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Philosophiae Doctor (PhD) Thesis 2012:14

Norwegian University of Life Sciences • Universitetet for miljø- og biovitenskap
Department of Chemistry, Biotechnology and Food Science
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Mitochondrial oxygen consumption and Myoglobin redox stability in Beef

Mitokondrielt oksygenforbruk og redokstilstand til myoglobin i storfekjøtt

Vinh Thanh Phung

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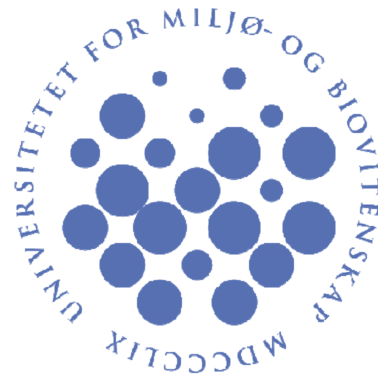
Ås 2012



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Preface

This thesis is submitted to the Department of Chemistry, Biotechnology and Food Science (IKBM) at the University of Life Sciences (UMB), Norway to attain the degree of Philosophiae Doctor (Ph.D.). The work consists of four scientific papers with a theoretical introduction and has been performed at IKBM in the period 2009 – 2012. Professor Bjørg Egelandsdal, Professor Erik Slinde, senior scientist Oddvin Sørheim and Post Doctor Jon Volden have supervised the work. The study was funded by Tine BA, Nortura BA, UMB and NFR project 11927515.

The work consists of a theoretical introduction and four scientific papers discussing the importance of mitochondria in sustaining color stability in *post mortem* muscle. The first paper studied the methods of myoglobin quantification and pre-processing of data. The importance of oxygen consumption by isolated mitochondria and permeabilized tissue in relation to meat color was determined in paper II. In Paper III the color stability as affected by inherent animal qualities was investigated. In Paper IV, various mitochondrial specific substrates and their role in retail packaged meat were tested for their ability to preserve color stability.

Dedication

To my parents

Acknowledgements

I would like to thank my family for the continuous support. You never grew tired of me, even though I was tiresome. Thank you for standing by me all the way.

I would like to thank my supervisor, Professor Bjørg Egelandsdal. I did not know what I was doing initially but you have been the light that shines my path, and I will forever be grateful of your guidance. I am also grateful to my co-supervisors Professor Erik Slinde, senior scientist Oddvin Sørheim and Post Doctor Jon Volden. You have inspired me to think outside the box and always strive to get a little bit further than yesterday. I thank you for all the advices, suggestions and discussions.

I'd also like to thank co-authors and friends for your support and contribution; Professor Tomas Isaksson, associate Professor Trygve Almøy, Post Doctor Kristian Liland, Dr. Linda Saga, Dr. Kim-Marius Moe, Dr. Heidi Grønnevik and master students Elise Sælid and Mamata Khatri. I also thank Kristin Saarem from Nortura SA and Berit Nordvi from Tine SA for constructive comments and discussions.

Oslo, March 2012

Vinh Phung

Abstract

Myoglobin is the main pigment in meat and exists in three predominant states; deoxymyoglobin (DMb), oxymyoglobin (OMb), and metmyoglobin (MMb). The different myoglobin states are dependent on the production of mitochondrial reducing equivalents. Several approaches were considered in order to elucidate the mitochondrial role as a natural antioxidant and the improvement of color stability in meat.

In paper I, a method to prepare the different myoglobin states and predictive model for myoglobin states was created. Three different myoglobin states were prepared by using oxygen partial pressure (OPP) instead of the traditional method using chemicals (CHEM). The samples were measured using selected wavelengths (SW) or multivariate reflectance mode (400-1100nm) with partial least square regression (PLS). Transformations of the spectra were done using Kubelka-Munk transformation or extended multiplicative scatter correction (EMSC). The OPP method with PLS and EMSC yielded the lowest prediction error for both DMb and OMb. The CHEM method remained as the best approach for creation of MMb.

