Study of meat qualities directly or indirectly related to mitochondria

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Acknowledgements

I choose the topic related to mitochondria because of my interest to have a deeper understanding about the organelle. Before starting my thesis, I had no idea about the relation of meat color to mitochondria. However, my interest started to rise later. Although consumers just see the color of the meat, after doing this thesis, I can also understand the reason behind change in meat color and different biochemical events that might have taken place in the meat. Studying about drip loss, cooking loss and tenderness was equally interesting. It was my life time experience and privilege to get chance to work in slaughterhouse at Rudshøgda, Norway during the sample collection for my thesis. The cleanliness and handling of the animals and meat products in slaughterhouse were very exciting to learn about.

Being a vegetarian myself, I always had an issue with torture animals had to face while they were slaughtered. But knowing the way of handling and slaughter of animals in Rudshøgda changed my view totally. I got chance to use different instruments and learned many techniques during my thesis, which I hope will help me in my future career in this field. With the continuous help from experienced and hard-working group, I was able to write paper about the calibration of myoglobin samples and co-author another paper related to mitochondria oxygen consumption rate and its link to color stability in muscle.

I want to give the main credit of my achievements during this thesis to my supervisor Prof. Bjørg Egelandsdal and my co-supervisor Vinh T Phung. Bjørg Egelandsdal was always the source of inspiration for me who helped me overcome many challenges during the thesis work and paper writing as well. I want to give a big thanks to Vinh T Phung for his friendly nature, constructive suggestions, comments and moral support while carrying out the entire task of the thesis and paper writing. It was a matter of privilege to get chance to work with Prof. Tomas Issakson who helped me with the statistical portion of data processing from calibration and color measurements samples. With his help, I learned to use Unscrambler software and various calculation techniques inside it. He was always helpful and supportive and gave good suggestions for making figures and tables for both thesis and paper.

I would also like to thank Oddvin Sørheim who helped us to work with new method called as OPP method which is used in both thesis and paper writing, during

calibration for color measurements in meat and also for helping to find details about it. His helps were always very inspiring and motivating. I would also like to thank Erik Slinde for his advice and help. I am very thankful to Rawank Jahn who helped me with the Warner Bratzler measurements.
Finally, I would like to thank my family and friends for the support and encouragement which they always gave me throughout my whole life.

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Prologue

I began my thesis by learning to isolate mitochondria from the muscles of beef. It took me some time to gain knowledge enough to carry out various steps of isolating mitochondria efficiently. The main experiment began with the collection of samples by me and my co-supervisor Vinh T Phung in the slaughterhouse at Rudshøgda, Norway. It took around five weeks to collect all the 41 samples. As we started on the first week, we had to do some trial of experiments for around a week to fix the experimental setup and to get habituated with the working environment in the slaughter house. My main work was to collect samples for color measurement, tenderness, drip loss and cooking loss, measure pH of muscles and isolate mitochondria for proteomic study. After successful completion of sample collection for 3 weeks in slaughterhouse, we worked at University of life Sciences (UMB) on those samples. We returned back and again collected the remaining samples and did further analysis as before.

Spectroscopic color analysis of the sample require good calibration model for prediction of myoglobin states of meat samples. Therefore, we started the calibration with the method that is popularly used all over the world (as explained in American Meat Science Association, AMSA guidelines). However, the method didn't prove to be satisfactory due to its various drawbacks during handling and processing. Therefore, my supervisor Prof. Bjørg Egelandsdal consulted other group members and decided to also use another method called oxygen partial pressure packaging (OPP) method for preparing a calibration model. The spectral measurements from both methods of calibration were processed using different statistical methods with the help of Prof. Tomas Issakson. The results with OPP method was promising compared to the classical method. Therefore, we worked together in a group to write a paper (I) titled; "New procedure for improving precision and accuracy of instrumental color measurements of beef". The paper has already been submitted to Journal of Meat Science and been positively reviewed.

By using the best calibration model for color measurement, we calculated myoglobin state change with time for all samples. Same samples were measured for color (variables L*, a*, b*) using Minolta as well. Tenderness, drip loss and cooking loss were also measured for the collected samples.

Further study of relationship of color change, slaughter variables and meat quality parameters were done by the PhD. Vinh T Phung. In his work the method of calculating myoglobin states was a prerequisite. I therefore co-author a second paper (II) titled; "Mitochondrial oxygen consumption in permeabilization fibers and its link to color stability in bovine *M. semimembranosus* muscle" that was written from the findings of Vinh's work and my thesis work. This paper has also been submitted to Journal of Meat Science.

There was collaboration between Vinh T Phung and myself during this whole project, so we have shared our data and results with each other. Since different work related to meat has been done during this period, my thesis is divided into three parts. The Oxygen consumption rate (OCR) data has been provided to me by Vinh T Phung which is used in part I and part III of my thesis. Part I deals with the tenderness, drip loss and cooking loss in muscles and their relationship to oxygen consumption rate of muscle after 3 weeks of chill storage whereas part III (taken from paper (II)) solely describes relationship between OCR and color change for 3 weeks stored muscle. However, part II (taken from paper (I)) compares the calibration methods and give us the best model to calculate the color in three weeks stored meat sample.

Hence my thesis is little away from the mainstream ones and contains one introduction whereas it has three parts with their own material and methods section, result and discussion sections and the conclusions. Due to the lack of time, we were not able to work with the proteomic part of mitochondria which was isolated early post mortem in the slaughterhouse. Hopefully, the samples can be used by a future Master student. The data from measurements such as, pH, tenderness, drip loss, cooking loss, Minolta values and more are documented in the Appendix section.

Abstract

Forty-one *M. semimembranosus* (S.M.) muscles of beef were collected from the local slaughterhouse in Norway (part I) for the study of meat quality and color stability. All the slaughter variables like pH, weight, temperature, fat content, animal group and so on were recorded. Piece B (cut from proximal side of S.M.) and Piece A (cut from distal side of S.M) were vacuum packaged in the slaughter house and were left for 3weeks in chilling room for maturation. A big S.M muscle (~5kg) was divided into various pieces for measurements that were carried out the in slaughterhouse, namely; pH, mitochondrial isolation, permeablization and oxygen consumption rate (OCR). Further measurements of the pieces were followed up three weeks later at our lab in IKBM, namely; tenderness, drip loss and cooking loss (using both piece A and B) and , color stability and OCR(using tissue from piece B). A small piece of S.M and *Longismus dorsi* (L.D.) muscle was used for pH measurements at time t0 (~4hrs postmortem), t1 (~8hrs postmortem) and t2 (~4hrs postmortem) with three replicate at different positions each time. Results from tenderness measurement showed that SM piece at proximal end (Piece B) was tendered than the distal end (Piece A). The rate of pH decline was different between the collected samples from number 1-20 and from 21-41. Most of the samples had good and acceptable amount of combined drip and cooking loss. Oxygen consumption rate (OCR) was measured on permeabilization fibers after 3weeks vacuum and chilled storage of the muscle. Similarly our results showed that, OCR could had some prediction ability for classifying the tender and tough group of Piece B from sample 21-41 but not for the 1-20 sample group. Regression of OCRs and WB values showed that removal of oxygen from the electron transport chain has a positive effect on tenderness.

For the spectral measurements of 3 weeks stored muscle, a calibration model (part II- taken from paper (I)) for myoglobin state was required. Fresh S.M muscles (vacuum packaged 4 days postmortem) were prepared to have deoxy (DMb), oxy (OMb) and metmyoglobin (MMb) state using Chemically induced myoglobin states (CHEM) method and oxygen partial pressure packaging (OPP) method. Absorbance spectra (400-1100nm) were made on the 24 sample surface (8 samples for each myoglobin state) with 4 replicate readings at different positions of each sample for each preparation method (OPP and CHEM). Absorbance spectra were transformed by using

different ways like using Kubelka-Munk (K/S) and extended multiplicative signal correction (EMSC).Calibration and validation model were made from transformed spectra by using either selected wavelengths (SW) or partial least square (PLS) regression. Post-transformation steps were used to normalize the myoglobin states so that, none of them were larger than one or negative and ensured that, sum of three states was not more than one at particular time. Low Root mean square error of cross validation (RMSECV) and high correlation coefficient (R_c) was used as criteria to choose the best calibration model for myoglobin states. Use of OPP preparation method with EMSC transformation and PLS regression on absorbance gave the best model for calibration.

The color stability of 3 weeks stored S.M muscle (Part III) were measured at different times from 0-7 days using absorbance spectra (400-1100nm) and L*, a* and b* values. The calibration models were used to predict the change in myoglobin states after exposure to atmosphere using the absorbance spectra for all samples. N-partial least square (N-PLS) regression between variations in L*a*b* values and myoglobin states to OCR was carried out. The DMb concentration predicted from the calibration model decreased with time and indicated that with an increase in time of exposure to atmosphere, DMb first transforms transiently to OMb and then to MMb, whose level rose concomitantly. The change in a* values with time and OCR were highly related. It was found that pH t0 and pH t1 are important for color stability of the meat. In three weeks stored muscle, complex II of mitochondria was the most stable complex in the electron transport system and often the only viable site for accepting reducing equivalents, therefore color stability could be related to its activity.

In conclusion, the activity of mitochondrial enzymes could be well related to color stability and also to tenderness for a subset of muscles that were characterized by a slower drop in pH early post mortem. No relation between mitochondrial enzyme activity and drip loss and cooking loss was found.

Abbreviations

CAC	Citric acid cycle
DMb	Deoxymyoglobin
ETF	Electron transferring flavoprotein
ETS	Electron transport system
IM	Inner membrane
IMS	Intermembrane space
MMb	Metmyoglobin
OM	Outer membrane
OMb	Oxymyoglobin

Table of Contents

1 Introduction

This work is based on the hypothesis that mitochondrial activities are important for meat quality variables such as color and tenderness. While a relationship between color and mitochondrial oxygen consumption has been assumed for some time (Hood 1980), it has not been quantitatively proven. The relationship between mitochondria and tenderness was more recently investigated as mitochondrial proteins involved in the removal of oxygen radicals were also found important for meat tenderization (Eismann, Huber et al. 2009; Jia, Veiseth-Kent et al. 2009), however, limited is known about the underlying mechanisms.

Difficulties regarding demonstrating these two relationships are:

- Lack of good methods for quantification of myoglobin states.
- Lack of efficient methods for shifting the dynamic range so that mitochondrial proteins can be analyzed among a large amount of contractile proteins (mitochondrial isolation).

This thesis addresses these points in 3 parts and since time did not allow for proteomics on isolated mitochondria so, I have included isolation procedure in Appendix.

Some theories related to the thesis are explained below.

1.1 Structure of muscle and their protein

Meat is composed of mainly three types of muscle (Sælid 2010);

- Smooth muscles - These are involuntary muscles and are found in the wall of organs such as stomach, intestine, blood vessels, bladder and so on.
- Cardiac muscles - Similar to smooth muscles, cardiac muscles are also involuntary. They are mainly found in the heart.
- Skeletal muscles - They are voluntary muscles which are responsible for the body movement and connected to the skeletal.

Skeletal muscle cells are well organized cells and are striated due to specialized myofibrils. Myofibrils are highly organized cylindrical structures which are made up of repeating units of sarcomere (Huff Lonergan, Zhang et al. 2010). These cells are

striated because of alternating dense protein bands called the A-band and less dense bands called the I-band in myofibrils. Z-lines bisect the I-bands and form structure of sarcomere between the two Z-lines. I-band is basically made up of a thin filament composed of mainly; actin, tropomyosin and troponin (Huff Lonergan, Zhang et al. 2010). A-band is made up of thick filaments whose main protein is myosin. Myosin tails form thick filaments and their globular head makes contact with actin in thin filament. Globular head catalyses hydrolysis of ATP and releases energy during contraction when myofibril shortens (Huff Lonergan, Zhang et al. 2010).

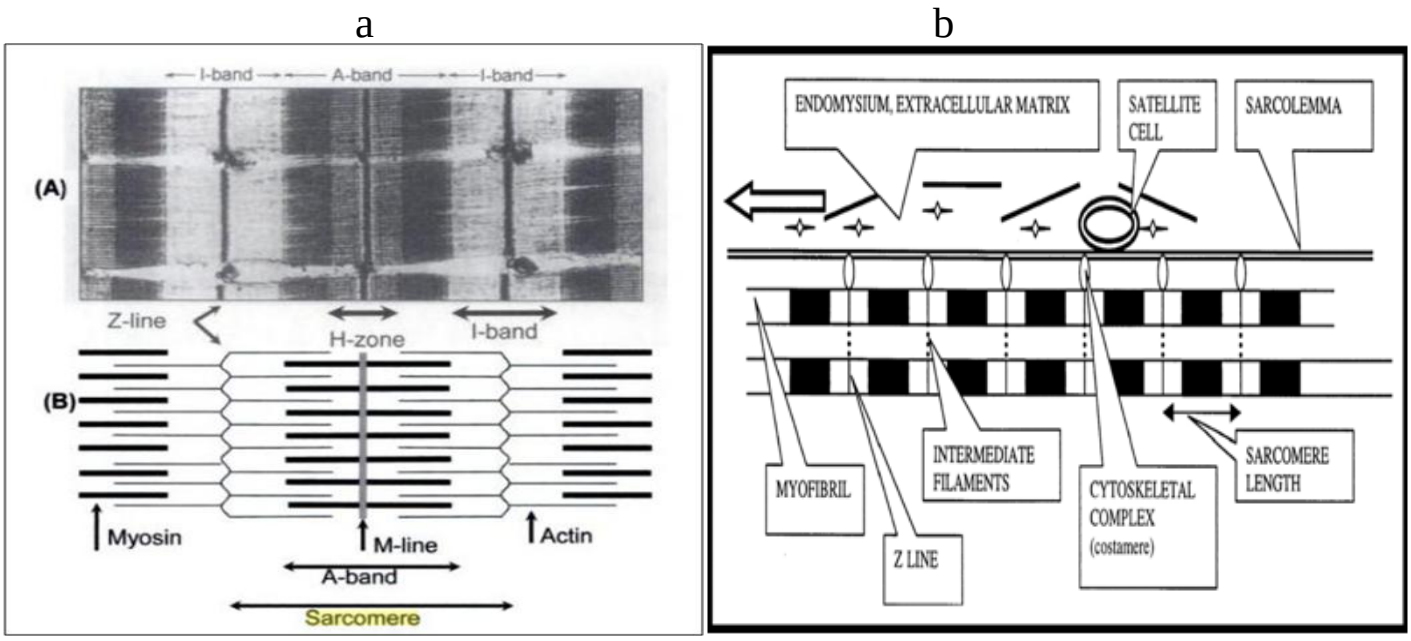

Figure 1: a) Electron micrographs of three myofibrils, (A) showing the thick (myosin) and thin (actin) filaments and the striped appearance, and the striped appearance, and a schematic overview (B) same as in **(**Macintosh, Gardiner et al. 2006) cross reference via (Sælid 2010). b) Extracellular matrix, sarcolemma and myofibrils. (De Deyne 2001). Different components of skeletal muscle such as myofibrils, z-line, intermediate filaments, costamere etc is shown in figure.

Skeletal muscle tissues are composed of water, protein and lipids. There are three types of muscle proteins (Karlsson, Klont et al. 1999) namely,

- Myofibrillar- This occupy 60% of the total protein in the muscle forming the largest fraction.
- Sarcoplasmic- They are 30% of the total consisting of myoglobin and enzymes involved in energy metabolism.

- Stroma proteins- These are 10% of all having collagen and elastin as main proteins. These are main components of connective tissues.

1.2 Meat quality parameters

Consumers make decision to purchase the meat based on its color, flavor, tenderness, odor, fat content and more. There are many extrinsic and intrinsic factors that affect the quality of the meat. Some factors that are most studied are: gender, breed, weight, age, fat content, postmortem pH, temperature, oxygen availability, lipid content, fatty acid composition, microbial load and many more. The above mentioned factors directly and indirectly affect the overall quality of the meat.

1.2.1 Meat color

Meat color is one of the most important factors in the field of meat quality study. Many extrinsic factors like light, oxygen availability, microbial growth, temperature and packaging and intrinsic factors like ultimate pH, rate of pH decline postmortem, sex, breed, muscle type, fiber type and antioxidants affects the meat color and its stability (Bekhit and Faustman 2005). One major factor which has direct relationship with meat color is the heme protein myoglobin. Other heme protein such as cytochrome C and hemoglobin can also affect meat color. Depending upon muscle and method to quantify hemoglobin, hemoglobin contributes between 6-16% of the total fresh meat color, as most of it is lost during exsanguinations (Bekhit and Faustman 2005). Many studies (Renerre and Labas 1987; Madhavi and Carpenter 1993; Arihara, Cassens et al. 1995; Sammel, Hunt et al. 2002) have shown the relationship between oxygen consumption and some partially undefined system named metmyoglobin reductase (MMR) as factors in deciding meat color postmortem. Color of meat also differs with muscle and fiber type. Some muscles are more consistent in color than others during postmortem storage. Color of meat also differs with different species as for example; color of beef meat is redder than pork meat. The important aspects of meat color will be studied later in the thesis.

1.2.2 Tenderness

Tenderness is an attribute that has direct link to the eating quality of the meat. Tough meat is generally disliked by the consumers. Tenderness of meat is affected by many

factors like postmortem time for maturation, temperature, pH, structure of muscle fiber, muscle types, species, genetic breeding and nutrition. Studies show that rate of tenderization are different for different species; For example: pork- 5 days, beef- almost 2 weeks and chicken- 2 days (Karlsson, Klont et al. 1999). As ATP level drops after death, lactate accumulates resulting in pH drop, and the cessation of ATP dependent proton pump causes the level of calcium to rise and form irreversible cross bridge between myosin head and actin (as shown in Figure 1), resulting in what is called rigor-mortis (stiffness of death) in the tissue (Huff Lonergan, Zhang et al. 2010). Due to this, sarcomere length decreases and muscle shortens and gets tough (Maltin, Balcerzak et al. 2003). The lowest amount of rigor shortening (10%) was observed at the pre-rigor temperature between 15-20°C whereas temperatures above and below this caused higher rate of shortening (Huff Lonergan, Zhang et al. 2010).

pH affects tenderization by raising the calcium level and hence activating the proteolytic enzyme system that plays an important role in making meat tender and studies also shows that a moderate rate of pH decline affects tenderness positively (Huff Lonergan, Zhang et al. 2010). The ultimate pH of meat also has some effect on meat tenderness. When the ultimate pH is high, it can be hypothesized that the glycogen level at the time of slaughter could be lower than normal resulting in low lactate production and hence increasing the tenderness. A reduction in glycolytic substrate availability causes more rapid ATP depletion and early rigor, allowing prolonged activity of proteases which makes meat tender by degradation of muscle protein (Maltin, Balcerzak et al. 2003). However, some studies reported that, such muscles with high ultimate pH are tougher than muscle with an ultimate pH of 5.6-5.8 (Maltin, Balcerzak et al. 2003). Similarly, meat with low ultimate pH is also tough as enzymes involved in postmortem tenderization are inhibited by acidification and also have increase drip loss (Maltin, Balcerzak et al. 2003).

Muscle fiber types also affect meat tenderness. Fiber types can roughly be divided into three groups on the basis of contractile and metabolic activities namely; slow-twitch oxidative, fast-twitch oxidative glycolytic and fast-twitch glycolytic (FG) (Maltin, Balcerzak et al. 2003). FG fibers have higher level of stored glycogen, so they have poorer eating quality compared to others (Maltin, Balcerzak et al. 2003) . Studies on *Longissmus* muscle at one day postmortem found that tenderness of meat also

differs with species and showed that meat of pork was tendered than beef, and beef was tendered than lamb(Koohmaraie, Whipple et al. 1991). After 1 to 14 days postmortem storage, tenderness improved more in lamb than in beef and pork showing that the rate of postmortem differed with species as well (Koohmaraie, Whipple et al. 1991). Tenderness is also affected by amount of collagen content in the muscle, as it forms non-reducible cross links in muscles and hence increases toughness in meat (Maltin, Balcerzak et al. 2003).

1.2.3 Water-holding capacity (WHC)

Drip loss in meat has been extensively studied as a factor behind big economic loss in meat industry. It has been estimated that more than 50% of the pork produced have unacceptably high drip losses (Huff-Lonergan and Lonergan 2005). Drip loss/ purge mainly contain water soluble proteins. Water in meat can be categorized into three parts according to Huff-Lonergan et al.,(2005) as;

Bound water which is present near proteins has reduced mobility and is present in small fraction and very little changes are observed in post-rigor muscle.

Entrapped water is held in attraction to bound water and changes with post-rigor changes. They are present in greater amount and may escape as drip/purge from the muscle.

Free water flows throughout tissue and are only observed when *entrapped water* are moved from its position.

WHC is affected by the net charge in muscle. When the postmortem pH decline reach a protein's isoelectric point, the internal positive and negative charges equalizes and the protein carries no net charge. The positive and negative groups in the protein become attracted to each other and expel excess water, resulting in a reduction of water that can be attracted and held by the protein(Huff-Lonergan and Lonergan 2005). The myofibril's structure becomes closely packed as repulsion is reduced due to a zero net charge in the protein (Huff-Lonergan and Lonergan 2005). Similarly, a rapid decline in pH and lower ultimate pH decreases the WHC of the muscle. A study

done in pig showed that examining initial development of pH was a good predictor of WHC in the muscle (Schafer, Rosenvold et al. 2002).

Water in muscle cells are mostly (85%) present in the myofibrils, which are arranged in thick and thin filaments. In post-rigor meat, the cross bridges are formed between thick and thin filament and reduces the space for water (Huff-Lonergan and Lonergan 2005). Water are normally moved from myofibrillar structure to extra-myofibrillar spaces within the muscle cell and expelled as a drip. If the linkage between costameres (Figure 1b) (the structural framework attaching myofibril to sarcolemma) remain intact in post rigor meat, the water will expel from the myofibrils and become collected in the extracellular space (Huff-Lonergan and Lonergan 2005). A proteolysis of cytoskeletal proteins in early post mortem (45min to 6h) by calpain proteases also has a positive effect on the WHC as water that is expelled from the intra myofibrillar spaces remains in the cell for a longer period of time. However, protein oxidation changes the conformation of calpain proteases and its affinity to cytoskeletal protein and hence affects WHC negatively (Huff-Lonergan and Lonergan 2005).

The initial temperature also affects WHC as it is correlated to initial pH and can account for variation in drip loss of pig at 24h postmortem (Schafer, Rosenvold et al. 2002).

1.3 Myoglobin states

The myoglobin is a globular protein with 150 amino-acid residues consisting of an iron containing heme group that forms 6 bonds. It has a prosthetic group that is located within the protein's hydrophobic pocket. Out of 6 bonds, it has 4 bonds with the pyrrole N, the 5^{th} bond coordinates with proximal histidine^{93} and 6^{th} reversibly binds with a small ligands such as O_2, H_2O, OH^-, NO or CO (Lindahl 2005; Mancini and Hunt 2005). The ligand attached to the 6^{th} bonding site and re-dox state of iron atom determines the color of myoglobin(Lindahl 2005). The myoglobin has three main states i.e. oxymyoglobin (OMb), metmyoglobin (MMb) and deoxymyoglobin (DMb). The main function of myoglobin in the muscle is to store oxygen and facilitates the diffusion from capillaries to the intracellular structures for the oxidative processes (Lindahl 2005).

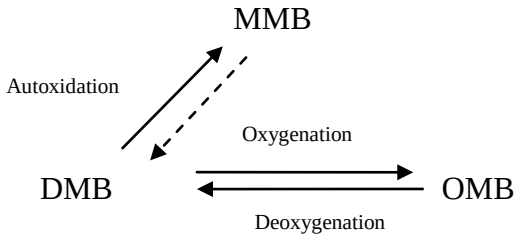

Figure 2: Interconversion of myoglobins (Lindahl 2005). The path from OMB to MMB is disputed.

OMb and DMb states have reduced ferrous (Fe^{2+}) iron state and have a cherry red and purple color, respectively. In living muscle, myoglobin is always in reduced form and actively transports and stores oxygen (Sammel, Hunt et al. 2002). The oxidized form of myoglobin is MMb which is normally reduced by MMb reducing system (MRS) in living cell. The DMb state has no ligand attached in its 6^{th} co-ordination position and converts to OMb when it is exposed to oxygen, and incorporates diatomic oxygen in its 6^{th} site (Lindahl 2005).

MMb is brownish in color and is not preferred by the consumers. In MMb, iron is in Fe^{3+} state and is bound to a water molecule. When DMb gets oxidized (autoxidation), MMb is formed as shown in Figure 2. The rate of autoxidation becomes high, when the partial oxygen pressure is 1mm Hg, and decreases with a declining partial oxygen pressure up to 30mm Hg (Lindahl 2005).

1.4 The structure and complexes of mitochondria

The mitochondria are central sub-cellular organelles which carry out metabolic reactions, synthesize ATP, involved in several signaling cascades, and alleviate oxidative damage. Mitochondria are membrane bound organelles which can replicate and differentiate independently from the cell. They have their own DNA and even synthesizes some of their own proteins (Frezza, Cipolat et al. 2007). The mitochondria grow and divide themselves and move in cell by associating with the cytoskeleton (Harner, Körner et al. 2011). Mammalian cells contain typically a few hundred to many thousand mitochondria, many of which has between 2-10 copies of the genome. The mitochondrial DNA (mtDNA) is circular and supercoiled. In humans, the mtDNA is 16,569 base pairs in length and has a collection of 37 genes, whereas 24 of which specify RNA molecules and the remaining 13 encodes proteins essential for the electron transport system (ETS) and ATP synthesis (Anderson, Bankier et al. 1981).

The myoglobin serves as oxygen reservoirs and oxygen transporter for mitochondria (Tang[a], Faustman et al. 2005). Furthermore, the mitochondria consume more than 80% of the oxygen available in the cell (Boveris, Oshino et al. 1972). The mitochondria have a complex membrane system and are actively involved in apoptosis and cellular ageing. They are basically involved in β-oxidation, citric acid cycle (CAC), biosynthesis of some metabolites of fatty acids, heme proteins and is also key regulator of apoptosis (Westermann 2010).

The structure of mitochondria is very complex. It has two membranes namely; outer membrane (OM) and inner membrane (IM) as shown in Figure 3. The IM and OM segregate the mitochondrial matrix from the cytosol. OM is made up of proteins and phospholipids and has porins which allows passage of small molecules and metabolites. However, for the passage of larger molecules, transporter proteins known as translocases are present. The OM has a smooth membrane whereas the IM has invaginations (Voet 2004). The invaginations of the IM are called cristae which is higher in number in organs containing higher respiration rates (e.g. heart) compared to the ones having lower respiration rates (e.g. liver)(Voet 2004). Inside the IM is a matrix which contains enzymes, substrate and cofactors for the Citric acid cycle (CAC). The IM allow free passage of molecules like O_2, CO_2 and H_2O but secludes ions such as ADP and protons. The inter-membrane space (IMS) is located between IM and OM, and makes up the electrochemical gradient in mitochondria.

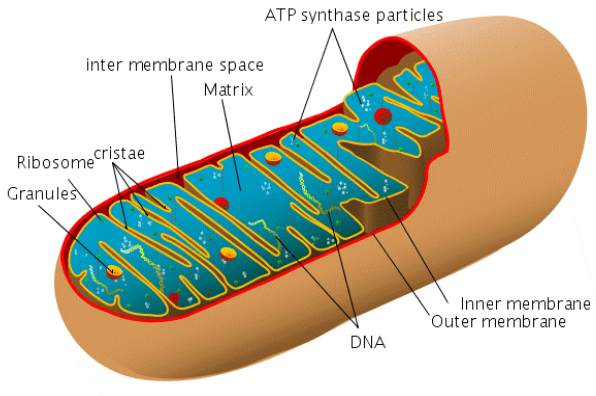

Figure 3: Detail structure of mitochondria (http://scienceblogs.com/worldsfair/Mitochondria.jpg).

There are five enzyme complexes in the mitochondrial ETS. The five complexes in mitochondria are (as shown in **Figure 4**):

- Complex I: NADH dehydrogense
- Complex II: succinate dehydrogenase
- Complex III: cytochrome C reductase/cytochrome bc_1
- Complex IV: cytochrome c oxidase
- Complex V: ATP synthase

Complex I, III and IV work as proton pump and pumps proton out of the IM and into the IMS. While complex II doesn't pump protons and complex V pumps proton reversibly (Kadenbach 2003) .There are two mobile electron carriers that play an important role in transferring electrons from one complex to the other, namely: ubiquione/ coenzyme Q (CoQ) and cytochrome C.

NADH binds to complex one of the ETS from the matrix side and transfers two electrons to flavin monomucleotide (FMN) present in complex I. The electrons are then transferred through 6-7 iron-sulfur clusters and finally to $CoQH_2$. In this process, 4 protons are pumped out from matrix to the IMS. Similarly, $FADH_2$ from CAC takes part in ETS. $FADH_2$ transfer a pair of electrons via complex II to $CoQH_2$. Since the reduction potential between CoQ and $FADH_2$ differs only slightly, this step doesn't require proton to be pumped out (Voet 2004).

Cytochrome C is an electron carrier residing on the cytosolic side, so $CoQH_2$ binds to the complex II on its cytosolic side. The electrons are transferred one at a time and oxidize cytochrome C. In this process 4 protons are pumped out to the IMS. The oxidized cytochrome C now transfers electrons to complex IV which passes to diatomic oxygen reducing it into two water molecules in the matrix side. In order to form two water molecules, 4 electrons are required, so two rounds of earlier steps are needed (Voet 2004). This step also requires protons to be pumped out to the IMS. Many protons are pumped out via complex I, complex III and complex IV and additionally 4 protons from the matrix are also used to produce water molecule. In total, a lack of protons on the matrix side increases and a proton gradient is established. In this stage, complex V of the ETS imports protons from the IMS to the

matrix. Complex V synthesizes ATP from ADP and inorganic phosphate by allowing import of protons to pass down its electrochemical gradient (Voet 2004).In this way, ATP is synthesized in mitochondria by the ATP synthase.

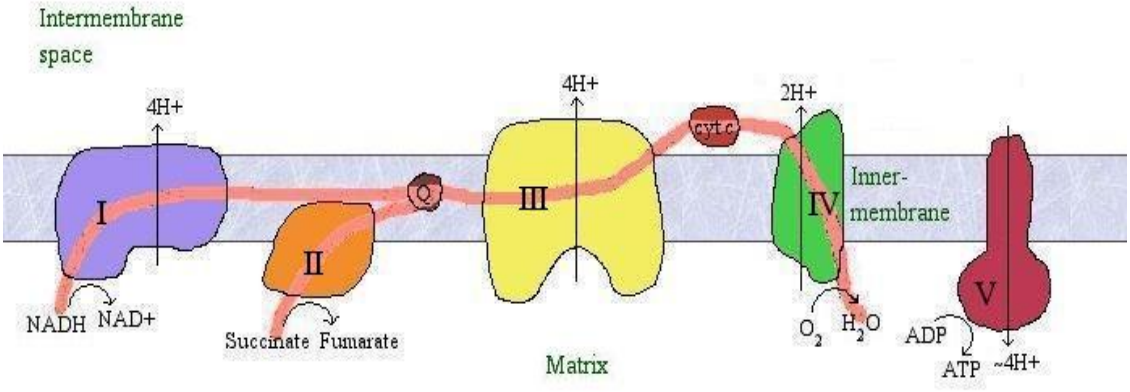

Figure 4: Complexes in mitochondria (http://www.biochem.arizona.edu/classes/bioc462/462bh2008/462bhonorsprojects/462bhonors2004/navratilovaz/BiochemBackground.htm).

1.5 Post-mortem change in enzymes of muscle

After slaughter, the blood supply to different parts of the body ceases, which also ceases the supply of oxygen. Oxygen is required for different purposes such as aerobic glycolysis and mitochondrial respiration. As there is no more oxygen supply, transition of glycolysis from aerobic to anaerobic form take place. The final product of aerobic glycolysis is pyruvate which is metabolized in the presence of oxygen through the CAC. However, in case of anaerobic glycolysis, the final product is lactate which upon accumulation creates an acidic environment by lowering the pH. A decrease in pH causes tissue shrinkage, protein denaturation and lower water holding capacity of the muscle (Werner, Natter et al. 2010).

Some key enzymes involved in metabolic processes in the muscle cells like glycogen phosphorylase and phosphofructokinase were found to be increased in different breeds of pig in research performed by Werner et al. (2010). The authors also found increase level of another enzyme fructose-2, 6-bisphosphate which is an allosteric activator of phosphofructokinase and lactate dehydrogenase slowly after slaughter. Increased level

of these enzymes might induce cells to increase energy production from glycolysis after slaughter by increasing the enzyme level to cope with the environment. Jia et al. (2006) also found a similar enzyme phosphofructokinase and other like, aldehyde dehydrogenase and enolase levels high due to ATP requirement in glycolysis in beef shortly after slaughter. The authors also found ATP- specific succinyl CoA synthetase beta subunit and isocitrate dehydrogenase 3 in high abundance from CAC. From another metabolic process NADP-dependent 3-hydroxybutyrate dehydrogenase which degrades amino-acid valine to produce succinyl-CoA (metabolic intermediate of CAC) also increased (Jia, Hildrum et al. 2006).

When extracellular matrix breaks down, the proteolytic system activates enzymatic degradation. The authors (Jia, Hildrum et al. 2006) found a decrease in enzymes like hyaluronidase, which degrades hyaluronic acid in the extracellular marix. The lower level of hyaluronidase means rapid proteolysis due to lower ATP level in the cell. Study of some stress proteins such as crystalline, HSP27 and HSP60 were found to increase after slaughter in the same research (Jia, Hildrum et al. 2006). HSP27 and crystalline proteins which are involved in protection of myofibrillar proteins from fragmentation decreased eventually during post-mortem storage in bovine muscle. Same stress protein HSP27 and another protein HSP20, level reached maximum at 4hours post-mortem and decreased eventually (Jia, Hildrum et al. 2006). The authors also found another protein lactoylglutathione lyase /glycolase I which catalyses pathway where methyglyoxal and glutathione is converted to S-lactoylglutathione and lactic acid is increased in early post mortem.

Myoglobin supplies stored oxygen to mitochondria in order to support oxidative metabolism before and after slaughter. The aerobic metabolism is high one hour after slaughter (Barksdale, Perez-Costas et al. 2010) as myoglobin tries to maintain partial oxygen pressure by supplying oxygen from itself. Barksdale et al. (2010) who worked on post-mortem human and mouse brain cell showed that mitochondria can withstand early degradation from proteolytic enzymes compared to other cellular structures. Similarly, Cheah (1971) found mitochondria in condensed form with clear OM, IM and distinct closely packed cristae when isolated after 0.5 and 96 hours postmortem from ox neck muscle, but after 120 and 144hours post mortem the mitochondria were swollen. Moreover, the work was supported by results from Tang et al. (2005) where

cardiac muscle was used. They found that, mitochondria were intact 2hours post mortem when visualized under electron microscopy, but after 96h there were visible fragments in mitochondria which were contradictory to Cheahs (1971) findings. However, the authors argued that the difference could be ultimate pH differences between the two muscles, as in case of cardiac muscle, the ultimate pH is reached after 2hours slaughter whereas in ox neck muscle it takes 48hours to reach ultimate pH.

The authors (Tang[a], Faustman et al. 2005) also found that, metabolic activities decreased 2 to 96hours post mortem at both pH 7.2 and 5.6 and the function of mitochondria is decreased but was not eliminated after 2-6hours. Even after 60days, the mitochondria still had the capacity to consume oxygen but at lower rates (Tang[a], Faustman et al. 2005). Most of the enzymes found in ETS were active even after 4 weeks post mortem, whereas, the major substrates of ETS were present at low concentration (Tang[a], Faustman et al. 2005). The potential of mitochondrial activity were also inhibited by decrease in pH. Some proteins like ubiquitin (a conjugating enzymes in the cell cycle and differentiation and SP-22 (which protects enzymes from oxidative damage in mitochondria) increases in muscle cells immediately after slaughter in bovine muscles (Jia, Hildrum et al. 2006). Another finding was a decrease in complex I activity within 40 minutes after slaughter as the reaction catalyzed by complex I is rate limiting and is important in regulation of oxidative phosphorylation (Werner, Natter et al. 2010). Cofilin, which is a substrate protein of mitochondrial ATP dependent proteinase similar to SP-22, had the expression level dropped immediate post-mortem (Jia, Hildrum et al. 2006) which could be due to a lack of ATP in the cell.

1.6 Present theory for oxygen consumption and MMb reduction in postmortem muscle

The theory for oxygen consumption and MMb reduction has been discussed in relation to the color stability as they are regarded as endogenous determinant of color stability in meat (McKenna, Mies et al. 2005). Many factors like temperature, humidity, partial oxygen pressure, pH, oxygen consumption rate, muscle type and lipid oxidation

affects MMb accumulation in post mortem meat (Madhavi and Carpenter 1993). The discoloration in meat is due to accumulation of MMb which can be eliminated if MMB could be reduced. A layer of MMb basically gets accumulated and slowly spread on the surface (Lindahl 2005). If OCR is low, more oxygen can penetrate inside the muscle and color could be more stable (McKenna, Mies et al. 2005).

The enzymes that are responsible for reducing MMb are DT-diaphorase, which is mainly present in the cytosol and MMb reductase, which is found in mitochondrial fractions (Tang[b], Faustman et al. 2005). MMb reduction via ETC is mediated by cytochrome b5 which is found mainly in mitochondria and also in microsomes (Tang[b], Faustman et al. 2005). The mitochondria plays important role in MMb reduction in meat by providing reducing co-factors such as NADH and $FADH_2$ (Bekhit and Faustman 2005). Therefore MMb reduction can take place even when only mitochondria are present. If the MMb reductase system works for long time, MMb gets reduced continuously and this prevents discoloration of meat.

Tang, Faustman et al. (2005) found that MMb were reduced by mitochondria when succinate was present, where succinate functions as a substrate for complex II of ETS. Myoglobin is a large sarcoplasmic protein and cannot pass through the mitochondrial membrane. Tang, Faustman et al. (2005) proposed that MMb reduction occurs via an ETS dependent pathway when electrons are transported from complex II (using $FADH_2$ as substrate). The electrons then pass to OM via electrostatic interaction to cytochrome b5 which is involved in MMb reduction in OM of mitochondria (Tang[b], Faustman et al. 2005). A reduction of MMb therefore occur outside the mitochondria through a transient contact (Livingston, McLachlan et al. 1985; Arihara, Cassens et al. 1995; Postnikova, Tselikova et al. 2009) or diffusion (Wittenberg and Wittenberg 2007).

According to Madhavi and Carpenter (1993), muscles with greater MMb reductase activity and lower OCR have extended color stability. Lower partial oxygen pressure and anaerobic conditions are necessary when MMb is reduced via the ETS dependent pathway in mitochondria (Tang[b], Faustman et al. 2005). When OCR is high, the electron availability for MMb reductase becomes less and hence might causes discoloration of the meat. Results from Madhavi and Carpenter (1993) showed that OCR decreased rapidly during the first 2 days post mortem which initially improved

color of meat until 7days post-mortem. ETS linked MMb reduction decreased with low pH, temperature and increases with time post mortem and mitochondrial density (Tang[b], Faustman et al. 2005). All in all, oxygen is the final electron acceptor in the ETS and anaerobic conditions that decrease OCR could help in accumulation of electrons and therefore works best in favor of meat color.

Experiments from 19 different bovine muscle concluded that muscles with low color stability also have low reducing ability, independent of the OCR in meat (McKenna, Mies et al. 2005). However, in order for a muscle to have a high color stability it should have a reducing ability that exceeds the OCR (McKenna, Mies et al. 2005).

1.7 Isolation of mitochondria

Mitochondria are studied by many researches in the field of medicine, evolution and meat sciences. Isolation of intact functional mitochondria is therefore of great importance for *in vitro* functional studies. Dounce, Witter et al. (1955) prepared a new method for mitochondria isolation from soft tissue by use of ground glass homogenizer. In their method, they used citric acid to lower the pH of homogenates to pH 6.2 in 0.44M sucrose. In the process, the isolation procedure used both low speed centrifugation and high speed centrifugation at different steps during isolation. Mitochondria can be found in various positions within the cell and therefore the isolation process should be different for isolating mitochondria from different organs. Subsarcolemmal mitochondria, which are beneath the sarcolemma, were treated with polytron tissue processor upon isolation whereas the remaining tissue , located between myofibrils, was digested with nargase to ease the isolation process from of interfibrillar mitochondria (Palmer, Tandler et al. 1977). This study showed that, both types of mitochondria hence isolated were intact but differed in some biochemical properties.

Skeletal muscles are tough and contain much connective tissues, collagens and cytoskeletal components. Isolation of mitochondria from skeletal muscle of hamster used nagarse to digest the muscles (Bhattacharya, Thakar et al. 1991). Mitochondria hence isolated had good respiratory and phosphorylation capabilities. However, isolation of mitochondria from brain cells using nagarse has been doubted. It has been shown that if nagarse was used during isolation procedures it does not become

completely and easily washed away (Wilson 1987). The problem comes to surface when SDS-PAGE is used, and the SDS buffer disrupts the inner membrane so that matrix proteins are released and membrane proteins are solubilized and become vulnerable to digestion by residual nagarse (Wilson 1987). Isolation of mitochondria from hard tissues can be done with nagarse but may come with a degree of protein degradation, while isolation from soft tissues can easily be done without digestive proteases (Frezza, Cipolat et al. 2007).

1.8 Proteins reported as important in tender meat

The proteinase system present in the muscle is known to have a prominent effect on meat tenderization after postmortem. The main proteinase system is the calpain system which contains two isoforms namely; μ-calpain and m-calpain (Huff Lonergan, Zhang et al. 2010). They require certain calcium concentration to get activate, which is commonly released from the mitochondria. μ-calpain gets activated earlier in postmortem as they require 5-10 μM calcium compared to m-calpain which requires 300-1000 μM calcium concentration (Huff Lonergan, Zhang et al. 2010). These enzymes get autolyzed in the process of getting activated. They affect the postmortem tenderization process positively by cleaving myofibrillar proteins such as nebulin and titin and intermediate protein desmin (Huff Lonergan, Zhang et al. 2010). There is an endogenous inhibitor of calpain system; calpastatin which requires calcium at lower concentration than calpain in order to get activated (Huff Lonergan, Zhang et al. 2010). Higher levels of this inhibitor in muscle make meat tougher. Studies in callipyge lamb which has a high level of calpastatin had reduced postmortem proteolysis and hence decreased meat tenderness (Kemp, Sensky et al. 2010).

Studies on the deep part of *Semimembranosus* muscle (DSM) and *Semimembranosus* at the surface (SSM) showed unequal activity of μ-calpain in different parts of muscle and showed higher degree of protein denaturation in DSM (Kim, Lonergan et al. 2010). The process lead to decreased proteolysis in DSM due to slow μ-calpain autolysis and hence resulted in decreased meat tenderness (Kim, Lonergan et al. 2010). The authors also found that there was minimal degradation of desmin and troponin-T degradation in DSM.

