrell, A.P., 1998. Hematocrit and blood oxygencarrying capacity. in: Perry, S.F., Tufts, B.L. (Eds.), Fish respiration. Academic Press, New York, USA, pp. 185–222.

Giardina, B., Mosca, D., De Rosa, M.C., 2004. The Bohr effect of haemoglobin in vertebrates: an example of molecular adaptation to different physiological requirements. Acta Physiol. Scand. 182, 229-244.

Gomez, A.H., He, Y., Pereira, A.G., 2006. Non-destructive measurement of acidity, soluble solids and firmness of Satsuma mandarin using Vis/NIR-spectroscopy techniques. J. Food Eng. 77, 313-319.

Gregory, N.G., 2005. Recent concerns about stunning and slaughter. Meat Sci. 70, 481-491.

Hankeln, T., Ebner, B., Fuchs, C., Gerlach, F., Haberkamp, M., Laufs, T.L., Roesner, A., Schmidt, M., Weich, B., Wystub, S., Saaler-Reinhardt, S., Reuss, S., Bolognesi, M., De Sanctis, D., Marden, M.C., Kiger, L., Moens, L., Dewilde, S., Nevo, E., Avivi, A., Weber, R.E., Fago, A., Burmester, T., 2005. Neuroglobin and cytoglobin in search of their role in the vertebrate globin family. J. Inorg. Biochem. 99, 110-119.

Hastein, T., Scarfe, A.D., Lund, V.L., 2005. Science-based assessment of welfare: aquatic animals. Rev. Sci. Tech. Off. Int. Epiz. 24, 529-547.

He, Y., Li, X.L., Shao, Y.N., 2006a. Discrimination of varieties of apple using near infrared spectra based on principal component analysis and artificial neural network model. Spectros. Spect. Anal. 26, 850-853.

He, Y., Feng, S.J., Deng, X.F., Li, X.L., 2006b. Study on lossless discrimination of varieties of yogurt using the Visible/NIR-spectroscopy. Food Res. Int. 39, 645-650.

Heia, K., Sivertsen, A.H., Stormo, S.K., Elvevoll, E., Wold, J.P., Nilsen, H., 2007. Detection of nematodes in cod (*Gadus morhua*) fillets by imaging spectroscopy. J. Food Sci. 72, E11-E15.

Hjeltnes, B., Erikson, U., Mejdell, C., Olsen, R.E., Slinde, E., Waagbø, R., 2010. Risikovurdering knyttet til bruk av gass, slag mot hode og strøm til bedøving av fisk. Uttalelse fra Faggruppe for dyrehelse og dyrevelferd i Vitenskapskomiteen for mattrygghet. Norwegian Scientific committee for Food Safety 09/810, pp. 1-21.

Hornsey, H.C., 1956. The colour of cooked cured pork. I.- Estimation of the nitric oxide-haem pigments. J. Sci. Food Agric. 7, 534 - 540.

References:

Huang, H.B., Yu, H.Y., Xu, H.R., Ying, Y.B., 2008. Near infrared spectroscopy for on/in-line monitoring of quality in foods and beverages: A review. J. Food Eng. 87, 303-313.

Huss, H.H., 1995. Biological aspects. in: Huss, H.H. (Ed.), Quality and quality changes in fresh fish, FAO Fisheries Technical Paper No. 348,. Food and Agriculture Organization, Roma, Italy, pp. 9-19.

Haard, N.F., 1992. Control of chemical-composition and food quality attributes of cultured fish. Food Res. Int. 25, 289-307.

Jensen, F.B., 2007. Nitric oxide formation from nitrite in zebrafish. J. Exp. Biol. 210, 3387-3394.

Johnston, I.A., 1980. Specialisations of fish muscle. in: Goldspink, D.F. (Ed.), Development and specialization of skeletal muscle. Seminar series (Society for Experimental Biology), Vol. 7, Cambridge University Press, Cambridge, UK, pp. 123–148.