The study in paper II was conducted on isolated mitochondria as well as permeabilized tissue to understand the mitochondrial activity in *post mortem* muscle and after freeze-storage. The results showed that complex II was more resistant to *post mortem* inactivation than complex I and the freeze-thaw cycle increased oxygen consumption. However, the freeze-thaw cycle reduced the mitochondrial oxygen consumption at low pH, indicating damage in the membrane, as verified by a cytochrome c addition.

Significant animal to animal differences in oxygen consumption of muscles were identified as presented in Paper III when muscles from 41 random cattle were followed from arrival at the abattoir to chill storage over three weeks. The early (4 hours *post mortem*) and late (3 weeks *post mortem*) oxygen consumption were measured and compared to color (L*a*b*, lightness, redness and yellowness, respectively) and myoglobin redox state changes. The results were related to an array of animal and muscle characteristics, where it was found that complex I respiration declined with increasing carcass temperature and time *post mortem*. It was intriguing to learn that an oxygen consuming side-reaction (ROX) most often increased under the same conditions.

In the last Paper, the synergy between mitochondrial metabolites on myoglobin redox states and color stability was investigated. Solutions were made containing combinations and pure forms of malate, glutamate, pyruvate, citrate and succinate. The various mixtures were added to the ground meat of *M. semimembranosus* and packaged in modified atmospheres containing high and low oxygen for 8 and 13 days, respectively. The mixtures' ability (combined with age and fat) to reduce metmyoglobin was surveyed. Results showed that a removal of oxygen in the headspace was crucial for myoglobin reduction and a mixture of 50% succinate and 50% glutamate-malate yielded mainly reduced myoglobin (DMb) in low oxygen packaging. High oxygen packaging yielded myoglobin in the form of OMb and was mediated by glutamate (or malate) with 0.02 kg/mol citrate. Furthermore, the concentration (0.05 mol/kg and 0.1 mol/kg) of the additives did not significantly affect color stability during the observation time.

Sammendrag (Norwegian abstract)

Myoglobin er det viktigste farge-pigmentet i kjøtt og kan eksistere i tre dominante former; deoxymyoglobin (DMb), oxymyoglobin (OMb) og metmyoglobin (MMb). Oksyderte myoglobin former er avhengig av mitokondrier for å bli redusert. Flere tilnærminger er brukt for å undersøke mitokondriets rolle som en naturlig antioksidant og farge-stabilisator i kjøtt.

En metode for å lage de ulike myoglobin-formene og lage en modell for prediksjon av myoglobin-tilstand, ble utviklet i Artikkel I. Det ble laget metoder for opparbeidelse av de tre myoglobin formene ved bruk av oksygen partialtrykk (OPP) i stedet for den tradisjonelle metoden som brukte kjemikalier (CHEM). Prøvene ble målt ved hjelp av utvalgte bølgelengder (SW) eller i refleksjonsmodus (bølgelengde 400-1100nm) etterfulgt av "partial least square" regresjon (PLS). Transformasjon av spektrene ble gjort ved hjelp Kubelka-Munk eller utvidet multiplikativ spredningskorreksjon (EMSC). OPP metoden kombinert med PLS og støykorreksjon ved EMSC ga den beste prediksjonen med lavest feilmargin for både DMb og OMb. CHEM-metoden ga best tilnærming for produksjon av MMb.

For å forstå mitokondrie aktivitet i tidlig *post mortem* muskel og etter fryse-lagring ble studien i artikkel II utført på både isolerte mitokondrier og permeabilisert vev. Resultatene viste at kompleks II var mer motstandsdyktig mot *post mortem* inaktivering enn kompleks I og at fryse-tine syklusen økte oksygenforbruket i mitokondrier. Imidlertid reduserte fryse-tine syklusen mitokondriets oksygenforbruk ved lav pH, noe som indikerer skader i den ytre mitokondrie-membranen, og dette ble verifisert ved å tilsette cytokrom c.

Betydelige dyr til dyr variasjoner i musklers oksygenforbruk ble funnet i artikkel III da 41 tilfeldige storfe ble undersøkt for oksygenforbruk fra ankomst til slakteriet fram til 3 ukers lagring i kjølerom. Tidlig (4 timer etter avliving) og sent (3 uker etter avliving) oksygenforbruk ble målt og sammenlignet med farge (L*, a*, b*, lyshet, rødhet og gulhet) og endringer i myoglobin. Resultatene ble knyttet til en rekke egenskaper ved dyr og muskler, hvor det ble funnet at oksygenforbruket i kompleks I falt med økende skrottemperatur og tid *post mortem*. Det var interessant å finne at en side-reaksjon (ROX) forbrukte oksygen og økte oksygenforbruket ved de samme vilkår som inaktiverte kompleks I.