Other proteinase systems are also involved in meat tenderization. The caspase system might be the one degrading and inactivating calpastatin in postmortem meat tenderization (Huff Lonergan, Zhang et al. 2010). They are a family of cysteine aspartate- specific proteases that has a main role in either apoptosis or inflammation (Kemp, Sensky et al. 2010). They are mainly involved in three pathways for apoptosis namely; extrinsic, intrinsic and endoplasmic reticulum mediated pathway. The last pathway has been related to stress and calcium homeostasis in the cell (Kemp, Sensky et al. 2010). Proteome analysis in tender and tough muscle found six proteins of the inner and outer mitochondrial membrane namely; prohibitin, mitofilin, elongation factor Tu, 2 voltage dependent anion selective channel (protein 1 and protein 2) and NADH- ubiquione oxido-reductase (Laville, Sayd et al. 2009). Similarly in the same research other proteins like actin, ketch-related protein 1, serum albumin, pyruvate kinase and adenylate kinase were also found abundant in tender group at day 0 indicating more extensive degradation. This could have been due to apoptosis where mitochondria gets fragmented and participated in caspase activation (Laville, Sayd et al. 2009).

Fragments of actin found in the tender group of muscles indicated active apoptosis as actin is the target of caspase (Laville, Sayd et al. 2009). More than 280 targets in myofibrillar and cytoskeletal proteins are identified and it has been hypothesized that slaughter and exsanguinations could have initiated apoptosis and hence caspase activity during early post mortem and have affected meat tenderization (Kemp, Sensky et al. 2010). A study in tender and tough meat found that most of the proteins are released during day 0 to day 5 in case of tender group whereas in tough meat, proteins were released within day 5 to day 21, indicating an important difference in postmortem ageing between them (Laville, Sayd et al. 2009).

Cathepsin L,B and enzymes of peptidases affected tenderization in beef positively at 8h postmortem as some studies also showed that cathepsin L has the ability to hydrolyse troponin T, I and C, nebulin, titin, tropomyosin in beef, rabbit and chicken myofibrils (Kemp, Sensky et al. 2010). Some bovine proteasomes that could be active in proteolyzing myofibrillar proteins like nebulin, myosin, actin and tropomyosin were detected 7 days postmortem when the pH was less than 6 and thereby affecting postmortem tenderization positively (Kemp, Sensky et al. 2010). Protein oxidation in

postmortem muscle is also known to have some effects on meat tenderization. . Oxidation of protein converts some amino acid residues like histidine to carbonyl derivatives and also forms intra/inter disulfide cross links and thus reduces the functions of proteins (Huff Lonergan, Zhang et al. 2010). The reduced function of proteins toughens the meat by causing changes in conformation of proteins like calpains (Huff Lonergan, Zhang et al. 2010). Protein oxidation could also result in the oxidation of myosin heavy chain and subsequently promotes its aggregation and altering muscle structure by degradation of titin, nebulin, filamin, desmin and troponin-T which are targets of proteolysis for μ-calpain (Huff Lonergan, Zhang et al. 2010).

Reactive oxygen species which are formed during metabolic and oxidative processes interacts with lipids and protein in postmortem muscle tissue forming carbonyl and decreasing sulfhydryl content in protein(Rowe, Maddock et al. 2004). Irradiation which is used as safety tool in foods accelerates oxidation and produces free radicals, therefore resulting in higher postmortem protein oxidation in sarcoplasmic and myofibrillar fraction of muscle tissues which are then associated with higher shear force values or lower tenderness in meat (Rowe, Maddock et al. 2004). Whereas high level of vitamin E is related to low rate of lipid oxidation and delayed MMb formation which finally increases rate of tenderization (Rowe, Maddock et al. 2004).

1.9 Meat color measurements

CIE L*a*b* values are mostly used to monitor meat surface color (Khliji, van de Ven et al. 2010). L* denotes brightness, where L*=0, means black color and L*=100, meaning white color. Similarly, a* value gives a range from red to green and b* value gives a range from yellow to blue. L*, a* and b* are called tristimulus parameters (Lindahl 2005). Using the reflectance and absorbance spectra, myoglobin states in meat sample can be measured. Different myoglobin states have different spectra. Myoglobin states can be calculated by using isobestic points for their spectra (Hunt, Acton et al. 1991). Isobestic points are the points where either 2 or 3 myoglobin states have same reflectance/absorbance (Lindahl 2005). According to American meat science association (AMSA) guidelines, at the wavelength of 525nm all the three states of myoglobin have the same absorbance (Hunt, Acton et al. 1991). Similarly,

474nm is the isobestic point for MMb and OMb, 610nm is isosbestic for MMB and DMb and 572nm is isobestic point for DMb and OMb (Hunt, Acton et al. 1991). At a particular time, the sum of values of myoglobin state is hypothesized to be one meaning, if the value of two myoglobin states are know, the third one can be assessed easily. Based on isobestic points and this hypothesis, Hunt et al. (1991) has presented formulas for calculating myoglobin states in meat. The formulas are given below:

$$Red_{Met} = \frac{\left(\frac{K/S_{474}}{K/S_{525\,cal,avg,Met}}\right) - \left(\frac{K/S_{474}}{K/S_{525\,sample}}\right)}{\left(\frac{K/S_{474}}{K/S_{525\,cal,avg,Met}}\right) - \left(\frac{K/S_{474}}{K/S_{525\,cal,avg,Red}}\right)} \quad \textbf{(Equation 1)}$$

$$Red_{Oxy} = \frac{\left(\frac{K/S_{474}}{K/S_{525\,cal,avg,Oxy}}\right) - \left(\frac{K/S_{474}}{K/S_{525\,sample}}\right)}{\left(\frac{K/S_{474}}{K/S_{525\,cal,avg,Oxy}}\right) - \left(\frac{K/S_{474}}{K/S_{525\,cal,avg,Red}}\right)} \quad \textbf{(Equation 2)}$$

$$Met_{Oxy} = \frac{\left(\frac{K/S_{572}}{K/S_{525\,cal,avg,Oxy}}\right) - \left(\frac{K/S_{572}}{K/S_{525\,sample}}\right)}{\left(\frac{K/S_{572}}{K/S_{525\,cal,avg,Oxy}}\right) - \left(\frac{K/S_{572}}{K/S_{525\,cal,avg,Met}}\right)} \quad \textbf{(Equation 3)}$$

$$Met_{Red} = \frac{\left(\frac{K/S_{572}}{K/S_{525\,cal,avg,Red}}\right) - \left(\frac{K/S_{572}}{K/S_{525\,sample}}\right)}{\left(\frac{K/S_{572}}{K/S_{525\,cal,avg,Red}}\right) - \left(\frac{K/S_{572}}{K/S_{525\,cal,avg,Met}}\right)} \quad \textbf{(Equation 4)}$$

$$Oxy_{Red} = \frac{\left(\frac{K/S_{610}}{K/S_{525\,cal,avg,Red}}\right) - \left(\frac{K/S_{610}}{K/S_{525\,sample}}\right)}{\left(\frac{K/S_{610}}{K/S_{525\,cal,avg,Red}}\right) - \left(\frac{K/S_{610}}{K/S_{525\,cal,avg,Oxy}}\right)} \quad \textbf{(Equation 5)}$$

$$Oxy_{Met} = \frac{\left(\frac{K/S_{610}}{K/S_{525\,cal,avg,Met}}\right) - \left(\frac{K/S_{610}}{K/S_{525\,sample}}\right)}{\left(\frac{K/S_{610}}{K/S_{525\,cal,avg,Met}}\right) - \left(\frac{K/S_{610}}{K/S_{525\,cal,avg,Oxy}}\right)} \quad \textbf{(Equation 6)}$$

Equations (1-6) are the detailed form of equations for calculating myoglobin states. According to AMSA guidelines, equation 1 and 2 should give the same result for calculating DMb (also called as reduced myoglobin-Red in equations) and same applies for other equations as well. In the equations, $K/S = (1-R)^2/2R$ are kubelka-Munk rations, where K=absorbance coefficient, S=scattering coefficient and R=

reflectance of the meat sample. Reflectance data are transformed into K/S values to make data linear and remove some scattering effects (Hunt, Acton et al. 1991).

In different laboratories, packaging material and instrumentation for myoglobin samples may vary, therefore calculation of DMb, OMb and MMb in visible range thus requires calibration. AMSA guidelines (Hunt, Acton et al. 1991) have given detailed information on preparing three pure myoglobin states which has been used all over the world. Meat with pure DMb, MMb and OMb (calibration samples) can also be produced with modified atmosphere or vacuum packaging and by adjusting partial pressure (Hunt, Sorheim et al. 1999). Another method by Krzywicki uses log(1/R) of incident light at four isosbestic points- 572nm, 525nm, 473nm and 730nm to obtain pure myoglobin states for calibration (Lindahl 2005).

1.10 Statistical methods

In order to avoid the disturbance of light scattering in spectroscopic data, Kubelka-Munk transformation is frequently used. Similarly, extended multiplicative signal correction (EMSC) can also be used, which removes complicated multiplicative and additive effects like light scattering in reflectance spectroscopy (Martens and Stark 1991). Regression tools are popularly used to find possible relationship between the variables. The method of partial least squares (PLS) regressions uses two matrices as Foe example, spectra and myoglobin as two matrices and find the relationship between them as it can analyze data with numerous noisy X-variables along with several Y-variable (Wold, Sjostrom et al. 2001). Therefore, PLS regression can help pick the specific wavelength giving lowest error for prediction of myoglobin states. Another regression method, N-PLS which is a multi-way regression method uses three and more modes (Bro 1996). The Y-variable (response) in N- PLS can have multi-way array responses unlike PLS regression which has either single matrix responses.

2 Part I- Early post mortem changes in oxygen consumption and its possible impact on quality variables like tenderness and WHC

The introductory part of this section is written above (Section 1).
In this section the oxygen consumption data were measured by Vinh T.Phung

The aim of this section was:

- To investigate the importance of early post mortem changes in oxygen consumption rate and relate these changes to tenderness as measured after a chill storage period.
- To relate early post mortem oxygen consumption rate (OCR) to cooking loss and drip loss.
- To investigate if the tenderness measured at the proximal end of *M. semimembranosus* would relate to the tenderness at the distal end of *M. semimembranosus.*

2.1 Material and methods

2.1.1 Animal samples and collection of slaughter variables

Forty-one cattle were used for the experiments. Among the forty-one cattle, 30 were Norwegian red bulls, 2 were Simmental, 1 was limousine, 1 was Angus, 3 were Charolais and 4 were Hereford. *M. semimembranosus* and *M.longissimus dorsi* muscles were collected from the above mentioned animals during five weeks (from early September to mid November, 2010) time period. Figure 5 represents the slaughter line in Norway (Nortura AS, Rudshøgda, Norway).

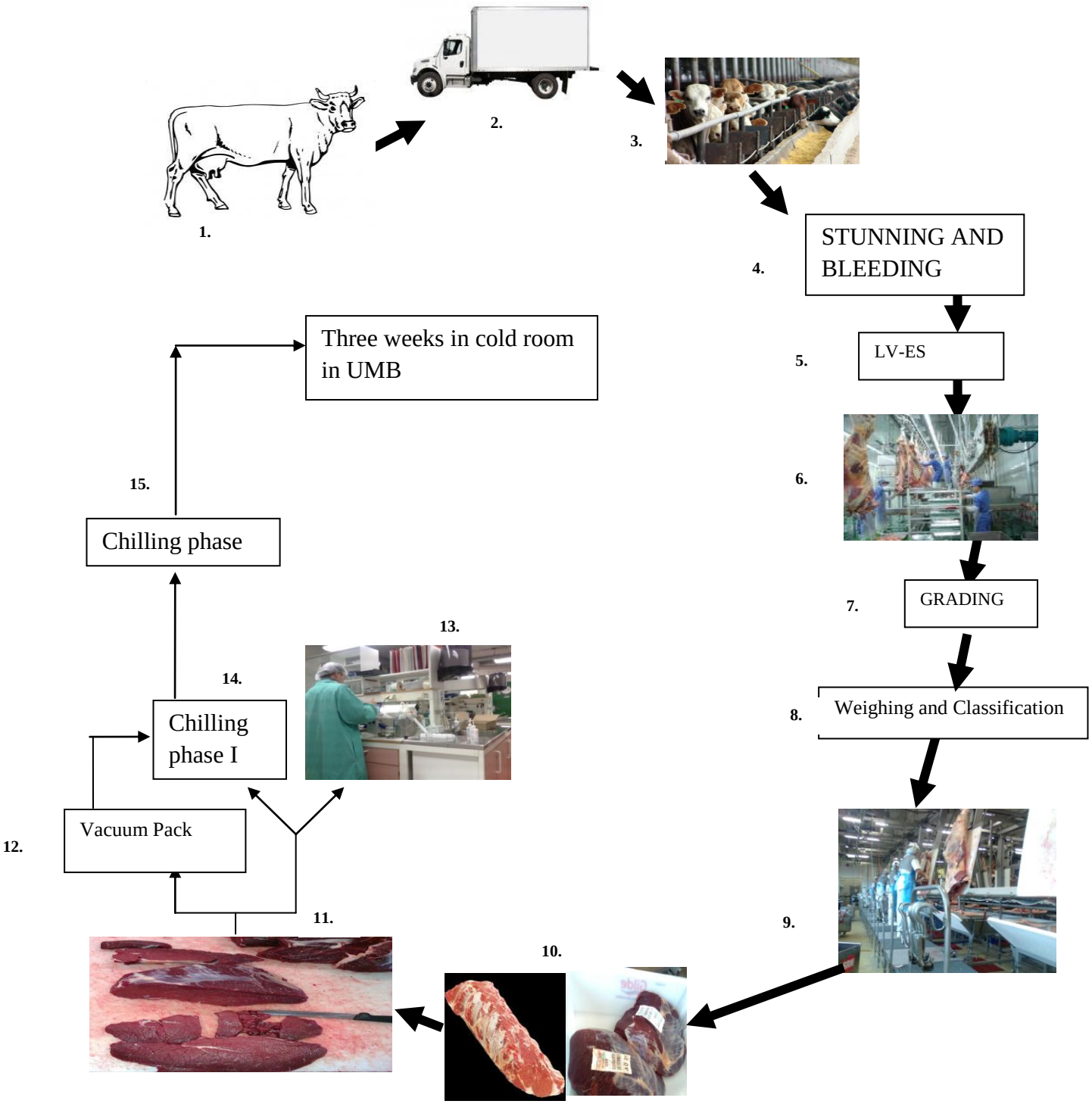

Figure 5: Different steps in Slaughter line in Nortura Rudshøgda, Norway till transportation to UMB, Ås, Norway. (1)Animals (http://www.supercoloring.com/wp-content/thumbnail/2008_11/cow-coloring-page.gif)chosen from farm, (2) animals transported (http://marinemammalconservancy.org/wp-content/uploads/2009/10/Truck-white1-300x169.jpg) to Slaughter house from different parts in Norway, (3) animals in individual pens, (4) Stunning and Bleeding of animals, (5) Low voltage electrical stimulation (LV-ES) applied on carcass(6) Exsanguinations and Eviscerations (http://image.made-in-china.com/2f1j00ivutMBSIlCkd/Cow-Slaughterhouse.jpg), (7) Grading (Split carcass into left and right side), (8) Weighing and Classification, (9) Hot- boning line, (10) *Longismus dorsi* (left)

(http://www.canadianbeef.info/ca/en/cb/muscle/rib.aspx) and *M.Semimembranosus* (right), (11) *M.Semimembranosus* cut into different pieces as described in Figure 6, (12)Vacuum Package Piece A and B in polyethylene bags, (13) Pieces for oxygen measurement taken to the Laboratory, (14) Chilling Phase I with temperature (6-11ºC); storing sample A and B and samples for pH measurements, (15) Chilling Phase II ; Piece A and B were taken next day morning from Chilling phase I, (16)Piece A and B transported in ice to 4°C cold room at UMB(Norwegian University of life sciences).

A. Time records are named according to Figure 5

Table 1: Naming of time records with respect to steps in Figure 5.

Time variables	**Steps on Figure 5**
Time from farm to abattoir (ToT)	1-3
Lairage time at abattoir before death (ToD)	3-4
Cutting time after death (ToC)	4-10
Time from death until OCR (ToDO)	4-13

B. Temperature measurement

Soon after obtaining *M. semimembranosus* muscle from the hot-boning line, the temperature was measured. The temperature was measured on the surface of the *M.semimembranosus* sample with the temperature logger (Ebro TLC 1598, Ebro Electronic GmbH & Co, Ingolstadt, Germany) for all 41 animals. The excised muscle piece for this measurement was from the posterior part of the animal body.

C. Sample division for different analysis

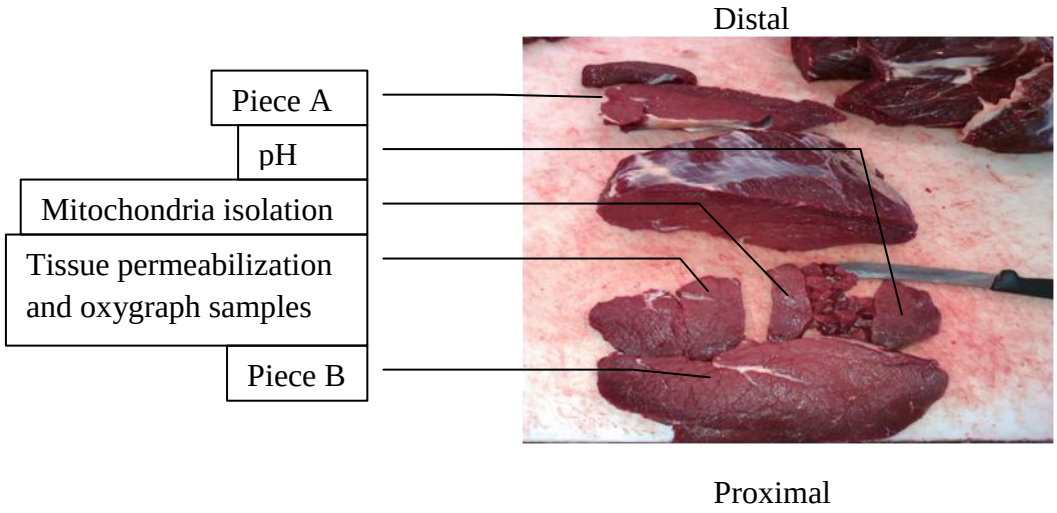

Figure 6: Division of *M.semimembranosus* pieces.

M.semimembranosus muscle was divided into five pieces for different usage namely, for pH measurement, mitochondria isolation, tissue permeabilization and oxograph samples and piece B as shown in Figure 6. Piece B which was cut from proximal side and was a larger than piece A that was cut from distal side of the *M.semimembranosus.* Piece A and B were weighed (called as W_1 later) and vacuum packaged (Type PA/PE, oxygen 30-40 cm^3/m, LogiCon Nordic A/S, Kolding Denmark) and transferred to chilling phase I and then placed for maturation as shown in Figure 5. Pieces taken for isolation of mitochondria and oxograph measurements were chosen to be close to each other. A small piece of *Longissimus dorsi* (LD) was also collected for pH measurements.

D. pH measurements of sample

The measurements of pH were taken in two different muscles namely; *M. semimembranosus* and *Longissimum dorsi* of the sample. Measurements were taken at time T0 (4hours after death), T1 (8 hours death/4hours after T0) and T2 (24 hours after T0) respectively. The samples were kept inside clean plastic bags in the chilling room I at approximately 10°C in the slaughter house. Holes were formed in the muscles just before the measurements with the help of clean forceps to insert the probe of the pH meter ((Portamess 913, Knick, Berlin, Germany). Three repeated measurements were made at different location on the muscle surface and were averaged later. The pH meter was calibrated every day.

E. OCR measurements *(This step was fully performed by V.T. Phung).*

i) *Permeabilization of tissue*: This step was carried out as soon as the muscle piece was obtained from the hot-boning line. All chemicals were of analytical grade and purchased from Sigma Chemicals Corp. (St. Louis, MO) except for pyruvate (Applichem, VWR, international AS, Norway) which was used in OCR measurements step explained below. Relaxing solution was used separate the muscle fibers. The solution contained 15 mM phosphocreatine, 10 mM Ca-EGTA (0.1 mM free calcium), 20 mM imidazole, 20 mM taurine, 6.6 mM $MgCl_2$, 50 mM K-2-(*N*-morpholino) ethanesulfonic acid, 0.5 mM dithiothreitol, and 5.8 mM ATP adjusted to pH 7.1. The separated muscle fibers were made for average length of 5.0mm having diameter of approximately 1.0mm. The tissue was further permeabilized in relaxing solution added with 0.052 mg/ml saponin for 30 min. Finally, they were washed for 10 minutes while in a shaker at 4^{o}C with respiration

medium. The respiration medium consisted of 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-methanesulfonate, 20 mM taurine, 10 mM KH_2PO_4 (Calbiochem, Darmstadt, Germany), 20 mM HEPES, 110 mM sucrose (Alfa Aesar, Karlsruhe, Germany), and 1.0 g/l BSA and was adjusted to pH 7.1.

ii) *OCR measurements*: The measurements were made two times, once after approximately 4-5 hrs postmortem and once after 3 weeks on chill stored samples. Here we will be discussing only the OCR measurements after 3 weeks. Measurements were made on oxygraph-2K instruments (Oroboros Instruments, Innsbruck, Austria) to carry out high resolution respirometry as explained by Gnaiger (2001).Using the permeablized tissue from step (i), OCR was measured at 20^{o}C and ~200 µM O_2 and were reported as (p mol O_2/sec)/mg protein.
All the following additions of different substrates were done in respiration medium as in step (i) malate and glutamate followed by beta-oxidation to an electron transferring flavoprotein (ETF) using octanoylcarnitine (Table 2). ADP was added to stimulate oxidative phosphorylation. Succinate which is substrate for complex II and rotenone was used to test respiration in complex II and malonic acid was used as an inhibitor for that complex. Antimycin A was used to inhibit complex III and uncoupling measurements were made by using p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP). Uncoupling was measured by FCCP addition and finally inhibition of complex III was inhibited by antimycin A.

Table 2: Abbreviations and end concentrations of chemicals used during OCR measurement. M; malate, OC; octanoylcarnitine, D; ADP, G; glutamate, S; succinate, F; FCCP, Rot; rotenone, Mna; malonic acid, Ana; antimycin A.

Seq.	Symbol	Chemical	Function/location	Working conc. [mM]	Coeff. Var* (Fresh)	Coeff. var* (Stored)
1	OCR_M	Malate	Complex I	4	NA	NA
2	OCR_{OC}	Octanoylcarnithin	ETF**	0.5	0.70	NA
3	OCR_D	ADP	ATP synthase	1.25	0.66	NA
4	OCR_G	Glutamate	Complex I	10	0.69	NA
5	OCR_S	Succinate	Complex II	5	0.65	0.71
6	OCR_F	FCCP	Uncoupler	0.5-1.5 x 10^{-3}	0.64	0.68
7	OCR_{Rot}	Rotenone	Complex I	2.5 x 10^{-3}	0.76	0.75
8	OCR_{Mna}	Malonic acid	Complex II	5	0.66	4.94
9	OCR_{Ana}	Antimycin A	Complex III	12.5 x 10^{-3}	NA	NA

* Coefficient of variation on the dispersion of measurements.
**ETF: electron transferring flavoprotein
NA: no response due to lack of activity. Antimycin A inhibited all oxygen consumption at complex III.

2.1.2 Chilled stored samples

These muscle samples collected in slaughter house were allowed to mature at 4°C in the chiller. The vacuum packaged Piece B was opened and further classified as shown in Figure 7 for later analysis.

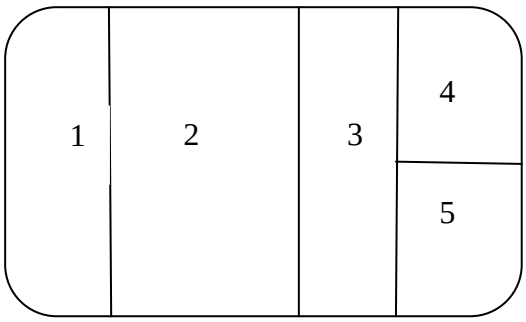

Figure 7: Piece B is distributed into five pieces, where piece 1-used for tenderness measurement, piece 2- color analysis, piece 3- oxygen consumption rate measurements and piece 4 and 5 for fiber-typing.

Piece 4 and 5 were not used during this thesis period due to the lack of time.

A. Different steps after opening vacuum packaged piece A and B

- Drip from piece A and B were collected in 5ml Eppendorf tubes for later use (not explained in thesis).
- Weight of both samples (called W_2) was measured excluding the drip.
- Piece B was cut into five pieces as shown in Figure 7.
- Color measurements were made within 5 minutes of opening of the vacuum packaged using piece B_2 (in Figure 7-piece 2) (further details will be given in part III (section-4) of this thesis).
- Piece B_1(in Figure 7- piece 1) and piece A, along with piece $B_{4\&5}$(in Figure 7-piece 4 and 5) were vacuum packaged using the Original Henkelman Vacuum 300 system (vacuum strength 9, type 300II, Hertogenbosch, The Netherlands) in separate polyacrylamide bags (SR 200 X 270PA, Maskegruppen, Vinterbro, Norway).
- Piece B_1 and piece A were cooked in hot water bath of 70°C for about 1hr till the internal temperature of these pieces reached 70°C. *M. semimembranosus* muscle piece of approximately same size as that of piece B_1 and piece A was used as a dummy sample. The temperature for all samples was tracked by injecting temperature logger (EB 1-2t-313, Ebro, Germany) in the dummy sample inside the hot water bath. After

the cooking process, these muscles were kept on ice to cool them down to room temperature.

- Piece $B_{1,}$ A and $B_{4\&5}$ were stored at -40°C and piece B_3 (in Figure 7-piece 3) was used for OCR measurements.
- Piece B_1 and piece A were kept in 4°C cold room one night before the measurements were made and were further thawed in water at room temperature (17°C).
- After thawing procedure, vacuum packaged of samples were open and weight of the sample along with liquid exudates formed after cooking (called as W_3) were taken first. The weight of the samples without liquid exudates (W_4) was also taken later.
- The remaining samples were used for tenderness measurements which are explained below (2.1.2 C).

B. Calculation of percentage Drip loss and cooking loss

Drip loss was the total loss in weight while comparing change in weight of muscle in slaughter house while collecting (explained above as W_1) and weight measured after removing drip from the samples three week later *i.e.* after three week storage in chilling room (explained above as W_2). Therefore percentage drip loss is given as;

Drip loss = $\frac{W1-W2}{W1} \times 100\%$

Drip loss for both piece A and B was calculated for all 41 samples.

Cooking loss was also calculated for all 41 samples in both piece A and $B_{1.}$Cooking loss is the measure of weight loss of samples after cooking compared to the weight before cooking.

Cooking loss =$\frac{W3-W4}{W3} \times 100\%$, where W_2 and W_4 are weight of samples explained above in step 2.1.2 A.

C. Warner-Bratzler shear force (WB) measurements

As explained above in step 2.1.2 A , the same cooked meat was used for this process. The meat sample was cut into at least 10 pieces for each individual piece A or Piece B_1 respectively. The cut pieces had size of approximately1 cm x 1 cm, length about 4 cm but somewhat adjusted to what was possible in order to control fiber direction and they were kept perpendicular to the fiber direction with the WB shear force device to cut it with the V shaped blade attached to the texture analyzer (Stable Micro Systems

Ltd, Surrey, UK)(Hildrum, Rodbotten et al. 2009) .All the 41 samples were measured for tenderness.

However, out of 41 samples, 3 randomly selected samples were also measured in another texture analyzer called Instron testing machine (Model 4202, Instron engineering Corporation, High Wycombe, UK) in Nofima which used program name Blue hill 2. This was done in order to compare the force measured on our Warner Bratzler shear measurements with the force measured using Nofima's Warner Bratzler shear measurements. This helped to find out the correct force that distinguishes tender and tough meat since Nofima has linked their Warner Bratzler shear measurements to consumer acceptance(Sivertsen, Kubberod et al. 2002). The agreement was relatively good, but our measurements at IKBM consistently gave more value than the measurements at Nofima. Studies also show that, even the use of standardize protocol may give different WB values in different institutions (Wheeler, Shackelford et al. 1997). All the WB measurements were made in Newton (N). The 10 replicated measurements for each sample were averaged.

2.1.3 Statistical analysis of the relationship between OCR and other slaughter variables

(The statistical analysis was performed with the aid of Vinh.T.Phung and Kristian Liland).

Measurement number 37 was removed as outlier in analysis of a* and b* values. One way ANOVA with random effects and Tukey's test for multiple comparisons were used to test for significant differences in OCR between additives as a result of animal variation. Minitab (version 15 and 16 from Minitab Inc., State College, PA, USA) was used for statistical calculations. Magnitude of effects is indicated as expected mean squares (σ^2). The data collected at the slaughter house for each animal (except $OCR_{(i)}$ -OCR in response to chemicals administered to mitochondria) where first subjected to a principal component analyses (weighting = 1/stand.dev) using The Unscrambler X 10.1 (CAMO Software, Oslo, Norway). Secondly the relationship between $OCR_{(i)}$ and individual animal descriptors was examined using both linear regression and stepwise regression (forward and backward) for final significant relationship. However, Principal component analysis (PCR) was then carried out first and served as an explorative screening method for removing variables that were highly correlated

before the stepwise regression was performed. The linear regression served as a supplementary technique to select an optimal variable for stepwise regression. The result from the stepwise linear regression is given as β-coefficients with their significance levels. The relationships between the changes in color with time and $OCR_{(i)}$ or individual slaughter variables were examined using NPLS. For myoglobin states and L*a*b* values NPLS was preferred as this method also provided information on the responses. NPLS can handle three or more modes (samples x variables x variables x ...) organized as a three-way (or more) array. Our X data are three-way having a sample mode (animals), a first variable mode (time), and a second variable mode (myoglobin states or L*a*b* channels). The Y data are either controllable variables (storage, transport e.t.c), uncontrollable variables (temperature, pH, ...), or OCR measurements. Using NPLS the X data are sequentially decomposed into orthogonal sets of score and loading vectors maximizing the covariance between the X data and the responses. The loading vectors have unit length and show how much weight is given to each variable in each mode for the current component. The percentage of MMb, OMb and DMb were obtained and is explained above in part III (section 4) of the thesis.

2.2 Result and Discussion

2.2.1 WB shear force, drip loss and cooking loss for 41 meat samples of 3 weeks aged meat

Table 3: Quality variables measured for all *M. semimembranosus* samples

Calculations	WB shear rate (N)	Drip loss (%)	Cooking loss (%)	Cooking + drip loss (%)
Max - Min. St. dev.	21.2-86.1 ±18.2	4.6-25.0 ±3.7	14.1-33.1±4.7	20.7 - 44.8 ±5.5

WB shear rate (N) was calculated for both piece A and B together and was averaged as shown in Table 3. The minimum force was 21.2N and the maximum force was 86.1 N with standard deviation of 18.2 N. This implied that, the samples covered a large tenderness range. Similarly, the drip and cooking loss were calculated separately and together as well to show the total weight loss in meat from the slaughter house to after being cooked. Also for these variables the covered range was quite large.

2.2.2 Drip loss and cooking loss (3 weeks aged meat)

The data for drip losses and cooking losses were split in three quality groups (Table 4) to give some indication of distribution.

Table 4: Distribution of sample (in percentage) based upon drip loss and cooking loss. Samples were divided as good, acceptable and bad of piece A and B of 41 animals. Assumptions for drip loss, loss<6%= Good; loss between 6-10%=Acceptable and loss>10%=Bad. Similarly, for cooking loss, Loss<20%= good; loss between 20-30%= Acceptable and loss> 30%= Bad.

Variables	Good (%)	Acceptable (%)	Bad (%)
Drip loss in piece A	51	32	17
Drip loss in Piece B	32	44	24
Cooking loss in piece A	19.5	68.5	12
Cooking loss in piece B	17	63.5	19.5

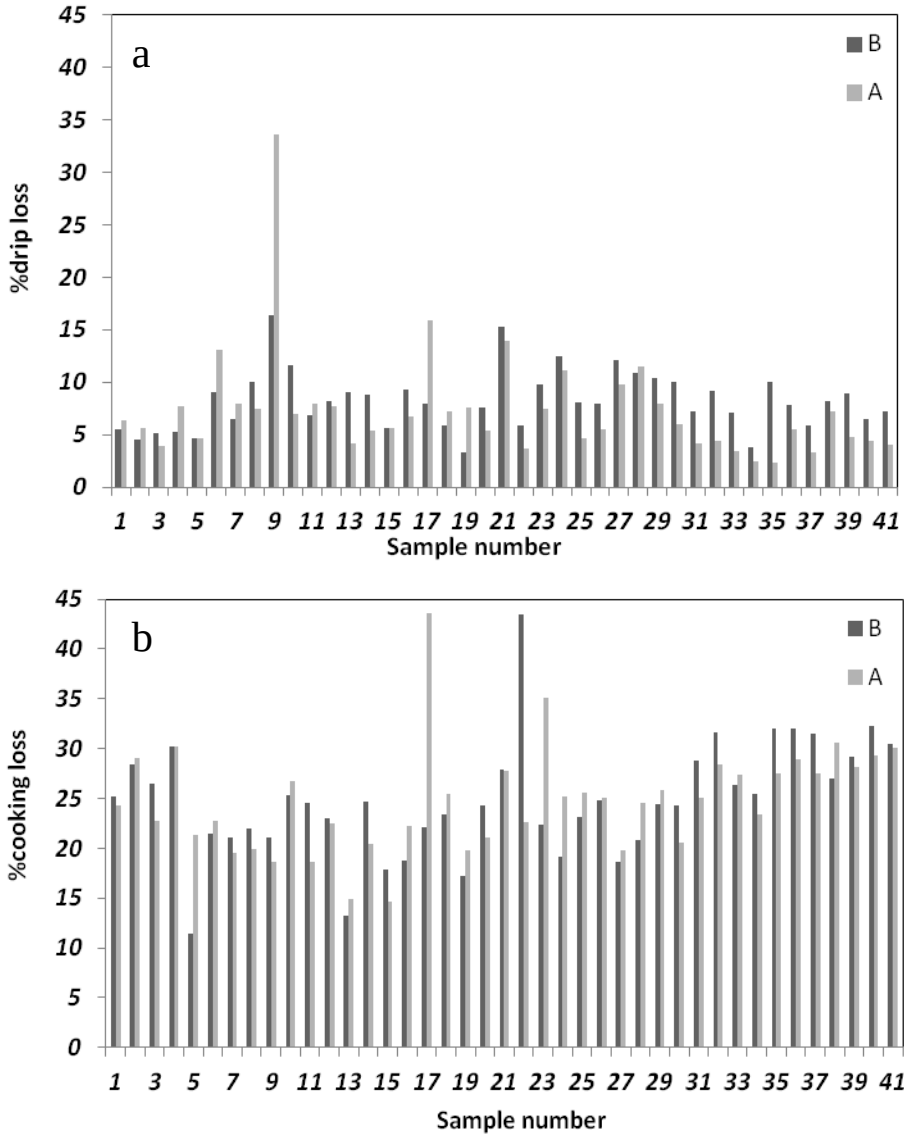

Figure 8: Percentage total loss in meat weight of 41 samples (a) percentage drip loss, b) percentage cooking loss; for all samples in piece A and B.

The above Figure 8 and Table 4 are the details obtained from the drip loss and cooking loss in Piece A and B of 41 samples. In Figure 8a, the drip loss in piece A was larger than in piece B in sample number 1-20, however this pattern was opposite in samples 21-41, where percentage drip loss was more for B rather than A samples. This could be due to the difference observed in other slaughter variables between the groups of animals from 1-20 and from 21-41, which is explained in the later parts. A single sample, sample number 9 showed a very high amount of drip loss of piece A and B, which might have been resulted due to experimental handling errors.

In Figure 8b, the cooking loss is distributed unevenly and no groups could be tracked from the results. However in some samples like, sample 17 and 23, cooking loss in piece A is very high compared to cooking loss in piece B; and also in sample 22,

cooking loss in B was higher than that of A. Sample 9, 17, 23 and 22 were assumed to be outliers which might have resulted from some experimental errors.

Table 4 was made to distinguish the samples as good, acceptable and bad. It was found that, more than 19% of the samples were in bad category, in piece B when both drip loss and cooking loss was observed. No absolute effect of weight was observed for drip loss and cooking loss in muscle. Our result was in agreement to results of Waritthitham et al.(2010) , which also showed no significant effect of weight of samples on WHC. High percentage of drip loss and cooking loss is a big disadvantage for the consumers as well to the company and our work showed that at least 76% of the samples were found in acceptable and good category in both case. In other words, it was found that, out of 41 samples less than 25% of them suffered from high drip and cooking loss.

2.2.3 WB measurements of 3 weeks aged samples from 41 animals

The value that separates the tender meat from tough was found to be 41N. From the Scatter plot in Figure 9, it was found that 18 samples were tender- 32% of total, 7 samples were tender in B but tough in A- 14% of the total and 2 samples were tender in A but tough in B- approx 4% of the total. When the muscle was cut in the slaughter house, piece A was cut from the distal side whereas piece B was cut from the proximal side. Our result suggested the fact that, piece B is more tender than piece A, which is in agreement to the earlier findings (Reuter, Wulf et al. 2002; Senaratne, Calkins et al. 2010). It was argued by them that tenderness varies in of *M. semimembranosus* muscles at different position and proximal ends were tendered than the distal ends.

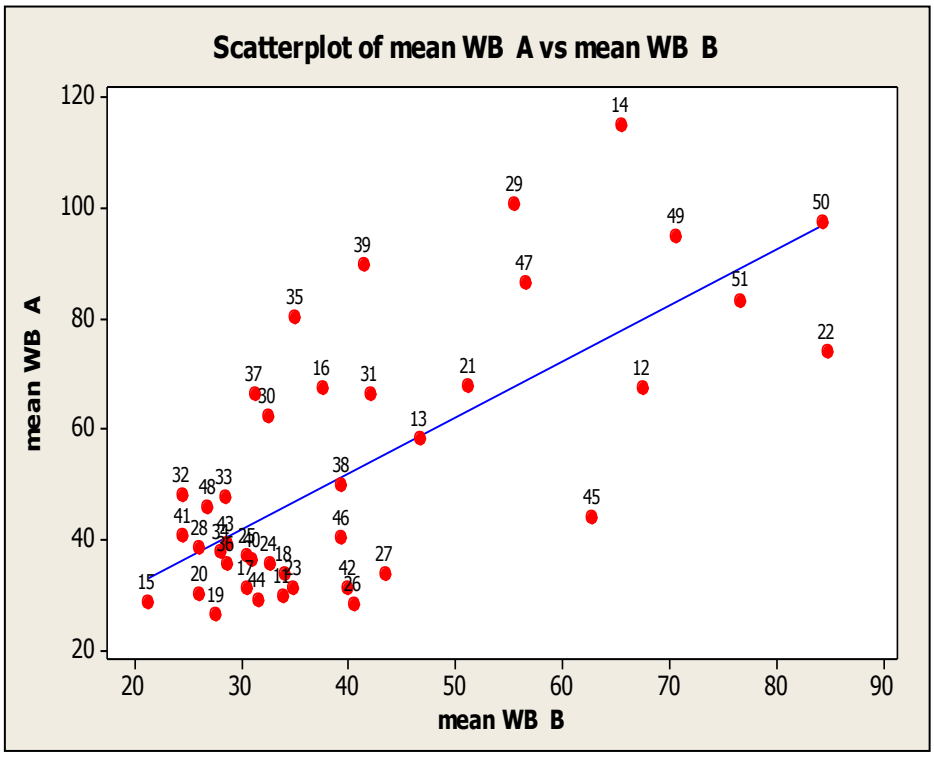

Figure 9: The scatter plot of mean WB peak force of piece A versus mean WB peak force of piece B in Newton (N). The blue line in between is the fitted regression line. The red dots are the sample and the number beside them is the sample numbers.

2.2.4 Difference between two groups in terms of slaughter variables

Table 5: Slaughter variables differed in samples from 1-20 and from 21-41.

Sample numbers	ToT (hrs)	Fat-grade	TODO (hrs)	pH 0	pH 1	Temp. at surface of *S.M*	Gender
1-20	2.23±0.34	7.05±0.53	3.6±0.1	6.26±0.05	6.04±0.05	33.07±0.66	0.25±0.10
21-41	3.00±0.46	5.52±0.70	4.5±0.2	6.48±0.05	6.16±0.05	30.01±0.51	0.65±0.10
p-value	0.19	0.09	0.001	0.003	0.11	0.001	0.006

Table 5 shows that there were a difference in ToDo, pH0 and Temperature at surface of the muscle. The first 20 samples collected where removed earlier, and despite this we observed a lower pH. This may indicate that these samples may in general been more efficiently stimulated than the group that came later. In retrospective it came to our knowledge that the electrical stimulation was subjecting more difficulties that

normally during the period where we collected meat samples. The temperature differences may be related to the same phenomenon, but possibly this could also be related to more efficient chilling later in the collection period; the last 20 samples were collected when the outside temperature was lower due to the merging winter.

2.2.5 Quality variables and relationship to OCR (i) measured after 3 weeks.

Table 6: Cross Validated relationship (correlation coefficient, R_c) of WB measurements and drip loss to OCR measurements after 3 weeks.

Variable	Series	OCR -3 weeks (R_c)
WB (N)	1-20	0.01 (PC1)
WB (N)	21-41	0.67(PC 7)
Drip loss (%)	1-20	0.04 (PC1)
	21-41	0.17 (PC1)

PLS regression with cross validation was run for WB , drip loss and cooking loss measurements to $OCR_{(i)}$, which were run separately for first 20 samples (1-20) and last 21samples (21-41) as shown in Table 6. In case of cooking loss, weak or no correlation was obtained (not shown in the table). No strong correlation was found for drip loss to the $OCR_{(i)}$ measurements after 3 weeks either. However, some correlation was found for the WB measurements to the difference in $OCR_{(i)}$ measured after 3 weeks. This result suggests that, the OCR and tenderness of meat are somehow related to each other.