Johnston, I.A., 1981. Structure and function of fish muscles. in: Day, M.H. (Ed.), Vertebrate locomotion: (the proceedings of a symposium held at the Zoological Society of London on 27 and 28 March 1980). Issue 48, Symposia of the Zoological Society of London, Academic Press, London, UK, pp. 71–113.

Johnston, I.A., 1983. Dynamic properties of fish muscle. in: Webb, P.W., Weihs, D. (Eds.), Fish Biomechanics. Praeger Publishers, New York, USA, pp. 36-67.

Karlsson, A., Lundstrom, K., 1991. Meat pigment determination by a simple and nontoxic alkaline hematin method - (an alternative to the Hornsey and the cyanometmyoglobin methods). Meat Sci. 29, 17-24.

Kranen, R.W., Van Kuppevelt, T.H., Goedhart, H.A., Veerkamp, C.H., Lambooy, E., Veerkamp, J.H., 1999. Hemoglobin and myoglobin content in muscles of broiler chickens. Poultry Sci. 78, 467-476.

Krzywicki, K., 1982. The determination of haem pigments in meat. Meat Sci. 7, (1), 29-36

Kristoffersen, S., Vang, B., Larsen, R., Olsen, R.L., 2007. Pre-rigor filleting and drip loss from fillets of farmed Atlantic cod (*Gadus morhua* L.). Aquac. Res. 38, 1721-1731.

Lambooij, E., Kloosterboer, R.J., Gerritzen, M.A., van de Vis, J.W., 2004. Head-only electrical stunning and bleeding of African catfish (*Clarias gariepinus*): assessment of loss of consciousness. Anim. Welfare. 13, 71-76.

Lambooij, E., Grimsbo, E., van de Vis, J.W., Reimert, H.G.M., Nortvedt, R., Roth, B., 2010. Percussion and electrical stunning of Atlantic salmon (*Salmo salar*) after dewatering and subsequent effect on brain and heart activities. Aquaculture. 300, 107-112.

Larsson, K., Almgren, A., Undeland, I., 2007. Hemoglobin-mediated lipid oxidation and compositional characteristics of washed fish mince model systems made from cod (*Gadus morhua*), herring (*Clupea harengus*), and salmon (*Salmo salar*) muscle. J. Agric. Food Chem. 55, 9027-9035.

Lee, M.H., Cavinato, A.G., Mayes, D.M., Rasco, B.A., 1992. Noninvasive short-wavelength near-infrared spectroscopic method to estimate the crude lipid-content in the muscle of intact rainbow-trout. J. Agric. Food Chem. 40, 2176-2181.

Lines, J.A., Robb, D.H., Kestin, S.C., Crook, S.C., Benson, T., 2003. Electric stunning: a humane slaughter method for trout. Aquac. Eng. 28, 141-154.

Lovdata, 2009. Lov om dyrevelferd (Animal welfare law). in: LMD (Ed.), LOV-2009-06-19-97. Lovdata, Oslo, Norway.

Love, R.M., 1975. Variability in Atlantic Cod (*Gadus morhua*) from Northeast Atlantic - Review of Seasonal and Environmental Influences on Various Attributes of Flesh. J. Fish. Res. Board Can. 32, 2333-2342.

Love, R.M., 1978. Dark colour in white fish flesh. Torry Advisory Notes - No. 76. Ministry of Agriculture, Fisheries and Food. Torry Research Station. HMSO Press, Edinburgh, UK.

References:

Maestre, R., Pazos, M,. Medina, I., 2009. Involvement of methemoglobin (MetHb) formation and hemin loss in the pro-oxidant activity of fish hemoglobins. J. Agric. Food Chem. 57, 7013-7021

Mancini, R.A., Hunt, M.C., 2005. Current research in meat color. Meat Sci. 71, 100-121.