Synergieffekten av mitokondrie-metabolitter på myoglobinets redoks-former og fargestabilitet ble undersøkt i artikkel IV. Det ble laget en løsning som inneholdt kombinasjoner av malate, glutamat, pyruvat, sitrat og succinat. De ulike løsningene ble blandet i kjøttdeigen fra *M. semimembranosus* og pakket i modifisert atmosfære. Pakningene inneholdt en høy eller en lav konsentrasjon av oksygen og kjøttet ble lagret i henholdsvis 8 og 13 dager. Blandingenes evne til å redusere myoglobin ble kartlagt. Resultatene viste at en fjerning av oksygen i pakkens frivolum var avgjørende for reduksjon av myoglobin (DMB) og en blanding av 50% succinate og 50% glutamat-malat ga mest redusert myoglobin ved lav konsentrasjon av oksygen i pakken. En høy konsentrasjon av oksygen i pakken ga myoglobin i form av OMb og ble stabilisert av glutamat (eller malat) med 0,02 mol/kg sitrat til stede. Videre var konsentrasjonene 0,05mol/kg og 0,1 mol/kg av tilsetningsstoffer ikke signifikant forskjellige med hensyn til fargestabilitet i det tidsrommet forsøket fant sted.

List of papers

I. Khatri, M., Phung, V. T., Isaksson, T., Sørheim, O., Slinde, E., & Egelandsdal, B. (2012) New procedure for improving precision and accuracy of instrumental color measurements of beef. *Meat Sci.* DOI: 10.1016/j.meatsci.2012.01.012

II. Phung, V. T., Sælid, E., Egelandsdal, B., Volden, J., & Slinde, E. (2011). Oxygen Consumption Rate of Permeabilized Cells and Isolated Mitochondria from Pork M. Masseter and Liver Examined Fresh and After Freeze-Thawing at Different pH Values. *J Food Sci, 76*(6), 929-936.

III. Phung, V. T., Khatri, M., Egelandsdal, B., & Slinde, E. (2012). Meat oxygen consumption and biological variation as an effect on color stability. Submitted *Meat Sci.*

IV. Phung, V. T, Bjelanovic, M., Langsrud, Ø., Slinde, Sørheim, Isaksson & Egelandsdal (2012). Ingredients to stimulate oxygen consumption in minced meat. Submitted *Meat Sci*.

Abbreviations

A	Absorbance
a*	Red or green
ADP	Adenosine di-phosphate
AMSA	American meat science association
Ana	Antimycin A
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
b*	Yellow or blue
DMb	Deoxymyoglobin
EMSC	Extended multiplicative scatter correction
ETS	Electron transport system
$FADH_2$	Reduced flavin adenine dinucleotide
FCCP	p-trifluoromethoxy carbonyl cyanide phenyl-hydrazone
K/S	Kubelka-Munk ratio
L*	Luminosity
M	Malate
MANOVA	Multivariate analysis of variance
MMb	Metmyoglobin
Mna	Malonic acid
$NADH_2$	Reduced nicotinamide adenine dinucleotie
OC	Octanoyl carnithine
OCR	Oxygen consumption rate
OMb	Oxymyoglobin
OXPHOS	Oxidative phosphorylation
PCA	Principal component analysis
PLS	Partial least square
Q	Ubiquinone
RMSECV	Root mean square error of cross validation
SW	Selected wavelength
TCA	Tricarboxylic acid
ToC	Time of cutting
Tod	Time of death
ToDo	Lairage time at abattoir
ToT	Time from farm to abattoir

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Paper I - IV

1 Theory

According to the Meat and Poultry Research Centre (Animalia) almost 50 kg of meat is consumed by each person per year in Norway (Animalia 2011). Furthermore, the consumption of meat in Norway has declined slightly since 2009, and more so for beef than other types of meat (Animalia 2011).