2.2.6 Predicated WB values for OCR (i) versus measured

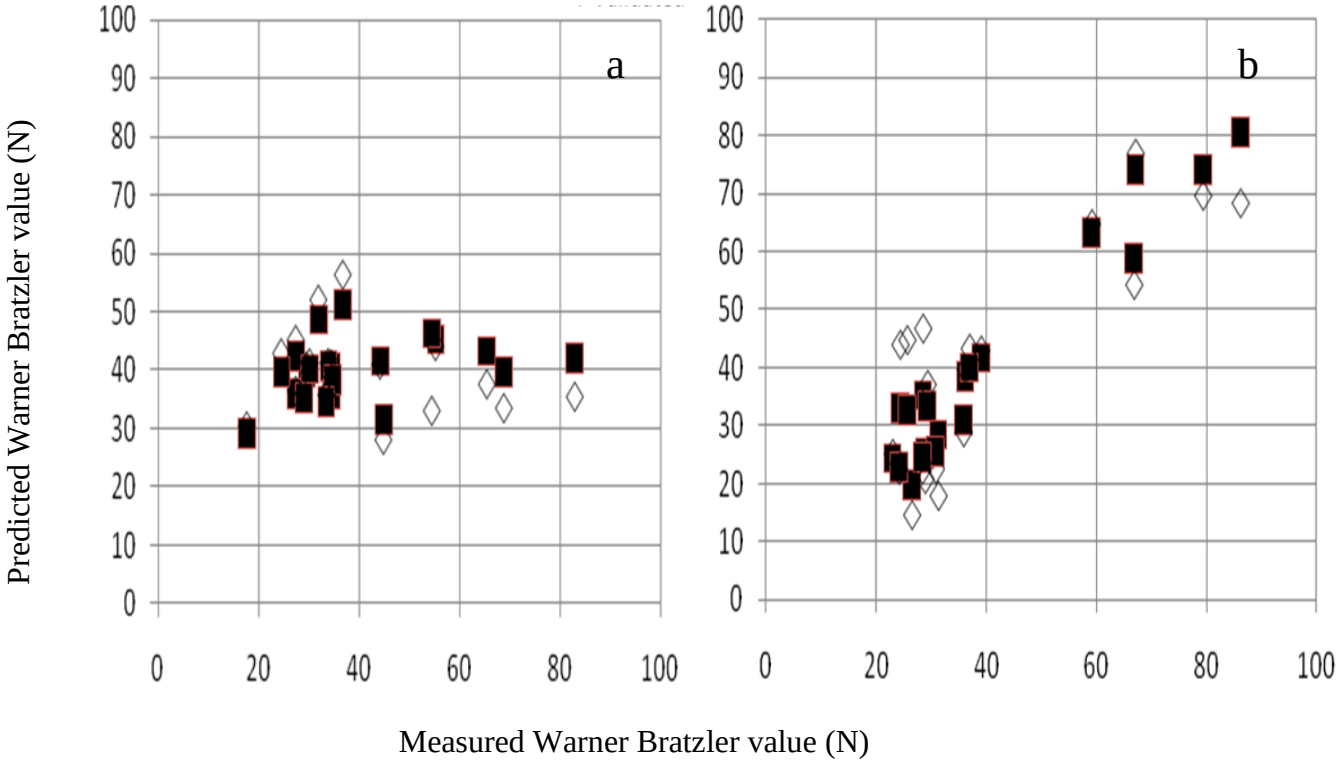

Figure 10: Predicted Warner Bratzler (WB) values of piece B from OCR (i) ■ versus measured where are measured and ◊ are the cross validated predicted samples. a) First 20 samples (1-20); b) last 21 samples (21-41).

When 41 samples of piece B were separated in a group of 1-20 and 21-41, clear indication of relationship between OCR and WB was observed in case of the later group (Figure 10b). From the OCR (i) in Figure 10 b, tender muscles were well separated from the tough muscles. The above result gives us clue that $OCR_{(i)}$ can be a predictor of tenderness of samples in case of sample group from 21-41.

2.2.7 Regression coefficient between protocol additives and WB values

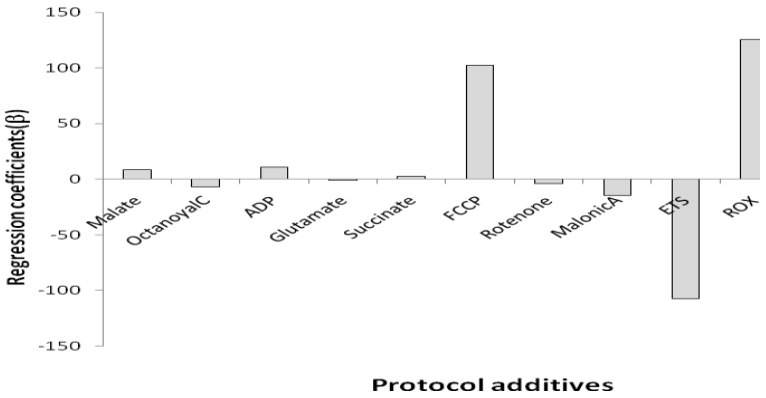

Figure 11: Regression coefficient between protocol additives and Warner Bratzler (WB) values.

The protocol additives in the Figure 11 are the additives used in $OCR_{(i)}$ measurements. High WB values meaning tough meat are correlated to high FCCP response, low ETS and high ROX which is as expected. High WB peak values correlated to low ETS. This would actually mean that when the removal of oxygen by ETS was high, more tender samples were measured. This would agree with the general belief (Huff Lonergan, Zhang et al. 2010) that reducing conditions would favor tenderization. FCCP was highly correlated to ETS which means that for the samples studied these variables measured the same. The relationship to ROX is difficult to explain as we do not know how this variable is explained. However, it is interesting to observe ROX may actually be detrimental to tenderization.

2.3 Conclusions

In our experiment, we used 41 *M.semimebranosus* muscles as piece A from distal side and piece B from proximal side directly collected from the slaughter house. We studied different meat quality variables like tenderness, drip loss and cooking loss and also its relation to oxygen consumption of muscle after 3 weeks maturation. The line differentiating tenderness and tough muscle was identified at 41N WB peak value measurements for our measurement system. Piece B was tendered than piece A; i.e. the position from where the steak is taken from affected the tenderness. $OCR_{(i)}$ rates in 3 weeks muscle were found to have some prediction ability of tenderness in group 21-41 in meat. We observed a difference in the pH decline rate for the first 20 samples compared to the last 21 samples. This may have influenced the postmortem tenderization process.

Most of the collected samples were in acceptable range in case of total drip loss and cooking loss documented. However, from our research we were not able establish any relationship between drip loss and cooking loss to $OCR_{(i)}$ in 3 week stored muscle and also to effects of difference in weight of samples.

3 Part II- Peparing a calibration model for predicting myoglobin states.

The introductory part to this section is written above. This part is written as an independent section since it forms the basis of the first publication (paper (I)) extracted from my thesis.

- The aim of this part was to make a calibration model from calculating different myoglobin states in beef *M. semimembranosus* muscle.
- Compare two methods of sample preparation for calibration, one using the AMSA guidelines (Hunt, Acton et al. 1991) and another method by altering the oxygen partial pressure (OPP) method and choose best one based upon prediction errors.
- Compare two method for calculating the calibration data, one using selected wavelengths (SW) as explained in AMSA guidelines (Hunt, Acton et al. 1991) and another using all the wavelengths by multivariate regression technique.

3.1 Material and Methods

3.1.1 Meat sample preparation:

The *M. semimembranosus* muscle samples were brought from local slaughter house (Fatland, Oslo, Norway). The carcass was deboned and muscles were vacuum packaged and kept on ice during transport and in cold room at 4°C and was used next day (4^{th} day of postmortem). Other variables like specific breed, age group and so on were not requested. For the whole calibration, muscles were taken from total 3 animals on different date. Muscles were cut into 48 steaks of approximately identical sizes. The thickness of sample was at least 15 mm as light may easily pass through the thin samples and give incorrect reading. AMSA guidelines also states that, the samples must be at least 12-15mm thick (Hunt, Acton et al. 1991). Muscle steaks were flat and the area of the surface was sufficient enough for the optical probe to be placed in four different positions. The muscle pieces were cut parallel to the fiber direction as it is the most practical approach. The 48 steaks were distributed into six parts (8 x 2 steaks each for DMb, MMb and OMb preparation using two different methods). The ultimate pH for *M.semimembranosus* at proximal side was 5.6. The pH varies along the muscles (Lee, Yancey et al. 2008).

3.1.2 Preparation of pure myoglobin states

Two methods were used to prepare the pure myoglobin states in the sample.

A. Chemically induced myoglobin preparation (CHEM) method as explained in AMSA guidelines (Hunt, Acton et al. 1991)

OMb: All the 8 steaks prepared for OMb preparation was placed in PE/PA bags with OTR of 30-40 $cm^3m^{-2}24h^{-1}$ at 23 ºC and 75 % RH (Maskegruppen, Vinterbro, Norway). The bags contain samples were kept on the ice and the measured temperature on the meat surface was 2°C. The samples were flushed with 100% oxygen for 10 minutes continuously. Samples were then wrapped with the low density polyethylene (LDPE) film (OTR 6500-8500 $cm^3m^{-2}24h^{-1}$ at 23 ºC and 75 % RH Toppits-glad, Melitta Group, Klippan, Sweden). The spectroscopic measurements were taken immediately.

MMb: The 8 steaks separated for MMb preparation were placed in 1.0% potassium ferricyanide (Merck eurolab, Proanalyser(P.A)). They were placed for 1 minute and drained properly. It was then blotted dry with the help of clean tissue paper and covered with LDPE film (Toppits-glad, Melitta group, Sweden) for 12 hours at 2-4°C to let it oxidize. It was then ready to scan.

DMb: Total 8 steaks were used to prepare DMb. The sample steaks were treated with 10% sodium dithionite (BDH, Prolab, Lutterworth, UK). Samples were drained and blotted dry using tissue paper. It was then vacuum packaged using the Original Henkelman Vacuum 300 system (vacuum strength 9, type 300II, Hertogenbosch, The Netherlands) in PE/PA bag (Maskegruppen) to reduce for 2 hours at room temperature. After 2 hours, it was quickly wrapped in LDPE film (Toppits-glad, Melitta group, Sweden) and was scanned.

B. OPP method

By making changes in packaging principals, different myoglobin states could be obtained. All steaks were packaged in Polimoon 511VG tray sealing machine (Promens, Kopavogur, Iceland).The tray packages were made of amorphous polyethylene terephthalate (APET) trays (Wipak Mulipet) with ethylene vinly alcohol (EVOH) top films (Wipak Biaxer) (both Wipak Oy, Natsola, Finland). Oxygen transmission rates for the tray and top film were 7 and 5 cm^3/m^2/24 h at 23 °C at 50 % relative humidity.

OMb: The trays containing steak for preparation of OMb were flushed with preblend 75 % O_2/ 25 % CO_2 from AGA, Oslo, Norway). Concentration of oxygen was

measured with a CheckMate 9900 (PBI Dansensor, Ringsted, Denmark). The samples were stored at 4°C and scanned next day (meaning after 24±2 hours).

MMb: The trays with MMb samples were first flushed with 60 % CO_2/ 40 % N_2 (preblend from AGA, Oslo, Norway). As MMb is formed at low partial oxygen pressure (Lindahl 2005), about 1.5% residual oxygen level was maintained. This was done by inserting air through syringes into the packages through sealing septas (Toray TO 125, Toray Engineering, Osaka, Japan). Samples were stored at 4°C and measurements were made only after 7 days (samples were allowed to convert into thick brown color).

DMb: Muscle steaks were vacuum packaged in PE/PA bags (Maskegruppen). The vacuum packaged samples were stored at 2-4°C for approximately 2 days (48 hours) and were scanned for color.

3.1.3 Color spectrum measurement on sample surface

Before all the measurements, all the samples were wrapped with LDPE film (Toppits-glad, Melitta Group) and was placed in high density polyethylene (HDPE) (Dyno 516, SWF Companies, Reedley, USA) black trays. The samples surface chosen for the measurement were the ones containing with minimum fat and connective tissues with surface temperature of 17±1°C (Würth Infrared thermometer Art no 08536007, Würth UK Ltd., United Kingdom). The instrument used for scanning the sample surface was FOSS NIRSystems (Model 6500, 0654-Oslo, transflectance modus with a 40 x 40mm^2 optiprobe ^{TM} system, 23 X 23 mm sensor area).The calibration of the instrument was done with the white ceramic plate having L*=101.01, a*= 1.74 and b*=5.3 from Minolta (Chroma meter CR-410, Konica Minolta, sensing inc. B8403706, Japan). NIRSystems could measure absorbance (log (1/R), where R is the reflectance) from 400-1100nm in 2nm steps, hence giving 350 spectra at a time for one measurement (which is an average of 32 scans and takes about 20 second).

Each sample steaks were scanned on four different positions on the surface. Therefore for each myoglobin preparation method (CHEM and OPP), there was 96 spectra i.e. 4 measurements (on sample surface) x 3 myoglobin states x 8 sample steaks. Hence, each myoglobin states had 32 spectra (4 measurements x 8 steaks) for each myoglobin preparation method.

3.1.4 Processing of spectra and statistical analysis (summarized in Figure 12)

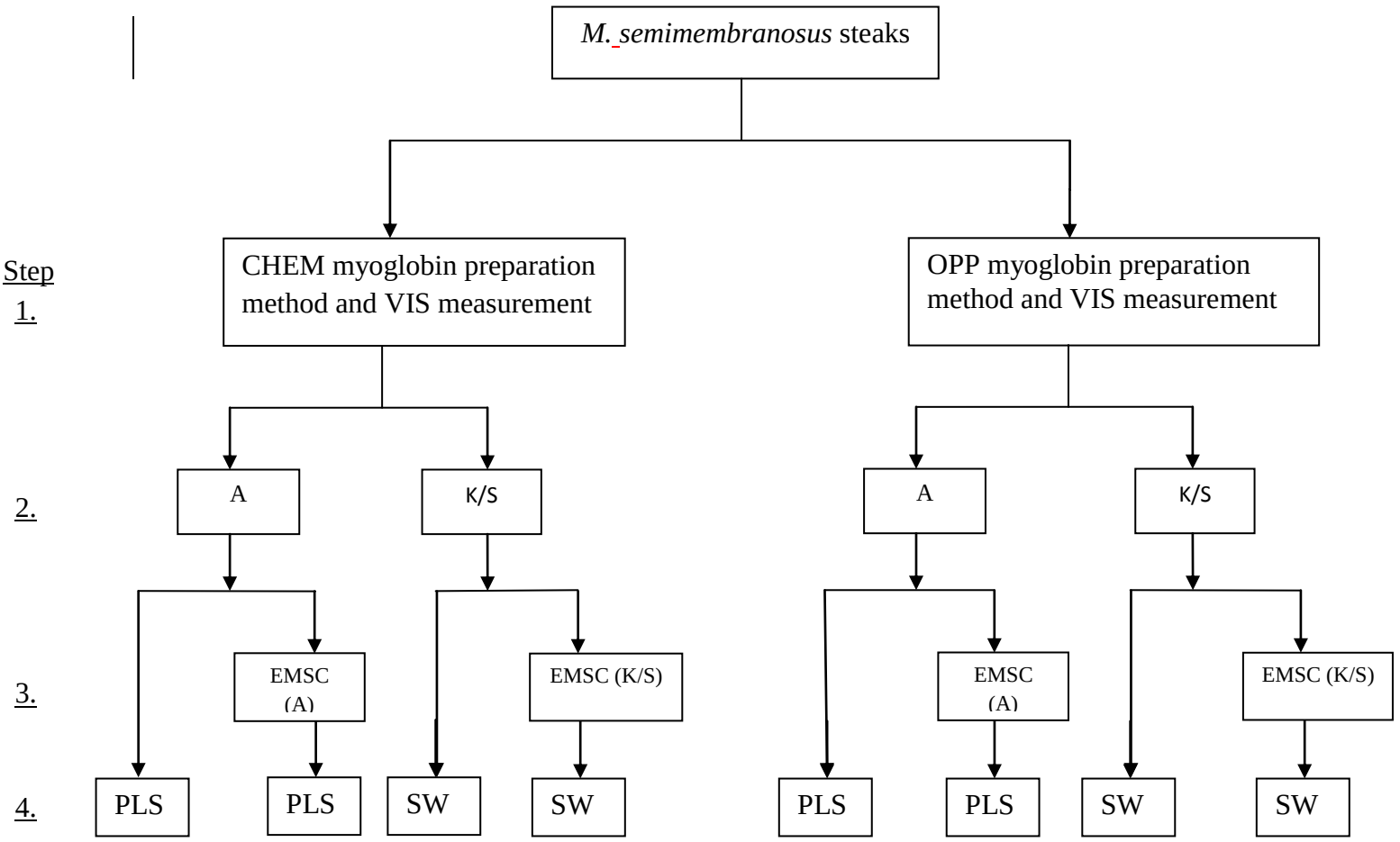

Figure 12: Flowchart showing the overall physical and statistical treatments done in calibration and chemically treated samples before normalization (for abbreviations see Materials and Methods).

Step1. Physical treatment for sample preparation either by OPP or CHEM method with visual (VIS) measurements
Step 2. Spectra transformation: Use of either absorbance (A) data or conversion into Kubelka-Munk (K/S).
Step 3. Pre-processing (with EMSC or no EMSC) with light scatter correction or no correction.
Step4. Calibration step: Absorbance data are calibrated by applying PLS regression and K/S data by selected wavelength (SW) as suggested in AMSA guidelines (Hunt, Acton et al. 1991)

A. Spectra Processing:

SW use from AMSA guidelines (Hunt, Acton et al. 1991):

The spectral data for all samples were in absorbance (A). They were converted into reflectance and to K/S ratios using The Unscrambler software version 9.7 (CAMO software AS, Oslo, Norway). Some ratios at wavelengths such as, 474nm, 525nm, 572nm and 610nm (Krzywicki 1982; Hunt, Acton et al. 1991) were saved and exported to MS-Excel 2007 (Redmond, Washington: Microsoft, Computer software) for further calculation.

They were used to calculate the percentage of myoglobin using formulas as shown in equation 1-8 in Introduction section. We used respective equations (two equations are given to calculate the same myoglobin state) to calculate different myoglobin states and decide to use the one with the best Correlation coefficient (R_c) and Root mean square error of cross validation(RMSCEV) for further processing. The total route of processing explained can be written as; from CIMS/OPP preparation method, A→R→K/S→SW.

Second option used was addition of another step called as extended multiplicative signal correction (EMSC)(Martens and Stark 1991) in between the route and can be written as; from CIMS/OPP preparation method, A→R→K/S→EMSC→SW. The K/S ratio was treated by using EMSC in The Unscrambler software version 9.7 (CAMO software AS, Oslo, Norway) using model and subtract options checked for channel number, squared channel number and squared spectrum in the software. The EMSC model was saved for later use in predicting real samples in part III (Section 4) of the thesis. From the EMSC treated data, the wavelengths were selected as earlier (one paragraph above).

PLS regression:

All the absorbance spectra from both preparation method were either treated with EMSC first or without treating with EMSC (as shown above in Figure 12) and were run through PLS-1 regression with cross validation, number of PLS factors=10, systematic validation with 4 samples (the repeated measurements) per segment for each myoglobin states. The EMSC model was saved like above. The result were saved and number of PLS factors were recorded. For particular myoglobin states, number of PLS factors was listed in column along with their scores in The Unscrambler software version 9.7 (CAMO software AS, Oslo, Norway) data sheet. For each myoglobin states, each specific data column was present in the Unscrambler result sheet. They

were exported to spreadsheet of Microsoft Excel, Version 2007 (Redmond, Washington: Microsoft, Computer software).

Overall route explained in this step can be written as; from CIMS/OPP preparation method, A→EMSC→PLS or from CIMS/OPP preparation method, A→PLS.

B. Post-transformation:

This step of post-transformation was done in spreadsheet of Microsoft Excel, Version 2007 (Redmond, Washington: Microsoft, Computer software) as all data were already exported from Unscrambler. Altogether three steps also called as normalization steps were carried out during post-transformation.

- $\hat{y}$ >1, corrected to $\hat{y}$=1; where $\hat{y}$ is the value of specific myoglobin state in present in the datasheet. If the value is greater than 1, it is changed and written as 1. The value 1 in the data sheet means that the presence of that specific myoglobin state in that sample is 100%.
- $\hat{y}$<0, corrected to $\hat{y}$=0. As the presence of specific myoglobin state in the sample can range from 0-1 (0-100%) and cannot have negative values.
- $\hat{y}_{OMb}+\hat{y}_{MMb}+\hat{y}_{DMb}=1$. Measurement of myoglobin sample at particular time can have three types of myoglobin namely, OMb, MMb and DMb and their sum should be one (meaning, when amount of all three forms of myoglobin states at particular instant is measured, it should be 100%).

After the above normalization steps, R_c and RMSCEV were calculated. The hypothesis behind this was, in pure or true sample, only one myoglobin state can present, For example: if the sample is prepared for pure OMb, the sample contains 100% OMb, and amount of DMb and MMb were zero. Therefore, R_c and RMSCEV were used as criteria for comparing myoglobin sample preparation method and calculation method. High value of R_c and low value for RMSCEV were picked for specific methods.

3.2 Results and Discussion

3.2.1 Comparison of spectra.

Spectra from OPP method (Figure 13c) had much distinct and compact spectra for all individual myoglobin states compared to that of CHEM method (Figure 13a).As

shown in Figure 13, after the treatment of spectra with EMSC, they had improved and the three myoglobin states showed much less deviation. The EMSC treatment made the DMb, MMb and OMb spectra more distinct (Figure 13b and d) than each other compared to the ones without EMSC (Figure 13a and c). This result is in agreement with the purpose of use of EMSC transformation method by Gallegher et al.(2005). Even after transformation of spectra with EMSC, OPP method gave more precise spectra (Figure 13d) compared to CHEM method (Figure 13b) for OMb and DMb. The OMb and DMb myoglobin states for most of the wavelength are not distinct and overlapped with each other from 500-900nm as shown in Figure 13b, however in Figure 13d, substantial difference can be viewed after 700nm. The problem with obtaining pure DMb has also been explained in AMSA guidelines (Hunt, Acton et al. 1991). Mostly as DMb rapidly picks up surface oxygen they get transformed into OMb and hence DMb becomes the mixture of OMb and DMb.

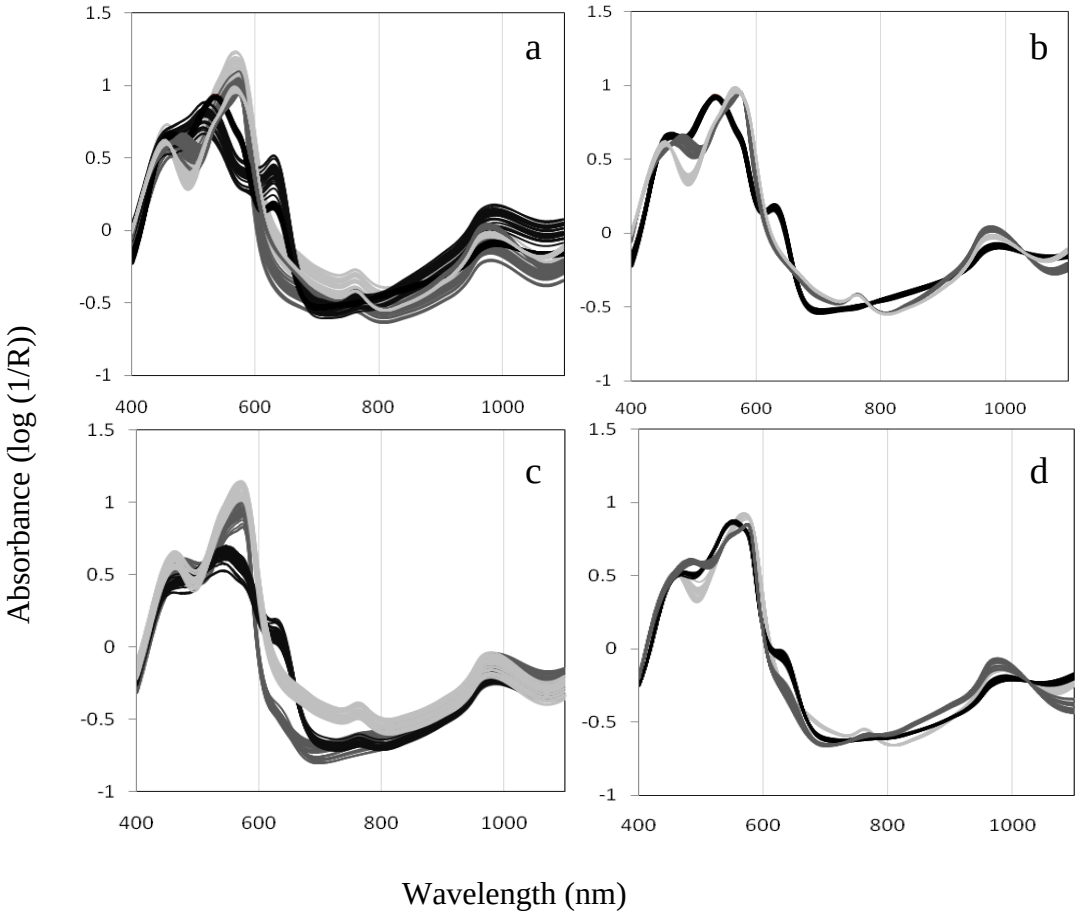

Figure 13: The absorbance (A= log(1/R), where R is the reflectance) spectra of the three different myoglobin states of *M. semimembranosus*; a) without and b) with extended multiplicative scatter correction (EMSC), using the CHEM method of sample preparation, c) without and d) with EMSC using the OPP method for preparing samples. ▬ denotes 100%MMb treatment ▬ denotes 100%

OMb treatment and denotes 100% DMb treatment. Each of the myoglobin states are presented by 32 spectra.

3.2.2 Comparison of Principal component analysis (PCA) plots

Principal component analysis describes whole data set and gives information on essential parameters with maximum information (Karadas and Kara 2012).Using PCA plot of Figure 14, we are trying to compare the method explained as in AMSA guidelines (Hunt, Acton et al. 1991)(route: spectra from CHEM method→A→R→K/S→PCA plot) to the OPP method of preparation using EMSC (route: spectra from OPP method→A→EMSC→PCA plot). Myoglobin states are well distributed from each other in Figure 14b compared to the ones in Figure 14a. Figure 14a is also in agreement with the results in 3.2.1 and can be stated that

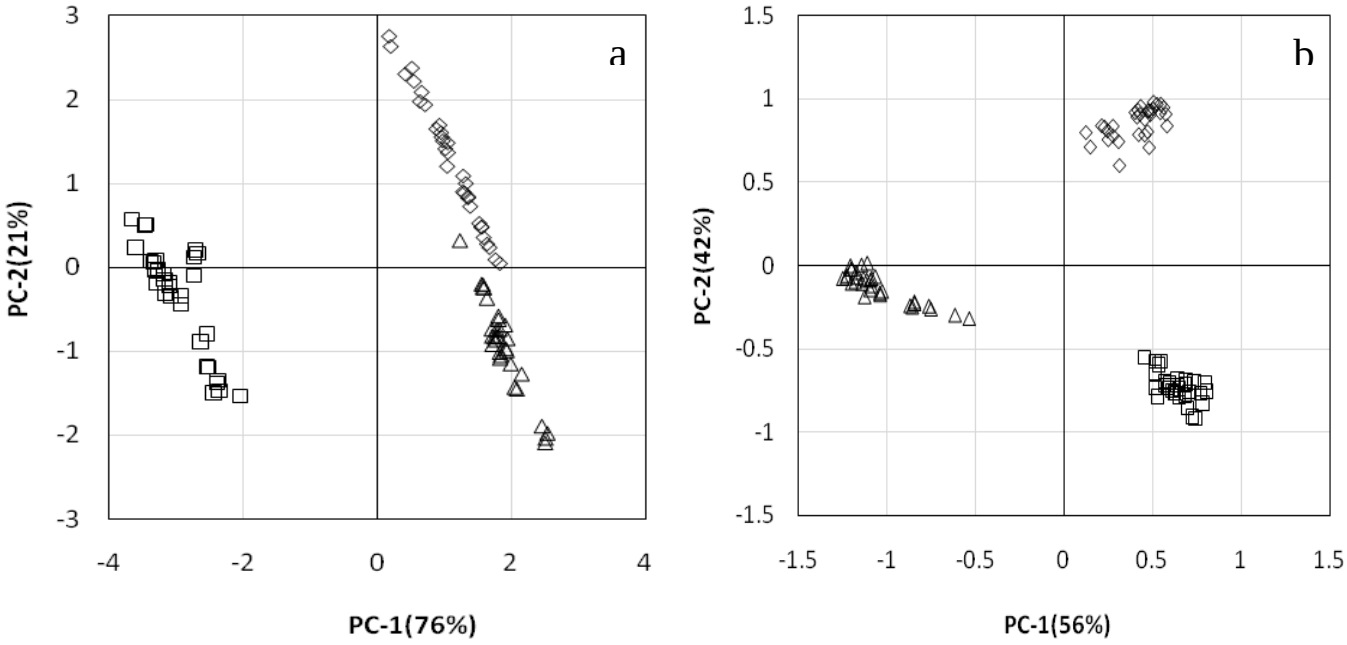

Figure 14: Principal component analysis (PCA) of myoglobin states. Explained variance of the two components was 97% (a) and 98% (b). PC-1 is principal component one and PC-2 is principle component two; a) CHEM (chemically induced myoglobin states) treatments and K/S spectroscopic transformation, b) OPP treatments of the meat and extended multiplicative scatter correction (EMSC) transformation. ◊ DMb (Deoxymyoglobin); □ MMb (Metmyoglobin); Δ OMb (Oxymyoglobin).

pure DMb samples have some OMb in it so they are not well separated. However, using the CHEM method along with EMSC transformation (Figure 14b) gave good cluster of OMb, DMb and MMb at three positions in the PCA plot. Results from Figure **14**b indicate that, each myoglobin state had the spectra that made them unique.

3.2.3 Regression comparisons

Table 7: Prediction errors expressed as root mean square error of cross validation (RMSECV in fractions) for the three different myoglobin states and the corrected correlation coefficient (R_c) between the true (prepared) state and predicted state. The calculation method was chosen according to the formula that yields lowest RMESCV for CHEM method. RMSECV and R_c refer to corrected predicted data (see the Material and Method section for details). Bold font indicates the most accurate predictions for each myoglobin state.

Myoglobin states	Treatments			RMSECV	No. PLS factors	R_c
	Physical[1]	Spectra prcocessing[2]	Calc. method (formula used)[3]			
DMb	OPP	A	PLS	0.060	3	0.994
		K/S	SW [OMb]	0.26	-	0.901
		EMSC(A)	**PLS**	**0.042**	2	**0.997**
		EMSC(K/S)	SW [OMb]	0.31	-	0.836
	CHEM	A	PLS	0.080	3	0.988
		K/S	SW [OMb]	0.18	-	0.960
		EMSC(A)	PLS	0.081	2	0.988
		EMSC(K/S)	SW [OMb]	0.21	-	0.932
OMb	OPP	A	PLS	0.072	1	0.992
		K/S	SW [DMb]	0.12	-	0.974
		EMSC(A)	**PLS**	**0.041**	3	**0.997**
		EMSC(K/S)	SW [MMb]	0.21	-	0.964
	CHEM	A	PLS	0.082	3	0.990
		K/S	SW [MMb]	0.16	-	0.947
		EMSC(A)	PLS	0.080	2	0.989
		EMSC(K/S)	SW [DMb]	0.21	-	0.924
MMb	OPP	A	PLS	0.069	3	0.996
		K/S	SW [DMb]	0.28	-	0.991
		EMSC(A)	PLS	0.039	3	0.997
		EMSC(K/S)	SW [DMb]	0.33	-	0.834
	CHEM	A	PLS	0.041	2	0.997
		K/S	SW [OMb]	0.079	-	0.993
		EMSC(A)	**PLS**	**0.029**	2	**0.998**
		EMSC(K/S)	SW [OMb]	0.39	-	0.928

1: How the beef was treated physically: Chemically induced myoglobin states (CHEM) and Oxygen Partial pressure Packaging (OPP).

2: Spectra transformed to: Absorbance (A), Kubelka-Munk (K/S), Extended Multiple Signal Corrected Absorbance (EMSC (A)) and Extended Multiple Signal Corrected Kubelka-Munk (EMSC (K/S)).

3. Calculation method used: Partial Lest Square (PLS) and Selected Wavelengths (SW) according to AMSA guidelines by Hunt et.al (1991) [formula used se eq. 1-8 in the introduction section 1.9above]. The formula was chosen to give the lowest prediction error.

A. CHEM method versus OPP method with K/S transformation and SWs.

The RMSECV values for DMb, OMb and MMb following the route: OPP→A→R→K/S→SW, were 0.26, 0.12 and 0.28 respectively. Similarly, RMSECV for DMb, OMb and MMb following route: CHEM→A→R→K/S→SW, were 0.18, 0.16 and 0.079 respectively, as shown in Table 7. From this result, we can say that OPP method is better option only for preparation of OMb and the possible reason could be the difference in incubation time between OPP and CHEM method. OPP preparation method used longer incubation time for OMb preparation than CHEM method, therefore thick layer of OMb could have formed on the sample giving better reading in OPP method. On the other hand, DMb preparation with both method was not satisfactory and had larger error than explained by Ledward (1970). He determined MMb in muscles using CHEM→K/S→SW route and reported error between (±) 0.05-0.07. The error for DMb was (±) 0.05 in his report which is lower than error indicated in our results. Ledward (1970) kept the DMb samples after reduction by dithionite under nitrogen gas until the spectral measurements were made and also used 20% dithionite of unknown incubation length for myoglobin to reduce which is different than preparation method explained in AMSA guidelines by Hunt et.al(1991). The difficulty in preparation of DMb has also been said in the AMSA guidelines. From this result, it can be said that, both CHEM and OPP method needs improvement in preparation of DMb. Another issue could be oxygenation of DMb during repackaging of sample with LDPE film (also known as oxygen permeable film).

B. PLS regression versus SW.

Using OPP→A→R→K/S→SW route, RMSECV for DMb, OMb and MMb were 0.26, 0.12 and 0.28 respectively whereas introducing PLS regression and eliminating SW and transformation of absorbance spectra in the route i.e. OPP→A→PLS improved RMSECV to 0.060, 0.072 and 0.069 for DMb, OMb and MMb respectively.

Introducing PLS regression in OPP method clearly improved the RMSECV and supports PLS over SW. The prediction errors after use of PLS regression decreased by more than 4 times for DMb and MMb while for OMb it decreased by more than 1.5 times for OPP method.

Similarly, in CHEM→A→R→K/S→SW route, RMSECV for DMb, OMb and MMb were 0.18, 0.16 and 0.079 respectively and similar as in above paragraph, changing the route of calculation to CHEM→A→PLS resulted RMSECV 0.080, 0.082 and 0.041 for DMb, OMb and MMb respectively. Use of PLS regression improved the results even for CHEM myoglobin preparation method and decreased prediction errors by almost 2 times for all myoglobin states.

Therefore, use of PLS regression should be the method of choice compared to SW along with K/S.

C. Transformation of spectra with EMSC.

RMSECV for DMb, OMb and MMb were 0.31, 0.21 and 0.33 respectively in OPP→A→R→K/S→EMSC→SW route. And, RMSECV or DMb, OMb and MMb were 0.21, 0.21 and 0.39 respectively in CHEM→A→R→K/S→EMSC→SW route. Even after the addition of transformation step in spectra processing, no improvements could be obtained in RMSECV values as compared to results in 3.2.3 A.

However, rather than the use of SW, when PLS regression was used, the results were improved after transformation with EMSC. The RMSECV were 0.042, 0.041 and 0.039 for DMb, OMb and MMb respectively in OPP→A→EMSC→PLS route. Even in CHEM→A→EMSC→PLS, RMSECV improved for DMB, OMb and MMb after using PLS regression with results, 0.081 0.080 and 0.029 respectively. In fact, CHEM method in this case showed slight improvement than OPP method for MMb while RMSECV for DMb and OMb supported OPP method.

From these results, it can be concluded that, use of EMSC was better option to PLS regression rather than SW.

3.2.4 Predictions and illustration of OMb using AMSA guidelines (CHEM→A→R→K/S→SW route) and OPP method with PLS regression

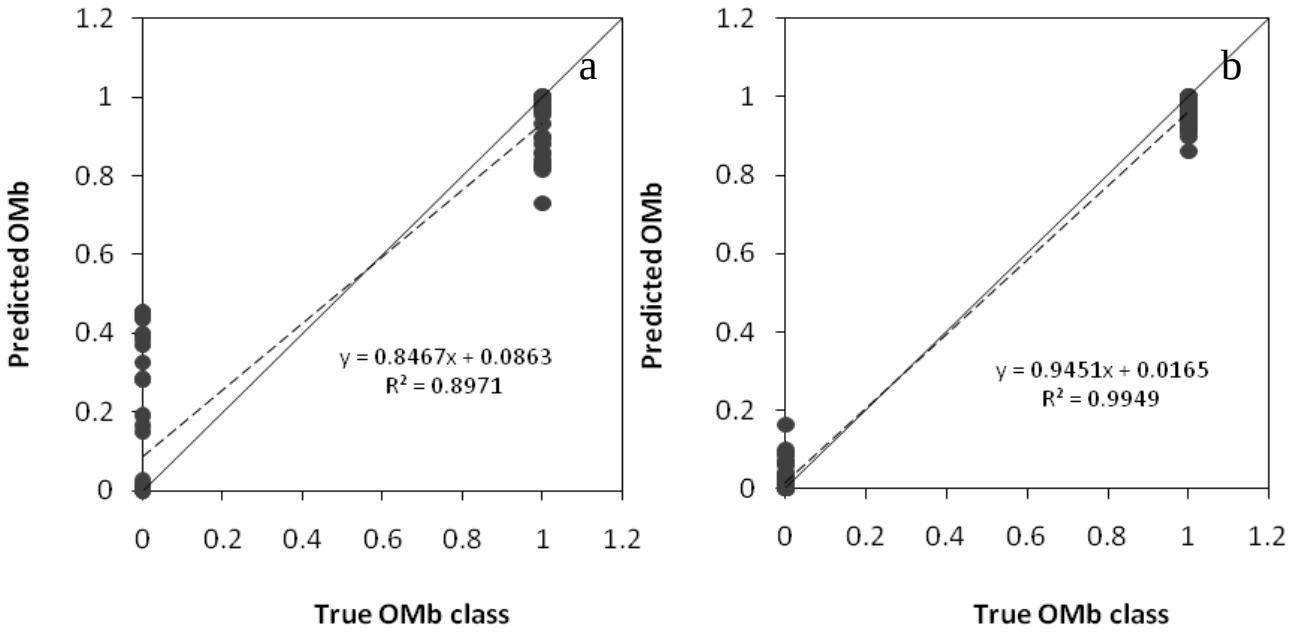

Figure 15: Corrected predicted oxymyoglobin (OMb) versus true (as prepared) OMb state; a) CHEM (K/S) SW data for OMb (correlation coefficient R_c=0.947); b) OPP (EMSC(A) PLS) for OMb (R_c=0.997). The diagonal line illustrates the target line (x=y). The dotted line is the fitted regression line.

Figure 15 illustrates the AMSA guidelines by Hunt et.al (1991) processing route i.e. CHEM→A→R→K/S→SW is compared to the OPP→A→EMSC→PLS processing route for OMb predictions. The data used for the illustrations are the ones that have gone through post-transformation. DMb, OMb and MMb have been transformed so their sum is one or they add up to total 100%. In true myoglobin class, 100% purity (prefect preparation and processing of data) means that the specific myoglobin state is predicted as one and the presence of two other myoglobin states is zero.

In Figure 15a, when the true class was zero (when OMb presence is suppose to be zero), the prediction could be as high as 0.04. This means even when no OMb (0%) was present; this method (CHEM→A→R→K/S→SW route) could predict the OMb presence as from 0-40%. Similarly, in pure state, when OMb should be 100%, this prediction range from almost 70-105%.

Prediction in Figure 15b was however different. When presence of OMb was zero, the prediction range from almost 0-18% and when OMb was 1(meaning 100%), the prediction range was from almost 85-100%. The illustration also supports better

prediction of OMb states when OPP→A→EMSC→PLS route was used as method for processing the calibration for myoglobins.

3.2.5 Regression coefficient

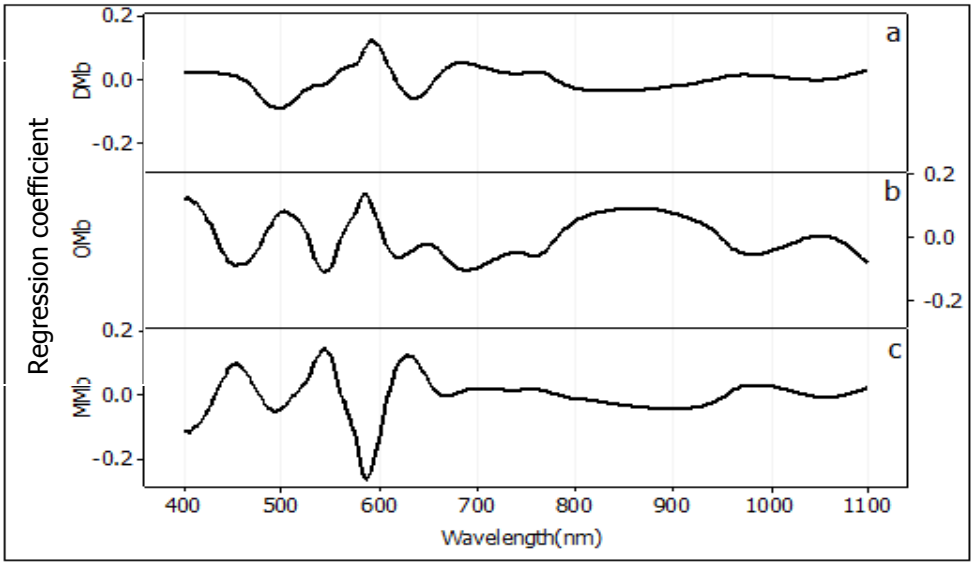

Figure 16: Partial least square (PLS) regression coefficients versus wavelength obtained when myoglobin states were related to spectra using calibration samples prepared with the OPP method and EMSC (A). a) Deoxymyoglobin, number of PLS factors was 2; b) Oxymyoglobin, number of PLS factors was 3; c) Metmyoglobin, number of PLS factors was 3.

DMb, OMb and MMb showed different magnitude for regression coefficient when OPP→A→EMSC→PLS was overall processing route as shown in Figure 16. This method conserved the individual specialty of each myoglobin states. The myoglobin stated had information in different parts and the particular wavelengths which were specific to all states were difficult to point. Since the information is spread in all parts of the spectra, the fact supports use of PLS regression (whole spectrum use) rather than the SW as in AMSA guidelines by Hunt et. al(1991).

3.3 Conclusion

The results from spectra and regression comparison clearly supports that EMSC should be the method of transformation technique. Processing route i.e. OPP→A→EMSC→PLS was the best one giving lowest prediction error (RMSECV) and high correlation coefficient (R_c) for all three myoglobin states except for MMb

which was slightly better in CHEM→ A→EMSC→PLS. However in both case, the route following EMSC and PLS became the method of choice. Therefore, use of all wavelengths (400-1100nm) and PLS regression are recommended in calibration method. However, the preparation of DMb was not satisfactory by both method (OPP and CHEM), so it is also recommended to improve the method for better preparation. Hence best calibration model for DMb and OMb prediction in samples is OPP→A→EMSC→PLS processing route and for MMb predictions is CHEM→ A→EMSC→PLS.

The EMSC model associated with the best calibration model was saved (explained in part II, Section 3.1) for myoglobin prediction in real samples which will be discussed in part III (Section 4).

4 Part III- Relating meat color and myoglobin states to oxygen consumption of 3 week chill stored muscles

The introduction to this part is written above. The work here relies on part II where a model has been created that make it possible to calculate the changes in myoglobin states of different muscles.

- The aim of this paper is to relate changes in color and myoglobin states to oxygen consumption measurements. The oxygen consumption measurements have been made by Vinh T. Phung.
- Since many of the experimental methods have been explained in parts I and I with how the samples were collected, this part is written like a scientific paper with less experimental details. The data presented here is included in a paper (II) that I co-author (after Appendix, status submitted)

4.1 Material and Methods

4.1.1 Meat samples and color measurement

The details about meat samples are explained in part I (Section 2.1). Color measurements were done after three weeks storage from the time of collection The piece for color measurement were of sufficient size for the optical probe to be placed in two positions and Minolta to be placed in three positions with thickness of at least 1.5 cm. The pH of samples recorded (in slaughter house) ranged between 5.44-5.78. The process for color measurement continues from section 2.1.1 C.