Maqsood, S., Benjakul, S., 2010. Comparative studies on molecular changes and pro-oxidative activity of haemoglobin from different fish species as influenced by pH. Food Chem. 124 (3), 875–883.

Maqsood, S., Benjakul, S., 2011,Comparative studies on molecular changes and pro-oxidative activity of haemoglobin from different fish species as influenced by pH. Food Chem. 124 (3), 875–883.

Marcinek, D.J., Bonaventura, J., Wittenberg, J.B., Block, B.A., 2001. Oxygen affinity and amino acid sequence of myoglobins from endothermic and ectothermic fish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 280, R1123-R1133.

McMurdy, J.W., Jay, G.D., Suner, S., Crawford, G., 2008. Noninvasive optical, electrical, and acoustic methods of total hemoglobin determination. Clin. Chem. 54, 264-272.

Michie, I., 2001. Causes of downgrading in the salmon farming industry. in: Kestin, S.C., Warriss, P.D. (Eds.), Farmed Fish Quality. Blackwell, Oxford, UK, pp. 405-406.

Midling, K.Ø., Mejdell, C., Olsen, S.H., Tobiassen, T., Aas-Hansen, Ø., Aas, K., Harris, S., Oppedal, K., Femsteinevik, Å., 2008. Slakting av oppdrettslaks på båt, direkte fra oppdrettsmerd, NOFIMA Rapport 6/2008, ISBN: 978-82-7251-637-5. NOFIMA AS, Tromso, Norway, pp. 59.

Mlcek, J., Sustova, K., Simeonovova, J., 2006. Application of FT NIR spectroscopy in the determination of basic chemical composition of pork and beef. Czech J. Anim. Sci. 51, 361-368.

Moorman, A.F.M., Christoffels, V.M., 2003. Cardiac chamber formation: Development, genes, and evolution. Physiol. Rev. 83, 1223-1267.

Morkore, T., Mazo, P.I., Tahirovic, V., Einen, O., 2008. Impact of starvation and handling stress on rigor development and quality of Atlantic salmon (*Salmon salar* L). Aquaculture. 277, 231-238.

Ni, Y.N., Gong, X.F., 1997. Simultaneous spectrophotometric determination of mixtures of food colorants. Anal. Chim. Acta. 354, 163-171.

Nicolai, B.M., Beullens, K., Bobelyn, E., Peirs, A., Saeys, W., Theron, K.I., Lammertyn, J., 2007. Nondestructive measurement of fruit and vegetable quality by means of NIR spectroscopy: A review. Postharvest Biol. Tec. 46, 99-118.

Nilsen, H., Esaiassen, M., 2005. Predicting sensory score of cod (*Gadus morhua*) from visible spectroscopy. Lwt-Food Sci. Technol. 38, 95-99.

Nilsen, H., Heia, K., Esaiassen, M., 2009. Basic composition: Rapid methodologies in: Nollet, L.M.L., Toldra, F. (Eds.), Handbook of Seafood and Seafood Products Analysis. CRC Press, New York, USA, pp. 121-138.

Nilsen, H., Esaiassen, M., Heia, K., Sigernes, F., 2002. Visible/near-infrared spectroscopy: A new tool for the evaluation of fish freshness? J. Food Sci. 67, 1821-1826.

Nilsen, H.A., Heia, K., 2009. VIS/NIR spectroscopy. in: Rehbein, H., Oehlenschlager, J. (Eds.), Fishery Products: Quality, Safety and Authenticity. Wiley-Blackwell, West Sussex, UK, pp. 89-104.

Olsen, S.H., Elvevoll, E.O., 2011. A pH induced shift in the hemoglobin spectra – A spectrophotometeric comparison of Atlantic cod (*Gadus morhua*) and mammalian haemoglobin. J. Agric. Food Chem. 59 (4), pp 1415–1422.