Meat color is the consumer's immediate impression of the meat and a factor that influences the purchasing decision more than any other quality. The visual impression is important as discoloration is believed to indicate bacterial contamination, rancidity, or spoilage which in turn reduces palatability and shelf life. The attractive color in meat is transitory due to the inevitable surface discoloration after exposure to air. Oxygen causes a series of reactions that are detrimental to color and taste, such as oxidation of myoglobin, protein and fatty acids (O'Grady, Monahan et al. 2001; Kim, Huff-Lonergan et al. 2010). Fresh meat products are subject to further deterioration during production, distribution, retail display and storage.

This work has touched upon several of the above mentioned processes. Prevention and alleviation of oxidative damage can be carried out by removing residual oxygen during packaging and provision of reductive substrates. We have therefore analyzed color stability in meat with respect to mitochondrial activity as this organelle both foster the ability to consume oxygen and production of reducing equivalents. Given mitochondria's role as an integral part of the cell, the use of mitochondria might therefore be the optimal approach toward color stability and oxidation in meat.

1.1 From muscle to meat

Norwegian cattle and pigs are normally slaughtered between 1.5 - 2 years (except for cows and breeding animals) and at 6 - 7 months of age, respectively. As animals get older their metabolism shifts from growth to accumulation of fat. The muscle fibre type, location of the muscle and capillary density of the particular muscles are important factors that influence the *post mortem* biochemical processes and therefore meat quality (Klont, Brocks et al. 1998).

There exist three types of muscle fibres, Type I and Type IIA and IIB. Type I fibres are slow twitch and oxidative fibres, which means that oxygen is consumed in the production of ATP. These fibres are red due to high myoglobin content and contain large amounts of mitochondria. Type II fibres are divided into oxidative (Type IIA) and glycolytic (Type IIB) fibres. Type IIA fibres are fast twitch, and similar to Type I, these fibres are red and contain large amount of mitochondria and myoglobin. In comparison, type IIB fibres are also fast twitch but may appear white as they contain low amount of myoglobin and mitochondria. The muscles employed in this study are oxidative muscles, containing mainly type I fibres in *M. masseter* (Phung, Saelid et al. 2011). *M. semimembranosus* contained mainly glycolytic fibres (>60%) and was dominantly anaerobic (Hunt and Hedrick 1977). See also Figure 1 for schematic representation of a muscle fibre.

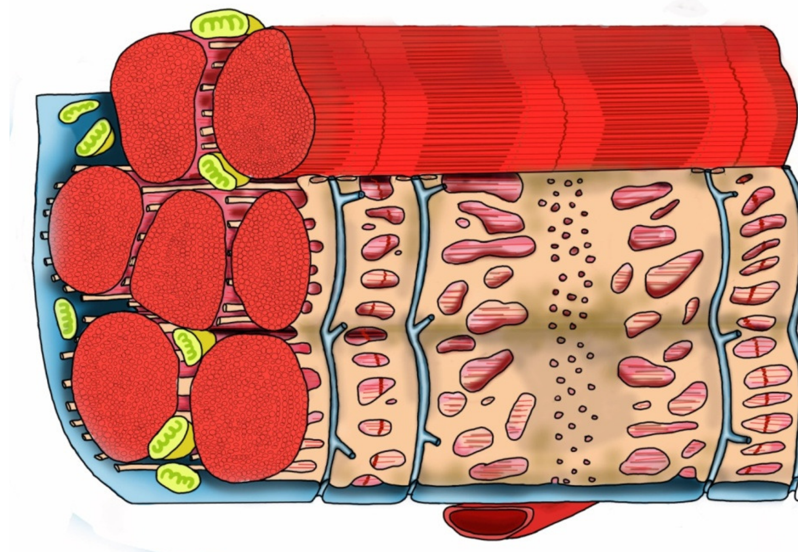

Figure 1. Schematic structure of a muscle fibre bundle. Mitochondria are yellow spheres dispersed between fibres. Artwork provided by Phung (2012).