As soon as the samples were opened from the vacuum package (type polyethylene/polyamide (PE/PA), oxygen transmission rate (OTR) of 30-40 $cm^3m^{-2}24^{-1}$at 23 ºC and 75 % RH LogiCon Nordic A/S, Kolding Denmark), they were wrapped with LDPE film (OTR 6500-8500 $cm^3m^{-2}24^{-1}$at 23 ºC and 75 % RH, Toppits-glad, Melitta group, Klippan, Sweden) and kept in high density polyethylene black trays (HDPE, Dyno 516, SWF Companies, Reedley, USA) up to 7 days. The samples were in contact with LDPE film only when measurements were made, otherwise LDPE films were kept approximately 2-3cm above the meat surface. The samples were analyzed for changes in myoglobin state due to exposure to atmosphere and light.

4.1.2 Instrumental measurements

Color measurements were made at time 0, 15, 30, 45, 60, 90, and 240 minutes and after 1 day, 2 days until 7 days from the opening time. The measurements were done on the surface at three positions with Minolta (Chroma meter CR-410, Konica Minolta, sensing inc. B8403706, Japan) giving L*, a*, b* values (CIE 1976) and at two positions with FOSS NIRSystems (Model 6500, 0654-Oslo, Norway). All the details were in similar way as explained in part II (section 3.1.3) for calibration samples with use of FOSS NIRSystems. The surface temperature of the samples was measured 17°C during measurements (samples had surface temperature of 17°Cfor less than 5 minutes). During this period the samples were stored under constant temperature cooler fluorescent lamp (Phillips Master TL-D 36W/830 H9 tube attached to tube-holder Phillips IKC 1/36, 1x 6 W-K) with light intensity of 800 lx in between measurement time.

4.1.3 Statistical analysis

A. Myoglobin samples data from FOSS NIRSystems

From part II of the thesis, best calibration model was chosen and was also saved in The Unscrambler software version 9.7 (CAMO software AS, Oslo, Norway).The myoglobin state of these samples were predicted using that predictive model. These data were transformed by use of best calibration model (which was done by choosing the existing model (saved model from part II- section 3.1.4) option in the Unscrambler software) for each individual myoglobin calculations. The result sheet for specific myoglobin states were exported to Microsoft Excel, Version 2007 (Redmond, Washington: Microsoft, Computer software). These data were post-transformed in using same steps as for calibration samples (section 3.1.4) and the two readings at each time for every sample were averaged. Percentage of DMb, OMb and MMb in each sample at particular time was obtained.

B. Myoglobin samples data from Minolta

The L*, a* and b* values at each time for all samples were recorded. The 3 replicate measurements at each time were later averaged to have single reading. Further analysis of L*, a* and b* values were done by Vinh.T.Phung and is explained in section 2.1.3.

C. Statistical Analysis

All the later statistical analysis is explained in 2.1.3.

4.2 Result and Discussion

4.2.1 Color change of M. Semimembranosus samples

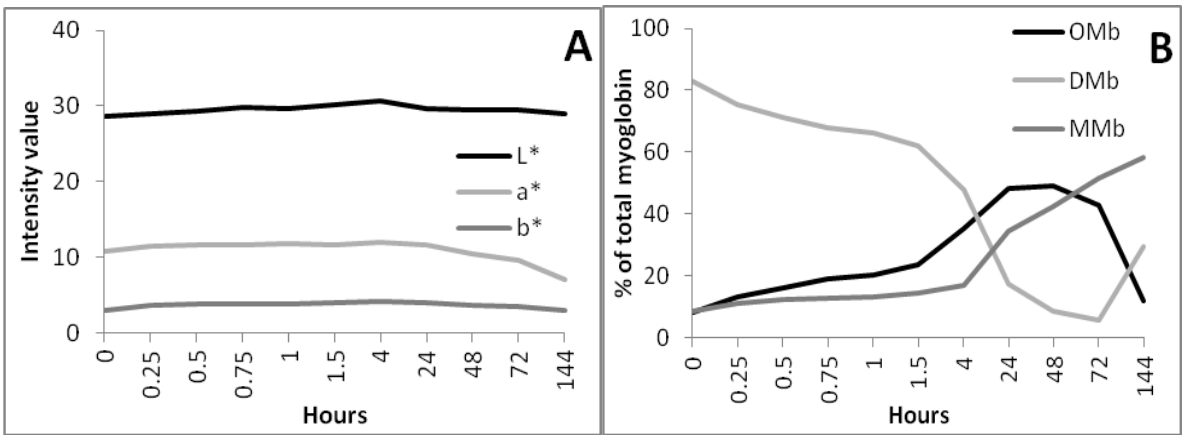

Figure 17: Average values showing (A) using Minolta to measure L*a*b* intensity and (B) myoglobin forms in % total myoglobin using NIR spectrophotometer (B). Samples were vacuum packaged and chill stored for three weeks. Measurements were made after exposure to air and continued until 144 hours.

The result presented in Figure 17 is data for color stability in 41 samples from Minolta and NIR measurements. Figure 17A are the change in L*, a* and b* values with time as samples were exposed to air. As shown in Figure 17A, a* value increased at first till 4 hours and decreased later. The b*value increased earlier and decreased later but L* values didn't changed much and were omitted from the further analysis.

More than 81% of the sample contained DMb at time 0 (0.5-2 min of vacuum package opening) and only 10% of OMb and MMb each were present in the sample. In Figure 17B, DMb was decreased (reduced) till first 1.5 hours and decreased rapidly beyond that. In Figure 17B, the sum of all myoglobin states at particular time is always 100%. As the vacuum package of the sample was opened, it rapidly gets oxygenated which is in agreement with Hunt et.al (1991) but remained stable for 48-72 hours. Later, the level of OMb decreased and as shown in Figure 17B; presence of more MMb in the sample can be observed. Eventually with the increase in time, more MMb are present in the sample. Figure 17B illustrates that, as the time passes most of the DMb transformed to OMb temporarily before getting oxidized to MMb and MMb level increased rapidly after 24h; which is in agreement to many findings (Hagler, Coppes et al. 1979; Livingston, McLachlan et al. 1985; Arihara, Cassens et al. 1995).

Compared to L*, a* and b* values, changes in myoglobin states could be well studied using NIR data. The result from 7th day is excluded from the analysis due to the excessive growth of bacteria.

4.2.2 The relationship between $OCR_{(i)}$ and color stability

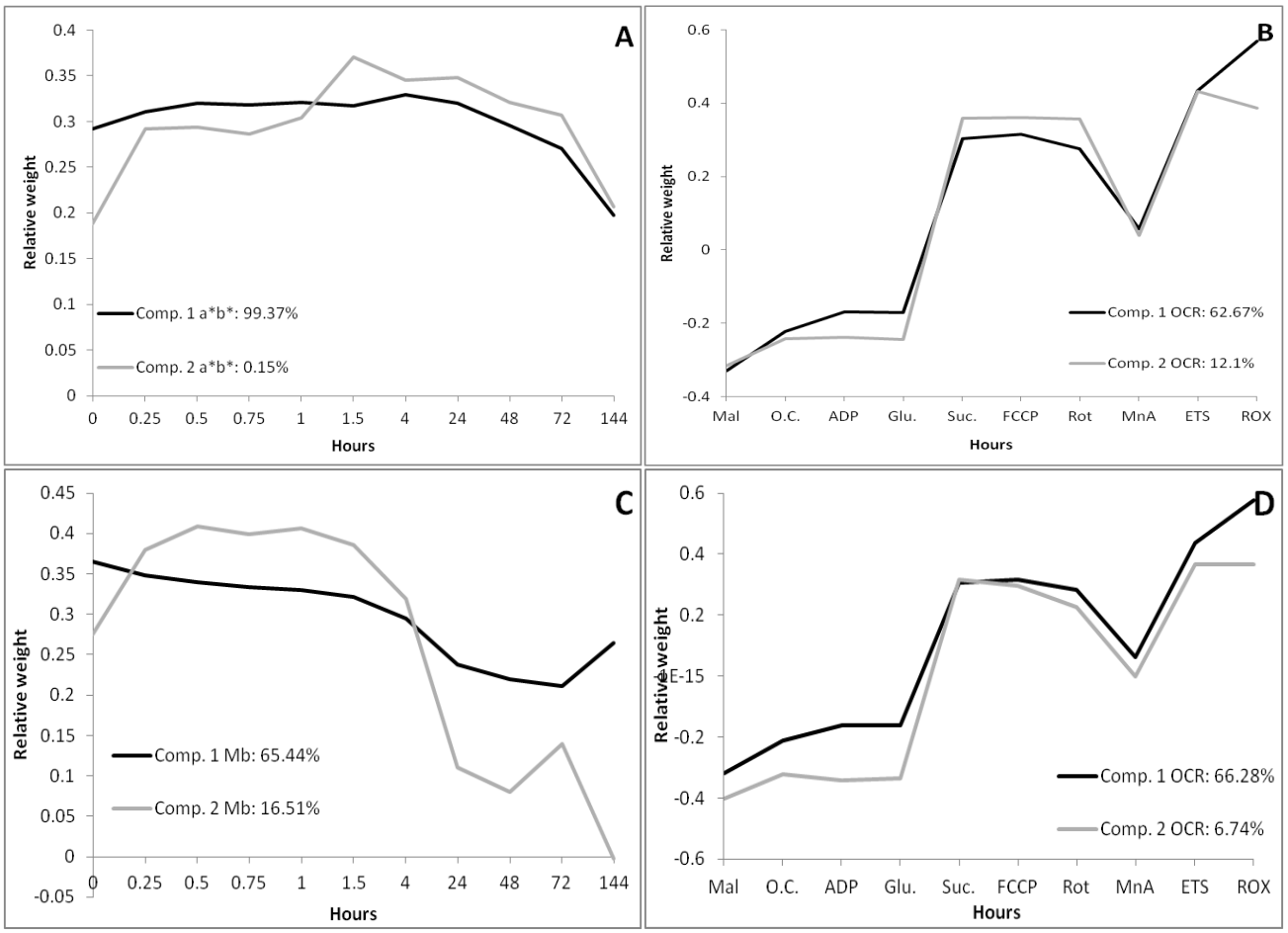

Figure 18: NPLS regression with loadings from two component NPLS regressions with response OCR (i). Panels A and B are based on light measurements (a*b*-time-animal), while panels C and D are based on % myoglobin (% myoglobin-time-animal). Component 1 is dominated by a* in panel A/B and DMb in panel C/D, while component 2 is dominated by b* in panel A/B and O/MMb in panel C/D. Oxygen consumption of *M. semimembranosus* after 3 weeks of storage was used.

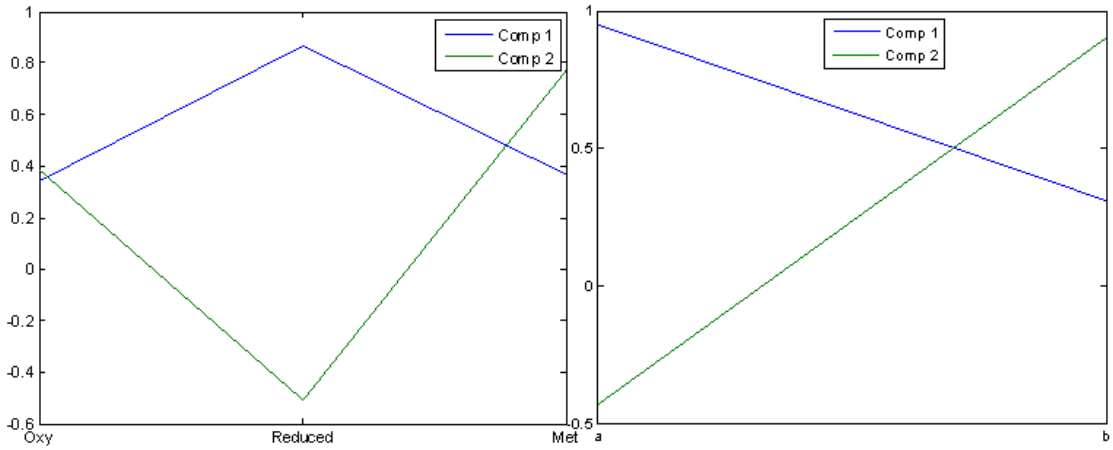

Figure 19: Right hand side is a* and b* values and left hand side is myoglobins.

Regression of OCR with Minolta variables (a*and b*) and with myoglobin states are shown in Figure. N-PLS component 1 was largely related to a* and component 2 to b* for Minolta measurements. Relative change in component 1 and 2 are shown in Figure 18A and the importance of different OCR protocol additives with change in component 1 and 2 are shown in Figure 18B. Component 1 explained 99.37% of variation in color of samples and component 2 explained 0.15%. Therefore component 1, which is largely related to a* (redness in meat) explained most of the variations (as shown in Figure 19B), redness was the dominating component that can describe variation in color with time. Similarly, 62.67% of variation in response to OCR protocol additives was explained by component 1 and 12.1% by component 2. Little changes in b* values were found after 1 day display in *M.semimembranosus* muscles by McKeena et al.(2005). Our result showed that b* values reached the peak at 1.5 hrs and decline slowly till 144h and a* values also decreased in similar way (Figure 18A). From Figure 18(A and B), it can be said that, change in color of the samples were explained by ETC system to remove oxygen present in mitochondria; because 99.5% variation in a* and b* was explained by 75% variation in OCR $_{(i).}$

In Figure Figure **19**B, N-PLS component 1 explained DMb and until 48h, component II explained OMb and after 48 h, it explained MMb in NIR measurements. Change in myoglobins with time is shown in Figure 18C and importance of different $OCR_{(i)}$ protocol additives with change in myoglobins are shown in Figure 18 D. Component 1

which predominantly related to DMb declined with time (Figure 18C). Component 1 explained 65.44% change in myoglobins with time and 66.28% of the variation OCR in inducible by protocol additives. Component 2 explained 16.51% of the change in myoglobins with time and 6.74% of the variation inducible in OCR by protocol additives. 82% of the change in myoglobin state of sample was explained by 75% of variation of OCR promoted by protocol additives.

4.2.3 Relationship between individual animal's descriptors (mainly pH) and color stability

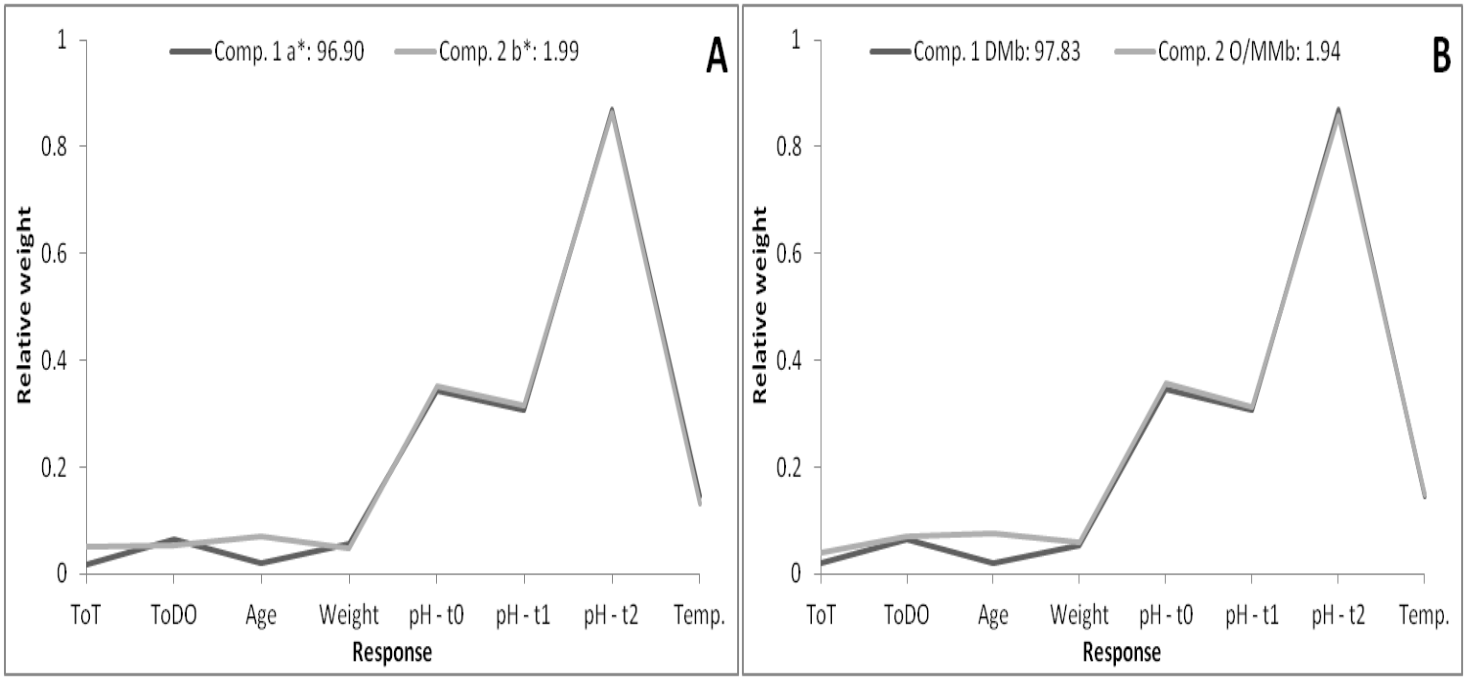

Figure 20: Response loadings from two component NPLS regressions with response animal descriptors. Panels A is based on Minolta measurements (a*b*-time-animal), while panels B is based on % myoglobin (% myoglobin-time-animal). Component 1 and 2 are dominated by a* and b* in panel A, respectively. Component 1 is DMb and contrasted to component 2 which is OMb/MMb in panel B. Loading weights are DMb 0.50, OMb -0.35 and MMb -0.80. Color stability/oxygen consumption of *M. semimembranosus* after 3 weeks of storage was used.

From Figure 20 (A and B), pH t0 and more especially pH t2 was most important for component for explaining a* and b* and myoglobin states. They are the postmortem pH measured on samples at different time interval. Compared to $OCR_{(i)}$ (section-4.2.2), component a* and DMb depended more on postmortem pH based on explained variance by these components. Previous studies has shown role of pH in color stability

and also in mitochondrial activity and protein denaturation (Joo, Kauffman et al. 1999).

4.2.4 Variation in $OCR_{(i)}$ of chill stored muscles of individual animals

Table 8: Effect of animal variation on the response to substrates of the ETS. Samples were allowed to mature for three weeks in a cooling room at 4°C.

Variable	O.C.	ADP	Glutamate	Succinate	FCCP	Rotenone	Mna	ETS	ROX
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.267	<0.001	<0.001
σ^2	0.23	0.31	0.24	2.89	2.19	3.31	0.01	2.50	0.12
σ^2_e	0.07	0.09	0.09	0.46	0.30	0.238	0.05	0.34	0.07
Min-Max (pool/sec/mg)	0 – 0.55	0 – 1.22	0 – 0.89	0.42 – 8.82	0.41 – 7.51	0.35 – 7.47	0 – 0.75	0.73 – 8.08	0 – 2.36

σ2: estimated variance due to animals; σ^2_e: Expected mean squares (EMS) error with random effect; O.C.= octanoylcarnitine; Mna= malonic acid; ETS= max obtained OCR activity; ROX= oxygen consuming side reactions:*Percentage reduction in absolute value.

Table 2 shows that $OCR_{(i)}$ was significantly different among individual animal's muscles. The measured $OCR_{(i)}$ of enzyme complexes in mitochondria varied for all additives of the protocol. The oxidation of succinate by succinate dehydrogenase (complex II) was the strongest and the most stable enzyme complex in beef mitochondria with aging (Table 8). In stored meat, complex I and β-oxidation were largely inactivated (as shown in Table 8, respiration activity of octanoyl carnithine is low) so when complex II was inhibited by malonic acid, complete inhibition of the ETS took place. The succinate respiration in many cases represented the maximum OCR capacity of the ETS in postmortem meat when ETS was uncoupled by FCCP.

4.3 Conclusion

Enzyme activities of e.g. complex I and beta oxidation are affected by the early muscle-to-meat transition after slaughter. Our results show that these processes are not involved in determining color stability as the activity of complex I was 61% reduced over 3 weeks chill storage (personal communication: Vinh T Phung). At this time complex II activity related dominantly to color stability as shown by measuring L*a*b* and myoglobin states. Ultimate pH had a large effect on color stability but was not a significant factor for $OCR_{(i)}$.

5 Future perspective

There are still many works that could be done further. Due to the lack of time, we were not able to do proteomic study of mitochondria isolated from the collected samples. Hence, it could be very interesting to see the relationship between change in different enzymes and slaughter variables. Looking at the fiber typing of the samples could be interesting as well as samples are also collected for this process. Drips are also collected for all the samples, so some studies can also be done based on this. Since we found some relationship between tenderness of meat and mitochondria's oxygen consumption rate, so more research could be done based upon this fact to find the exact connection between them. This could help in deeper understanding about the different aspects in meat and help meat industry.

6 References

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7 Appendix I

Mitochondria Isolation

Chemicals: Isolation buffer(phosphate buffer)
K_2HPO_4 5mM/KH_2PO_4 2mM, sucrose 0.25M, EDTA 10mM – adjust to pH 7
Equipments
Scissors, forceps, one 500mL Erlenmeyer flask, pipette and pipette tips of 5ml, 1ml, 100-1000µl, 20 Eppendorf tubes. Homogenisator 3ml(small) **Chemicals:** 10mM EDTA, 1x PBS, **Trypsin**: Make 0.05% trypsin in 10mL of ice-cold PBS containing 10mM EDTA. Or 5mg of trypsin dissolved in 10mL of cold PBS with 29.2g EDTA. Extraction buffer-B (5 x)- 100mM MOPS, pH 7.5, containing 550mM KCl and 5mM EGTA

1. Take fresh meat and isolate muscle (remove collagens and connective tissues)
2. Weigh out 10g from it.
3. Wash twice with 10ml Phosphate buffer (PBS) containing EDTA.
4. Minch the muscle sample well.
5. Incubate the muscle sample in 10ml PBS containing EDTA and 0.05% trypsin for 30 minutes in ice.
6. Add albumin to the solution to make it 10mg/ml and mix it properly and leave for few (5 min) minutes.
7. Remove the solution and Wash the muscle with 10ml PBS EDTA one time.
8. Now, add 5ml extraction buffer-B (1 x) to 5g muscle sample i.e. 10ml extraction buffer-B to 10g muscle.
9. Homogenize 5g muscle sample each in 3ml volume homogenizer(reach only once to the bottom).Note- Do not make the homogenizer tube hot, because it will form more gels and interrupt the mitochondria extraction .
10. Filter the mixture of muscle with extraction buffer B (1 x) from the white cloth for times.
11. Transfer the filtrate to Eppendrof tubes (800µl in each tube).
12. Centrifuge at 600Xg for 5 minutes and keep the supernatant.
13. Centrifuge the supernatant at 11,000Xg for 10 minutes.

14. Aspirate and keep the pellet.
15. Store the mitochondria pellet in liquid nitrogen.

8 Appendix II

Slaughter Variables for 41 animals

Animal number	ToD	ToC	ToDO	Age	Gender	Weight	Fat	Class	Animal category
1	13.35	15.30	3.09	16	0	324	7	4	1
1	13.35	15.30	3.09	16	0	324	7	4	1
2	0.42	1.55	3.58	21	0	234	6	9	1
2	0.42	1.55	3.58	21	0	234	6	9	1
3	14.22	15.27	3.30	80	1	292.8	8	6	6
3	14.22	15.27	3.30	80	1	292.8	8	6	6
4	24.23	25.23	3.47	25	0	373.8	9	6	1
4	24.23	25.23	3.47	25	0	373.8	9	6	1
5	15.33	16.34	3.47	70	1	324.8	11	6	6
5	15.33	16.34	3.47	70	1	324.8	11	6	6
6	14.09	15.32	4.40	15	0	317.3	9	6	1
6	14.09	15.32	4.40	15	0	317.3	9	6	1
7	23.44	25.03	4.10	18	0	240.1	7	6	1
7	23.44	25.03	4.10	18	0	240.1	7	6	1
8	20.21	21.21	3.32	18	0	295.4	4	6	1
8	20.21	21.21	3.32	18	0	295.4	4	6	1
9	16.54	18.45	4.30	18	0	350.1	5	2	1
9	16.54	18.45	4.30	18	0	350.1	5	2	1
10	23.23	24.21	3.30	17	0	280.7	7	4	1
10	23.23	24.21	3.30	17	0	280.7	7	4	1
11	18.05	20.03	4.37	83	1	222.9	4	9	6
11	18.05	20.03	4.37	83	1	222.9	4	9	6
12	2.34	4.00	4.46	16	0	300.7	9	6	1
12	2.34	4.00	4.46	16	0	300.7	9	6	1
13	16.42	17.56	3.32	67	1	242.5	9	9	6
13	16.42	17.56	3.32	67	1	242.5	9	9	6
14	19.23	20.37	5.15	17	0	282.8	5	5	1
14	19.23	20.37	5.15	17	0	282.8	5	5	1
15	22.8	23.19	4.18	15	0	287.3	5	6	1
15	22.8	23.19	4.18	15	0	287.3	5	6	1
16	20.49	22.90	3.31	19	0	308.7	5	4	1
16	20.49	22.90	3.31	19	0	308.7	5	4	1
17	17.25	18.41	3.32	27	0	355.3	9	6	1
17	17.25	18.41	3.32	27	0	355.3	9	6	1
18	21.41	22.39	3.00	30	0	275.4	5	7	3
18	21.41	22.39	3.00	30	0	275.4	5	7	3
19	13.48	15.10	3.12	76	1	351	12	4	6
19	13.48	15.10	3.12	76	1	351	12	4	6
20	11.56	13.45	4.42	16	0	337.7	5	5	1
20	11.56	13.45	4.42	16	0	337.7	5	5	1

21	13.26	14.47	5.04	31	1	269.3	5	6	4
21	13.26	14.47	5.04	31	1	269.3	5	6	4
22	18.51	20.13	4.32	39	1	298.1	11	7	4
22	18.51	20.13	4.32	39	1	298.1	11	7	4
23	20.24	21.36	5.57	18	0	249.9	5	6	1
23	20.24	21.36	5.57	18	0	249.9	5	6	1
24	17.01	18.01	3.16	36	1	211.7	6	9	6
24	17.01	18.01	3.16	36	1	211.7	6	9	6
25	18.08	20.02	4.57	42	1	258.1	10	7	4
25	18.08	20.02	4.57	42	1	258.1	10	7	4
26	20.31	22.06	5.36	116	1	363	5	3	6
26	20.31	22.06	5.36	116	1	363	5	3	6
27	14.36	15.53	4.24	51	1	297.1	9	7	6
27	14.36	15.53	4.24	51	1	297.1	9	7	6
28	16.2	17.31	4.18	24	1	176.2	4	8	4
28	16.2	17.31	4.18	24	1	176.2	4	8	4
29	17.38	18.57	5.21	40	1	177.6	2	9	4
29	17.38	18.57	5.21	40	1	177.6	2	9	4
30	2.08	3.18	4.35	25	0	294	4	6	2
30	2.08	3.18	4.35	25	0	294	4	6	2
31	18.27	19.57	4.40	17	0	333.8	7	6	1
31	18.27	19.57	4.40	17	0	333.8	7	6	1
32	20.39	22.19	6.12	46	0	484.5	2	1	2
32	20.39	22.19	6.12	46	0	484.5	2	1	2
33	18.09	19.20	3.02	14	0	232.1	4	7	1
33	18.09	19.20	3.02	14	0	232.1	4	7	1
34	17.5	19.23	4.21	28	1	277.7	7	6	5
34	17.5	19.23	4.21	28	1	277.7	7	6	5
35	19.18	20.33	5.14	32	1	207.8	2	8	4
35	19.18	20.33	5.14	32	1	207.8	2	8	4
36	21.55	23.01	3.03	44	0	516.3	9	3	2
36	21.55	23.01	3.03	44	0	516.3	9	3	2
37	17.26	18.41	4.59	29	1	187.4	2	9	4
37	17.26	18.41	4.59	29	1	187.4	2	9	4
38	11	12.36	6.39	28	1	294	12	6	5
38	11	12.36	6.39	28	1	294	12	6	5
39	0.5	1.50	4.46	14	0	136.4	2	7	3
39	0.5	1.50	4.46	14	0	136.4	2	7	3
40	15.49	17.49	4.45	45	1	227.8	2	10	4
40	15.49	17.49	4.45	45	1	227.8	2	10	4
41	15.57	17.57	6.26	29	1	201.5	6	8	5
41	15.57	17.57	6.26	29	1	201.5	6	8	5

animal number	pH - t0	pH - t1	pH - t2	Temp surface S.M
1	5.92	5.82	5.44	40.50
1	5.92	5.82	5.44	40.50
2	5.89	5.81	5.56	37.00
2	5.89	5.81	5.56	37.00
3	6.34	6.28	5.56	36.50
3	6.34	6.28	5.56	36.50
4	6.28	6.16	5.66	36.50
4	6.28	6.16	5.66	36.50
5	6.42	6.37	5.78	34.40
5	6.42	6.37	5.78	34.40
6	6.03	5.53	5.45	32.50
6	6.03	5.53	5.45	32.50
7	6.38	6.19	5.54	31.70
7	6.38	6.19	5.54	31.70
8	6.53	6.42	5.60	29.80
8	6.53	6.42	5.60	29.80
9	6.39	6.13	5.67	29.10
9	6.39	6.13	5.67	29.10
10	5.98	5.90	5.48	31.20
10	5.98	5.90	5.48	31.20
11	6.24	6.03	5.79	28.20
11	6.24	6.03	5.79	28.20
12	6.16	5.96	5.47	31.20
12	6.16	5.96	5.47	31.20
13	6.20	6.15	5.51	33.50
13	6.20	6.15	5.51	33.50
14	6.64	5.67	5.51	32.80
14	6.64	5.67	5.51	32.80
15	6.49	6.33	5.43	32.50
15	6.49	6.33	5.43	32.50
16	6.58	6.48	5.53	34.70
16	6.58	6.48	5.53	34.70
17	6.33	6.14	5.56	32.90
17	6.33	6.14	5.56	32.90
18	6.40	5.91	5.60	32.60
18	6.40	5.91	5.60	32.60
19	6.14	5.90	5.53	33.60
19	6.14	5.90	5.53	33.60
20	5.82	5.62	5.52	30.20
20	5.82	5.62	5.52	30.20
21	6.74	6.66	5.60	33.30
21	6.74	6.66	5.60	33.30
22	6.44	6.16	5.69	30.50

22	6.44	6.16	5.69	30.50
23	6.15	5.73	5.41	31.90
23	6.15	5.73	5.41	31.90
24	6.35	5.88	5.50	29.60
24	6.35	5.88	5.50	29.60
25	6.33	6.15	5.55	29.60
25	6.33	6.15	5.55	29.60
26	6.15	5.83	5.45	33.10
26	6.15	5.83	5.45	33.10
27	6.81	6.59	5.43	32.90
27	6.81	6.59	5.43	32.90
28	6.84	6.47	5.55	33.20
28	6.84	6.47	5.55	33.20
29	6.59	5.98	5.44	24.80
29	6.59	5.98	5.44	24.80
30	6.08	5.83	5.47	28.20
30	6.08	5.83	5.47	28.20
31	6.27	6.16	5.57	29.60
31	6.27	6.16	5.57	29.60
32	6.58	6.27	5.56	33.10
32	6.58	6.27	5.56	33.10
33	6.52	6.44	5.52	29.00
33	6.52	6.44	5.52	29.00
34	6.47	6.05	5.64	27.40
34	6.47	6.05	5.64	27.40
35	6.48	6.18	5.55	28.90
35	6.48	6.18	5.55	28.90
36	6.36	6.08	5.55	29.40
36	6.36	6.08	5.55	29.40
37	6.50	6.36	5.60	29.80
37	6.50	6.36	5.60	29.80
38	6.65	6.28	5.68	31.20
38	6.65	6.28	5.68	31.20
39	6.40	5.74	5.45	27.90
39	6.40	5.74	5.45	27.90
40	6.77	6.36	5.54	26.80
40	6.77	6.36	5.54	26.80
41	6.91	6.59	5.63	28.70
41	6.91	6.59	5.63	28.70

9 Appendix III

L* a* and b* values for 41 sample (numbered from 11-51).

L*

sample	Replicate	Time(hrs)											
		0	0.25	0.5	0.75	1	1.5	4	24	48	72	142	167
1	1	30.08	30.49	28.2	28.85	28.2	33.35	30.09	31.47	30.46	29.87	29.67	32.24
1	2	30.49	30.45	28.42	29.74	30.41	30.84	28.72	28.09	31.02	31.97	29.98	32.34
1	3	30.49	28.09	28.87	32.28	28.85	30.84	30.01	30.57	30.24	27.43	31.27	31.78
2	1	30.45	31.77	28.08	32.54	30.56	33.1	30.65	32.20	31.53	29.32	30.87	32.60
2	2	30.55	31.01	27.86	30.78	32.27	29.33	33.91	30.92	32.20	28.65	32.26	31.48
2	3	28.11	30.46	27.68	35.93	30.56	30.86	33.91	30.92	29.88	30.83	28.22	30.39
3	1	22.99	27.92	29.02	29.2	29.45	28.36	31.36	27.86	26.31	24.82	26.76	26.75
3	2	29.93	30.07	28.24	30.19	26.17	28.33	26.46	26.47	26.67	26.37	25.69	26.46
3	3	27.02	27.99	28.24	30.19	26.17	27.53	29.02	24.61	23.98	27.73	28.37	28.38
4	1	28.99	28.49	26.92	27.88	26.81	35.14	31.08	28.34	30.26	30.49	27.55	30.15
4	2	27.92	25.06	25.08	29.93	28.8	30.06	30.64	28.66	30.10	29.59	27.61	27.27
4	3	28.99	29.66	26.92	29.93	26.81	27.87	31.08	28.06	28.43	30.25	27.66	28.09
5	1	26.91	25.49	24.94	25.13	27.8	28.05	24.90	28.84	26.04	26.97	27.01	28.51
5	2	28.82	25.07	24.97	25.92	26.36	28.21	27.14	27.64	29.82	26.55	25.06	27.36
5	3	29.36	30.47	23.38	28.42	28.03	29.56	30.46	25.99	26.57	22.38	29.97	26.42
6	1	31.85	31.7	34.23	34.27	33.74	35.65	33.63	33.01	31.62	30.00	35.03	30.56
6	2	32.82	34.51	35.11	36.82	31.44	36.16	33.39	32.15	33.09	33.89	35.04	28.69
6	3	33.1	32.07	34.48	32.48	32.95	35.15	35.12	33.47	32.67	31.81	35.24	30.15
7	1	28.77	30.86	29.11	30.85	30.63	31.9	26.45	27.18	27.98	26.73	27.70	28.14
7	2	26.79	30.15	31.78	31.07	31.67	30.35	27.31	26.76	28.11	25.56	28.00	28.48
7	3	28.85	28.51	28.82	32.46	28.88	29.99	26.52	27.71	27.06	28.47	27.04	28.41
8	1	31.72	29.52	28.1	28.16	28.61	32.86	28.28	29.03	29.81	29.25	31.11	28.70
8	2	26.82	28.35	26.46	29.9	28.23	32.87	30.89	28.50	29.62	29.33	27.05	28.19
8	3	28.43	27.85	27.1	32.08	31.99	29.36	27.31	28.46	27.60	27.46	27.19	25.78
9	1	30.48	27.85	31.58	29.59	31.27	31.34	32.35	28.76	28.39	29.90	28.69	28.03
9	2	28.97	28.63	31.88	30.98	31.45	33.84	30.90	27.07	29.62	30.05	31.05	28.07
9	3	31.76	28.55	32.32	31.77	31.88	31.24	30.63	29.81	29.84	29.84	26.19	28.30
10	1	30.82	32.23	33.43	32.41	32.71	33.17	32.23	30.89	33.65	31.45	30.18	28.15
10	2	30.06	30.94	33.1	33.46	30.39	29.85	32.73	29.54	31.11	29.62	30.50	26.82
10	3	31.15	30.19	32.34	32.91	31.34	30.23	31.70	31.15	31.49	29.01	30.87	26.85
11	1	27	29.5	29.91	30.61	26.36	28.83	27.68	30.63	29.78	29.15	30.25	27.41
11	2	26.91	28.56	26.86	26.42	27.38	27.7	27.79	27.79	28.89	27.13	28.23	27.09
11	3	25.56	26.31	26.07	30.4	28.62	27.45	26.61	28.41	27.37	26.74	27.84	28.36
12	1	28.61	32.86	33.33	33.2	34.4	34.05	34.08	32.28	32.61	33.43	31.67	30.69
12	2	32.02	30.47	32.36	31.16	33.35	34.94	32.63	33.43	30.53	31.87	30.11	29.71
12	3	33.12	33.61	28.44	33.05	32.15	33.76	33.33	33.36	31.40	31.34	29.58	31.46
13	1	29.2	27.21	29.03	30.87	31.31	30.72	32.46	31.03	28.90	26.85	27.68	27.41
13	2	28.49	27.14	26.45	28.62	29.87	29.45	30.77	29.02	26.12	25.64	27.89	27.12
13	3	26.47	27.95	27.22	28.54	31.11	30.42	30.74	27.96	27.88	28.46	27.87	26.11

14	1	28.2	29.68	32.07	31.81	31.43	31.43	33.09	32.97	28.56	31.64	30.69	30.50
14	2	30.09	27.85	29.23	28.64	32.13	28.15	29.27	29.38	31.60	29.32	30.00	30.47
14	3	28.64	33.18	31.09	30.77	30.5	31.73	31.78	31.06	31.69	30.05	29.11	29.70
15	1	27.58	29.91	31.93	28.96	33.87	29.42	33.47	31.33	32.18	26.83	30.86	30.29
15	2	26.61	30.77	35.47	32.17	27.9	27.04	27.86	29.26	28.47	31.60	28.95	28.27
15	3	29.68	27.77	27.76	27.43	27.12	28.36	33.14	29.55	25.69	30.98	30.77	26.68
16	1	28.3	30.38	28.14	28.18	28.02	29.63	31.73	29.58	32.54	30.95	27.96	28.33
16	2	28.25	27.76	29.04	28.32	27.89	27.57	28.85	28.64	31.33	29.74	30.67	28.47
16	3	28.58	28.52	28.24	29.09	28.49	27.64	27.61	27.55	31.33	29.74	30.02	28.02
17	1	26.38	27.95	31.66	27.3	27.92	29.77	31.80	26.01	27.68	27.03	27.06	29.47
17	2	26.54	26.51	26.8	28.42	27.97	28.18	31.33	28.67	28.15	27.50	27.66	27.61
17	3	27.12	26.37	29.72	27.48	27.37	31.43	27.45	29.44	29.62	28.97	28.51	28.65
18	1	27.92	28.22	28.15	29.5	30.77	28.05	27.44	28.87	28.43	28.81	27.37	28.31
18	2	29.23	27.11	28.34	29.52	29.62	30.01	31.59	29.31	29.37	29.75	27.80	28.46
18	3	29.28	29.58	30.1	28.78	29.66	29.4	29.61	28.86	27.21	27.59	30.63	29.81
19	1	25.28	28.24	31.25	29.4	31.61	32.29	31.72	28.65	28.15	27.64	28.90	27.01
19	2	26.75	27.14	24.66	28.01	26.99	29	26.14	29.17	28.67	28.16	28.74	26.51
19	3	25.16	28.61	27.07	28.36	26.29	27.67	26.57	28.16	27.66	27.15	24.69	26.32
20	1	27.2	26.27	27.95	26.45	27.54	27.43	30.56	27.73	29.66	31.58	26.40	27.74
20	2	30.22	26.27	26.62	26.86	27.58	27.51	28.84	27.61	29.54	31.46	26.60	28.53
20	3	29.53	26.18	28.08	26.54	27.68	27.83	28.02	28.87	30.80	32.72	26.67	29.12
21	1	27.99	31.78	31.49	30.13	32.64	28.81	34.03	32.54	32.53	31.50	33.17	30.51
21	2	32.49	26.77	34.58	33.32	28.89	29.38	29.95	28.46	30.87	31.75	29.61	27.51
21	3	29.78	27.55	32.9	31.18	30.33	28.28	32.70	31.21	29.48	32.46	34.14	26.21
22	1	28.46	27.85	27.23	24.88	26.92	25.89	31.84	29.49	27.15	28.65	25.56	26.90
22	2	27	26.37	26.71	25.13	25.77	25.92	28.92	26.57	27.37	26.26	25.68	28.41
22	3	28.8	27.97	28.53	27.07	25.83	26.08	30.32	27.97	26.03	27.27	27.12	27.27
23	1	30.66	31.55	28.06	31.02	31.03	30.8	32.23	29.77	29.36	29.36	28.94	31.89
23	2	29.71	31.99	28.94	31.75	32.14	32.95	33.55	31.09	31.29	30.02	29.43	29.83
23	3	30.3	28.85	28.68	28.93	27.64	29.96	32.75	30.29	29.42	29.73	28.72	31.88
24	1	26.54	29.72	33.23	33.19	31.95	32.02	32.12	29.47	30.25	30.23	29.63	29.05
24	2	27.91	33.2	28.9	31.74	32.95	29.68	33.84	32.79	29.76	29.74	30.19	28.50
24	3	29.12	28.9	31.8	29.5	30.28	30.65	29.72	30.53	30.46	32.33	28.34	31.36
25	1	26.67	28.16	29.91	30.88	33.01	32.74	37.12	30.76	32.11	31.75	31.96	25.35
25	2	25.89	30.35	30.13	31.23	29.68	34.71	31.27	30.54	31.81	31.45	32.11	29.54
25	3	28.33	33.36	28.62	28.81	30.62	31.03	35.62	29.58	29.85	29.49	33.95	35.11
26	1	28.93	29.21	28.65	28.99	28.49	28.75	30.74	29.67	29.92	30.43	28.42	27.42
26	2	27.52	27.44	27.64	27.95	27.2	28.81	32.16	27.30	28.90	29.41	25.35	27.28
26	3	28.98	28.19	28.26	27.4	30.13	27.94	30.23	30.64	28.72	29.23	28.15	28.49
27	1	25.35	25.77	26.73	31.27	27.66	28.28	26.34	26.38	27.06	27.74	27.26	25.33
27	2	25.35	29.38	24.71	25.21	30.23	27.07	28.47	27.12	27.80	28.48	25.60	25.11
27	3	27.83	27.04	27.36	24.84	27.67	29.71	28.47	27.06	27.74	28.42	25.78	25.03
28	1	31.78	32.41	32.58	32.1	32.61	32.41	31.04	30.28	29.95	29.63	28.54	27.54
28	2	29.66	30.83	31.67	31.8	29.07	30.81	29.87	31.43	31.10	30.78	29.57	28.44
28	3	30.31	30.14	30.12	31.27	34.48	30.08	31.93	32.08	31.75	31.43	26.19	29.01
29	1	30.51	29.85	32.6	30.06	32.77	33.11	32.85	31.23	31.94	32.64	31.67	29.91