Olsen, S.H., Sorensen, N.K., Stormo, S.K., Elvevoll, E.O., 2006. Effect of slaughter methods on blood spotting and residual blood in fillets of Atlantic salmon (*Salmo salar*). Aquaculture. 258, 462-469.

Olsen, S.H., Sorensen, N.K., Larsen, R., Elvevoll, E.O., Nilsen, H., 2008. Impact of pre-slaughter stress on residual blood in fillet portions of farmed Atlantic cod (*Gadus morhua*) - Measured chemically and by Visible and Near-infrared spectroscopy. Aquaculture. 284, 90-97.

References:

Olson, K.R., 1996. Secondary circulation in fish: Anatomical organization and physiological significance. J. Exp. Zool. 275, 172-185.

Olsson, G.B., Seppola, M.A., Olsen, R.L., 2007. Water-holding capacity of wild and farmed cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) muscle during ice storage. Lwt-Food Sci. Technol. 40, 793-799.

Osborne, B.G., 2001. Near-infrared spectroscopy in food analysis. in: Meyers, R.A. (Ed.), Encyclopedia of Analytical Chemistry: Applications, Theory, and Instrumentation. John Wiley & Sons Ltd, Chichester, UK.

Pazos, M., Medina, I., Hultin, H.O., 2005. Effect of pH on hemoglobin-catalyzed lipid oxidation in cod muscle membranes in vitro and in situ. J. Agric. Food Chem. 53, 3605-3612.

Pazos, M., Andersen, M.L., Skibsted, L.H., 2009. Efficiency of Hemoglobin from Rainbow Trout, Cod, and Herring in Promotion of Hydroperoxide-Derived Free Radicals. J. Agric. Food Chem. 57, 8661-8667.

Pellegrini M., Giardina B., Verde C., Carratore V., Olianas A., Sollai L., Sanna M.T., Castagnola M., di Prisco G., 2003. Structural–functional characterisation of the cathodic haemoglobin of the conger eel, *Conger conger*: molecular modelling study of an additional phosphate-binding site. Biochem. J. 372, 679-686.

Pelster, B., Decker, H., 2004. The Root effect - a physiological perspective. Micron. 35, 73-74.

Pelster, B., Randall, D., 1998. The physiology of the Root effect. in: Perry, S.F., Tufts, B.L. (Eds.), Fish respiration. Academic Press, New York, USA, pp. 113–139.

Poli, B.M., Parisi, G., Scappini, F., Zampacavallo, G., 2005. Fish welfare and quality as affected by pre-slaughter and slaughter management. Aquac. Int. 13, 29-49.

Prevolnik, M., Candek-Potokar, M., Skorjanc, D., 2004. Ability of NIR spectroscopy to predict meat chemical composition and quality - a review. Czech J. Anim. Sci. 49, 500-510.

Qiao, J., Ngadi, M.O., Wang, N., Gariepy, C., Prasher, S.O., 2007. Pork quality and marbling level assessment using a hyperspectral imaging system. J. Food Eng. 83, 10-16.

Randall, D.J., 1970. The circultatory system. in: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, Vol. 4. Academic Press New York, USA, pp. 133–168.

Reich, G., 2005. Near-infrared spectroscopy and imaging: Basic principles and pharmaceutical applications. Adv. Drug Deliv. Rev. 57, 1109-1143.

Richards, M.P., 2000. Contributions of blood and blood components to lipid oxidation in fish muscle. Ph.D thesis. University of Massachusetts Amherst. Ohio, USA.

Richards, M.P., Hultin, H.O., 2000. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: A possible role for deoxyhemoglobin. J. Agric. Food Chem. 48, 3141-3147.

Richards, M.P., Hultin, H.O., 2002. Contributions of blood and blood components to lipid oxidation in fish muscle. J. Agric. Food Chem. 50, 555-564.