29	2	30.96	28.98	30.06	30.32	29.05	31.17	28.40	28.88	29.59	30.29	29.68	31.33
29	3	27.65	31.89	29.58	31.02	30.37	30	32.85	29.39	30.10	30.80	31.73	31.69
30	1	27.02	28	30.99	29.14	29.51	31.84	31.64	30.36	29.66	28.97	29.19	29.17
30	2	29.98	27.82	29.7	32.31	31.21	29.34	32.81	31.16	30.46	29.77	29.55	29.36
30	3	30.75	27.81	28.66	30.06	28.8	33.55	31.97	30.98	30.28	29.59	28.12	29.56
31	1	27.6	30.11	28.98	28.62	28.5	28.87	30.49	30.74	28.91	29.05	29.34	28.74
31	2	30.31	28.89	28.5	32.12	29.45	28.9	28.62	29.59	29.17	29.31	28.39	28.48
31	3	29.73	30.44	28.97	30	29.35	28.96	30.62	30.40	29.45	29.59	31.45	29.11
32	1	26.61	25.87	29.36	24.81	29.43	26.17	34.55	31.80	29.37	29.74	26.94	28.34
32	2	26.49	26.43	29.49	29.13	30.36	26.72	33.34	27.04	26.46	26.83	28.47	27.24
32	3	28.58	29.92	30.11	28.14	26.31	26.46	32.72	25.41	25.87	26.24	26.24	28.69
33	1	32.77	32.12	32.19	30.91	29.57	32.15	32.78	31.40	31.54	33.21	30.98	32.70
33	2	31.91	32.39	33.27	32.91	28.77	33.47	35.48	34.10	32.66	32.45	31.83	31.64
33	3	32.79	30.36	32.9	34.4	30.58	31.3	33.80	32.42	32.29	32.38	31.96	31.34
34	1	28.09	29.82	28.67	31.41	30.4	31.72	32.38	32.05	28.09	29.80	28.53	28.88
34	2	27.19	28.09	31.59	32.42	28.78	29.47	33.80	33.47	29.85	29.09	29.90	29.51
34	3	26.89	30.33	30.23	27.53	30.37	31.06	29.19	28.86	29.31	29.28	28.79	29.00
35	1	29.59	28.6	30.46	31.84	32.13	31.89	34.73	33.62	33.11	31.47	29.20	29.69
35	2	27.89	26.83	28.89	30.17	30.59	30.85	32.64	31.53	31.94	32.42	29.45	28.07
35	3	26.77	27.73	29.78	30.36	31.66	30.11	29.82	28.71	30.85	30.57	29.51	29.16
36	1	29.42	30.19	29.45	28.86	31.06	31.13	31.66	30.80	31.21	30.03	29.05	27.58
36	2	26.61	30.62	31	32.2	31.63	34.45	33.15	32.29	31.12	30.88	24.53	23.06
36	3	29.17	30.87	29.83	28.23	28.44	30.66	28.88	28.02	28.04	27.01	30.25	28.78
37	1	29.1	29.23	28.75	28.55	27.69	29.52	29.34	28.89	28.82	27.83	28.13	28.48
37	2	27.76	28.16	27.87	27	28.51	29.19	29.29	28.84	28.03	28.35	27.48	27.83
37	3	27.43	28.96	28.56	27.96	29.05	29.02	29.20	28.75	28.63	27.44	28.53	28.88
38	1	26.34	26.04	27.01	26.93	27.7	30.12	29.17	28.17	30.03	31.42	30.57	28.44
38	2	28.24	30.7	29.65	31.22	29.33	32.83	30.62	29.62	30.82	30.65	32.36	30.23
38	3	26.94	26.64	28.83	27.94	30.82	27.8	32.69	31.69	27.56	28.87	31.83	29.70
39	1	29.1	28.84	30.32	33.38	30.94	34.61	30.44	30.27	31.33	29.43	27.27	24.56
39	2	31.23	32.54	33.6	28.53	29.76	28.47	30.02	29.69	30.75	33.89	26.29	29.76
39	3	28.18	28.3	27.53	32.06	29.75	30.63	31.53	28.01	29.07	30.17	30.53	27.30
40	1	26.89	27.32	28.48	28.3	28.3	30.09	30.98	29.27	28.64	26.43	28.22	27.98
40	2	26.7	26.83	27.64	25.38	28.4	26.37	28.78	26.18	25.55	28.73	27.58	27.42
40	3	26.16	28.04	26.36	27.79	27.06	28.9	29.99	30.69	30.06	27.44	28.65	28.98
41	1	25.54	27.55	26.71	25.74	28.02	25.93	28.34	27.49	27.38	27.45	27.12	26.85
41	2	25.59	26.12	26.22	25.76	26.76	25.47	25.66	27.43	27.32	27.24	27.08	27.89
41	3	25.66	26.57	26.94	26.81	29.3	26.73	27.93	27.62	27.51	27.24	27.76	27.82

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Sample	replicate	Time(hrs)											
		0	0.25	0.5	0.75	1	1.5	4.00	24.00	48.00	72.00	142.00	167.00
1	1	12.41	11.93	15.51	15.42	15.84	12.36	15.02	11.94	12.02	11.89	8.55	4.91
1	2	11.93	12.44	15.03	13.84	12.96	14.09	15.18	12.68	12.05	11.44	8.45	4.99
1	3	11.93	13.93	14.39	14.14	14.78	14.09	15.05	13.89	13.23	11.43	7.48	5.57
2	1	16.24	11.51	14.1	10.39	11.53	9.99	13.34	12.52	11.23	11.33	7.24	7.51

2	2	11.26	10.49	13.7	10.97	10.64	12.96	11.67	13.28	11.67	11.71	6.77	7.27
2	3	11.05	10.25	13.83	9.06	11.53	12.05	11.67	13.28	11.23	10.69	8.76	8.66
3	1	10.52	9.41	8.49	8.23	9.35	8.56	8.24	9.11	8.69	9.27	5.21	6.13
3	2	9.52	8.4	9.96	8.23	9.19	8.35	9.52	9.36	8.89	8.63	5.43	6.15
3	3	8.67	9.35	9.96	8.23	9.19	9.14	8.25	10.85	10.35	8.09	5.55	5.84
4	1	10.4	10.59	12.63	11.74	11.44	8.06	11.79	10.51	8.56	7.98	5.49	4.29
4	2	9.41	12.28	11.78	10.19	11.6	12.04	11.65	10.16	8.61	9.84	6.03	5.10
4	3	10.4	10.68	12.63	10.19	11.44	12.02	11.79	11.03	10.06	7.75	5.06	6.96
5	1	7.34	8.54	8.93	8.97	8.55	9.61	10.56	9.62	8.77	7.26	6.84	6.72
5	2	7.95	8.38	8.65	8.19	8.99	8.2	10.73	9.37	8.17	8.20	6.68	7.32
5	3	6.57	8.21	9.88	8.74	9	8.67	9.48	10.76	8.42	9.54	5.44	6.52
6	1	14.47	15.25	12.91	13.01	14.32	13.86	15.36	14.24	13.40	14.46	9.18	7.97
6	2	13.61	14.16	13.35	13.32	16.5	13.54	15.68	15.19	14.14	11.63	9.23	8.74
6	3	13.07	14.88	14.09	15.16	15.52	14.32	14.67	13.74	13.09	12.85	9.24	7.79
7	1	10.99	10.04	10.94	10.16	11.23	10.09	11.06	12.69	10.50	10.32	7.10	8.15
7	2	11.47	11.14	10.2	11.8	10.97	11.71	10.77	12.37	10.30	10.37	6.97	8.23
7	3	10.66	11.66	12.31	10.53	11.78	11.2	10.81	12.43	10.97	9.03	7.24	8.33
8	1	9.41	11.18	12.1	11.88	12.14	9.98	13.11	12.84	11.17	9.96	6.69	7.57
8	2	11.58	11.97	13.12	11.3	11.92	10.83	12.38	13.16	10.95	10.48	7.79	7.32
8	3	11.58	11.96	12.41	10.93	10.82	11.9	14.67	12.91	13.24	8.62	7.44	8.35
9	1	12.82	14.56	13.54	14.78	13.46	13.27	15.09	16.46	15.05	13.14	7.70	9.63
9	2	13.96	14.78	13.9	13.61	14.27	13.13	15.11	16.71	15.33	12.79	7.30	9.82
9	3	11.84	12.47	13.14	14.23	13.98	13.59	15.11	15.89	15.14	11.51	8.50	8.80
10	1	14.25	14.28	14.2	15.65	15.74	14.77	16.44	17.43	13.57	14.63	10.78	10.90
10	2	14.84	15.94	15.21	15.49	18.71	18.15	16.29	18.64	15.85	16.28	10.90	11.19
10	3	13.65	17	15.29	15.84	16.07	17.88	17.98	17.45	15.93	15.24	10.76	11.21
11	1	10.75	10.51	10.48	10.13	12.89	11.85	13.12	9.64	9.15	8.41	6.95	8.24
11	2	11.42	11.34	12.29	13.11	12.57	12.64	13.21	10.87	9.34	9.44	7.98	8.41
11	3	12.04	12.68	12.87	10.91	12.07	12.76	13.45	10.81	11.20	9.48	8.02	7.51
12	1	11.69	10.37	10.61	10.57	10.24	10.99	11.36	11.30	9.91	8.69	7.06	7.60
12	2	9.95	12.09	11.13	11.91	11.14	10.1	12.39	10.20	10.13	8.94	7.31	7.84
12	3	9.77	10.21	13.63	11.22	12	11.13	11.58	11.17	9.94	9.44	7.81	7.10
13	1	7.87	9.57	9.02	8.45	8.47	8.87	8.72	8.65	8.48	9.09	5.47	6.22
13	2	8.1	9.78	10.57	9.7	9.56	9.51	9.62	9.39	9.73	9.75	5.51	6.47
13	3	9.12	9.33	10.29	9.6	8.83	8.88	9.69	9.63	9.34	8.84	5.39	6.63
14	1	12.9	11.47	11.08	11.25	11.33	11.24	10.84	9.53	11.09	9.29	8.67	9.00
14	2	10.89	13.77	13.38	13.92	11.22	13.97	14.41	11.73	9.46	10.84	9.01	8.59
14	3	12.57	9.91	11.82	12.08	12.18	11.58	11.66	10.56	9.87	9.72	8.94	8.66
15	1	11.62	10.75	10.46	11.66	9.58	12.04	10.05	11.00	9.91	12.37	6.83	8.00
15	2	11.99	10.7	9.28	10.89	12.66	13.27	13.63	12.01	11.85	10.49	7.08	8.99
15	3	11.47	12.43	12.69	12.84	13.25	12.23	10.99	12.14	13.37	11.60	6.98	9.59
16	1	13.56	12.77	15.14	14.98	14.87	14.1	13.16	12.86	12.20	11.77	9.75	8.80
16	2	13.66	13.83	13.99	15.33	14.98	14.88	15.11	13.59	12.99	12.56	8.27	9.56
16	3	13.12	13.99	15.38	15.24	14.55	15.33	16.02	14.72	12.99	12.56	9.34	9.00
17	1	11.94	12.24	11.35	13.3	13.02	12.02	11.07	13.88	12.01	10.16	8.94	8.59
17	2	11.93	12.44	12.91	12.93	12.68	12.78	11.99	12.11	11.62	9.77	8.92	9.55

17	3	11.78	13.08	11.27	13.16	12.98	10.66	13.56	11.91	11.07	9.22	8.50	9.52
18	1	9.23	11.02	10.99	10.14	9.27	10.75	11.12	9.85	9.04	6.20	6.78	7.21
18	2	9.33	11.5	11.16	10.36	10.11	10.24	9.92	9.65	8.89	6.05	6.59	7.69
18	3	9.06	10.3	10.28	11.2	9.85	10.41	10.88	9.77	9.50	6.66	5.63	7.63
19	1	7.67	7.59	7.04	7.4	7.16	6.53	7.52	8.59	8.15	7.71	3.88	6.89
19	2	7.41	8.21	8.98	8.21	8.48	7.75	9.59	8.08	7.64	7.20	4.11	6.91
19	3	8.08	7.66	8.08	8.07	9.06	8.45	10.21	8.39	7.95	7.51	4.71	7.45
20	1	10.1	12.89	12.12	13.03	12.74	13.05	12.26	15.30	12.76	10.22	7.61	8.07
20	2	9.05	12.76	12.74	13.21	12.89	13.17	14.00	15.05	12.51	9.97	7.70	7.95
20	3	9.37	12.99	11.88	13.34	13.18	13.12	14.31	14.07	11.53	8.99	7.75	7.50
21	1	8.49	7.51	7.83	8.46	7.79	9.69	8.21	7.96	7.25	7.25	4.42	4.96
21	2	6.82	9.79	7.51	8.34	9.28	9.88	9.98	9.73	8.16	7.32	5.30	5.89
21	3	7.86	9.16	7.93	8.32	9.83	9.56	8.31	8.06	8.24	7.27	4.54	5.67
22	1	9.28	9.89	11.34	12.96	11.56	12.25	9.33	9.35	10.63	8.09	7.51	7.71
22	2	10.26	11.34	11.61	12.43	12.87	12.63	10.41	10.43	10.11	8.65	7.99	7.82
22	3	9.26	10.22	10.52	11.03	11.46	13.09	11.19	11.21	10.81	9.57	7.55	8.85
23	1	12,18	12.31	14.23	13.68	13.24	14.02	13.86	13.37	12.45	10.99	7.17	7.98
23	2	12.08	12.5	13.6	13.56	13.44	13.24	13.51	13.02	11.43	10.72	6.69	8.26
23	3	12.3	13.8	14.92	15.02	15.64	14.96	13.92	13.43	11.94	10.77	7.28	7.98
24	1	10.98	9.88	8.84	8.82	9.03	9.12	9.63	9.67	8.43	7.43	6.00	7.83
24	2	10.17	8.8	10.43	9.59	9.01	10.06	9.27	8.65	8.79	7.79	5.51	8.26
24	3	9.84	10.62	9.45	10.3	10.18	10.04	10.65	9.55	7.66	6.66	6.52	6.45
25	1	10.06	10.22	9.89	9.64	8.88	9	8.30	9.84	8.28	7.97	5.97	8.81
25	2	10.39	9.26	9.55	9.5	10.03	8.06	10.06	9.86	9.15	8.84	6.10	7.94
25	3	9.23	8.08	9.92	9.94	9.41	9.49	8.98	10.28	9.78	9.47	5.77	6.92
26	1	9.82	11.03	11.94	12.29	12.31	12.68	12.78	12.00	10.75	9.49	5.92	8.39
26	2	10.98	11.94	12.87	13.08	14.1	12.57	11.41	12.75	11.02	9.76	6.61	8.19
26	3	10.26	11.57	12.29	12.93	12.38	13.6	12.45	12.23	12.09	10.83	6.05	8.36
27	1	9.13	9.51	9.32	8.39	9.08	8.96	10.32	9.14	8.03	6.93	6.54	7.02
27	2	9.01	8.06	10.53	10.57	8.97	9.6	10.17	9.29	8.18	7.08	7.07	7.07
27	3	8.48	9.2	9.04	11.35	9.46	9.29	10.17	10.05	8.94	7.84	7.24	7.17
28	1	8.4	8.52	8.43	8.89	8.9	8.82	9.90	8.94	8.24	7.53	5.87	5.91
28	2	9.08	9.61	9.49	9.24	10.55	9.77	10.76	8.63	7.93	7.22	5.80	5.87
28	3	8.76	9.6	9.9	9.5	8.72	9.72	10.05	8.17	7.47	6.76	6.53	6.33
29	1	9.82	10.13	9.75	10.9	9.9	9.81	10.08	9.20	8.52	7.85	6.91	6.93
29	2	10.65	11.5	11.38	11.25	12.21	11.35	12.80	10.77	10.09	9.42	7.54	7.18
29	3	10.84	9.62	10.89	10.44	11.1	11.19	9.97	9.97	9.29	8.62	7.02	7.34
30	1	12,35	13.03	11.2	13.35	13.03	11.86	12.87	12.33	11.27	10.21	6.23	6.06
30	2	10.36	13.04	12.66	11.35	11.94	13.52	12.64	11.92	10.86	9.80	5.93	6.37
30	3	10.69	13.26	13.06	12.77	13.63	11.4	12.73	12.28	11.22	10.16	6.33	6.22
31	1	12.73	12.73	13.91	13.83	13.85	14.32	14.13	14.57	14.05	13.18	8.04	7.40
31	2	11.2	13.42	13.92	11.93	14.04	14.78	14.66	14.16	13.64	12.77	8.00	8.32
31	3	11.94	12.17	14.19	12.82	13.92	14.51	14.11	13.64	13.56	12.69	6.58	7.25
32	1	11.33	13.15	11.78	14.87	10.74	15.07	9.49	10.84	12.46	9.77	10.29	8.25
32	2	11.21	12.75	11.8	10.8	11.32	13.63	11.27	14.31	14.24	11.55	8.44	8.73
32	3	10.43	9.42	10.62	12.21	13.71	12.7	10.93	13.76	13.99	11.30	7.88	8.71

33	1	11.15	11.83	12.34	12.43	13.34	13	14.03	14.50	13.25	11.97	8.46	7.98
33	2	10.66	11.44	11.72	11.88	12.93	12.01	12.46	12.93	12.51	12.81	7.32	8.53
33	3	11.39	12.52	12.21	11.37	13.23	13.7	13.76	14.23	12.62	12.47	8.21	8.72
34	1	10.22	10.61	11.5	10.06	11.02	10.66	10.70	7.95	8.38	7.44	6.77	5.92
34	2	10.51	11.22	10.25	9.73	11.15	11.19	10.15	7.40	8.41	7.03	6.71	5.89
34	3	10.41	10.59	10.56	11.95	10.77	10.85	11.76	9.01	7.74	7.65	6.52	5.97
35	1	12.12	14.28	12.84	12.41	11.53	2.38	11.83	10.79	10.69	9.96	6.64	7.86
35	2	14.53	14.43	14.86	14.35	13.8	13.9	12.85	11.94	10.85	10.07	6.54	8.06
35	3	12.85	15.17	12.64	12.49	12.13	13.19	14.42	13.49	10.72	10.26	6.46	8.30
36	1	9.76	10.21	10.97	11.53	11.16	11.19	11.80	10.35	9.04	8.84	5.44	3.77
36	2	11.33	9.4	11.34	9.52	9.69	9.27	10.58	9.13	8.91	8.34	6.96	5.29
36	3	9.08	10.5	10.26	12.66	12.71	11.89	13.05	11.60	9.97	9.79	5.16	3.49
37	1	11.47	12.61	13.31	13.43	14.24	13.5	14.56	12.25	2.89	2.98	7.30	5.11
37	2	12.31	13.28	13.9	14.54	14.37	13.61	14.35	12.04	2.69	2.74	7.17	4.98
37	3	12.63	13.28	13.52	14.93	13.42	13.88	14.41	12.10	2.95	3.12	7.25	5.06
38	1	10.33	10.69	10.82	10.79	11.08	9.19	10.18	9.03	7.32	6.22	5.21	5.42
38	2	9.65	9.41	10.02	9.17	9.43	8.94	9.81	8.66	7.69	6.36	5.20	5.41
38	3	9.93	10.58	9.84	10.11	9.57	10.12	9.35	8.20	7.99	7.44	4.54	4.75
39	1	11.66	12.93	12.02	12.06	12.15	10.93	13.16	11.25	9.51	8.88	7.13	6.97
39	2	11.55	12.12	11.76	13.08	13.03	13.43	13.21	11.82	10.08	7.51	7.73	6.30
39	3	12.84	13.14	13.37	11.68	12.78	13.05	12.96	12.43	10.69	8.73	6.17	6.93
40	1	11.7	12.9	12	11.98	12	11.39	11.98	12.20	11.34	11.78	8.49	7.28
40	2	12.27	12.85	12.24	13.27	12.09	12.91	13.13	13.83	12.97	10.60	9.01	8.31
40	3	12.51	12.23	13.22	12.03	12.93	11.96	12.62	11.72	10.86	11.35	8.25	7.18
41	1	11.43	11.86	12.71	13.26	11.92	13.71	13.06	12.72	11.72	11.04	7.29	5.13
41	2	11.69	12.16	12.77	13.5	12.27	13.38	13.96	12.77	11.77	11.17	7.04	4.76
41	3	11.73	12.41	12.24	12.86	12.57	13.28	13.44	12.79	11.79	11.06	6.71	4.48

b*

sample	replicate	Time(hrs)											
		0	0.25	0.5	0.75	1	1.5	4.00	24.00	48.00	72.00	142.00	167.00
1	1	3.19	3.15	5.48	5.58	5.66	4.12	5.57	4.35	4.61	4.57	4.25	3.55
1	2	3.15	4.09	5.41	4.82	4.63	5.06	5.48	4.66	4.72	4.33	3.90	3.75
1	3	3.15	4.51	4.45	5.14	5.37	5.06	5.46	5.08	4.81	5.41	3.45	3.56
2	1	6.31	3.96	4.58	3.7	3.85	3.25	4.88	4.88	4.20	4.41	3.00	2.52
2	2	3.17	3.42	4.78	3.56	3.46	4.43	4.34	4.99	4.68	4.79	3.30	2.91
2	3	2.66	3.28	4.86	3.09	3.85	4.09	4.34	4.99	5.02	4.17	3.30	2.72
3	1	2.88	2.06	2.57	2.43	2.87	2.59	2.53	2.67	2.59	2.47	1.39	1.11
3	2	3.23	2.71	3.17	2.6	2.55	2.42	2.88	2.62	2.71	2.55	1.90	1.60
3	3	2.38	2.94	3.17	2.6	2.55	2.74	2.05	3.21	3.09	2.37	1.79	1.00
4	1	2.64	2.96	4.06	3.73	3.38	2.28	4.01	3.19	2.72	2.68	2.80	2.31
4	2	2.06	3.67	3.19	3.08	3.63	3.9	3.84	2.96	2.78	3.59	1.93	1.61
4	3	2.64	3.32	4.06	3.08	3.38	3.66	4.01	3.17	3.41	2.37	2.23	2.45
5	1	1.5	1.72	2.07	2.15	2.01	2.62	3.21	2.86	2.44	2.01	2.18	2.46
5	2	1.8	1.85	2.03	1.84	2.25	2.08	3.01	2.91	2.54	2.18	2.12	2.04
5	3	1.53	2.09	2.04	2.32	2.48	2.26	2.75	3.16	2.57	2.67	1.97	2.10

6	1	5.31	6.01	4.97	5.09	5.99	5.52	6.39	6.03	5.60	5.97	4.32	3.92
6	2	4.63	5.33	4.91	5.18	6.19	5.49	5.85	5.74	5.38	4.77	4.40	3.71
6	3	4	5.27	5.37	5.56	5.59	5.26	6.14	5.36	5.54	5.93	4.38	3.76
7	1	2.68	3.03	3.47	3.2	3.72	3.14	2.30	4.34	3.68	3.71	3.18	2.54
7	2	2.8	3.54	3.2	3.89	3.51	3.78	2.36	4.24	3.38	3.73	2.95	2.68
7	3	2.46	3.53	3.96	3.38	3.67	3.54	2.16	4.15	3.77	3.08	3.09	2.70
8	1	2.29	2.9	3.61	3.52	3.67	2.92	4.38	4.58	3.99	3.61	2.32	1.95
8	2	2.95	3.31	3.77	3.36	3.69	3.58	4.03	4.43	3.83	3.32	2.53	1.82
8	3	2.82	3.04	3.63	3.5	3.22	3.54	4.84	4.36	4.36	3.00	2.31	2.14
9	1	3.89	4.74	4.7	5.24	4.62	4.59	5.61	6.05	5.35	4.95	2.59	2.08
9	2	4.03	4.8	4.92	4.55	4.96	4.72	5.57	5.85	5.64	4.44	3.07	2.34
9	3	3.08	3.88	4.55	5.13	4.92	4.82	5.60	5.83	5.49	3.82	3.07	2.14
10	1	4.71	5.28	5.3	6.18	6.1	5.68	6.51	6.90	5.48	5.74	4.48	4.42
10	2	4.6	5.98	5.8	5.94	7.54	7.26	6.64	7.54	6.51	6.70	4.58	4.60
10	3	4.6	6.22	5.46	6.32	6.25	7.16	7.21	7.21	6.56	6.28	4.44	4.55
11	1	2.57	3.12	3.17	2.97	3.9	3.73	4.05	2.82	2.73	2.59	2.23	2.03
11	2	2.77	3.22	3.65	4.04	3.76	3.8	4.12	3.28	2.92	2.84	2.48	1.77
11	3	3.02	3.73	3.64	3.35	3.6	3.74	4.35	3.21	3.34	3.13	2.77	1.99
12	1	3.4	3.61	3.83	3.63	3.68	3.78	3.92	3.82	3.49	3.21	4.15	4.09
12	2	2.83	4.02	3.8	4.08	3.82	3.43	4.34	3.31	3.38	2.84	3.78	4.10
12	3	3.07	3.51	4.69	3.96	4.14	3.9	3.99	3.70	3.31	3.33	4.27	3.48
13	1	1.97	2.72	2.63	2.46	2.52	2.64	2.69	2.64	2.57	2.86	2.16	1.99
13	2	1.97	2.81	3.18	2.9	2.98	2.89	2.95	2.74	2.88	3.03	2.19	2.10
13	3	2.16	2.65	3.14	2.77	2.74	2.71	2.99	2.74	2.81	2.74	2.19	1.95
14	1	3.75	4.09	4.1	4.21	4.21	4.06	3.84	3.68	4.03	3.77	3.64	4.15
14	2	3.44	5.01	4.68	5.05	4.15	5.19	5.17	4.35	3.53	4.13	3.77	3.84
14	3	3.64	3.45	4.39	4.4	4.46	4.32	4.17	3.58	3.89	3.61	3.75	3.99
15	1	2.95	3.18	3.37	3.57	3.12	3.92	3.36	3.69	3.40	4.38	2.38	1.99
15	2	3.2	3.29	3.05	3.58	4.06	4.26	4.59	3.98	4.08	3.79	3.21	2.27
15	3	4.09	3.78	3.86	3.96	4.23	3.72	3.74	4.08	4.53	4.94	2.50	2.81
16	1	4.48	4.77	5.95	5.92	5.69	5.66	5.45	5.10	5.10	4.89	3.87	3.63
16	2	4.47	5.13	4.9	6.19	5.86	5.54	5.47	5.12	5.51	5.30	3.90	3.80
16	3	3.92	5.05	5.93	6.16	5.1	6.03	6.54	5.76	5.51	5.30	3.97	3.55
17	1	3.42	4.32	4	4.58	4.45	4.09	3.70	4.90	4.23	3.73	2.50	2.78
17	2	3.5	4.02	4.25	4.67	4.42	4.54	4.36	4.00	4.10	3.60	2.64	2.47
17	3	3.57	4.48	4.03	4.54	4.27	3.5	4.57	4.14	3.79	3.29	2.37	2.53
18	1	2.11	3.29	3.45	3.3	2.83	3.33	3.47	3.28	2.78	2.64	2.88	2.23
18	2	2.25	3.51	3.54	3.3	3.18	3.28	3.14	3.10	2.90	2.76	2.41	2.22
18	3	2.33	3	3.31	3.63	3	3.35	3.47	3.14	3.04	2.90	2.04	2.12
19	1	1.67	2.14	1.86	2.29	2.12	1.85	2.00	2.29	2.22	2.14	1.87	1.81
19	2	1.66	2.03	2.19	2.24	2.19	2.16	2.39	2.17	2.10	2.02	1.94	1.69
19	3	1.63	1.78	2.19	2.1	2.33	2.08	2.73	1.95	1.88	1.80	1.88	2.22
20	1	1.85	3.77	3.65	3.84	3.92	4.07	3.94	5.23	4.69	4.14	2.52	2.72
20	2	2.26	3.72	3.78	3.99	3.98	4.11	4.65	5.08	4.54	3.99	2.62	2.90
20	3	2.1	3.84	3.52	4	4.05	4.11	4.74	4.66	4.12	3.57	2.61	2.68
21	1	2.42	2.47	2.49	2.68	2.46	3.09	2.66	2.65	2.67	2.68	2.65	3.16

21	2	2.13	2.67	2.51	2.9	2.79	2.9	2.93	2.92	2.78	2.49	3.14	3.00
21	3	2.29	2.83	2.43	2.68	3.34	3.01	2.47	2.46	2.67	2.61	2.89	3.40
22	1	2.43	3.02	3.57	4.18	3.74	4.02	2.99	2.94	3.61	2.92	2.67	1.99
22	2	2.74	3.59	3.68	3.94	4.32	4.22	3.16	3.11	3.41	3.13	2.69	2.23
22	3	2.39	3.12	3.31	3.35	3.46	4.34	3.72	3.67	3.65	3.55	2.33	2.08
23	1	3.33	4.34	5.02	4.88	4.64	4.99	5.08	4.90	4.83	4.61	3.66	2.82
23	2	3.63	4.42	4.85	5.04	4.84	4.84	5.07	5.11	4.57	4.58	4.22	3.06
23	3	3.5	4.65	5.39	5.54	5.7	5.61	5.23	5.27	4.73	4.50	4.10	3.01
24	1	2.6	2.82	2.68	2.65	2.62	2.68	2.75	2.81	2.52	2.29	1.59	1.58
24	2	2.47	2.73	2.92	2.81	2.72	2.75	2.81	2.56	2.66	2.43	1.53	1.70
24	3	2.53	2.99	2.68	2.94	2.85	2.81	2.95	2.71	2.35	2.12	1.78	1.61
25	1	2.7	3.14	3.09	3.03	2.8	2.77	2.57	3.08	2.58	2.64	2.78	1.93
25	2	2.61	2.77	2.76	2.9	2.82	2.28	3.06	3.05	2.97	3.03	2.93	1.81
25	3	2.36	2.39	2.65	2.73	2.77	2.74	2.74	3.07	3.12	3.18	2.77	1.97
26	1	2.82	3.51	3.96	4.12	4.06	4.22	4.21	3.86	3.64	3.34	2.93	3.28
26	2	3.05	3.71	4.32	4.34	4.89	4.38	3.74	4.11	3.70	3.40	2.90	3.19
26	3	3.01	3.6	3.99	4.29	4.2	4.71	4.00	4.15	4.20	3.90	2.95	3.34
27	1	2.44	2.8	2.74	2.7	2.64	2.39	3.05	2.67	2.54	2.41	2.81	3.14
27	2	2.26	2.49	2.96	3.09	2.85	2.71	3.07	2.73	2.60	2.47	3.22	3.32
27	3	2.28	2.7	2.66	3.18	2.84	2.81	3.07	2.95	2.82	2.69	3.27	3.34
28	1	2.25	2.64	2.57	2.72	2.73	2.66	3.12	2.83	2.68	2.52	2.81	2.80
28	2	2.34	2.92	3.01	2.85	3.19	3.02	3.36	2.88	2.73	2.57	2.80	2.83
28	3	2.25	2.89	3.04	2.97	2.87	2.87	3.12	2.73	2.58	2.42	3.08	2.97
29	1	2.7	3.1	3.23	3.47	3.33	3.24	3.44	3.09	3.12	3.15	3.55	3.51
29	2	3.04	3.68	3.65	3.82	4.11	4	4.48	3.81	3.84	3.87	3.57	3.57
29	3	2.84	3.05	3.36	3.38	3.47	3.51	3.34	3.26	3.29	3.32	3.45	3.48
30	1	3.07	3.84	3.6	4.44	4.4	4.05	4.53	4.20	4.00	3.80	4.04	4.08
30	2	2.62	3.96	4.06	3.73	4.02	4.57	4.41	4.05	3.85	3.65	3.88	4.11
30	3	2.62	3.91	4.12	4.21	4.57	3.77	4.42	4.22	4.02	3.82	4.16	3.98
31	1	4.23	4.96	5.56	5.58	5.62	5.96	6.01	6.27	6.09	5.94	5.73	5.48
31	2	3.62	5.27	5.36	4.75	5.88	6.23	5.91	6.09	5.95	5.80	5.62	4.87
31	3	4.02	4.5	5.7	4.9	5.56	5.89	5.92	5.83	5.75	5.60	5.30	5.34
32	1	3.76	4.34	4.55	5.13	3.68	5.25	3.34	3.83	4.86	3.92	3.77	2.96
32	2	2.91	4.78	4.5	3.43	4.34	5.02	4.14	5.39	4.94	4.00	2.53	3.23
32	3	3.46	3.02	3.86	4.36	4.81	3.8	3.98	4.27	5.38	4.44	1.96	3.61
33	1	3.61	4.26	4.71	4.46	4.86	5.13	5.68	5.71	5.30	4.92	3.98	1.99
33	2	2.9	3.76	3.97	4.51	4.23	4.11	4.65	4.68	5.28	4.69	3.23	2.26
33	3	3.75	4.65	4.65	4.01	5	5.46	5.73	5.76	4.72	5.16	3.80	2.19
34	1	2.79	3.46	3.67	3.27	3.65	3.58	3.48	2.27	2.43	2.22	2.56	2.41
34	2	2.8	3.4	3.36	2.88	3.17	3.61	3.38	2.17	2.58	1.81	2.57	2.45
34	3	2.55	3.42	3.04	3.66	3.42	3.3	3.41	2.20	1.72	2.21	2.22	2.72
35	1	3.64	5.14	4.56	4.37	4.03	4.51	4.30	4.03	3.94	3.82	3.20	2.73
35	2	4.46	4.73	5.33	5.24	5.01	5.2	4.55	4.28	4.06	3.89	2.88	2.67
35	3	3.34	5.34	4.18	4.11	3.94	4.47	5.01	4.74	3.67	3.68	2.45	2.82
36	1	2.56	3.17	3.36	3.59	3.59	3.58	3.90	3.28	2.98	2.93	1.64	0.81
36	2	3.04	2	3.58	2.88	2.81	2.76	3.21	2.59	2.61	2.56	2.01	1.18

36	3	2.12	2.97	2.88	4.04	4.03	3.63	4.25	3.63	3.01	3.17	1.33	0.50
37	1	2.83	3.86	4.2	4.22	4.42	4.28	4.84	4.74	3.29	3.28	2.21	1.39
37	2	2.88	3.97	4.25	4.5	4.64	4.34	4.65	4.55	3.09	3.04	2.06	1.24
37	3	2.96	4.11	4.24	4.86	4.34	4.4	4.70	4.60	3.35	3.42	2.34	1.52
38	1	2.94	3.29	3.38	3.38	3.78	2.83	3.13	2.76	2.37	2.09	1.88	1.84
38	2	2.71	3.05	3.09	2.92	2.9	2.98	3.16	2.79	2.60	1.96	1.99	1.95
38	3	2.8	2.94	2.92	2.9	3	3	2.92	2.55	2.40	2.54	1.43	1.39
39	1	2.72	4.1	4.01	4.37	4.09	4.02	4.84	3.99	3.50	3.36	1.52	0.66
39	2	2.98	4.18	4.21	4.3	4.45	4.64	4.60	4.27	3.78	2.84	1.72	0.86
39	3	3.16	4.08	4.31	3.93	4.18	4.72	4.70	4.28	3.79	3.18	1.34	0.74
40	1	2.93	3.85	3.73	3.78	3.76	3.68	4.13	4.15	3.91	4.05	3.02	2.64
40	2	2.96	3.85	3.71	4	3.82	4.04	4.43	4.56	4.32	3.74	3.07	2.41
40	3	2.95	3.67	4.01	3.52	3.95	3.71	4.22	4.01	3.77	4.01	2.88	2.48
41	1	2.49	3.35	3.77	4.11	3.58	4.34	4.22	4.04	3.66	3.40	3.04	2.70
41	2	2.61	3.39	3.89	4.12	3.75	4.12	4.40	4.09	3.71	3.59	2.95	2.81
41	3	2.7	3.43	3.59	3.91	4.01	4.17	4.37	4.04	3.66	3.46	2.86	2.45

10 **Appendix IV**

% Drip loss and cooking loss for 41 samples

Sample	% drip loss		%cooking loss	
	B	A	B	A
1	5.46	6.42	25.17	24.31
2	4.53	5.64	28.39	29.07
3	5.17	3.94	26.45	22.81
4	5.27	7.69	30.27	30.16
5	4.64	4.64	11.49	21.28
6	9.06	13.06	21.45	22.77
7	6.47	8.00	21.11	19.48
8	10.08	7.45	22.04	19.89
9	16.41	33.64	21.03	18.60
10	11.63	7.00	25.27	26.71
11	6.88	8.00	24.56	18.65
12	8.20	7.77	23.07	22.48
13	9.11	4.20	13.28	14.85
14	8.79	5.34	24.72	20.38
15	5.63	5.58	17.86	14.65
16	9.25	6.79	18.74	22.30
17	7.99	15.94	22.15	43.54
18	5.85	7.23	23.45	25.42
19	3.33	7.58	17.19	19.79
20	7.63	5.36	24.27	21.05
21	15.26	13.90	27.90	27.75
22	5.87	3.68	43.51	22.68
23	9.76	7.42	22.43	35.07
24	12.53	11.15	19.18	25.21
25	8.03	4.64	23.09	25.56
26	7.95	5.57	24.78	25.04
27	12.12	9.76	18.60	19.78
28	10.91	11.50	20.81	24.52
29	10.39	7.96	24.47	25.82
30	10.08	5.97	24.28	20.60
31	7.18	4.20	28.75	25.02
32	9.20	4.41	31.61	28.39
33	7.15	3.39	26.36	27.34
34	3.86	2.49	25.43	23.39
35	10.05	2.30	32.05	27.52
36	7.86	5.49	32.01	28.91
37	5.85	3.30	31.54	27.57
38	8.23	7.19	27.05	30.65
39	8.89	4.78	29.21	28.11

40	6.44	4.44	32.32	29.30
41	7.19	4.02	30.52	30.03

11 Appendix V

Warner Bratzler shear force measurements for 41 samples

Warner Bratzler values

Sample	A	B
1	29.68351	33.85852
2	67.11096	67.45767
3	58.09776	46.70466
4	114.8814	65.48971
5	28.62284	21.25152
6	67.25565	37.56081
7	31.01299	30.47511
8	33.44469	34.05136
9	26.33105	27.64757
10	29.9431	26.11881
11	67.7065	51.19668
12	73.6936	84.7627
13	30.94641	34.8575
14	35.33984	32.65758
15	36.79541	30.53744
16	28.22775	40.5224
17	33.56794	43.423
18	38.40028	26.06384
19	100.551	55.53036
20	62.2229	32.52847
21	66.28141	42.05451
22	47.90664	24.48902
23	47.4211	28.50484
24	37.71693	28.15141
25	79.87912	35.06725
26	35.52153	28.61601
27	66.00353	31.25953
28	49.8208	39.3409
29	89.62214	41.45225
30	36.19149	31.06467
31	40.62482	24.56054
32	31.05545	40.00413
33	39.19771	28.67308
34	28.99926	31.55917
35	43.82573	62.7577
36	40.31638	39.3438
37	86.14541	56.52818
38	45.49259	26.85402
39	94.5346	70.60302

40	97.32021	84.30881
41	83.14121	83.14121

12 **Appendix VI**

Different states of myoglobins in 41 samples with time

sample	replicate	Time(hrs)											
Oxymyoglobin		0	0.25	0.5	0.75	1	1.5	4	24	48	72	142	167
1	1	0.01	0.09	0.16	0.18	0.22	0.24	0.40	0.54	0.46	0.44	0.24	0.03
1	2	0.06	0.01	0.04	0.12	0.16	0.24	0.33	0.47	0.43	0.44	0.24	0.02
2	1	0.00	0.03	0.08	0.05	0.06	0.07	0.18	0.36	0.36	0.32	0.00	0.00
2	2	0.00	0.03	0.03	0.04	0.04	0.01	0.12	0.32	0.36	0.32	0.00	0.00
3	1	0.40	0.40	0.38	0.42	0.42	0.45	0.53	0.52	0.57	0.56	0.12	0.03
3	2	0.34	0.36	0.36	0.42	0.41	0.40	0.50	0.51	0.51	0.50	0.11	0.02
4	1	0.14	0.27	0.31	0.42	0.42	0.41	0.59	0.57	0.54	0.55	0.17	0.11
4	2	0.17	0.26	0.26	0.31	0.38	0.39	0.60	0.58	0.54	0.55	0.14	0.14
4	1	0.15	0.16	0.16	0.18	0.19	0.23	0.29	0.37	0.44	0.40	0.10	0.02
5	2	0.19	0.15	0.17	0.17	0.18	0.23	0.28	0.37	0.48	0.48	0.10	0.05
5	1	0.00	0.00	0.00	0.00	0.00	0.11	0.14	0.45	0.44	0.47	0.19	0.00
6	2	0.00	0.00	0.00	0.00	0.00	0.04	0.16	0.39	0.47	0.46	0.20	0.00
6	1	0.11	0.14	0.18	0.18	0.18	0.22	0.06	0.39	0.42	0.42	0.07	0.03
7	2	0.12	0.15	0.15	0.17	0.19	0.19	0.05	0.39	0.41	0.39	0.05	0.00
8	1	0.02	0.09	0.11	0.11	0.13	0.14	0.28	0.48	0.50	0.39	0.00	0.00
8	2	0.02	0.07	0.11	0.10	0.10	0.08	0.24	0.43	0.49	0.40	0.00	0.00
9	1	0.00	0.05	0.08	0.11	0.17	0.15	0.36	0.61	0.62	0.58	0.00	0.00
9	2	0.00	0.01	0.06	0.08	0.15	0.17	0.32	0.59	0.60	0.59	0.00	0.00
10	1	0.00	0.07	0.10	0.21	0.15	0.28	0.44	0.66	0.62	0.62	0.13	0.00
10	2	0.00	0.03	0.14	0.16	0.21	0.24	0.44	0.65	0.63	0.63	0.13	0.00
11	1	0.19	0.23	0.27	0.31	0.28	0.35	0.36	0.47	0.47	0.41	0.00	0.00
11	2	0.14	0.22	0.26	0.26	0.32	0.31	0.33	0.44	0.46	0.39	0.00	0.00
12	1	0.10	0.15	0.27	0.30	0.28	0.40	0.38	0.53	0.51	0.48	0.11	0.00
12	2	0.09	0.14	0.27	0.28	0.34	0.42	0.42	0.54	0.50	0.47	0.14	0.00
13	1	0.09	0.19	0.24	0.29	0.31	0.32	0.45	0.52	0.53	0.50	0.12	0.07
13	2	0.06	0.19	0.22	0.27	0.28	0.31	0.42	0.52	0.53	0.49	0.12	0.10
14	1	0.00	0.06	0.12	0.10	0.18	0.15	0.37	0.44	0.42	0.38	0.36	0.32
14	2	0.00	0.07	0.10	0.10	0.15	0.16	0.31	0.42	0.42	0.38	0.36	0.33
15	1	0.04	0.07	0.09	0.13	0.12	0.15	0.28	0.48	0.48	0.49	0.09	0.00
15	2	0.02	0.08	0.10	0.10	0.10	0.18	0.24	0.49	0.46	0.47	0.09	0.00
16	1	0.00	0.00	0.09	0.20	0.06	0.26	0.30	0.52	0.50	0.58	0.06	0.00
16	2	0.00	0.04	0.00	0.15	0.18	0.19	0.27	0.46	0.48	0.57	0.05	0.00
17	1	0.19	0.28	0.32	0.35	0.36	0.40	0.58	0.63	0.61	0.13	0.01	0.00
17	2	0.17	0.27	0.31	0.32	0.37	0.43	0.53	0.62	0.61	0.14	0.01	0.01
18	1	0.10	0.27	0.30	0.31	0.32	0.31	0.41	0.52	0.50	0.27	0.10	0.12
18	2	0.11	0.25	0.30	0.31	0.31	0.28	0.36	0.51	0.48	0.27	0.10	0.12
19	1	0.20	0.22	0.32	0.33	0.34	0.38	0.48	0.57	0.65	0.51	0.25	0.11