Richards, M.P., Nelson, N.M., Kristinsson, H.G., Mony, S.S.J., Petty, H.T., Oliveira, A.C.M., 2007. Effects of fish heme protein structure and lipid substrate composition on hemoglobin-mediated lipid oxidation. J. Agric. Food Chem. 55, 3643-3654.

Richards, M.P., Aranda Roman, IV., He, C., Phillips G.N. Jr., 2009. Effect of pH on structural changes in Perch hemoglobin that can alter redox stability and heme affinity. J. Aquat. Food Prod. Tech. 18, 416-423

Robb, D., Whittington, P., 2004. Fish Welfare: a quality issue, In: Fish Farming International, Vol. 31, No 1. January, pp. 28.

Robb, D.H.F., Kestin, S.C., 2002. Methods used to kill fish: Field observations and literature reviewed. Anim. Welfare. 11, 269-282.

References:

Robb, D.H.F., Roth, B., 2003. Brain activity of Atlantic salmon (*Salmo salar*) following electrical stunning using various field strengths and pulse durations. Aquaculture. 216, 363-369.

Robb, D.H.F., Phillips, A.J., Kestin, S.C., 2003. Evaluation of methods for determining the prevalence of blood spots in smoked Atlantic salmon and the effect of exsanguination method on prevalence of blood spots. Aquaculture. 217, 125-138.

Robb, D.H.F., Wotton, S.B., McKinstry, J.L., Sorensen, N.K., Kestin, S.C., 2000. Commercial slaughter methods used on Atlantis salmon: determination of the onset of brain failure by electroencephalography. Vet. Rec. 147, 298-303.

Rodkey, F.L., 1967. Kinetic aspects of cyanmethemoglobin formation from carboxyhemoglobin. Clin. Chem. 13, 2-&.

Roth, B., Torrissen, O.J., Slinde, E., 2005a. The effect of slaughtering procedures on blood spotting in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Aquaculture. 250, 796-803.

Roth, B., Slinde, E., Robb, D.H.F., 2006. Field evaluation of live chilling with CO2 on stunning Atlantic salmon (*Salmo salar*) and the subsequent effect on quality. Aquac. Res. 37, 799-804.

Roth, B., Slinde, E., Robb, D.H.F., 2007a. Percussive stunning of Atlantic salmon (*Salmo salar*) and the relation between force and stunning. Aquacult. Eng. 36, 192-197.

Roth, B., Birkeland, S., Oyarzun, F., 2009a. Stunning, pre slaughter and filleting conditions of Atlantic salmon and subsequent effect on flesh quality on fresh and smoked fillets. Aquaculture. 289, 350-356.

Roth, B., Imsland, A., Moeller, D., Slinde, E., 2003. Effect of electric field strength and current duration on stunning and injuries in market-sized Atlantic salmon held in seawater. N. Am. J. Aquac. 65, 8-13.

Roth, B., Johansen, S.J.S., Suontama, J., Kiessling, A., Leknes, O., Guldberg, B., Handeland, S., 2005b Seasonal variation in flesh quality, comparison between large and small Atlantic salmon (*Salmo salar*) transferred into seawater as 0+or 1+smolt. Aquaculture 250, 830–840.

Roth, B., Obach, A., Hunter, D., Nortvedt, R., Oyarzun, F., 2009b. Factors affecting residual blood and subsequent effect on bloodspotting in smoked Atlantic salmon fillets. Aquaculture. 297, 163-168.

Roth, B., Schelvis-Smit, R., Stien, L.H., Foss, A., Norwedt, R., Imsland, A., 2007b. Exsanguination of turbot and the effect on fillet quality measured mechanically, by sensory evaluation, and with computer vision. J. Food Sci. 72, E525-E531.

Ruis, M.A.W., Bayne, C.J., 1997. Effects of acute stress on blood clotting and yeast killing by phagocytes of rainbow trout. J. Aquat. Anim. Health. 9, 190-195.