19	2	0.19	0.25	0.28	0.29	0.29	0.37	0.45	0.56	0.64	0.52	0.21	0.11
20	1	0.00	0.08	0.11	0.12	0.15	0.17	0.31	0.57	0.65	0.05	0.10	0.00
20	2	0.00	0.03	0.07	0.07	0.08	0.12	0.26	0.54	0.67	0.02	0.10	0.00
21	1	0.07	0.13	0.19	0.22	0.24	0.29	0.43	0.30	0.45	0.41	0.14	0.06
21	2	0.06	0.12	0.18	0.20	0.23	0.29	0.38	0.29	0.44	0.41	0.15	0.07
22	1	0.23	0.32	0.32	0.35	0.35	0.38	0.48	0.40	0.52	0.37	0.08	0.06
22	2	0.23	0.27	0.30	0.33	0.33	0.36	0.46	0.41	0.51	0.37	0.07	0.05
23	1	0.00	0.04	0.00	0.07	0.14	0.16	0.33	0.01	0.40	0.29	0.00	0.00
23	2	0.00	0.00	0.00	0.05	0.09	0.06	0.35	0.00	0.34	0.26	0.00	0.00
24	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.39	0.20	0.10	0.00
24	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.41	0.38	0.19	0.10	0.00
25	1	0.11	0.12	0.00	0.08	0.10	0.12	0.21	0.55	0.54	0.62	0.04	0.00
25	2	0.09	0.07	0.00	0.00	0.08	0.09	0.14	0.54	0.53	0.63	0.05	0.00
26	1	0.12	0.16	0.21	0.22	0.20	0.18	0.31	0.63	0.59	0.60	0.10	0.05
26	2	0.10	0.15	0.14	0.20	0.18	0.15	0.27	0.61	0.58	0.60	0.09	0.04
27	1	0.23	0.27	0.31	0.36	0.35	0.39	0.47	0.52	0.58	0.17	0.08	0.00
27	2	0.25	0.27	0.31	0.35	0.34	0.38	0.46	0.51	0.58	0.16	0.08	0.00
28	1	0.06	0.14	0.17	0.21	0.22	0.24	0.37	0.45	0.47	0.38	0.14	0.00
28	2	0.06	0.13	0.17	0.20	0.21	0.24	0.37	0.44	0.47	0.36	0.13	0.00
29	1	0.00	0.00	0.01	0.05	0.07	0.11	0.27	0.38	0.08	0.23	0.00	0.00
29	2	0.00	0.00	0.00	0.03	0.04	0.08	0.24	0.36	0.13	0.23	0.00	0.00
30	1	0.05	0.12	0.18	0.24	0.26	0.33	0.50	0.58	0.53	0.57	0.10	0.00
30	2	0.06	0.11	0.20	0.22	0.26	0.32	0.48	0.57	0.52	0.57	0.11	0.00
31	1	0.00	0.00	0.00	0.00	0.00	0.06	0.23	0.39	0.43	0.36	0.00	0.00
31	2	0.00	0.00	0.00	0.00	0.02	0.00	0.15	0.36	0.39	0.38	0.00	0.00
32	1	0.12	0.20	0.26	0.36	0.36	0.42	0.56	0.59	0.63	0.51	0.00	0.00
32	2	0.08	0.25	0.28	0.30	0.34	0.38	0.53	0.62	0.64	0.43	0.00	0.00
33	1	0.00	0.00	0.00	0.04	0.02	0.06	0.14	0.48	0.53	0.43	0.00	0.00
33	2	0.00	0.00	0.00	0.02	0.01	0.03	0.23	0.49	0.39	0.49	0.00	0.00
34	1	0.17	0.26	0.29	0.36	0.35	0.38	0.53	0.53	0.48	0.49	0.41	0.38
34	2	0.17	0.25	0.29	0.34	0.34	0.37	0.54	0.53	0.50	0.49	0.42	0.39
35	1	0.09	0.19	0.24	0.24	0.28	0.33	0.47	0.58	0.53	0.52	0.15	0.05
35	2	0.10	0.18	0.24	0.27	0.30	0.31	0.45	0.59	0.55	0.51	0.12	0.04
36	1	0.18	0.27	0.33	0.37	0.39	0.40	0.54	0.62	0.56	0.58	0.38	0.26
36	2	0.18	0.24	0.32	0.35	0.35	0.41	0.55	0.64	0.58	0.59	0.38	0.24
37	1	0.07	0.21	0.23	0.26	0.28	0.32	0.50	0.57	0.55	0.56	0.42	0.40
37	2	0.09	0.18	0.22	0.25	0.28	0.34	0.49	0.59	0.56	0.58	0.43	0.40
38	1	0.00	0.05	0.04	0.18	0.10	0.17	0.23	0.32	0.34	0.33	0.15	0.03
38	2	0.00	0.05	0.08	0.15	0.08	0.17	0.22	0.32	0.31	0.32	0.15	0.00
39	1	0.00	0.00	0.00	0.01	0.03	0.04	0.25	0.38	0.33	0.37	0.01	0.00
39	2	0.00	0.00	0.00	0.00	0.00	0.07	0.25	0.34	0.33	0.36	0.01	0.00
40	1	0.07	0.14	0.15	0.16	0.18	0.20	0.36	0.53	0.55	0.50	0.17	0.00
40	2	0.07	0.12	0.13	0.17	0.16	0.19	0.36	0.52	0.55	0.51	0.17	0.00

41	1	0.10	0.18	0.22	0.25	0.29	0.31	0.44	0.56	0.59	0.56	0.23	0.03
41	2	0.10	0.17	0.23	0.26	0.28	0.32	0.45	0.57	0.60	0.56	0.23	0.00
sample	replicate						Time(hrs)						
Deoxymyoglobin		0	0.25	0.5	0.75	1	1.5	4	24	48	72	142	167
1	1	0.99	0.91	0.84	0.82	0.78	0.76	0.59	0.21	0.12	0.14	0.00	0.00
1	2	0.94	0.99	0.96	0.88	0.84	0.76	0.67	0.32	0.21	0.08	0.00	0.00
2	1	1.00	0.97	0.92	0.95	0.94	0.93	0.82	0.56	0.51	0.30	0.73	1.00
2	2	1.00	0.97	0.97	0.96	0.96	0.99	0.88	0.60	0.38	0.32	0.71	1.00
3	1	0.35	0.33	0.35	0.32	0.31	0.31	0.25	0.15	0.07	0.07	0.37	0.81
3	2	0.37	0.37	0.35	0.30	0.34	0.34	0.25	0.12	0.10	0.08	0.37	0.83
4	1	0.86	0.73	0.62	0.54	0.58	0.51	0.39	0.18	0.08	0.04	0.10	0.50
4	2	0.77	0.68	0.67	0.62	0.54	0.55	0.33	0.13	0.08	0.05	0.16	0.31
4	1	0.75	0.75	0.73	0.72	0.71	0.68	0.58	0.38	0.24	0.23	0.68	0.82
5	2	0.71	0.75	0.74	0.73	0.71	0.68	0.60	0.38	0.20	0.16	0.72	0.78
5	1	1.00	1.00	1.00	1.00	1.00	0.89	0.85	0.26	0.02	0.00	0.02	0.31
6	2	1.00	1.00	1.00	1.00	1.00	0.96	0.84	0.28	0.02	0.00	0.07	0.24
6	1	0.83	0.80	0.74	0.75	0.74	0.65	0.92	0.34	0.14	0.05	0.26	0.70
7	2	0.83	0.80	0.77	0.75	0.72	0.69	0.90	0.34	0.16	0.08	0.28	0.69
8	1	0.98	0.91	0.89	0.89	0.87	0.86	0.72	0.36	0.24	0.14	0.71	0.99
8	2	0.98	0.93	0.89	0.90	0.90	0.92	0.76	0.40	0.24	0.20	0.75	0.96
9	1	1.00	0.95	0.92	0.89	0.83	0.85	0.64	0.26	0.17	0.04	0.48	0.97
9	2	1.00	0.99	0.94	0.92	0.85	0.83	0.68	0.27	0.18	0.07	0.44	0.97
10	1	1.00	0.93	0.90	0.79	0.85	0.72	0.55	0.15	0.08	0.05	0.31	0.68
10	2	1.00	0.97	0.86	0.84	0.79	0.76	0.56	0.15	0.10	0.04	0.30	0.70
11	1	0.81	0.77	0.73	0.69	0.72	0.65	0.54	0.20	0.04	0.00	0.93	0.96
11	2	0.86	0.78	0.74	0.74	0.68	0.69	0.57	0.21	0.05	0.00	0.92	0.95
12	1	0.90	0.85	0.73	0.70	0.72	0.60	0.51	0.13	0.00	0.00	0.00	0.39
12	2	0.91	0.86	0.73	0.72	0.66	0.57	0.48	0.15	0.00	0.00	0.00	0.41
13	1	0.78	0.66	0.61	0.57	0.54	0.52	0.36	0.08	0.00	0.00	0.13	0.54
13	2	0.81	0.67	0.65	0.58	0.58	0.54	0.39	0.10	0.00	0.00	0.14	0.51
14	1	0.92	0.84	0.76	0.79	0.68	0.70	0.44	0.06	0.00	0.00	0.00	0.00
14	2	0.95	0.84	0.81	0.80	0.72	0.71	0.48	0.07	0.00	0.00	0.00	0.00
15	1	0.91	0.88	0.87	0.82	0.81	0.78	0.62	0.19	0.00	0.00	0.58	0.90
15	2	0.94	0.89	0.86	0.84	0.85	0.76	0.62	0.18	0.01	0.00	0.58	0.89
16	1	0.99	0.95	0.84	0.72	0.84	0.63	0.57	0.08	0.04	0.10	0.33	0.69
16	2	0.98	0.94	0.94	0.81	0.75	0.75	0.54	0.10	0.04	0.08	0.33	0.70
17	1	0.77	0.67	0.63	0.60	0.58	0.54	0.31	0.11	0.05	0.00	0.71	0.77
17	2	0.79	0.69	0.65	0.63	0.58	0.51	0.37	0.14	0.07	0.00	0.80	0.77
18	1	0.80	0.63	0.60	0.58	0.56	0.55	0.42	0.12	0.05	0.00	0.30	0.58
18	2	0.80	0.65	0.59	0.57	0.57	0.59	0.47	0.14	0.06	0.00	0.31	0.59
19	1	0.62	0.61	0.51	0.51	0.50	0.46	0.34	0.15	0.13	0.08	0.00	0.67
19	2	0.64	0.59	0.55	0.56	0.54	0.47	0.38	0.17	0.14	0.08	0.00	0.68
20	1	1.00	0.88	0.87	0.88	0.83	0.81	0.66	0.27	0.35	0.00	0.30	0.93

20	2	1.00	0.97	0.93	0.93	0.92	0.88	0.73	0.31	0.33	0.00	0.43	0.94
21	1	0.65	0.61	0.51	0.49	0.49	0.39	0.21	0.00	0.00	0.00	0.00	0.00
21	2	0.68	0.63	0.54	0.52	0.51	0.39	0.26	0.00	0.00	0.00	0.00	0.00
22	1	0.67	0.57	0.57	0.54	0.54	0.51	0.37	0.00	0.08	0.12	0.53	0.73
22	2	0.67	0.62	0.59	0.56	0.56	0.52	0.40	0.00	0.06	0.13	0.56	0.75
23	1	1.00	0.96	1.00	0.93	0.84	0.80	0.52	0.00	0.00	0.00	0.00	0.80
23	2	1.00	1.00	1.00	0.95	0.90	0.91	0.56	0.00	0.00	0.00	0.00	0.79
24	1	0.55	0.37	0.16	0.04	0.00	0.00	0.00	0.16	0.04	0.00	0.39	0.94
24	2	0.53	0.29	0.18	0.16	0.00	0.00	0.00	0.17	0.06	0.00	0.35	0.93
25	1	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.03	0.00	0.31	0.90
25	2	0.39	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.04	0.00	0.25	0.90
26	1	0.43	0.07	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.30	0.70
26	2	0.45	0.12	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.42	0.69
27	1	0.56	0.48	0.49	0.46	0.41	0.36	0.27	0.06	0.07	0.00	0.46	0.82
27	2	0.57	0.51	0.51	0.49	0.43	0.41	0.30	0.08	0.06	0.00	0.46	0.80
28	1	0.83	0.73	0.68	0.62	0.59	0.57	0.36	0.06	0.00	0.00	0.00	0.00
28	2	0.84	0.73	0.68	0.64	0.60	0.56	0.36	0.07	0.00	0.00	0.00	0.00
29	1	0.89	0.88	0.85	0.78	0.75	0.63	0.34	0.00	0.00	0.39	0.00	0.00
29	2	0.92	0.88	0.89	0.82	0.77	0.69	0.40	0.01	0.00	0.37	0.00	0.00
30	1	0.91	0.85	0.79	0.73	0.70	0.61	0.40	0.14	0.05	0.03	0.00	0.00
30	2	0.91	0.86	0.78	0.75	0.70	0.63	0.44	0.15	0.06	0.03	0.00	0.00
31	1	1.00	1.00	1.00	1.00	1.00	0.94	0.71	0.41	0.22	0.27	0.00	0.02
31	2	1.00	1.00	1.00	1.00	0.98	1.00	0.84	0.48	0.33	0.25	0.00	0.00
32	1	0.82	0.73	0.68	0.54	0.58	0.47	0.26	0.18	0.07	0.00	0.92	0.91
32	2	0.83	0.65	0.64	0.61	0.58	0.49	0.33	0.15	0.07	0.00	0.91	0.92
33	1	1.00	1.00	1.00	0.96	0.98	0.94	0.80	0.30	0.21	0.16	0.31	1.00
33	2	1.00	1.00	1.00	0.98	0.99	0.97	0.77	0.31	0.14	0.22	0.40	1.00
34	1	0.72	0.63	0.59	0.50	0.52	0.47	0.28	0.10	0.07	0.01	0.00	0.00
34	2	0.71	0.64	0.59	0.53	0.53	0.49	0.27	0.10	0.06	0.02	0.00	0.00
35	1	0.89	0.79	0.74	0.74	0.69	0.63	0.46	0.10	0.00	0.00	0.19	0.89
35	2	0.89	0.81	0.74	0.70	0.66	0.65	0.48	0.08	0.01	0.00	0.26	0.89
36	1	0.75	0.69	0.62	0.56	0.54	0.54	0.36	0.09	0.03	0.00	0.08	0.15
36	2	0.78	0.69	0.64	0.58	0.58	0.48	0.35	0.09	0.06	0.00	0.09	0.20
37	1	0.92	0.78	0.75	0.71	0.68	0.63	0.37	0.03	0.00	0.00	0.00	0.01
37	2	0.91	0.82	0.77	0.73	0.69	0.62	0.40	0.07	0.00	0.00	0.00	0.03
38	1	0.90	0.84	0.84	0.69	0.78	0.70	0.60	0.24	0.11	0.09	0.38	0.65
38	2	0.90	0.84	0.81	0.74	0.80	0.69	0.61	0.26	0.15	0.12	0.36	0.74
39	1	1.00	1.00	1.00	0.99	0.97	0.96	0.68	0.17	0.00	0.00	0.79	1.00
39	2	1.00	1.00	1.00	1.00	1.00	0.93	0.69	0.19	0.00	0.00	0.79	1.00
40	1	0.93	0.86	0.85	0.84	0.81	0.79	0.57	0.18	0.04	0.00	0.25	0.45
40	2	0.93	0.88	0.87	0.83	0.83	0.79	0.56	0.20	0.04	0.00	0.25	0.45
41	1	0.89	0.81	0.76	0.74	0.69	0.65	0.49	0.19	0.06	0.02	0.00	0.00
41	2	0.89	0.83	0.77	0.73	0.71	0.65	0.49	0.18	0.06	0.03	0.00	0.00

sample	replicate	Time(hrs)											
Metmyoglobin		0	0.25	0.5	0.75	1	1.5	4	24	48	72	142	167
1	1	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.25	0.42	0.41	0.76	0.97
1	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.35	0.48	0.76	0.98
2	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.14	0.38	0.27	0.00
2	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.26	0.36	0.29	0.00
3	1	0.25	0.28	0.28	0.26	0.27	0.25	0.22	0.33	0.36	0.37	0.51	0.16
3	2	0.29	0.27	0.29	0.27	0.26	0.26	0.25	0.37	0.38	0.42	0.51	0.15
4	1	0.00	0.00	0.06	0.04	0.00	0.08	0.02	0.25	0.38	0.41	0.73	0.39
4	2	0.06	0.06	0.07	0.07	0.07	0.07	0.07	0.29	0.38	0.40	0.69	0.55
4	1	0.10	0.09	0.10	0.10	0.10	0.09	0.13	0.25	0.32	0.38	0.22	0.16
5	2	0.10	0.09	0.10	0.11	0.11	0.09	0.12	0.25	0.31	0.36	0.18	0.16
5	1	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.29	0.54	0.53	0.79	0.69
6	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.51	0.54	0.73	0.76
6	1	0.06	0.06	0.08	0.07	0.08	0.12	0.02	0.27	0.43	0.53	0.67	0.27
7	2	0.05	0.06	0.07	0.07	0.09	0.12	0.05	0.27	0.44	0.53	0.66	0.31
8	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.26	0.47	0.29	0.01
8	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.27	0.40	0.25	0.04
9	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.22	0.38	0.52	0.03
9	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.22	0.34	0.56	0.03
10	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.30	0.33	0.56	0.32
10	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.28	0.33	0.57	0.30
11	1	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.33	0.49	0.59	0.07	0.04
11	2	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.35	0.50	0.61	0.08	0.05
12	1	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.34	0.49	0.52	0.89	0.61
12	2	0.00	0.00	0.00	0.00	0.00	0.01	0.11	0.31	0.50	0.53	0.86	0.59
13	1	0.13	0.15	0.14	0.14	0.15	0.15	0.19	0.40	0.47	0.50	0.74	0.39
13	2	0.12	0.13	0.13	0.15	0.14	0.15	0.18	0.38	0.47	0.51	0.73	0.39
14	1	0.08	0.10	0.12	0.11	0.14	0.15	0.19	0.50	0.58	0.62	0.64	0.68
14	2	0.05	0.09	0.09	0.11	0.13	0.13	0.21	0.51	0.58	0.62	0.64	0.67
15	1	0.05	0.04	0.04	0.04	0.07	0.06	0.10	0.33	0.52	0.51	0.33	0.10
15	2	0.03	0.03	0.04	0.06	0.05	0.06	0.14	0.33	0.53	0.53	0.33	0.11
16	1	0.01	0.05	0.08	0.09	0.10	0.11	0.13	0.40	0.46	0.32	0.61	0.31
16	2	0.02	0.02	0.06	0.04	0.07	0.07	0.18	0.44	0.47	0.35	0.63	0.30
17	1	0.05	0.05	0.04	0.05	0.06	0.06	0.11	0.26	0.34	0.87	0.27	0.23
17	2	0.03	0.05	0.04	0.04	0.06	0.06	0.10	0.25	0.33	0.86	0.20	0.21
18	1	0.10	0.10	0.10	0.11	0.12	0.13	0.17	0.36	0.45	0.73	0.60	0.29
18	2	0.10	0.10	0.10	0.11	0.12	0.13	0.16	0.35	0.46	0.73	0.60	0.28
19	1	0.18	0.17	0.17	0.15	0.16	0.16	0.17	0.28	0.22	0.41	0.75	0.22

19	2	0.18	0.16	0.17	0.15	0.16	0.15	0.17	0.27	0.22	0.40	0.79	0.21
20	1	0.00	0.04	0.02	0.00	0.02	0.02	0.03	0.17	0.00	0.95	0.61	0.07
20	2	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.15	0.00	0.98	0.47	0.06
21	1	0.28	0.26	0.29	0.29	0.28	0.32	0.36	0.70	0.55	0.59	0.86	0.94
21	2	0.26	0.26	0.28	0.27	0.26	0.32	0.36	0.71	0.56	0.59	0.85	0.93
22	1	0.10	0.10	0.11	0.11	0.11	0.11	0.15	0.60	0.40	0.51	0.39	0.21
22	2	0.10	0.10	0.11	0.11	0.11	0.12	0.13	0.59	0.43	0.50	0.38	0.20
23	1	0.00	0.00	0.00	0.00	0.02	0.04	0.15	0.99	0.60	0.71	1.00	0.20
23	2	0.00	0.00	0.00	0.00	0.01	0.03	0.10	1.00	0.66	0.74	1.00	0.21
24	1	0.45	0.63	0.84	0.96	1.00	1.00	1.00	0.41	0.57	0.80	0.50	0.06
24	2	0.47	0.71	0.82	0.84	1.00	1.00	1.00	0.41	0.56	0.81	0.55	0.07
25	1	0.56	0.88	1.00	0.92	0.90	0.88	0.79	0.36	0.43	0.38	0.65	0.10
25	2	0.53	0.93	1.00	1.00	0.92	0.91	0.86	0.36	0.43	0.37	0.70	0.10
26	1	0.45	0.77	0.79	0.78	0.80	0.82	0.69	0.29	0.41	0.40	0.60	0.25
26	2	0.44	0.73	0.86	0.80	0.82	0.85	0.73	0.28	0.42	0.40	0.49	0.27
27	1	0.21	0.24	0.20	0.18	0.24	0.25	0.26	0.42	0.35	0.83	0.47	0.18
27	2	0.18	0.22	0.18	0.16	0.23	0.22	0.24	0.41	0.36	0.84	0.46	0.20
28	1	0.11	0.13	0.15	0.17	0.19	0.20	0.27	0.49	0.53	0.62	0.86	1.00
28	2	0.10	0.13	0.15	0.16	0.18	0.20	0.26	0.49	0.53	0.64	0.87	1.00
29	1	0.11	0.12	0.14	0.17	0.18	0.26	0.39	0.62	0.92	0.38	1.00	1.00
29	2	0.08	0.12	0.11	0.15	0.19	0.23	0.37	0.63	0.87	0.40	1.00	1.00
30	1	0.04	0.03	0.02	0.04	0.04	0.06	0.10	0.29	0.42	0.40	0.90	1.00
30	2	0.04	0.03	0.02	0.02	0.05	0.05	0.08	0.29	0.42	0.39	0.89	1.00
31	1	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.20	0.35	0.37	1.00	0.98
31	2	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.16	0.28	0.37	1.00	1.00
32	1	0.06	0.07	0.06	0.10	0.06	0.11	0.18	0.23	0.30	0.49	0.08	0.09
32	2	0.09	0.10	0.08	0.09	0.08	0.13	0.13	0.23	0.29	0.57	0.09	0.08
33	1	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.21	0.26	0.41	0.69	0.00
33	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.47	0.29	0.60	0.00
34	1	0.11	0.12	0.12	0.14	0.13	0.15	0.19	0.37	0.45	0.49	0.59	0.62
34	2	0.12	0.12	0.12	0.14	0.14	0.15	0.19	0.37	0.44	0.49	0.58	0.61
35	1	0.01	0.02	0.02	0.02	0.03	0.04	0.07	0.32	0.47	0.48	0.66	0.06
35	2	0.01	0.00	0.02	0.03	0.03	0.04	0.07	0.32	0.44	0.49	0.63	0.07
36	1	0.06	0.04	0.05	0.07	0.07	0.06	0.10	0.29	0.40	0.42	0.54	0.59
36	2	0.04	0.07	0.05	0.07	0.07	0.10	0.10	0.27	0.36	0.41	0.53	0.56
37	1	0.00	0.01	0.02	0.03	0.04	0.05	0.12	0.40	0.45	0.44	0.58	0.59
37	2	0.00	0.00	0.01	0.02	0.03	0.05	0.11	0.34	0.44	0.42	0.57	0.57
38	1	0.10	0.10	0.12	0.13	0.12	0.13	0.17	0.44	0.55	0.59	0.47	0.32
38	2	0.10	0.10	0.11	0.11	0.11	0.14	0.17	0.43	0.54	0.57	0.49	0.26
39	1	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.45	0.67	0.63	0.21	0.00
39	2	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.47	0.67	0.64	0.20	0.00
40	1	0.00	0.00	0.00	0.00	0.02	0.02	0.08	0.29	0.42	0.49	0.58	0.55
40	2	0.00	0.00	0.00	0.00	0.01	0.02	0.07	0.28	0.41	0.49	0.58	0.55

41	1	0.02	0.01	0.02	0.02	0.02	0.04	0.07	0.26	0.35	0.42	0.77	0.97
41	2	0.01	0.00	0.01	0.02	0.01	0.03	0.06	0.25	0.35	0.42	0.77	1.00

Highlights

- Accurate measurements of pure oxy-, deoxy- and metmyoglobin are needed
- Myoglobin forms in beef made with chemicals, or partial oxygen pressure adjustment
- Data at specific wavelengths, or the 400-1100 nm spectrum combined with multivariate regression used
-Calculation of myoglobin states; same prediction error for all myoglobin states
- Myoglobin prediction was greatly improved using the all wavelengths method

New procedure for improving precision and accuracy of instrumental color measurements of beef

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Abstract

The surface layers of steaks from bovine *M. semimembranosus* were prepared to have deoxy- (DMb), oxy- (OMb) and metmyoglobin (MMb) states using either chemicals (CHEM) or oxygen partial pressure packaging (OPP). Ninety-six samples for each method were measured in reflectance mode (400-1100 nm). The reflectance spectra were transformed in different ways by using: Kubelka-Munk transformation (K/S), absorbance (A) and extended multiplicative scatter correction (EMSC). Transformed spectra of prepared pure states were used to make calibration and validation models of MMb, DMb and OMb using either selected wavelengths (SW) or partial least square (PLS) regression. Finally, the predicted myoglobin states were normalized to ensure that the sum of states was one and had no negative values or values larger than one. The CHEM(K/S) SW and the OPP EMSC(A) PLS methods predicted MMb, DMb and OMb with root-mean-square errors of cross validation (RMSECV) equal to 0.08, 0.16 and 0.18 (range 0-1) and 0.04, 0.04 and 0.04 (range 0-1), respectively. Multivariate calibrations (i.e. PLS) outperformed the univariate calibrations (*i.e.* SW). The OPP method of preparation of pure states gave clearly better prediction results compared to the CHEM method for the states DMb and OMb of fresh meat surfaces.

Keywords: Myoglobin state, Reflectance, Kubelka-Munk transformation, Partial least square regression, Extended multiplicative scatter corrections.

1. Introduction

Meat color is an important quality variable that affects consumers' willingness to purchase and re-purchase meat. The importance of meat color has called for extensive research into the mechanism of color stability. Oxygen consumption rate and metmyoglobin reductase activity (MRA) are assumed to be the two most important endogenous factors that determine the shift between myoglobin states and meat surface color (Bekhit & Faustman, 2005; McKenna et al., 2005; Renerre & Labas, 1987).

In order to understand the effect of additives to meat, packaging variables and variation in endogenous compounds to color stability, numerous color measurements are a necessity. CIE L*a*b* values (CIE, 1976) are often used to monitor meat surface color over time (Feldhusen, Warnatz, Erdmann, & Wenzel, 1995; Hopkins, Khliji, van de Ven, Lamb, & Lanza, 2010; Tapp, Yancey, & Apple, 2011). Also ratios between the reflectance obtained at selected wavelengths are used for monitoring fresh meat color changes R_{630nm}/R_{580nm} (American Meat Science Association, *i. e.* AMSA, 1991). The ratio gives an indication of the change in oxymyoglobin (OMb) and deoxymyoglobin (DMb) to metmyoglobin (MMb). The Kubelka-Munk transformation to K/S values (Kubelka, 1948), and specifically $K/S_{610nm} - K/S_{525nm}$ is used to measure the proportion of OMb on ground beef surfaces and to characterize discoloration (Mancini, Hunt, & Kropf, 2003). However, in order to calculate the three states of DMb, OMb and MMb from reflectance measurements in the visible range (400-700 nm), an instrumental calibration is recommended where pure states (*i .e*. DMb, OMb and MMb) are produced and measured with the type of sample, packaging material and instrumentation to be used in the main experiment. This means that all laboratories doing these types of measurements should set up their own calibration before they can calculate the states of myoglobin of unknown samples.

Adding the chemicals sodium dithionite and potassium ferricyanide (Wilson, Ginger, Schweigert and Aunan, 1959) is known to produce DMb and MMb, respectively. OMb is readily formed by flushing the surface DMb of meat with 100% oxygen. These three methods, called Chemically Induced Myoglobin States (CHEM), are all well established for measurements of myoglobin states (AMSA, 1991). It might also be possible that these solvents could dilute the meat surface and thereby give a surface different from the samples to be tested later. Alternatively, meat with DMb, OMb and MMb can be produced with modified atmosphere or vacuum packaging by adjusting the partial pressure of oxygen (O_2) to ~zero, low or high concentrations, respectively (Taylor, Down, & Shaw, 1990; Hunt, Sørheim, & Slinde 1999). The latter group of packaging methods will be called Partial Oxygen Pressure Packaging (OPP).

Specific wavelengths are used for calculations of the three different states of myoglobin (Krzywicki, 1979; AMSA, 1991); here called the selected wavelength (SW) method. Conversion to Kubelka-Munk K/S values is recommended to improve on linearity and scattering disturbances before assessing the relative content of OMb and MMb at the surface of beef (Francis & Clydesdale, 1975).

Krzywicki (1982) has published an alternative method to assess the relative fractions of DMb, OMb and MMb based on the variable log(1/R) (R is reflectance) of incident light at 572, 525, 473 and 730nm. The latter wavelength is used as an objective measure of the sample's lightness and scattering. This method is not flexible since specific wavelengths are defined and the method uses molar absorbance coefficients for myoglobin, which may differ among different animal species. Recommending specific wavelengths for all systems is intuitively limiting as light scattering, packaging material and in particular instrument wavelength calibration may call for a

"softer" mathematical approach. In principle we question the existence of true isobestic points in meat systems where we do not know the path of light.

The method of partial least squares regression (PLS) (Martens and Næs, 1989) finds fundamental relations between two matrices (*spectra* and *myoglobin redox state*). A PLS model will try to find the multidimensional direction in the *X* (spectra) space that explains the maximum multidimensional variance direction in the *Y (myoglobin state)* space. PLS regression is particularly well suited when the matrix of predictors has more variables than observations, and when there is multi co-linearity among *X* values. This also means that the inherent nature of this regression method is to pick the wavelengths of the spectra that will predict the myoglobin states with the lowest error.

Reflectance spectra are also frequently transformed to improve on linearity and reduce the contribution from scattering and in essence to make the spectra look more like transmission spectra. The Kubelka-Munk transformation (Kubelka, 1948) is frequently used for this purpose. Another method (Martens and Stark, 1991) to handle diffuse reflectance spectra is called extended multiplicative scattering (EMSC) and is used to isolate and remove complicated multiplicative and additive effects, such as those caused by light scattering in reflectance spectroscopy.

The aim of this study was to determine a calibration model that would predict all myoglobin states on the surface of the samples under investigation *i.e.* bovine *M. semimembranosus* steaks. Since the DMb, OMb and MMb states can be formed by adjusting the partial oxygen pressure in the headspace, the aim was to compare the traditional CHEM method regarding preparation of different states with the use of specific packaging techniques named the OPP method. We also compared two spectral transforming techniques: the K/S transformation and the EMSC method.

Finally we compared the calculation principles using SW as explained by AMSA (1991) with the multivariate calibration method called PLS regression. The purpose of the work was simply to identify which methodological approach would give the lowest prediction errors for all three myoglobin states.

2. Materials and Methods

2.1 *Raw material*

2.1.1 Calibration and chemically treated samples

Fresh (3 days *post mortem*) vacuum packed beef *M. Semimembranosus* (SM) was obtained from a local slaughter house (Fatland, Oslo, Norway). The carcass was deboned, and the muscles were collected and kept on ice or at 4°C until used the next day, *i.e*. the 4th day *post mortem*. A specific breed or age group was not requested. Muscles (one side) from three different animals were collected and the samples were distributed among the states to be prepared. Each muscle was cut into 8 steaks of approximately identical size and of at least 1.5 cm thickness and further subdivided randomly for the further preparation. True replicates were secured. The steaks were of sufficient size for the optical probe to be placed in four positions on the meat surface. The steaks were cut parallel to the fiber direction. The SM typically has ultimate pH 5.6 measured on proximal sides. The actual pH varies, however, across the muscle.

2.1.2 Additional samples

Six fresh beef *SM* were obtained from a local slaughter house (Nortura, Rudshøgda, Norway). These samples were vacuum-packed and stored for three weeks at 4°C. The pH of samples varied from 5.44 to 5.78. The sample size and fiber directions were the same as in the calibration and chemically treated samples described below. Immediately after removal (0.5 to 1 min) from the vacuum bags (type polyethylene/polyamide (PE/PA), oxygen transmission rate (OTR) of 30-40 $cm^3m^{-2}24$ h^{-1} at 23 ºC and 75 % RH, LogiCon Nordic A/S, Kolding Denmark), the steaks were wrapped with low density polyethylene (LDPE) film (OTR 6500-8500 $cm^3m^{-2}24$ h^{-1} at 23 ºC and 75 % RH, Toppits-glad, Melitta Group, Klippan, Sweden) and kept in high density polyethylene black trays (HDPE, Dyno 516, SWF Companies, Reedley, USA) up to 7 days. The gas to meat ratio was approximately 1: 30. The samples were analyzed for changes in myoglobin state on the surface due to air and light (see below) exposure. The test set of 6 additional samples for prediction were randomly picked from a larger set of 41 samples.

2.2 Preparation of muscle samples having defined myoglobin states using the CHEM and OPP methods for calibration and chemically treated samples

2.2.1 CHEM method

The CHEM method of AMSA (1991) with minor changes was used for preparation of different myoglobin states:

OMb: The steaks were placed in PE/PA bags with OTR of 30-40 $cm^3m^{-2}24$ h^{-1}at 23 ºC and 75 % RH (Maskegruppen, Vinterbro, Norway) on ice (meat surface at 2°C) and were flushed with 100% oxygen for 10 minutes. The meat was then wrapped in LDPE film (Toppits-glad, Melitta Group) before spectroscopic measurements.

MMb: The steaks were treated with 1% potassium ferricyanide (Merck Eurolab) for 1 minute, drained, blotted dry, packed in LPDE film (Toppits-glad, Melitta group, Sweden) to oxidize at 2-4°C for 12 hours and measured.

DMb: The steaks were treated with 10% sodium dithionite (BDH, Prolab, Lutterworth, UK) for 2 min, drained, blotted dry and vacuum packed using the Original Henkelman Vacuum 300 system (vacuum strength 9, type 300II, Hertogenbosch, The Netherlands) in PE/PA bags (Maskegruppen) to reduce for 2 hours at room temperature. The meat was then immediately wrapped in LDPE (Toppits-glad, Melitta group) and measured for surface color as specified by AMSA (1991).

2.2.2 OPP method

The OPP method for preparation of different myoglobin states used different packaging principles to obtain the different states. Steaks for OMb and MMb were packed on a Polimoon 511VG tray sealing machine (Promens, Kopavogur, Iceland). The trays were made of amorphous polyethylene terephthalate trays (Wipak Mulipet) with ethylene vinyl alcohol top films (Wipak Biaxer) (both Wipak Oy, Natsola, Finland). OTR for the tray and top film were 7 and 5 $cm^3m^{-2}24\ h^{-1}$ at 23 °C at 50 % RH, respectively.

OMb: The trays were flushed with 75 % O_2/ 25 % CO_2 (preblend from AGA, Oslo, Norway). Measurements were taken the next day (24 ± 2 hrs).

MMb: The packages for MMb were flushed with 60 % CO_2/ 40 % N_2 (preblend from AGA, Oslo, Norway). O_2 levels of 1.5 % for induction of MMb in the packages were obtained by inserting syringes with air through self sealing septas (Toray TO 125, Toray Engineering, Osaka, Japan). Color measurements were made on different time intervals/days until day7 (see below).

DMb: *M. semimembranous* muscle steaks were vacuum packed in PE/PA bags (Maskegruppen) and stored at 2-4°C for 48±2 hours before taking color measurements.

2.3 Color spectrum measurements

2.3.1 Calibration and chemically treated samples

Areas for spectroscopic measurements were selected to minimize the influence of fat and larger connective tissues. All the steaks were covered with LDPE film (Toppits-glad, Melitta Group) and were in direct contact with the film only when the probe was placed on the steak (otherwise film was not directly in contact and was 2-3 cm above the meat surface). Steaks were kept in high density polyethylene (Dyno 516, SWF Companies, Reedley, USA) black trays. The steaks were measured at 17 ±1°C surface temperature.

FOSS NIRSystems (Model 6500, 0654-Oslo, transflectance modus with a 40 x 40mm^2 optiprobe ^{TM} system, 23 X 23mm sensor area) was used for the color measurements. Absorbance (A= log (1/R)) was calculated between 400-1100nm in 2nm steps giving 350 spectral variables. The instrument was calibrated using a white ceramic plate (from Minolta measurements: L*=101.01, a*= 1.74 and b*=5.3) as a reference before all meat measurements. Each spectral measurement is an average of 32 scans, and takes about 20 secs. For each calibration sample, four random readings at different locations on the surface were taken and were averaged. Finally, for each myoglobin state (DMb, OMb and MMb) and preparation method (CHEM and OPP) there were 32 (1 state x 4 repeated measurements x 8 different steaks) spectra. Each of the CHEM and the OPP method consisted of altogether 96 (3 states x 4 repeated measurements x 8 steaks) spectra.

2.3.2 Additional samples

Two random readings at different locations on the surface per samples were taken at 17°C (steaks had surface temperature of 17°C for less than 5 minutes) and were averaged for the test set. The readings of surface colour were taken at time 0 and further after 0.25, 0.5, 0.75, 1, 1.5, 4, 24, 48, 72, 142 and 167 hours. The steaks were illuminated in a constant temperature cooler at 4°C under a Phillips Master TL-D 36W/830 H9 tube (color code 3000K) attached to tube-holder Phillips IKC 1/36, 1x 6 W-K with light intensity of 800 lx between measurement times.

2.4 *Processing of spectral data and statistical analysis*

2.4.1 Calibration and chemically treated samples

AMSA (1991) formulas using selected wavelength (SW): Reflectance data at selected wavelengths, which are isobestic for two or three myoglobin states were used (AMSA, 1991). Absorbance spectra were first converted to reflectance and then to the Kubelka-Munk ratio K/S using $K/S= (1-R)^2 /2R$ where R is the absolute reflectance, in The Unscrambler software version 9.7 (CAMO software AS, Oslo, Norway).

The data at wavelengths 474nm, 525nm, 572nm and 610nm were saved (Kryzwicki, 1979; AMSA, 1991). These data were exported to Microsoft Excel, Version 2007 (Redmond, Washington: Microsoft, Computer software) and were used to calculate percentage of the different myoglobin states using the SW formulas given by AMSA (1991). From the calibration data, the average values at SW were obtained for each myoglobin states. Myoglobin states could be calculated using two SW formulas, as shown below (for OMb):

$$OMb_{DMb} = \frac{\left(\frac{K/S_{610}}{K/S_{525}\,cal,avg,DMb}\right)-\left(\frac{K/S_{610}}{K/S_{525}\,sample}\right)}{\left(\frac{K/S_{610}}{K/S_{525}\,cal,avg,DMb}\right)-\left(\frac{K/S_{610}}{K/S_{525}\,cal,avg,OMb}\right)} \qquad \text{(eq. 1)}$$

$$OMb_{MMb} = \frac{\left(\frac{K/S_{610}}{K/S_{525}\,cal,avg,MMb}\right)-\left(\frac{K/S_{610}}{K/S_{525}\,sample}\right)}{\left(\frac{K/S_{610}}{K/S_{525}\,cal,avg,MMb}\right)-\left(\frac{K/S_{610}}{K/S_{525}\,cal,avg,OMb}\right)} \qquad \text{(eq. 2)}$$

The SW method uses two formulas for measuring the myoglobin states of the same sample. For example, as shown above, OMb can be calculated using calibration data from MMb and DMb states. Both formulas were used by us to calculate the myoglobin states. The formula giving the lowest RMSCEV (root mean square error of cross validation) and the higher R_c (correlation coefficient) were selected.

Partial least square (PLS) regressions: First all the spectral data in absorbance form were run through PLS regression with cross validation, number of PLS factors=10, systematic validation with 4 samples (the repeated measurements) per segment for each myoglobin forms. The result was saved and number of PLS factors was recorded. Secondly, the spectral data were treated by using extended multiplicative signal correction (EMSC) (Martens and Stark, 1991). The EMSC was performed using model and subtract options of the software and then run through PLS regression as previously done. The EMSC models were saved for later use in predicting states of additional samples. R_c and RMSECV from each state were used as criteria for best fit.

The number of PLS factors was listed in columns with their scores for each particular myoglobin state in The Unscrambler software version 9.7 (CAMO software AS, Oslo, Norway) data spreadsheet. Each specific data column belonging to a specific myoglobin state was exported to Microsoft Excel, Version 2007 (Redmond, Washington: Microsoft, Computer software) from the Unscrambler result list. All the data of three myoglobin states were post transformed in Microsoft Excel, Version 2007. Details regarding the processing of data are given in Figure 1 and Table 1.

2.4.2 Additional samples

After selecting the calibration method with the lowest prediction error, the myoglobin states of the additional samples were calculated using the predictive model. We compared the OPP method of sample preparation that used the EMSC model followed by PLS regression (OPP EMSC(A) PLS), where A is the absorbance and the CHEM method using the SW as described in AMSA (1991). Since there were two random replicated readings at each time for each additional sample, the predicted outcome of the spectra was averaged.

Finally, the data of calibration and chemically treated samples and additional samples were normalized and further corrected. This transformation after calculation of states consisted of three steps: (1) $\hat{y}$ >1, corrected to $\hat{y}$=1; (2) $\hat{y}$<0, corrected to $\hat{y}$=0; and (3) $\hat{y}_{OMb}+\hat{y}_{MMb}+\hat{y}_{DMb}=1$.

3. Results and Discussion

253 *3.1 Comparison of spectra and PCA plots from OPP and CHEM methods*

254 Spectra in Figure 2 a-b show the distinct spectral patterns of the three prepared myoglobin states
255 using the two preparation methods (OPP and CHEM). The EMSC adjusted the replicates for
256 both preparation methods*; i.e.* after EMSC the replicates of the three myoglobin states showed
257 less deviation (Figure 2 c-d). The difference between the two preparation methods of states also
258 became less apparent after EMSC. This result is in agreement with the purpose of this
259 transformation method (Gallagher, Blake, & Gassman, 2005). The CHEM method gave less
260 precise replicates for OMb and DMb states, and the spectra were overlapping at most
261 wavelengths (Figure 2c) between 500-900nm whereas in Figure 2d, there are substantial
262 differences ≥700nm. The guidelines of the CHEM method also inform the user that maintaining
263 a pure DMb state in the meat surface is difficult (AMSA, 1991). The DMb on the surface will
264 rapidly pick up oxygen and become transformed into OMb. Thus the intended pure DMb will be
265 a mixture of DMb and OMb and its spectra become more similar to that of OMb.
266
267 Figure 3 shows how the two methods used separated between different states of myoglobin in a
268 PCA plot. Figure 3a (CHEM method with K/S spectroscopic transformation) shows that all three
269 myoglobin states were not clearly separated from each other. OMb and in particular the DMb
270 state were neither well reproduced nor well separated in the PCA plot. However, the three
271 different states of myoglobin were well separated by the OPP method with EMSC of spectra into
272 three clusters, indicating that each state had spectra that made them unique (Figure 3b).
273
274 *3.2 Regression comparisons*

3.2.1 CHEM versus OPP

As shown in Table 1, modelling with data according to the CHEM method followed by spectroscopic K/S transformation using SW (called CHEM (K/S) SW) gave RMSECV equal to 0.18, 0.16 and 0.08 for the DMb, OMb and MMb states, respectively. Ledward (1970) determined MMb of muscle using CHEM (K/S) SW and reported an error between (±)0.05-0.07. He indicated a comparable error (±0.05) for DMb. This is contradictory to the large error reported here for DMb. It appears that his DMb sample could have been kept under nitrogen gas by Ledward (1970) after dithionite reduction until spectral measurements. He also used 20% dithionite of unknown incubation length for reduction of myoglobin. The AMSA guidelines (AMSA, 1991) using the CHEM (K/S) SW method do not indicate an expected error for any state, but the guidelines caution about the difficulties that will be experienced for the DMb state. Table 1 indicated that OMb was not very accurately determined. However, when using transformation to K/S values for the OPP method followed by using SW (*i.e.* OPP (K/S) SW) RMSECV was 0.26 for DMb and 0.12 and 0.28 for OMb and MMb, respectively. Actually this suggested that the OPP method was a better preparation option than the CHEM method only for OMb. This was probably due to the fact that the incubation time was longer than the time suggested by the AMSA guidelines and thus a thicker OMb layer was formed. The result also indicated that both preparation methods used for DMb needed improvement. In our case, the repackaging with oxygen permeable film was a step that introduced oxygenation. It could be better to prepare DMb without repackaging.