Saenz, C., Hernandez, B., Alberdi, C., Alfonso, S., Dineiro, J.M., 2008. A multispectral imaging technique to determine concentration profiles of myoglobin derivatives during meat oxygenation. Eur. Food Res. Technol. 227, 1329-1338.

Saguer, E., Fort, N., Regenstein, J.M., 2006. Fish (rainbow trout) blood and Its fractions as food ingredients. J.Aquat. Food Prod. Technol. 15, 19-51.

Sakai, T., Ohtsubo, S., Minami, T., Terayama, M., 2006. Effect of bleeding on hemoglobin contents and lipid oxidation in the skipjack muscle. Biosci. Biotechnol. Biochem. 70, 1006-1008.

Sanchez-Zapata, E., Perez-Alvarez, J.A., Fernandez-Lopez, J., Barber-Valles, J.X., 2010. Descriptive study of reflectance spectra of hake (*Merluccius australis*), salmon (*Salmo salar*) and light and dark muscle from tuna (*Thunnus thynnus*). J. Food Quality. 33, 391-403.

Sandblom, E., Axelsson, M., 2007. The venous circulation: A piscine perspective. Comp. Biochem. Phys. A. 148, 785-801.

References:

Sandblom, E., Farrell, A.P., Altimiras, J., Axelsson, M., Claireaux, G., 2005. Cardiac preload and venous return in swimming sea bass (*Dicentrarchus labrax* L.). J. Exp. Biol. 208, 1927-1935.

Satchell, G.H., 1992. The venous system. in: Hoar, W.W., Randall, D.J., Farrell, A.P. (Eds.), The cardiovascular system, Fish Physiology. Vol. 12A. Academic Press, San Diego, Calif, USA, pp. 141-179.

Schubring, R., 2008. Use of "filtered smoke" and carbon monoxide with fish. J. Verbr. Lebensm. 3, 31-44.

Scotter, C.N.G., 1997. Non-destructive spectroscopic techniques for the measurement of food quality. Trends Food Sci. Technol. 8, 285-292.

Shahidi, F., Metusalach, Brown, J.A., 1998. Carotenoid pigments in seafoods and aquaculture. Crit. Rev. .Food Sci. Nutr. 38, 1-67.

Sigernes, F., Lorentzen, D.A., Heia, K., Svenoe, T., 2000. Multipurpose spectral imager. Appl. Optics. 39, 3143-3153.

Sivertsen, A.H., Kimiya, T., Heia, K., 2011. Automatic freshness assessment of cod (*Gadus morhua*) fillets by Vis/Nir spectroscopy. J. Food Eng. 103, 317-323.

Sivertsen, A.H., Chu, C.K., Wang, L.C., Godtliebsen, F., Heia, K., Nilsen, H., 2009. Ridge detection with application to automatic fish fillet inspection. J. Food Eng. 90, 317-324.

Skjervold, P.O., Fjaera, S.O., Ostby, P.B., 1999. Rigor in Atlantic salmon as affected by crowding stress prior to chilling before slaughter. Aquaculture. 175, 93-101.

Skjervold, P.O., Fjaera, S.O., Ostby, P.B., Einen, O., 2001. Live-chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). Aquaculture. 192, 265-280.

Snell, S.M., Marini, M.A., 1988. A convenient spectroscopic method for the estimation of hemoglobin concentrations in cell-free solutions. J. Biochem Bioph. Meth. 17, 25-34.

Soldatov, A.A., 2006. Organ blood flow and vessels of microcirculatory bed in fish. J. Evol. Biochem. Phys. 42, 243-252.

Sorensen, N.K., Tobiassen, T., Carlehög, M., 2004. How do handling and killing methods affect ethical and sensory quality of farmed Atlantic salmon. in: Sakaguchi, M. (Ed.), More Efficient Utilization of Fish and Fisheries Products. Elsevier, Amsterdam, pp. 301–307.