3.2.2 Transformation of the spectra before calculation of myoglobin states

Combining EMSC with spectra transformed to K/S values (EMSC(A) (K/S) SW) was not an efficient transformation as all RMSECV increased (Table 1).When specific wavelengths were not used for predicting myoglobin states as in PLS regression, the results were very different (Table 1). The CHEM method (CHEM PLS(A)) now gave RMSECV equal to 0.08, 0.08 and 0.04 for DMb, OMb and MMb (range 0-1), respectively. The OPP method (OPP PLS(A)) gave RMSECV equal to 0.06, 0.07 and 0.07 for DMb, OMb and MMb, respectively. This suggested that restricting the calculation of DMb, OMb and MMb to specific wavelengths actually doubled the prediction error compared to the values stated for PLS in Table 1. Scatter correction was an adequate transformation before calculation of states.

3.2.3 SW versus whole spectra with PLS regression

Removing the wavelength constraints largely eliminated the difference between the two preparation methods except for the preparation method of MMb prepared as suggested by AMSA Guideline (AMSA, 1991); using CHEM (ferricyanide) seemed preferable to using OPP (low oxygen packaging). It is important to stress that the lower error obtained using PLS regression makes it more relevant also to actually predict DMb and OMb in experiments. When we included EMSC(A) before the PLS regression (PLS EMSC(A)); the CHEM method gave RMSECV equal to 0.08, 0.08 and 0.03 and the OPP method gave 0.04, 0.04 and 0.04 for DMb, OMb and MMb, respectively. Surprisingly only the prediction of MMb clearly improved following EMSC(A) added to the CHEM method (from 0.041 to 0.029; Table 1). The OPP method now gave the same low RMSECV for all forms. It should be pointed out that an RMSECV of 0.04 means that 95% of the samples will be predicted within an error of ±2 RMSECV, *i.e.* ±0.08. This is, however, a vast improvement from the errors of ±2 x 0.18 (DMb);

±2 x 0.16 (OMb) or ±2 x 0.08 (MMb) obtainable by using transformation to K/S values and restricting the analysis to specific wavelengths.

The OPP method of sample preparation that used EMSC transformation along with PLS regression (OPP EMSC(A) PLS) gave best correlation and low RMSECV for all three forms of myoglobin. However, the spectra from CHEM preparation with revised transformation using EMSC and PLS regression instead of K/S and SW gave slightly better result for MMb, although the difference may not be significant. The fact that the OPP method gave good predictions for DMb, it should be mentioned that the time allocated for myoglobin reduction (48 hrs) may not always be optimal.

3.3 Illustration of the predictability of OMb for two models

All the data predicted for DMb, OMb, and MMb states were transformed so they added up to one. This ultimate transformation, numerically adjusted all states so the sum of three states of myoglobin always equalled one. Similarly, true OMb class, the experimentally attempted pure OMb state of the three states was defined as 1. The true OMb class was defined as zero when either pure DMb or MMb was attempted. Figure 4a gives the relationship between the prepared fraction of the OMb state and the correspondingly predicted value using the CHEM (K/S) SW method (*i.e.* as suggested by AMSA, 1991) for calculating states. The data shows higher deviation, compared to the OPP EMSC(A) PLS method (Figure 4b). This was apparent when OMb was 0, meaning the condition where DMb or MMb prevailed. In that case the more extreme predictions were that the fraction of the OMb state was above 0.4. This result was nevertheless in agreement with Table 1 that suggested an RMSECV of 0.16 for OMb. With such

a magnitude for RMSECV, values above 0.3 will sometimes be predicted since 1% of the prediction will be 3 RMSCEV(0±3x.0.16) away from the true value. Deoxymyoglobin was however, the state predicted with the most samples > 105% deoxymyoglobin, or as a state with the most samples < 0.95, for an assumingly pure state. Minimum and maximum deviation from 1 was 0.79-1.10 for deoxymyoglobin, respectively.

3.4 Prediction of myoglobin in additional samples using CHEM and OPP methods

The model with lowest prediction error (OPP EMSC(A) PLS) was used here to demonstrate the predictability of additional samples(can also be called as test set). In addition, a comparison with the predictions from CHEM (K/S) SW model (Figure 5) was made. Figure 5a and 5b show, as expected, that there was some resemblance between MMb fraction as determined by the CHEM and the OPP method. The highest fraction of MMb state was in both cases 0.7 (Figure 5a and 5b for one sample). However, during the first observation hours the CHEM (K/S) SW model predicted that the steak contained 20-30% MMb (Figure 5a), while the OPP EMSC(A) PLS model suggested that the MMb content was 5% (Figure 5b). This relatively large absolute difference observed for one sample (Figure 5 a and b) regarding MMb fraction still remained for six samples (Figure 5c and d), but the relative changes were fairly similar. The six average samples might give time change in redox states with respect to myoglobin redox state stability however, that might not be the case for one single sample. Therefore, the reason for using one and then six sample set was to demonstrate the robustness of the prediction.The difference that we observed here for MMb fractions is quite possible since the difference in RMSECV of the OPP EMSC(A) PLS model was 0.04 (the R_c= 0.997) for all states while the CHEM (K/S) SW model predicted MMb with an RMSECV of 0.079 (the R_c= 0.993) (Table 1). In addition, the normalization to one of all states will introduce some further difference since DMb and OMb

were not well predicted by the CHEM (K/S) SW method. The OPP method gave an expected gradual reduction in DMb (Figure 5b and 5d) just after the steak was taken out from the vacuum packaging and an expected equally gradual increase in OMb during the first few hours of atmosphere exposure. Figure 5b also demonstrated why it is difficult to measure DMb if repackaging is done; since changes in myoglobin state took place within few minutes. There were some resemblences regarding changes in OMb with time for the two methods, while the changes in DMb with time were completely different. The fact that the prediction obtained from the OPP EMSC(A) PLS seemed in accordance with expectations concerning myoglobin behaviour with atmosphere exposure indicated that OPP EMSC(A) PLS was a better method for calculating myoglobin states than the CHEM (K/S) SW method. The increase in DMb towards the end of the storage period under air was due to growth of aerobic bacteria (results not shown).

3.5 Regression coefficients

The magnitudes of the regression coefficients given in Figure 6 showed very different features for the three different myoglobin states. This meant, as expected, that the three different states had information in different parts of the spectra. However, Figure 6 also clearly illustrated, that it was not straight forward to pin-point four different wavelengths that were characteristic for all states. It seemed that more wavelengths could be needed, *i. e.* the entire spectrum could be needed in order to get a good regression model. Finally, we do not know if the minimum wavelengths that we would pick for prediction from our dataset would be identical to those picked in a future attempt on a slightly different dataset with other scattering properties and larger variability in myoglobin levels. Thus we think that our EMSC(A) PLS approach using all

important parts of the spectrum for prediction will be more robust if a low standard error of prediction is the goal.

3.6. Practical implications

The calculations made here require access to statistical computer programs and also some experience in data handling. However, when there is a need for better accuracy in amounts of DMb and OMb levels in the sample, instead of obtaining just their combined amounts, the more elaborate procedure used here is recommendable. In addition, our calculation principle also seemed to give relevant prediction errors for DMb despite the fact that preparing pure DMb is difficult. Our general recommendation is, however, to use the OPP method (metmyoglobin from the CHEM method could be an exception) for preparing the three states of myoglobin in beef, and to apply EMSC(A) to absorbance spectra in a multivariate regression method such as PLS.

4. Conclusion

The prediction error results for all the three states of myoglobin used for comparing the two physical preparation methods, clearly showed that OPP EMSC(A) PLS gave lower prediction errors compared to CHEM (K/S) SW. Extended multiplicative signal correction of the absorbance data gave lower prediction error compared to only using Kubelka-Munk (K/S) transformation. Finally, the use of all wavelengths (400-1100nm) and a multivariate regression method (PLS regression), gave clearly lower prediction errors compared to the use of the

406 selected wavelengths and the use of the formula given in the AMSA guidelines. No single
407 wavelengths could be identified from the PLS regression coefficient that are unique for the three
408 states of myoglobin. It seemed that the several regions in the whole spectra from 400-1100nm
409 contributed to the predictive ability for the three states of myoglobin.

410

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417

418

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Figure

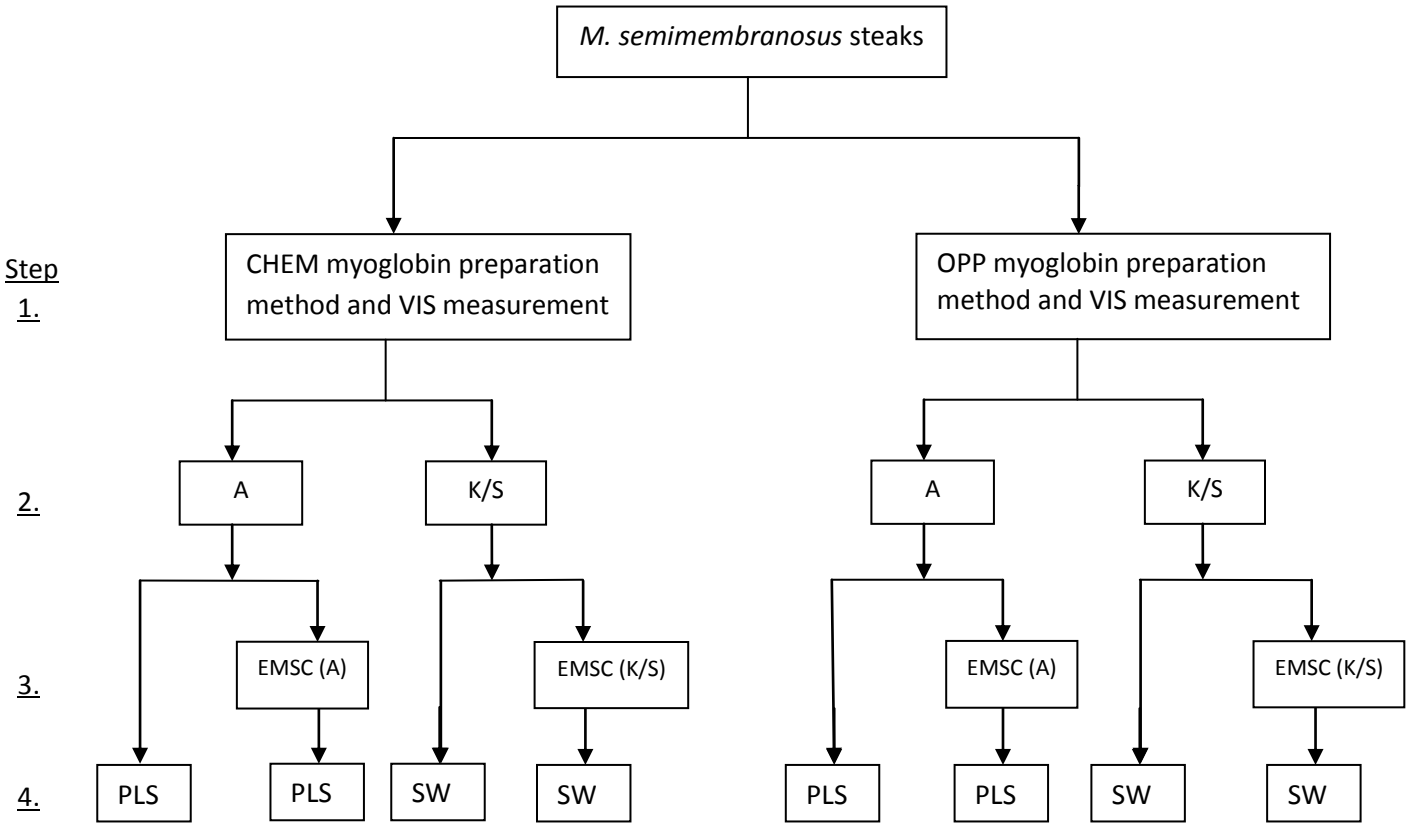

Figure 1

Figure

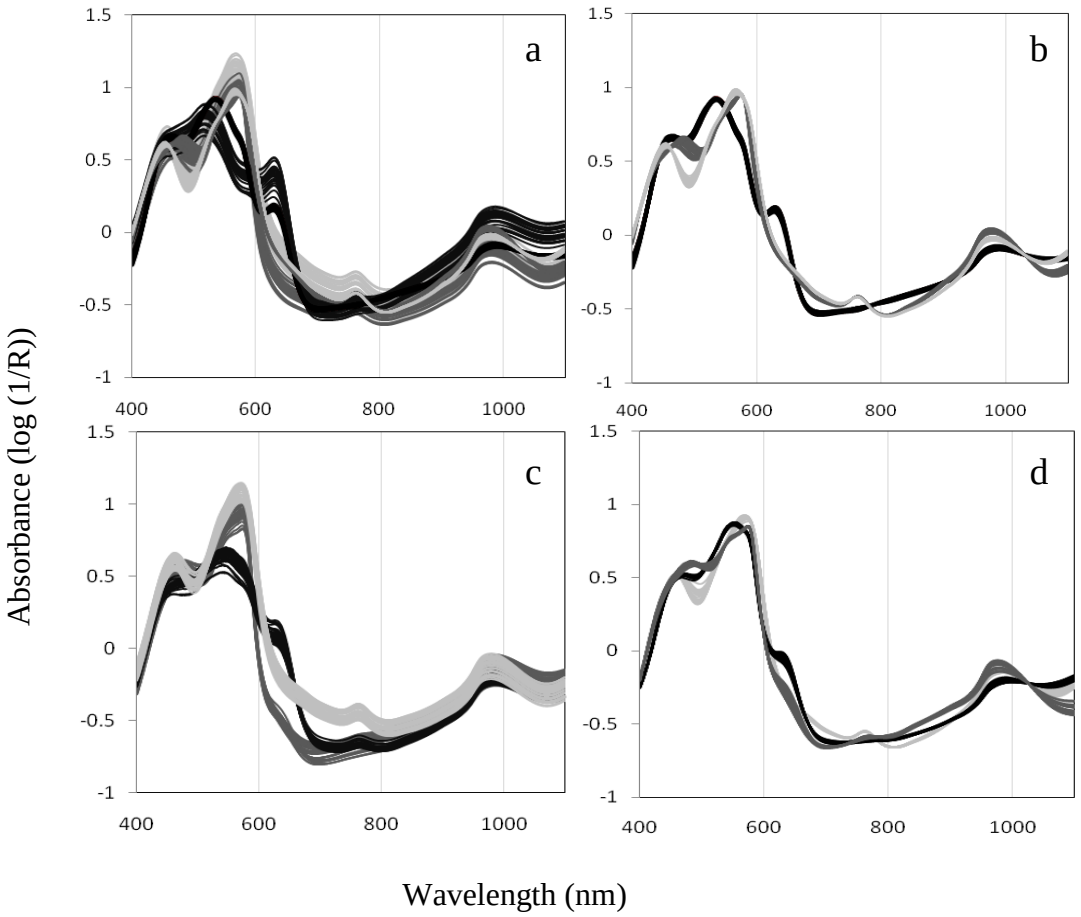

Figure 2

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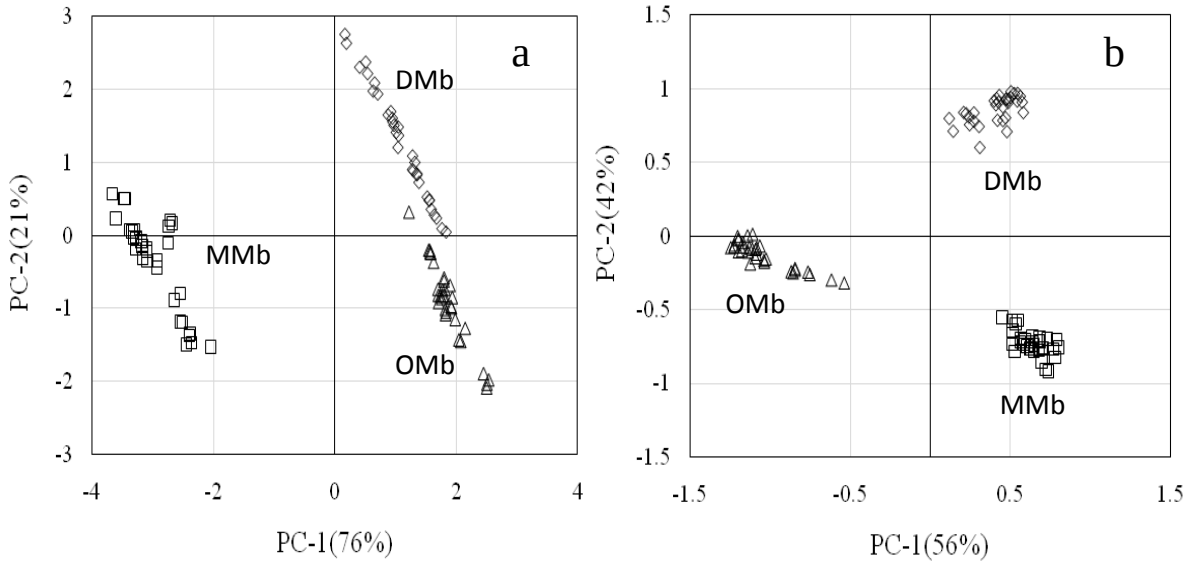

Figure 3

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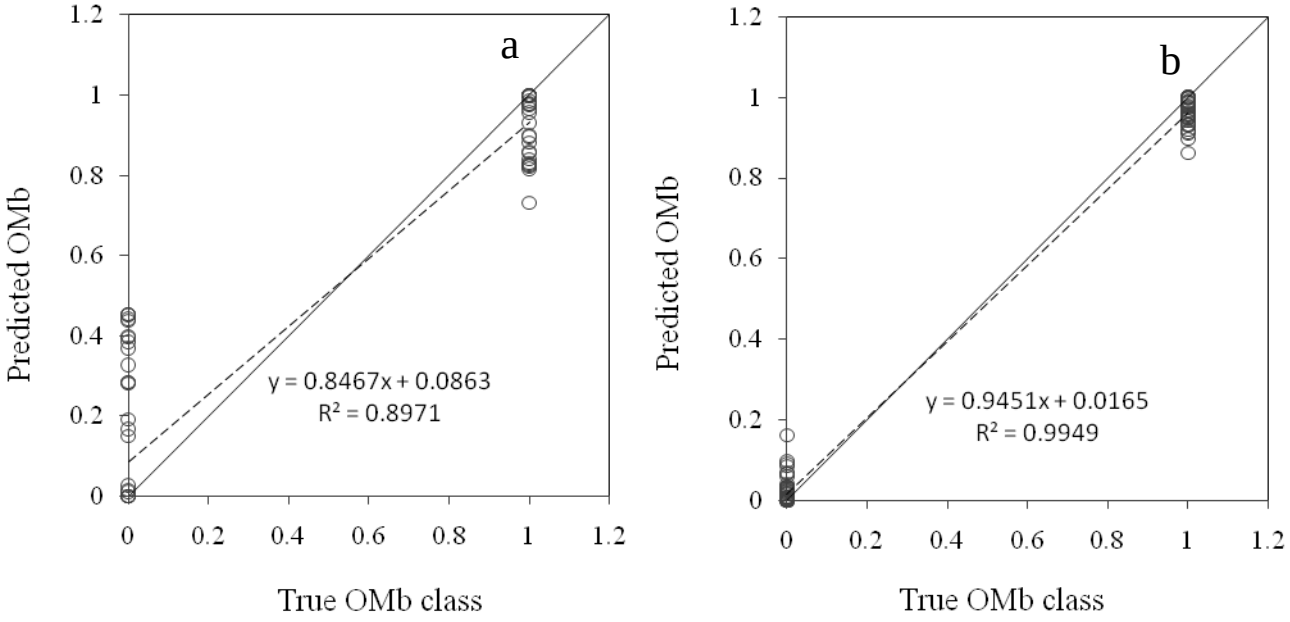

Figure 4

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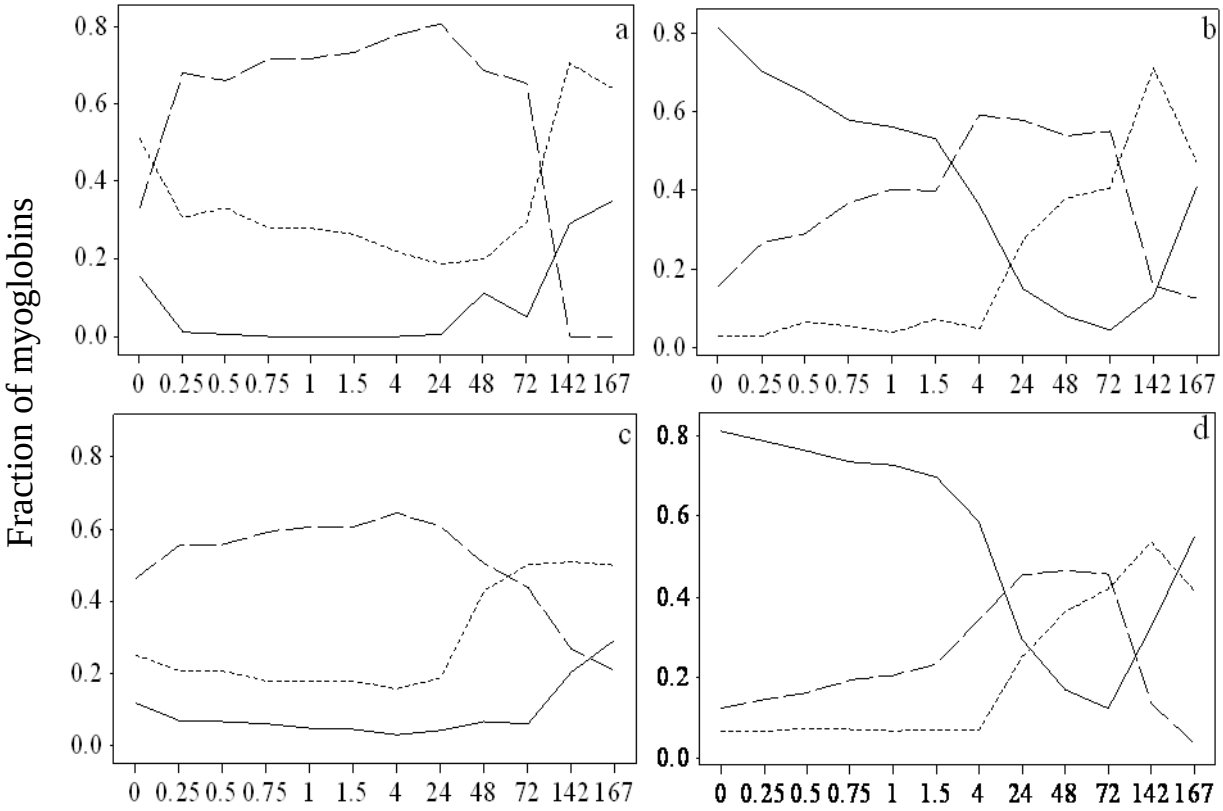

Figure 5

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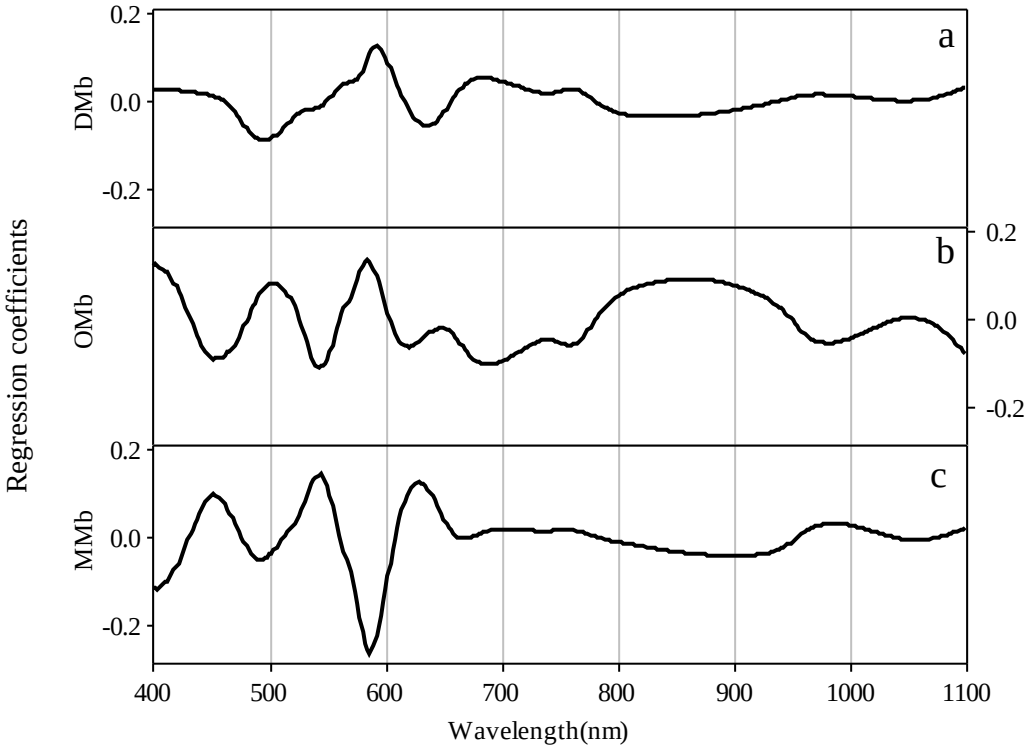

Figure 6

Figure captions

Figure 1. Flowchart showing the overall physical and statistical treatments done in calibration and chemically treated samples before normalization (for abbreviations see Materials and Methods): Step1-Physical treatment for sample preparation either by OPP or CHEM method with visual (VIS) measurements; Step 2-Spectra transformation: Use of either absorbance (A) data or conversion into Kubelka-Munk (K/S); Step 3-Pre-processing (with EMSC or no EMSC)/ with light scatter correction or no correction; Step 4-Calibration: Absorbance data are calibrated by applying PLS regression and K/S data by selected wavelength (SW) as suggested in AMSA guidelines.

Figure 2. The absorbance (A= log(1/R), where R is the reflectance) spectra of the three different myoglobin states of *M. semimembranosus*; a) without and b) with extended multiplicative scatter correction (EMSC), using the CHEM method of sample preparation**,** c) without and d) with EMSC using the OPP method for preparing samples**.** ▬ denotes 100%MMb treatment ▬ denotes 100% OMb treatment and ▬ denotes 100% DMb treatment. Each of the myoglobin states are presented by 32 spectra.

Figure 3. Principal component analysis of myoglobin states. Explained variance of the two components was 97% (a) and 98% (b). PC-1 is principal component one and PC-2 is principle component two; a) CHEM (chemically induced myoglobin states) treatments and K/S spectroscopic transformation, b) OPP treatments of the meat and extended multiplicative scatter correction (EMSC) transformation. ◊ DMb (Deoxymyoglobin); □ MMb(Metmyoglobin); Δ OMb (Oxymyoglobin).

Figure 4. Corrected predicted oxymyoglobin (OMb) versus true (as prepared) OMb state; a) CHEM (K/S) SW data for OMb (correlation coefficient R_c=0.947); b) OPP (EMSC(A) PLS) for OMb (R_c=0.997). The diagonal line illustrates the target line (x=y). The dotted line is the fitted regression line.

Figure 5. Predicted fraction of myoglobin states (—— deoxy myoglobin, - - - - oxymyoglobin and ····· metmyoglobin) versus storage time after vacuum packaged steaks were repackaged in low density polyethylene film. At each time point, reflectance was measured using two different

methods: a) prepared according to CHEM method with transformation to Kubelka-Munk (K/S) using selected wavelength (SW) (called CHEM (K/S) SW), one sample used; b) OPP preparation method with extensive multiplicative scattering on the absorance spectra (called EMSC(A)) before Partial least square (PLS) regression, *i.e*. OPP EMSC(A) PLS, one sample used; c) prepared as in 5a, average of 6 samples; d) prepared as in 5b, average of 6 samples.

Figure 6. Partial least square (PLS) regression coefficients versus wavelength obtained when myoglobin states were related to spectra using calibration samples prepared with the OPP method and EMSC(A). a) Deoxymyoglobin, number of PLS factors was 2; b) Oxymyoglobin, number of PLS factors was 3; c) Metmyoglobin, number of PLS factors was 3.

Table

***Table 1**. Prediction errors expressed as root mean square error of cross validation (RMSECV, in fractions) for the three different myoglobin states and the corrected linear correlation coefficient (R_c) between the true (prepared) state and predicted state. RMSECV and R_c refer to corrected predicted data (see the Material and Method section for details). Bold font indicates the most accurate predictions for each myoglobin state.*

Myoglobin states	Treatments			RMSECV	No. PLS factors	R_c
	Physical[1]	Spectra processing[2]	Calc. method (formula used)[3]			
DMb	OPP	A	PLS	0.060	3	0.994
		K/S	SW [OMb]	0.26	-	0.901
		EMSC(A)	**PLS**	**0.042**	2	**0.997**
		EMSC(K/S)	SW [OMb]	0.31	-	0.836
	CHEM	A	PLS	0.080	3	0.988
		K/S	SW [OMb]	0.18	-	0.960
		EMSC(A)	PLS	0.081	2	0.988
		EMSC(K/S)	SW [OMb]	0.21	-	0.932
OMb	OPP	A	PLS	0.072	1	0.992
		K/S	SW [DMb]	0.12	-	0.974
		EMSC(A)	**PLS**	**0.041**	3	**0.997**
		EMSC(K/S)	SW [MMb]	0.21	-	0.964
	CHEM	A	PLS	0.082	3	0.990
		K/S	SW [MMb]	0.16	-	0.947
		EMSC(A)	PLS	0.080	2	0.989
		EMSC(K/S)	SW [DMb]	0.21	-	0.924
MMb	OPP	A	PLS	0.069	3	0.996
		K/S	SW [DMb]	0.28	-	0.991
		EMSC(A)	PLS	0.039	3	0.997
		EMSC(K/S)	SW [DMb]	0.33	-	0.834
	CHEM	A	PLS	0.041	2	0.997
		K/S	SW [OMb]	0.079	-	0.993
		EMSC(A)	**PLS**	**0.029**	2	**0.998**
		EMSC(K/S)	SW [OMb]	0.39	-	0.928

1: How the beef was treated physically: Chemically Induced Myoglobin states (CHEM) and Oxygen Partial pressure Packaging (OPP).
2: Spectra transformed to: Absorbance (A), Kubelka-Munk (K/S), Extended Multiple Signal Corrected Absorbance (EMSC(A)) and Extended Multiple Signal Corrected Kubelka-Munk (EMSC(K/S)).
3. Calculation method used: Partial Least Square (PLS) and Selected Wavelengths (SW) according to AMSA 1991 guidelines [formula used se eq. 1 and 2 in the Material and method section above]. The formula was chosen to give the lowest prediction error.

Intended for Meat Sci.

Mitochondrial oxygen consumption in permeabilised fibers and its link to colour stability in bovine *M. semimembranosus* muscle

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Abstract

Slaughter and carcass variables were recorded for 41 cattle. *M. semimembranosus* was removed ~3.5 hours *post mortem*. The oxygen consumption rate (OCR) was measured early *post mortem* (EPM, ~ 4 hrs) and after 3 weeks' storage. Colour stability measurements were performed on stored slices of *M. semimembranosus* (0–167 hrs) exposed to air using reflectance spectra (400–1100nm) and the colour coordinates L*, a* and b*.

Significant individual EPM differences in OCR were found for mitochondrial complexes I and II. OCR_{EPM} of complex I declined with increased temperature and time, while residual oxygen-consuming side reactions (ROX) increased *post mortem*. OCR of stored muscles was dominated by complex II respiration. A three-way regression between colour variables collected upon air exposure and OCR of 3 week-old fibres revealed a relationship between OCR and complex II, but also between OCR and ROX. Complex I and β-oxidation were not involved in *post mortem* colour stability largely due to their inactivation.

Keywords: oxygen consumption rate, colour stability, chill storage, mitochondrial complexes

1. Introduction

Meat colour is important to consumers' priorities. Colour in meat is defined largely by myoglobin that exists in the three dominant states metmyoglobin (MMb), oxymyoglobin (OMb) and deoxymyoglobin (DMb). These states are interconverted depending on oxygen, enzymes and antioxidants.

According to McKenna, Mies, Baird, Pfeiffer, Ellebracht and Savell (2005), colour stability is affected by the same factors. Different muscles have different colour stability depending on the age and weight of the animal. Increased age and weight will lead to an accumulation of myoglobin and darker colour (Sookhareea, Taylor, Woodford, Dryden, & Shorthose, 1995). *M. semimembranosus* is a large muscle with intermediate colour stability (Hood, 1980; McKenna, et al., 2005; O'Keeffe & Hood, 1982). Meat with the highest oxygen consumption rate (OCR) often has the poorest colour stability (Renerre & Labas, 1987). It has been shown in pigs that the same muscle from different breeds may exhibit different enzyme activities, fibre type composition and lean meat values (Werner, Natter, & Wicke, 2010).

Meat colour is influenced by mitochondrial oxygen consumption and enzymatic-reducing ability (Arihara, Cassens, Greaser, Luchansky, & Mozdziak, 1995; Livingston, McLachlan, La Mar, & Brown, 1985; O'Keeffe & Hood, 1982). A deficiency in nutrient and oxygen delivery occurs in *post mortem* meat due to the cessation of blood flow. This results in deterioration of the mitochondrial electron transport system (ETS) and an increase in reactive oxygen species (ROS) (Lenaz, 2001). Mouse NADH-ubiquinone oxidoreductase (complex I) plus fatty acid oxidation were recently reported as the first enzyme complexes in the ETS to be inactivated *post mortem*

(Barksdale, et al., 2010; Werner, et al., 2010). OCR in meat is mediated by mitochondria utilising some Krebs cycle substrates *in vivo* for consuming oxygen in the production of ATP.

The slaughter process starts with transportation from farm to abattoir, and insufficient lairage time before slaughter may cause pre-slaughter stress leading to increased anaerobic glycogen metabolism and hence carcass temperature early *post mortem* with a concomitant fall in pH (Rosenvold & Andersen, 2003; Schafer, Rosenvold, Purslow, Andersen, & Henckel, 2002; Simela, Webb, & Frylinck, 2004; Stoier, Aaslyng, Olsen, & Henckel, 2001). A low meat pH has been reported to decrease colour stability in beef and pork (Ledward, Dickinson, Powell, & Shorthose, 1986; Monin & Sellier, 1985) but may increase stability in other species such as goat (Simela, et al., 2004). Farouk and Swan (1998) and Young (1999) reported that a high rigor temperature decreased the endogenous oxygen consumption rate (OCR) and colour stability, while increasing the immediate redness in beef. Early *post mortem* studies on slaughter procedure, colour stability and mitochondrial stability are limited. This is also the case for research that shows quantitatively the direct impact of mitochondrial complexes on meat colour.

The aim of this work was to: 1) elucidate differences between individual animals in terms of oxygen consumption response to chemicals administered to mitochondria (OCR_i); 2) measure the changes in OCR_i with time of chilled storage; 3) elucidate how the activities of the different complexes of mitochondria contribute to colour stability when meat is stored; 4) understand the importance of different slaughterhouse variables on OCR_i ; 5) relate OCR_i quantitatively to changes in myoglobin states and L*, a*, b* values collected over time.

2. Materials and methods

2.1. *Meat samples*

Bovine *M. semimembranosus* and *M. longissimus dorsi* muscles from 41 animals were collected from a slaughter line (Nortura SA, Rudshøgda, Norway). The intention was to collect animals representing the types of specimen that normally arrive at this slaughterhouse. Thirty Norwegian Red Cattle (NRF), 2 Simmental, 1 Limousine, 1 Angus, 3 Charolais and 4 Hereford animals were collected. Due to the strong dominance of Norwegian Red Cattle at Norwegian slaughterhouses, we have not split our analysis into breeds but kept animals as individual specimen.

The animals were low voltage stimulated at 85 V, duration 35 sec, pulse duration 5 ms and pulse pause 65 ms (Carometec A/S, Herlev, Denmark), and were then hot-boned approx. 2 hours *post mortem*.

2.2. Data describing each individual animal

Time variables**:** Transportation time from farm to abattoir (ToT); Lairage time at abattoir before death (ToD); Hot-boning/cutting time after death (ToC); Time from death to OCR measurements (ToDO).

pH-measurements: pH of muscles were recorded by inserting the probe (Portamess 913, Knick, Berlin, Germany) t0= once the meat was available at the slaughter line, (pH-t0 ;~4 hours *post mortem*), after 8 h (pH-t1; 8 hours *post mortem*) and after 24 h(pH-t2; 24 hours *post mortem*).

Temperature: A slice of beef was excised from the posterior of *M. semimembranosus* of each animal. The temperature (Ebro TLC 1598, Ebro Electronic GmbH & Co, Ingolstadt, Germany) of the *M. semimembranosus* was recorded on the meat surface immediately after cutting the section to be used for subsequent analysis (called Temp). A slice was vacuum-packed in polyamide bags, (oxygen transmission rate 30-40 cm^3/m^2/24 h/atm at 23 °C and 0 % RH, LogiCon Nordic A/S, Kolding, Denmark) and transferred to storage at 10°C and subsequently to 4°C within 16 hours for 3 weeks' storage.

2.3. Analytical measurements

Colour measurements: After three weeks of storage a slice from *M. semimembranosus* of each animal was covered with oxygen-permeable film (Toppits-glad, Melitta Group, Helsingborg, Sweden) during the colour measurement period. During this period the test samples were stored under a fluorescent lamp (Phillips Master TL-D 36W/830 H9, Phillips, Oslo, Norway) with a light intensity of 800 lx. The colour of all samples was measured with Konica Minolta Chroma meter CR 410 (Konica Minolta Sensing Inc., Osaka, Japan) (3 replicates) giving L*a*b* (lightness, redness and yellowness, respectively) values (CIE, 1976) and visible and near infrared reflectance (400–1100nm, 2 measurements on each slice) with FOSS NIRSystems (Model 6500, Oslo, Norway) in transflectance mode with an interactance probe making use of fibre optics (40x40mm^2 optiprobe ™ system). Absorbance (A= log1/R) was measured between 400–1100nm in 2nm steps giving 350 spectral variables. The instrument was calibrated using a ceramic plate as a reference before all measurements. Each spectral measurement is an

average of 32 scans and takes about 20 secs. The measurements were taken at the following times: 0, 15, 30, 45, 60, 90 and 240 minutes and 1, 2 and 7 days. During this period the slices were exposed to air. The temperature was recorded simultaneously, and the colour measurements were taken at a surface temperature of 17°C (Würth Infrared thermometer, type 08536007, Würth UK Ltd., Kent, United Kingdom). Percentages of MMb, OMb and DMb were calculated according to (Khatri, et al., In press) with a method that uses a Partial Least Square (PLS) prediction model obtained from reflectance spectra (wavelengths 400–1100nm) of meat samples having dominantly one fraction of myoglobin.

Tissue permeabilisation: The muscle fibers were permeabilised prior to OCR measurements. The fibres were separated in a relaxing solution containing 15 mM phosphocreatine, 10 mM Ca-EGTA (0.1 mM free calcium), 20 mM imidazole, 20 mM taurine, 6.6 mM $MgCl_2$, 50 mM K-2-(*N*-morpholino) ethanesulfonic acid, 0.5 mM dithiothreitol, and 5.8 mM ATP adjusted to pH 7.1. Muscle fibres were separated to an approximate length of 5.0 mm with a diameter of approx. 1.0 mm. The tissue was permeabilised in the same relaxing solution containing 0.052 mg/ml saponin for 30 mins. The samples were subsequently washed for 10 min. at 4^{o}C with shaking in the respiration medium (see OCR measurements below).

Oxygen consumption rate (OCR): OCR measurements were carried out after ~5 hours *post mortem* and after 3 weeks' chilled storage. All chemicals were of analytical grade and purchased from Sigma Chemicals Corp. (St. Louis, MO), except for pyruvate (Applichem, VWR International AS, Norway). High-resolution respirometry was carried out with Oroboros Oxygraph-2K instruments (Oroboros Instruments, Innsbruck, Austria) as described by Gnaiger (2001). The OCR of permeabilised tissue was measured at 20^{o}C and ~200 μM O_2. The closed

chambers had a volume of 2.1 ml and results are reported as (pmol O_2/sec)/mg protein. Injection of substrates was done with a Hamilton syringe of 10µl–50µl.

The OCR measurements were carried out by stimulating complex I respiration with malate and glutamate. β-oxidation was carried out using octanoylcarnitine, which donates electrons to an electron transferring flavoprotein (ETF) (Table 1). Oxidative phosphorylation was stimulated by the addition of ADP. Complex II respiration was tested by succinate after rotenone addition. Inhibition of complex II was achieved by adding malonic acid. Uncoupling was measured by FCCP addition, and finally inhibition of complex III was antimycin A. A schematic representation of the functional sites of the substrates is shown in Figure 1. Background oxygen consumption after antimycin A inhibition (Gnaiger, 2008) is recognised as residual oxygen-consuming side reactions (ROX). ETS_{max} is the maximum OCR of the ETS without background adjustment and after uncoupling. The medium consisted of 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-methanesulfonate, 20 mM taurine, 10 mM KH_2PO_4 (Calbiochem, Darmstadt, Germany), 20 mM HEPES, 110 mM sucrose (Alfa Aesar, Karlsruhe, Germany) and 1.0 g/l BSA. The respiration medium had pH 7.1.

2.4 *Statistical analysis*

The experiment consisted of 82 OCR measurements (2 replicates x 41 animals). One-way ANOVA with animal as random effects was used to test for significant differences in OCR between animals with respect to OCR_i responses to substrates. Minitab (versions 15 and 16 from Minitab Inc., State College, PA, USA) was used for statistical calculations. Magnitudes of effects of animal (A) are indicated as estimated variance components $\hat{\sigma}_A^2$.

A principal component analysis (PCA, The Unscrambler X 10.1; CAMO Software, Oslo, Norway) was first carried out with all variables and served as an explorative screening method for identifying both animal and OCR variables that were highly correlated before a stepwise regression was performed. Weighting (1/stand.dev) of all variables was used. A subset of variables was selected based on clustering from the PCA score plot for subsequent use in stepwise regression.