SSB, 2010. Record year for Norwegian salmon. Statistics Norway Oslo, Norway, http://www.ssb.no/english/subjects/10/05/fiskeoppdrett_en/.

Standal, D., Utne, I.B., 2007. Can cod farming affect cod fishing? A system evaluation of sustainability. Mar. Policy. 31, 527-534.

Steinke, J.M., Shepherd, A.P., 1992. Effects of temperature on optical absorbance spectra of oxyhemoglobin, carboxyhemoglobin, and deoxyhemoglobin. Clin. Chem. 38, 1360-1364.

Stormo, S.K., 2009. Anisakid rundworm larvea - Detection of parasites in Cod muscle and the freeze tolerance of Sealworm (*Pseudoterranova decipiens*). Ph.D thesis. University of Tromsø, Tromsø, Norway.

Stormo, S.K., Sivertsen, A.H., Heia, K., Nilsen, H., Elvevoll, E., 2007. Effects of single wavelength selection for anisakid roundworm larvae detection through multispectral imaging. J. Food Protect. 70, 1890-1895.

Summers, A.P., 2004. Biomechanics - Fast fish. Nature. 429, 31-33.

Synowiecki, J., Heeley, D.H., Shahidi, F., 1992. Purification and characterization of myoglobin from harp seal (*Phoca groenlandica*). J. Agric. Food Chem. 40, 714-718.

Tavares-Dias, M., Oliveira, S.R., 2009. A review of the blood coagulation system of fish. Braz. J. Biosci. 7 205-224.

References:

Terlouw, E.M.C., Arnould, C., Auperin, B., Berri, C., Le Bihan-Duval, E., Deiss, V., Lefevre, F., Lensink, B.J., Mounier, L., 2008. Pre-slaughter conditions, animal stress and welfare: current status and possible future research. Animal. 2, 1501-1517.

Thorarensen, H., Gallaugher, P.E., Kiessling, A.K., Farrell, A.P., 1993. Intestinal blood-flow in swimming chinook salmon (*Oncorhynchus tshawytscha*) and the effects of hematocrit on blood-flow distribution. J. Exp. Biol. 179, 115-129.

Tretsven, W.I., Patten, B.G., 1981. Effect of arterial incisions on the amount of bleeding and flesh quality of rainbow-trout. Mar. Fish. Rev. 43, 16-18.

Tzeng, D.Y., Berns, R.S., 2005. A review of principal component analysis and its applications to color technology. Color Res. Appl. 30, 84-98.

Uddin, M., Okazaki, E., Turza, S., Yumiko, Y., Tanaka, M., Fukuda, Y., 2005. Non-destructive visible/NIR spectroscopy for differentiation of fresh and frozen-thawed fish. J. Food Sci. 70, C506-C510.

Undeland, I., Kristinsson, H.G., Hultin, H.O., 2004. Hemoglobin-mediated oxidation of washed minced cod muscle phospholipids: Effect of pH and hemoglobin source. J. Agric. Food Chem. 52, 4444-4451.

van de Vis, H., Kestin, S., Robb, D., Oehlenschlager, J., Lambooij, B., Munkner, W., Kuhlmann, H., Kloosterboer, K., Tejada, M., Huidobro, A., Ottera, H., Roth, B., Sorensen, N.K., Akse, L., Byrne, H., Nesvadba, P., 2003. Is humane slaughter of fish possible for industry? Aquac. Res. 34, 211-220.

Vareltzis, P., Hultin, H.O., Autio, W.R., 2008. Hemoglobin-mediated lipid oxidation of protein isolates obtained from cod and haddock white muscle as affected by citric acid, calcium chloride and pH. Food Chem. 108, 64-74.

Vazquez, G.R., Guerrero, G.A., 2007. Characterization of blood cells and hematological parameters in *Cichlasoma dimerus* (Teleostei, Perciformes). Tissue & Cell. 39, 151-160.

Verde, C., Vergara, A., Giordano, D., Mazzarella, L., Di Prisco, G., 2007. The Root effect - a structural and evolutionary perspective. Antarc. Sci. 19, 271-278.

Volkel, S., Berenbrink, M., 2000. Sulphaemoglobin formation in fish: A comparison between the haemoglobin of the sulphide-sensitive rainbow trout (*Oncorhynchus mykiss*) and of the sulphide-tolerant common carp (*Cyprinus carpio*). J. Exp. Biol. 203, 1047-1058.

Warriss, P., 1976. Quantitative-determination of hemoglobin in ovine muscles. Anal. Biochem. 72, 104-112.

Warriss, P.D., Rhodes, D.N., 1977. Hemoglobin concentrations in beef. J. Sci. Food Agric. 28, 931-934.

Warriss, P.D., Wilkins, L.J., 1987. Exsanguination of meat animals. in: Carter, H.E., Carter, V.R. (Eds.), Pre-slaughter Stunning of Food Animals. Royal Society for Prevention of Cruelty to Animals, Horsham, Sussex, UK, pp. 150–158.

Williams, T.M., Wortby, G.A.J., 2002. Anatomy and Pysiology: the challenge of aquatic living. in: Hoelzel, R.A. (Ed.), Marine mammal biology: an evolutionary approach. Blackwell Publishing, Oxford, UK, pp. 73-97.

Wilson, R.H., Tapp, H.S., 1999. Mid-infrared spectroscopy for food analysis: recent new applications and relevant developments in sample presentation methods. Trac-Trend. Anal. Chem. 18, 85-93.

Wold, J.P., Isaksson, T., 1997. Non-destructive determination of fat and moisture in whole Atlantic salmon by near-infrared diffuse spectroscopy. J. Food Sci. 62, 734-736.

Wold, J.P., Johansen, I.R., Haugholt, K.H., Tschudi, J., Thielemann, J., Segtnan, V.H., Narum, B., Wold, E., 2006. Non-contact transflectance near infrared imaging for representative on-line sampling of dried salted coalfish (bacalao). J. near Infrared Spec. 14, 59-66.

References:

Workman, J.J., 1999. Review of process and non-invasive near-infrared and infrared spectroscopy: 1993-1999. Appl. Spectrosc. Rev. 34, 1-89.

Yam, K.L., Papadakis, S.E., 2004. A simple digital imaging method for measuring and analyzing color of food surfaces. J. Food Eng. 61, 137-142.

Yang, X.J., Albrecht, E., Ender, K., Zhao, R.Q., Wegner, J., 2006. Computer image analysis of intramuscular adipocytes and marbling in the longissimus muscle of cattle. J. Anim. Sci. 84, 3251-3258.

Yoshikawa, H., Kawai, F., Kanamori, M., 1994. The Relationship between the Eeg and Brain Ph in Carp, Cyprinus-Carpio, Subjected to Environmental Hypercapnia at an Anesthetic Level. Comp. Biochem. Phys. A. 107, 307-312.

Yoshikawa, H., Yokoyama, Y., Ueno, S., Mitsuda, H., 1991. Electroencephalographic Spectral-Analysis in Carp, *Cyprinus carpio*, Anesthetized with High-Concentrations of Carbon-Dioxide. Comp. Biochem. Phys. A. 98, 437-444.

Zijlstra, W.G., Buursma, A., 1997. Spectrophotometry of hemoglobin: Absorption spectra of bovine oxyhemoglobin, deoxyhemoglobin, carboxyhemoglobin, and methemoglobin. Comp. Biochem. Phys. B. 118, 743-749.

Zijlstra, W.G., Buursma, A., van Assendelft, O.W., 2000. in: Zijlstra, W.G., Buursma, A., van Assendelft, O.W. (Eds.), Visible and near Infrared absorption spectra of human and animal haemoglobin. VSP Publishing, Utrecht, the Netherlands, pp. 55-187.