The relationships between OCR_i and individual animal descriptors were obtained through stepwise regression (forward and backward) for identification of significant relationships (Minitab, version 16 from Minitab Inc., State College, PA, USA). The approach served the purpose of identifying significance levels for groups of variables.

The result from the stepwise linear regression is given as regression coefficients with their significance levels.

Through MATLAB (version 2011a, The MathWorks, Inc., Natick, Massachusetts, USA) the relationships between changes over time in colour variables L*, a*, b* and myoglobin states and OCR_i or individual slaughter variables were examined using N-way partial least squares (NPLS) (Bro, 1996). NPLS is a multi-way generalisation of partial least squares (PLS) (Martens & Naes, 1989). Most variants of PLS work on X data in two modes (samples x variables) organised as a matrix. NPLS can handle three or more modes (samples x variables x variables x ...) organised as a three-way (or more) array. The response (Y) in PLS regression is either a single vector or a matrix of responses. In NPLS this can also be a multi-way array of responses. Our X data is three-way with a sample mode (animals), an observation time mode and the colour (L*, a*, b*) or myoglobin state modes. The Y data were either the controllable plus uncontrollable

slaughterhouse variables (storage times, transport times, temperature, pH, ...) or OCR_i measurements. The X data are sequentially decomposed into orthogonal sets of score and loading vectors maximising the covariance between the X data and the responses (Y). The loading vectors have unit lengths and show how much weight is given to each variable in each mode for the current component. Since the scale of the Y data differs, these were weighted according to 1/st.dev. This makes the variables easier to compare, but can hide the differences in variation. **3.**

Results and Discussion

3.1 Variation in OCR_i among fresh and chill-stored muscles of individual animals

Table 2 shows that OCR in both stored and fresh meat varied between individual animals and between the different substrates used (except for the malonate effect on stored samples, $\hat{\sigma}_A^2$ = 0.01 and $\hat{\sigma}_e^2$ = 0.05). OCR_G and OCR_S divided samples into 3 significantly (P< 0.05) different groups (Tukey's test), while most samples were divided in two groups. Of all oxygen-consuming reactions, ROX was the only one that increased after storage and occurred only when biological material was present.

The enzyme complex most vulnerable to *post mortem* inactivation was complex I of the ETS; OCR_G was reduced by 61% after 3 weeks, while OCR_S was only reduced by 13% (Table 2). Werner, Natter and Wicke (2010) reported similar results for pigs where complex I activity increased immediately *post mortem* and then fell dramatically after 12 hours, while other ETS complexes such as complex IV remained constant *ante* and *post mortem* (24 hrs before and 12 hrs after slaughter). High complex I activity early *post mortem* may therefore be responsible for an immediate rise in carcass temperature and glycolysis (Farouk & Swan, 1998; Young, et al., 1999). Furthermore, findings by Nowak, Clifton and Bakajsova (2008) have shown that

mitochondrial respiration on succinate also stimulated activity of complex I even 4 hours after isolation. The oxidation of succinate by succinate dehydrogenase (complex II) was the strongest and the most stable enzyme complex in mitochondria with aging (Table 2). Uncoupling of the ETS by FCCP (ETS_{max}) showed that succinate respiration in many cases represented max OCR capacity of the ETS, thus ATP synthesis was already uncoupled in *post mortem* meat (Table 2). We observed a small increase in OCR after rotenone addition. This may be due to electrons from succinate oxidation being prevented from reverse electron transport (RET) to complex I, subsequently shifting to a forward flow to complex IV. RET occurs in fresh meat and to an even greater degree in stored meat after rotenone inhibition (0–2.36 pmol/sec/mg, Table 2). Complex I inactivation and RET from complex II to complex I are known to stimulate production of ROS (Capel, et al., 2005; Kushnareva, Murphy, & Andreyev, 2002; Pitkanen & Robinson, 1996), which in turn may lead to increased MMb formation and lipid oxidation (Tang, et al., 2005). Blocking of complex II by malonic acid confirms that oxygen consumption was largely determined by complex II (Table 2). This is especially apparent in stored meat where complex I and β-oxidation were largely inactivated, and thus blocking of complex II resulted in an almost complete inhibition of the ETS (Table 2). RET can, however, be prevented if the membrane potential is destroyed, *e.g.* by uncoupling using FCCP or an inhibitor that destroys the membrane potential (Scholes & Hinkle, 1984). It is therefore peculiar when rotenone elicits an increase in OCR when it is used after FCCP in our protocol, but this could be due to an exceedingly large RET at complex I and possibly also the release of ROS from complex III at this point (St-Pierre, Buckingham, Roebuck, & Brand, 2002).

OCR_{OC} of aged muscle was reduced with 51% compared with fresh muscle (Table 2). Stimulation by octanoylcarnitine increased respiration both in fresh and sometimes in stored

meat as the electron entry occurs through electron-transferring flavoproteins, independent of complex I and II, to coenzyme Q. It was not possible to discern the importance of either complex I or $FADH_2$ mediated OCR as a substrate combination of both malate and octanoylcarnitine are necessary to drive β-oxidation at full capacity (Lumeng, Bremer, & Davis, 1976).

Table 2 shows that the average overall activity of the ETS in stored meat was 22% lower than in fresh meat. The reduction in OCR_S appeared smaller (Table 2), but altogether the reduction in OCR_{Rot} and OCR_F suggested that a reduction of around 20% in max OCR could be expected with storage. Furthermore, after 3 weeks the respiration activity was strongly reduced in malate and octanoyl carnithin activity, and so complex II was the main electron-contributing site of the ETS. As much as 90% of OCR can be inhibited by the complex II inhibitor malonic acid in 3 week-aged meat compared to 72% in fresh meat.

3.2.Relationship between OCR_i and individual animals' descriptors

Figures 2a and 2b show how the various variables collected at the slaughterhouse related to each other and to OCR_i measured on fresh permeabilised muscle fibres and after 3 weeks' chilled storage of the muscle. Figure 3 shows the above statement concerning the close link between ETS and OCR_S, but also between OCR_{FCCP} and OCR_{Rot}. OCR_G and OCR_{ADP} clustered together after 4–5 hours and after 3 weeks' storage (Figs. 3A and B). OCR_G changed from being positively related to OCR_S in fresh muscle to being negatively related in stored muscle ($P<0.001$). OCR_{OC} did not cluster with any variable in fresh muscle but was close to OCR_G and OCR_{ADP} in aged muscle. HIence, early and late *post mortem* related oxygen consumption was different.

Tables 3 and 4 give relationships between OCR_i and variables selected in the stepwise regression. The tables indicate that temperature, ToDo and pH were parameters with great variations at the slaughter line. Surprisingly, the pH profile was not very important early *post mortem* for OCR_i and had no significant relevance for meat that had been chill-stored for 3 weeks.

Fresh muscle

OCR_i related most strongly to temperature and weight (Table 3). The group ETS and OCR_S, OCR_{FCCP} and OCR_{Rot} all showed a positive increase in OCR with increased weight and a reduction (except OCR_{FCCP}) with increased temperature. Weight and surface temperature were correlated ($r = 0.32$; $P = 0.04$). Both variables were, however, significantly related to several OCR_i independent of how the variables entered in the stepwise regression. But surface temperature related stronger to OCR_i than did carcass weights.

The sum of glutamate+malate activity (and ADP activity) declined over time after death (ToDO; Table 3) but otherwise showed the same relationship to carcass weight and surface temperature as the OCR_S cluster.

Slaughterhouse variables (temperature, pH and times) / weight and age: The temperature profile of the *M. semimembranosus* was the most critical variable for maintaining good oxygen consumption (Table 3). All enzyme complexes of the ETS were largely affected by carcass surface temperature, indicating increased anaerobic metabolism and glycolysis. Although the effect of ToDo was significantly related to OCR_G, the surface temp. was causing much more variation in OCR_G than ToDO early *post mortem* in our data (due to its large variation).

High temperature due to high carcass weight and thick fat insulation will result in a slower chilling rate, this in turn promoted complex I inactivation. However, large carcasses *per se* did not reduce OCR_G and OCR_S. Similarly, high carcass weight stimulated higher respiration on succinate (0.0156; Table 3) and, to a smaller extent, higher respiration on glutamate (0.0105; Table 3).

Stored muscle

The relationships between ToDO (time of OCR measurements after death) and age were the strongest for OCR_i in 3 weeks chill-stored muscle. The group ETS and OCR_S, OCR_{FCCP} and OCR_{Rot} all showed a positive increase in OCR with increased age and a reduction with increased time after death (Table 4). ToDo and age were uncorrelated (P=0.31), as were weight and age (P=0.29).

The glutamate/malate activity (and ADP activity) declined with age (Table 4). Complex I appeared related to the time of transport (Table 4). The influence of ToT reflected the only relationship between a stress variable and OCR_i, and long transport times reduced OCR_G.

OCR_{OC} decreased with age and weight (Table 4), while the only oxygen consumption process that had a positive trend with storage was ROX.

Slaughterhouse variables: OCR_S related positively to age (Table 4). Vitorica, Cano, Satrustegui and Machado (1981) showed that complex II activity increased 1.7 times in old animals and mice (of 29 months) that had a OCR deficit of 29% compared with young animals (mice of 9 months) (Jones & Brewer, 2010). In old animals low cytochrome c levels prevent normal upregulation of

294 respiration, and the conditions are abolished upon substrate supplementation (Jones & Brewer,
295 2009; Phung, Saelid, Egelandsdal, Volden, & Slinde, 2011). However, the former authors
296 confirmed that old animals contain the same number of mitochondria as young animals and that
297 the loss in activity is due to a less functional cytochrome c oxidase (complex IV).

298 The effect of ToDo on OCR_S suggested that rapid chilling *early post mortem* may be beneficial
299 for higher oxygen consumption at a later stage and hence affect colour stability positively. This
300 could actually be contrary to conditions that would support tenderisation.

301 The inactivation of ATP synthesis and complex I with age (Table 4) could be due to an
302 accumulation of mitochondria respiring on complex II rather than complex I, which is more
303 robust against age, oxygen limitation and freeze storage (Galkin, Abramov, Frakich, Duchen, &
304 Moncada, 2009; Jones & Brewer, 2010; Phung, et al., 2011). Furthermore, mitochondria in older
305 specimen are believed to have higher OCR in order to compensate for a decreased efficiency in
306 ATP production (Jones & Brewer, 2010). Furthermore, it can also be seen as a defence
307 mechanism when tissue temperature was temporarily high due to elevated *post mortem*
308 glycolysis (above 37°C). We hypothesize that an increase in SDH (complex II) activity may
309 occur as a compensatory effect for loss of complex I activity.

310 The cause of ROX is not known. The actual magnitude for ROX indicated that this effect could
311 be important in aged meat. Further investigations are needed.

312

313 *3.3.Colour changes in M. Semimembranosus exposed to air*
314

315 Measurement number 37 was removed as an outlier in the analysis of a* and b* values. Figure
316 3A shows that a* increased gradually and then decreased after 4 hours. Similarly, the DMb form
317 remained reduced until 1.5 hours and then decreased rapidly (Fig. 3B). B* values increased and
318 then decreased, while both a* and b* had characteristics of blooming in meat. L* values were
319 omitted from the NPLS analysis as they changed little with time.

320 The NIR spectrophotometer provided more details regarding the colour transitions in meat. The
321 general trend was that DMb fraction gradually declined with time and transformed temporarily
322 into OMb before becoming oxidised to MMb (Fig. 3B). Figure 3B shows that the DMb form
323 remained reduced until 1.5 hours and then decreased rapidly; similar to the increase in a* values..
324 The MMb fraction increased from 4 hours (Fig. 3B). This trend was in agreement with Arihara
325 (1995) and others (Hagler, Coppes, & Herman, 1979; Livingston, et al., 1985), where a transition
326 from DMb to MMb may occur rapidly with an OMb intermediate. It was apparent that the
327 reduction in DMb on average preceded changes in a* (Figs. 3A and B). Reduced myoglobin
328 reached a minimum after 3 days (72 hours) and for a* and b* after 6 days (144 hours). This
329 showed that assessing colour by measuring myoglobin forms will show changes earlier than
330 when measuring a* or b*. L*a* and b* data depicted the surface colour and texture and may
331 therefore be more informative for judging colour as a whole than are measurements of
332 myoglobin forms.

333

334 *3.4 Relationship between OCR_i and colour stability*

335 In respect of Minolta measurements, NPLS component 1 related largely to a* (Fig. 4A), while
336 NPLS component 2 related largely to b* values. Figure 4A shows progress in components 1 and

2 with time, and Figure 4B shows the relative importance of the different substrates in the OCR protocol regarding explained changes in a* (~NPLS component 1) and b* (~NPLS component 2). The first NPLS component (Fig. 4A) described 99.37% of the colour variation and 62.67% of the chemical response variation (*i.e.* of the variation in the OCR_i protocol). This component was dominated by a*, meaning that redness was the single most important variable for describing the variation in colour over time. The first component (Fig. 4A) rose slightly up to four hours, then dropped more strongly up to 144 hours and then levelled off. The second component described only 0.15% of the colour variation but 12.1% of the chemical response variation. The component had a more distinct rise in b* level up to 4 hours and also a slow decline until 144 hours (Fig. 4A). In comparison, McKenna et al. (2005) found few changes in b* values in *M .semimembranonsus* after 1 day's display. We show here that b* values increased substantially until peaking between 1.5–4 hours, followed by a concomitant decrease in a* values (Fig. 4A). Figures 4A and B thus supported that 99.5% of the variation in a* and b* was explained by 75% of the variation in OCR_i. This demonstrated that the colour changes in muscle exposed to air were explained by the ability of ETS to remove oxygen.

Two NPLS components were also calculated from reflectance measurements; a third NPLS component could be related to light scattering. The two NPLS components both consisted of combinations of myoglobin states. Our component 1 nevertheless described predominantly DMb, while component 2 described largely OMb and gradually converted to MMb after 48 hours . Figure 4C shows the average change of DMb (comp. 1) and OMb/MMb (comp. 2) with time and the importance of the different substrates in the OCR protocol regarding explained changes in the myoglobin states (Fig. 4D).

Since component 1 of Figure 4C was dominated by DMb, it made sense that component 1 seemed to decline strongly after the same time period as component 2. Component 2 was a combination of OMb and MMb (loading weight -0.71 and -0.37 for MMb and OMb respectively) but represented more of MMb than of OMb as this form was more important than the oxyform in the regression model (Figs. 4C and 4D). The plateau of OMb/MMb (Fig. 4C) occurred earlier (as supported by Fig. 3B) than what was measured by a* and b* for both components 1 and 2 but still had similar shapes. The first NPLS component of Figures 4C and 4D described 65.44% of the myoglobin forms and 66.28% of the OCR_i. With regard to the 2^{nd} component, 16.51% was explained by myoglobin forms and 6.74% was OCR_i response. Altogether 75% of the variation in OCR_i explained 82% of the variation in myoglobin states. The apparently weaker relationship between myoglobin states and OCR_i than between variables a*, b* and OCR_i was at least partly due to the fact that myoglobin states are not accurately predicted (Khatri, et al., In press).

Figures 4B and 4D show that the correlated group of variables OCR_S, OCR_{FCCP}, OCR_{Rot} and OCR_{ETS} (see Fig. 2) was important in explaining changes in a*, b* and myoglobin states. Total ETS OCR was ranked with the highest nominal importance (Fig. 4). This variable and its related variables all pointed to the importance of complex II for removing oxygen. The relationship with OCR_S should be expected since complex II was the only complex with substantial activity *post mortem* (except complex IV, which was not directly tested).

Our OCR_i measurements were only intended for ranking activities of the complexes/enzymes and not for quantitatively representing data for the activities in the sample since the ultimate pH varied in meat. However, not much variation in OCR_S between pH 5.5 and 6.0 using permeabilised pork fibres (Phung, et al., 2011) was found. Furthermore, the actual value for OCR_S between pH 5.5–6.0 was 50–60% of the value at pH 7.1 (Phung, et al., 2011). The data

also suggested that although there are only low amounts of ETS substrates after 3 weeks of chilled storage, there are still some substrates supporting ETS through complex II or other complexes, at least for a few hours after atmosphere exposure.

The other OCR_i group, OCR_G, OCR_{ADP} and OCR_{OC} (Fig. 2), was not very important for colour and was mainly involved in the transition from muscle to meat. However, it appeared that high OCR_S promoted the presence of reduced myoglobin, while any activity in the OCR_G and OCR_{OC} group did the opposite. Thus complex I activity would be regarded as a colour destabiliser. In Figures 4B and D the substrates of OCR_G, OCR_{ADP}, OCR_{OC} are presented as factors that have a relatively large negative weight for colour. However, the small absolute values of these OCR after three weeks (Table 2) make them less influential than OCR_S, for example. In Figure 4 the weighting of variables makes it easier to see the variables with opposing effects on colour changes. Removing the weighting would clearly indicate the importance as: OCR_S and related variables > ROX >> OCR_G and related variables.

Finally, high OCR by ROX maintained DMb for longer (Figs. 4B and 4D). ROX was on average a small contributor to OCR (Table 2). However, it was of importance as the oxygen-consuming side reactions increased with storage time and the substrates that were used. More importantly, ROX continued to consume oxygen even when the mitochondrial ETS had ceased to function due to inhibitors. Results showed that oxygen consumption by ROX only occurred when biological materials were present and not in isolated mitochondria, for example (results not shown). ROX may therefore gradually begin to play a more important role as meat ages when mitochondria are non-functional and internal substrates are depleted.

3.5 Relationship between individual animals' descriptors and colour stability

405
406 The NPLS regression method was also used to relate animal descriptors to the 3 directions: a*,
407 b*-time-animal number (Figs. 5A and B) or the three directions: % myoglobin state-time-animal
408 number (Fig. 5C and D) respectively. The data in Figs. 5B and D used weighted (1/st.dev)
409 animal descriptors.

410 Unweighted animal descriptors suggested that weight and age affected colour stability in
411 accordance with Table 4 that identified these variables as important for OCR_i (Figs. 5A and 5C).
412 However, when weighting was introduced (Figs. 5B and 5D), pH- t2 (ultimate *post mortem* pH)
413 was identified as important for colour stability. Components 1 and 2 described 99.5% of the
414 variation in a* and b* (X-block) and 98.9% of the variation in animal descriptors (Y-block, Fig
415 5B, weighted animal descriptors). For myoglobin states, these were 74.6% for components 1 and
416 2 of the variation (X) and 99.8% for components 1 and 2 of the variation in animal descriptors
417 (Fig. 5D). Fig. 5 indicated that high *post mortem* pH-t2 (ultimate pH) was positive for high a*
418 values and DMb states (the larger components).

419 According to Hood (1980) and McKenna et al. (2005), colour stability is barely affected by pH.
420 However, colour stability is severely affected by OCR in muscle. This means that OCR_i
421 measurements at the specific pH of the muscle should have given an even stronger relationship
422 between OCR_i and colour stability than those reported in Fig. 4. pH plays a role in colour
423 stability and can also affect mitochondrial activity and protein denaturation (Joo, Kauffman,
424 Kim, & Park, 1999).

425 Higher surface temperature early *post mortem* affected colour stability (Fig. 5).The temperature
426 effect was not identified as a most important effect independent of regression method (Fig. 5),
427 however. This is in agreement with Table 4. Quick cooling is important for colour and OCR

428 through maintaining complex II activity as suggested above. However, it may not be important to
429 keep an active complex I, thus optimal chilling could be important for optimal colour stability.
430 The colour stability after 3 weeks possibly reflected both the slaughterhouse environment (pH-
431 t2) and intrinsic variables of each animal such as weight.

432

433 **Conclusion**

434 The enzyme activities of complex I and β-oxidation, for example, are involved in the early
435 muscle-to-meat transition after slaughter. Our results show that these systems are not dominant
436 in determining colour stability. Early *post mortem* treatment, such as slow chilling, reduced the
437 activity level of complex I. After 3 weeks of chilled storage 61% reduction in complex I activity
438 was found. At this time complex II activity related predominantly to colour stability as shown by
439 measuring changes in L*, a*, and b* values and myoglobin states. Carcass weight, age and
440 ultimate pH affected colour stability, but ultimate pH was not a significant factor for OCR. After
441 3 weeks chill storage OCR was related to carcass age and weight and to early *post mortem*
442 cooling profile. A hitherto unknown factor, ROX, is important for the oxygen consumption in
443 meat and was the only factor with an increased OCR with stored meat.

444
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Figure text

Figure 1.

Overview of the ETS with substrates and inhibitors at their active locations. Reactions generating reducing equivalents are shown with thick arrows, redox reactions are shown with medium arrows, the flow of electrons are shown with thin arrows. Inhibitors are shown with a circular X together with a black arrow. (See also Table I) Mitochondrial ETS complexes are marked with Roman numerals. ETF: electron transferring flavoprotein, C: cytochrome c, Q: ubiquinone. Modified from (Kanehisa ,1995)

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Figure 2.

Score plot from principal component analysis (PCA) using weighted (1/st.dev.) animal variables and OCR_i of *M. semimembranosus*. Each point reflects measurements taken on *M. semimembranosus* of 41 animals where A) is fresh and B) is after 3 weeks of chill storage. The box denotes response at max OCR and of complex II; the circle denotes responses of complex I and β-oxidation.

Figure 3.

Average values showing: (A) L*, a*, b* values. Left-side vertical scale is for a* and b*; right-side vertical scale is for L*. (B) myoglobin states in % total myoglobin using NIR spectrophotometer. Samples were vacuum-packed and chill-stored for three weeks. Sample 37 was removed as an outlier in analysis of a* and b*. Timescale shows time of measurements after exposure to air and continued for 144 hours.

Figure 4.

NPLS regression with loadings from two component NPLS regressions with response OCR(i). Panels A and B are based on light measurements (a*b*-time-animal), while panels C and D are based on % myoglobin (% myoglobin-time-animal). Component 1 is dominated by a* in panel A/B and DMb in panel C/D, while component 2 is dominated by b* in panel A/B and O/MMb in panel C/D. Oxygen consumption of *M. semim*. after 3 weeks of storage was used. Sample 37 was removed as an outlier in analysis of a* and b*. Explained variance in Y-block is written on

panels. Timescale shows time of measurements after exposure to air and continued for 144 hours.

Figure 5.

Weights (loadings) from two components obtained by NPLS regressions using animal descriptors as response. Panels A and B are based on Minolta measurements (a*, b*-time-animal), while panels C and D are based on % myoglobin state (% myoglobin state-time-animal). Panels B and D are based on weighted (1/st.dev) responses. Components 1 and 2 are dominated by a* and b* in panel B respectively. Component 1 was largely DMb, while component 2 was OMb/MMb in panel D. Colour stability/oxygen consumption of *M. semimembranosus* after 3 weeks of storage was used. Explained variance in Y-block is written on panels.

Figure1

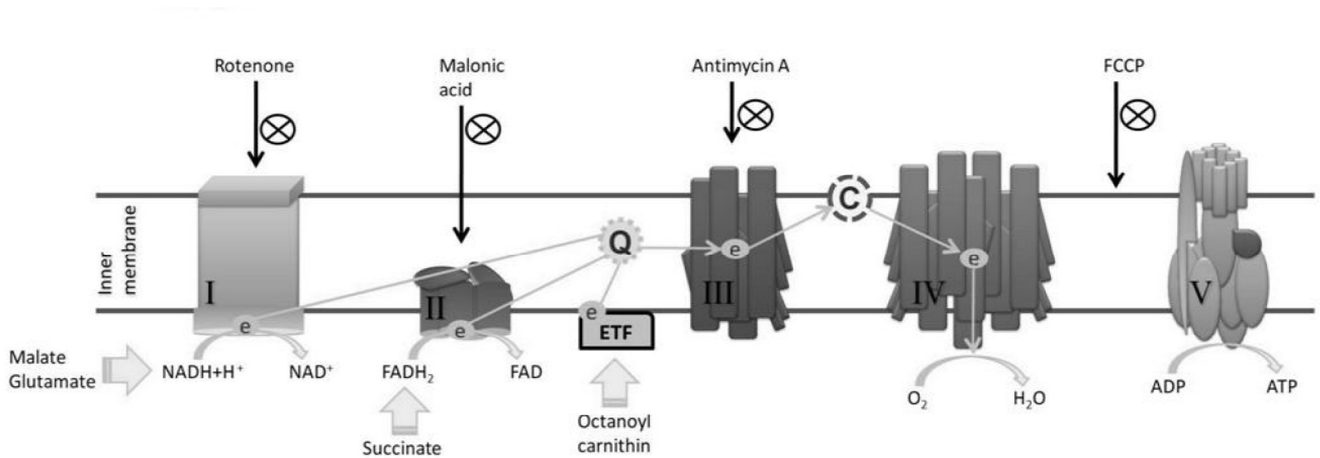

Fig. 1.

Figure2

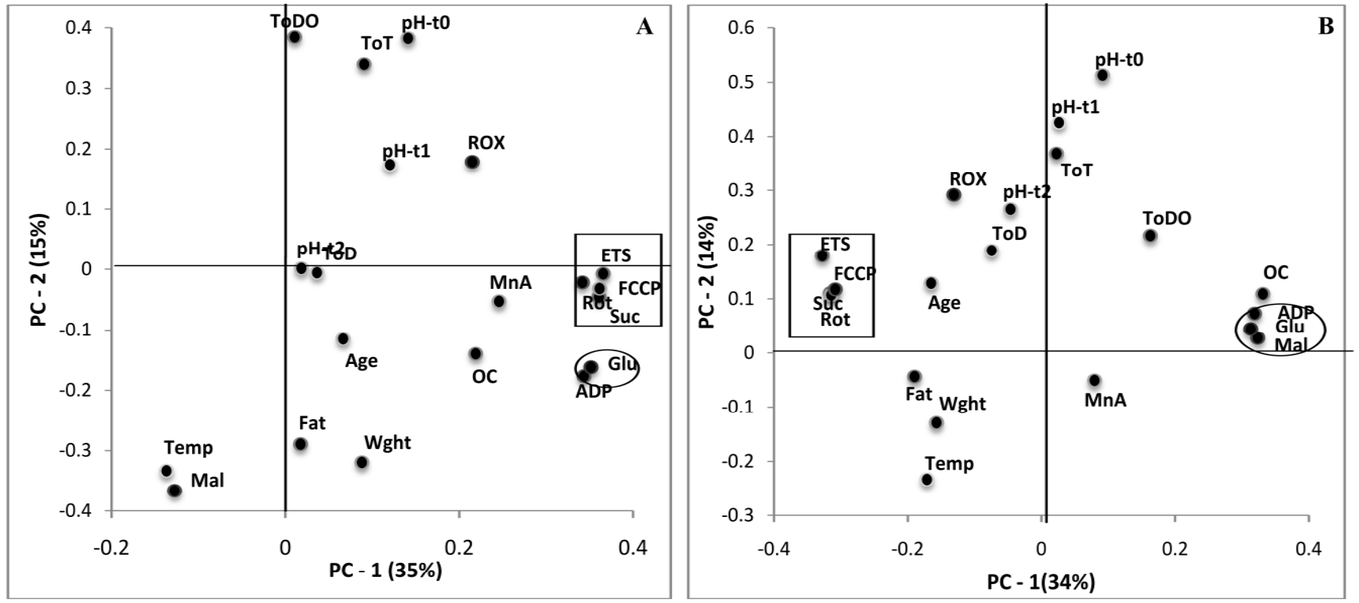

Fig. 2.

Figure3

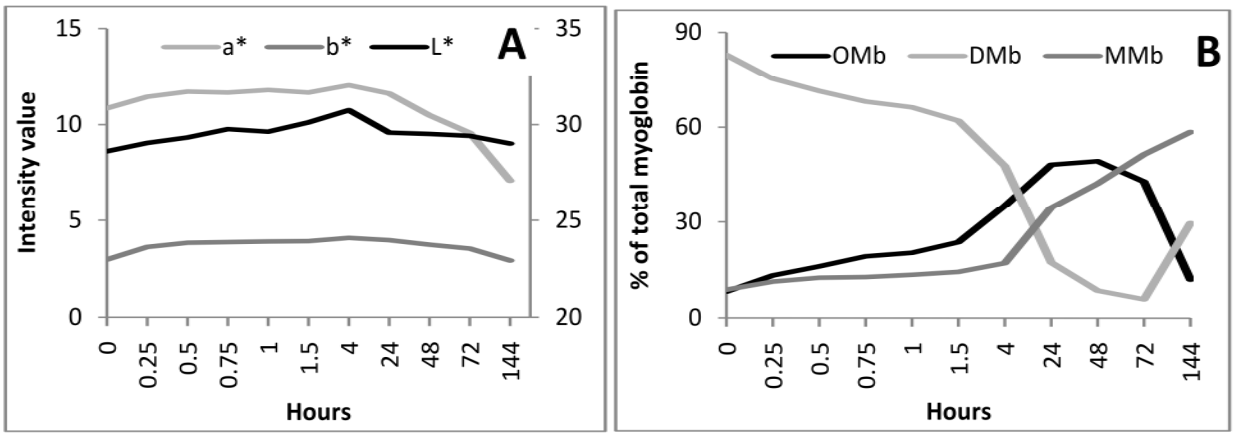

Fig. 3.

Figure4

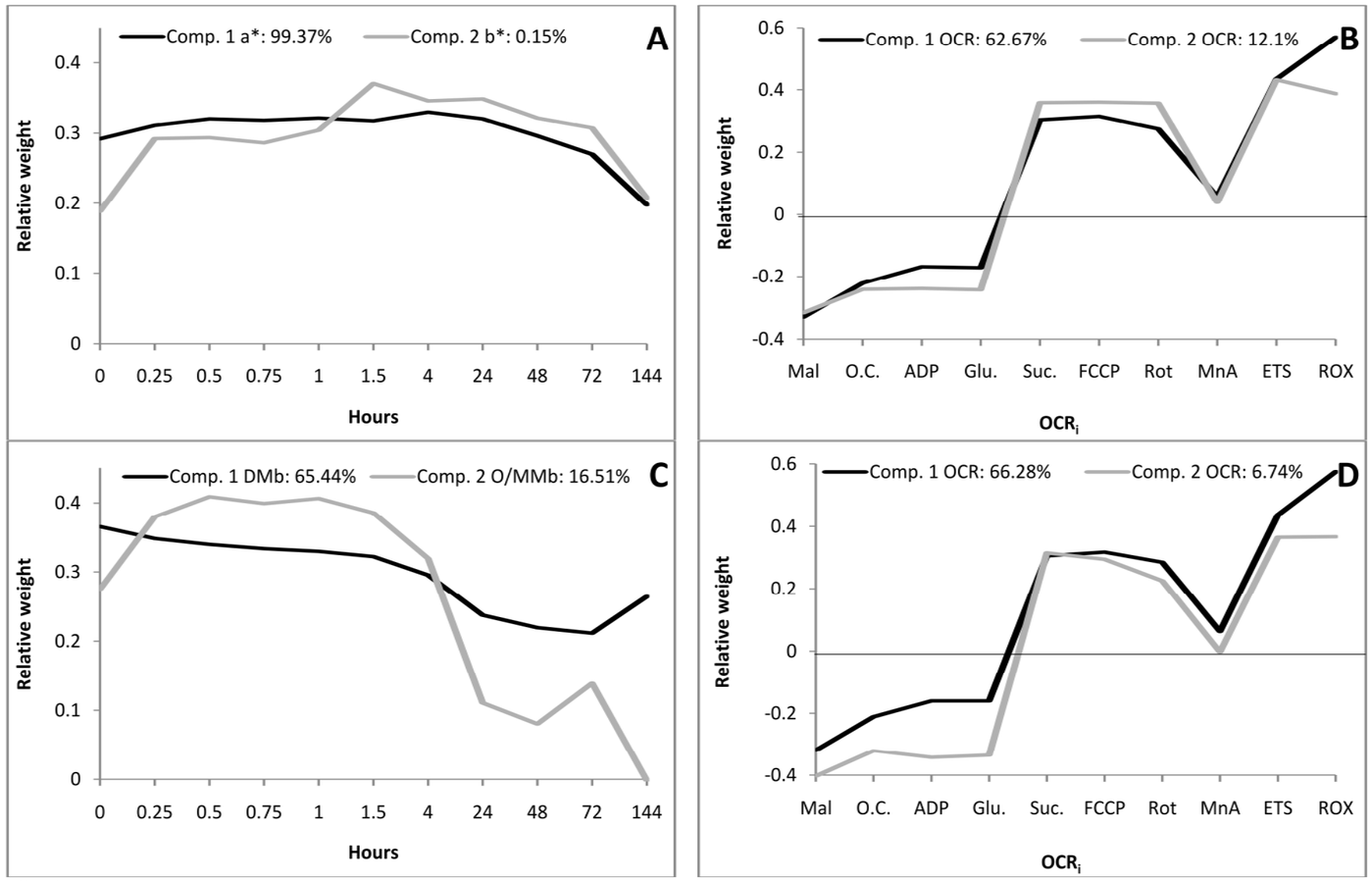

Fig. 4.

Figure5

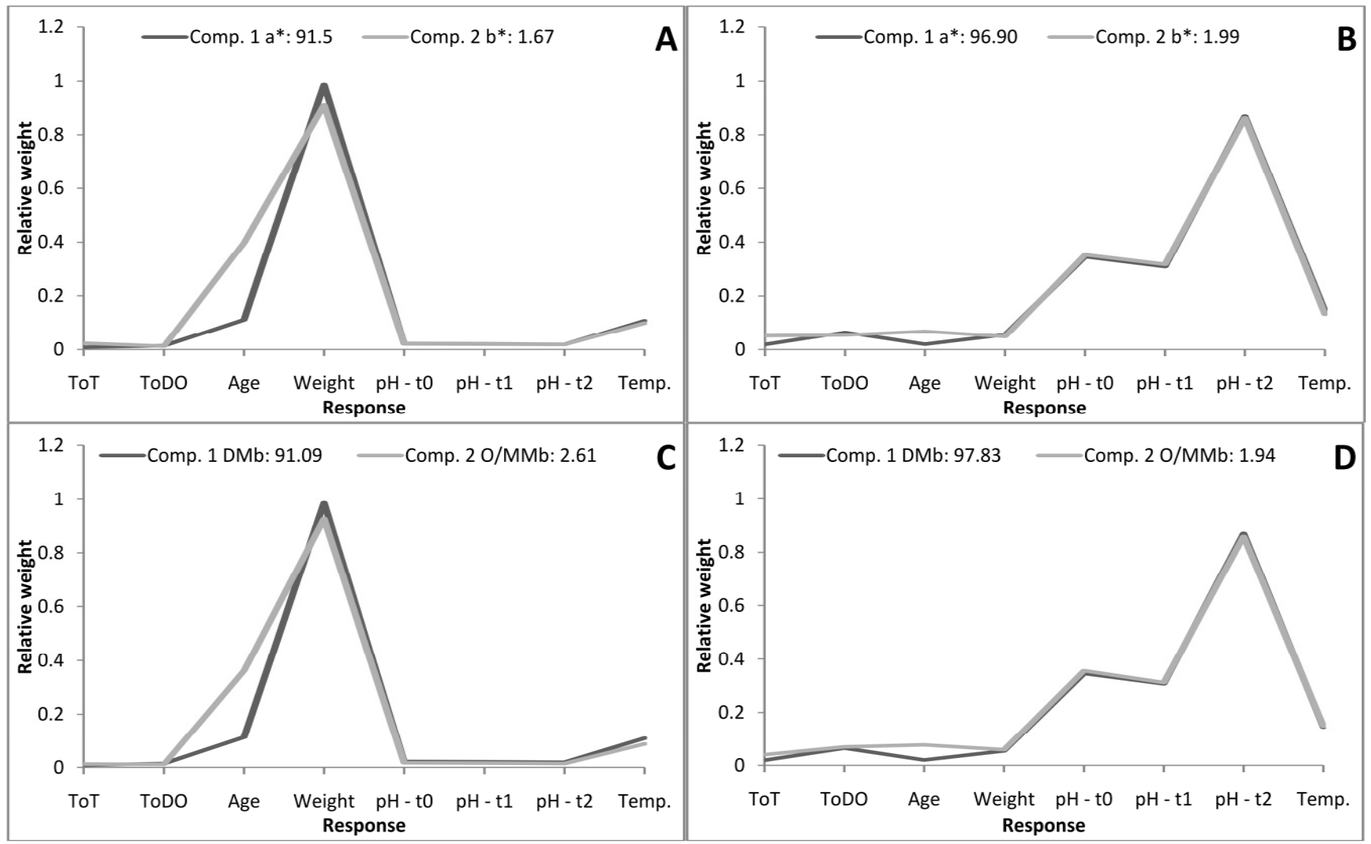

Fig. 5.

Table1

Table 1

Abbreviations and end concentrations of substrates and inhibitors used during OCR measurements. Measurements were taken with volumes of 2.1 ml at 37°C adjusted to pH 7.1. (See also Fig 1)

Seq.	Chemical	Symbol	Function	Location	Working conc. [mM]
1	Malate	OCR_M	Substrate	Complex I	4.00
2	Octanoyl carnithine	OCR_{OC}	β-oxidation	ETF*	0.50
3	ADP	OCR_{ADP}	Oxidative phosphorylation	Complex V	1.25
4	Glutamate	OCR_G	Substrate	Complex I	10.0
5	Succinate	OCR_S	Substrate	Complex II	5.00
6	FCCP	OCR_F	Uncoupler	Complex V	0.5-1.5 x 10^{-3}
7	Rotenone	OCR_{Rot}	Inhibitor	Complex I	2.5 x 10^{-3}
8	Malonic acid	OCR_{Mna}	Inhibitor	Complex II	5 .00
9	Antimycin A	OCR_A	Inhibitor	Complex III	12.5 x 10^{-3}

*ETF: electron transferring flavoprotein

Table2

Table 2

OCR measurement in fresh and stored samples of *M. semimembranosus* fibres added substrates and inhibitors. Fresh samples were taken from the slaughter line and measured immediately (~4 hrs after death). Stored samples were allowed to mature for three weeks at 4°C and were then measured after the package was opened. All variables except for ETS_{max} and ROX are mitochondrial substrates.

	Variable	Malate	O.C.	ADP	Glutamate	Succinate	FCCP	Rotenone	Mna	ETS_{max}	ROX
Fresh	Min-Max (pmol/sec/mg)	0 – 1.99	0 – 3.47	0.34 – 6.50	0.35 – 6.49	0.64 –11.78	0.65 –11.30	0.55 –12.30	0.10 – 2.57	1.48 – 12.51	0 – 1.84
	$\hat{\sigma}_A^2$	0.53[a]	0.58	2.50	2.80	7.01	6.48	8.95	0.36	7.47	0.10
	$\hat{\sigma}_e^2$	0.08	0.31	0.90	0.77	1.57	1.59	1.64	0.12	1.72	0.05
Stored	Min-Max (pmol/sec/mg)	0 – 0.36	0 – 0.55	0 – 1.22	0 – 0.89	0.42 – 8.82	0.41 – 7.51	0.35 – 7.47	0 – 0.75	0.73 – 8.08	0 – 2.36
	$\hat{\sigma}_A^2$	0.16	0.23	0.31	0.24	2.89	2.19	3.31	0.01	2.50	0.12
	$\hat{\sigma}_e^2$	0.10	0.07	0.09	0.09	0.46	0.30	0.238	0.05	0.34	0.07
ΔOCR	% ΔOCR	(-3 %)*	(-51%)	(-59%)	(-61%)	(-13%)	(-20%)	(-11%)	(-72%)	(-22%)	(+0.02%)
	$\hat{\sigma}_A^2$	0.18	0.54	2.21	2.81	10.46	9.10	13.63	0.39	9.78	0.10
	$\hat{\sigma}_e^2$	0.18	0.42	0.98	0.84	1.61	1.55	1.46	0.13	1.68	0.11

$\hat{\sigma}_A^2$: estimated variance due to animals; $\hat{\sigma}_e^2$: estimated variance due to noise; O.C. = octanoylcarnitine; Mna = malonic acid; ETS = max obtained OCR activity; ROX = oxygen-consuming side reactions: *Percentage reduction in absolute value. ETS_{max} = max OCR of the ETS without ROX subtracted as background. [a] All animal effects were significant ($p \leq 0.001$) except for the Mna response of stored muscle ($p = 0.27$).

Table3

1

1 **Table 3**
2 Estimated regression coefficients between different OCR_i measured approx. 4 hrs[1] *post mortem* and individual slaughter variables (stepwise regression).

	Weight (α × kg)[x]	**Temp. surface (α × °C)**	**ToDO (α × time)**	**pH-t1 (α × pH)**	**% expl. variance**	**Mean OCR_i± st. dev (α)**
Malate	n.s.	0.084*	n.s.	n.s.	42.4	0.32±0.78
Octanoyl C	n.s.	n.s.	n.s.	n.s.	21.2	1.34±0.97
ADP	0.0097**	-0.245**	-0.59*	n.s.	44.0	2.78±1.83
Glutamate	0.0105**	-0.284**	-0.58*	n.s.	47.6	2.72±1.88
Succinate	0.0156**	-0.39**	n.s.	n.s.	52.4	4.50±2.91
FCCP	0.0149**	0.37**	n.s.	n.s.	52.9	4.41±2.83
Rotenone	0.019**	-0.43**	n.s.	3.8*	53.9	4.25±3.24
Malonic A	n.s.	-0.09**	n.s.	n.s.	35.3	1.04±0.076
ETS	0.0162**	-0.42**	n.s.	n.s.	54.6	5.50±3.02
ROX	0.00142*	-0.043**	n.s.	n.s.	42.0	1.09±0.37

3 [1]These were removed from the slaughter line's hot-boning area as quickly as possible. **P<0.01; *P<0.05; n.s. not significant. ToDO: time of death until OCR
4 measurement, pH-t1: pH at time t1, expl. var: explained variance, st. dev: standard deviation; [x] **α** =pmol/(s×mg).

Table4

1

Table 4

Estimated regression coefficients between different OCR_i measured after 3 weeks *post mortem* chill storage and individual slaughter variables (stepwise regression).

	Weight (α × kg) [x]	**Age (α × months)**	**ToDO (α × time)**	**ToT (α × time)**	**% expl. variance**	**Sample mean ± st. dev (α)**
Malate	-0.0028**	-0.0061*	n.s.	n.s.	43.7	0.01±0.02
Octanoyl C	-0.0025*	-0.0059*	n.s.	n.s.	48.8	0.06±0.04
ADP	-0.0028*	-0.0085*	0.24**	n.s.	46.7	0.09±0.03
Glutamate	n.s.	-0.007*	n.s.	-0.098*	44.4	0.08±0.03
Succinate	n.s.	0.029**	-0.74**	n.s.	51.0	2.56±1.82
FCCP	n.s.	0.019*	-0.69**	n.s.	48.2	2.30±1.57
Rotenone	n.s.	0.026*	-0.77**	n.s.	50.1	2.49±1.87
Malonic Λ	n.s.	n.s.	n.s.	n.s.	21.1	0.05±0.03
ETS	n.s.	0.024*	-0.59*	n.s.	54.8.	3.38±1.67
ROX	0.0017*	n.s.	n.s.	0.094**	47.2	1.07±0.44

The meat was stored at 4°C. **P<0.01; *P<0.5; n.s. not significant; [x] **α** =pmol/(s×mg).

ToDO: time of death until OCR measurement, ToT: time of transport from farm to abattoir, st. dev: standard deviation.