When muscle is transformed to meat, the blood flow with nutrient and oxygen delivery in the body ceases. As a result, an array of processes is interrupted and new ones initiated (Figure 2). The animal loses all regulation of the central nervous system (CNS), maintenance of antioxidants and substrates and the intracellular oxygen becomes depleted. The muscle has lost its communication with the external environment and shifts from aerobic to anaerobic metabolism. Anaerobic metabolism produces small amounts of ATP and the carcass temperature declines. As oxygen is no longer available, the metabolism on glycogen and an impeded blood

flow leads to the accumulation of lactic acid, which in turn decreases the pH. A normal pH in *post mortem* muscle 24 hours after death is between 5.5 - 5.8 and protein denaturation and inactivation occur. Internal substrates and ATP are gradually depleted and an onset of *rigor mortis* follows. In beef and lamb, the time from death to *rigor mortis* is normally between 6 – 12 hours, while in pork it can range from 1 – 6 hours (Parker 2003). If the carcass is cooled too rapidly, the cold temperature may induce an irreversible contraction of the muscle known as cold shortening, resulting in the meat being up to 5 times tougher than normal. The reduction potential in meat is low due to a depletion of substrate and antioxidants, which in turn lead to the initiation and accumulation of protein and fatty acid oxidation and reactive oxygen species (ROS). A common condition in stress susceptible pigs is a rapid decline in pH which may reach the ultimate pH within one hour, while the carcass is still warm. The meat can be recognized with a pale color, soft texture and an exuding surface (PSE).

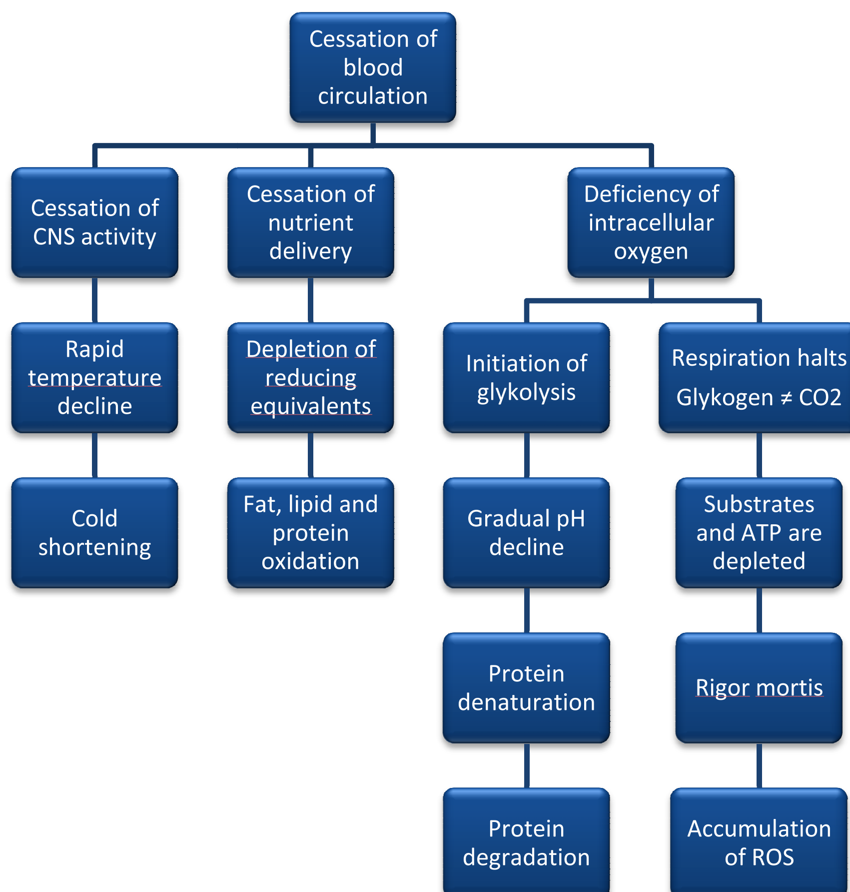

Figure 2. From muscle to meat.

1.1.1 Tissues relevant to the study

Post mortem muscle varies in color ranging from dark purple to pale light gray *e.g*. breast muscle of chicken are pale or white due to low amount of myoglobin, whereas the leg muscle is darker due to twice as much myoglobin. The muscles used in this work were oxidative and dark red, containing large amounts of myoglobin and mitochondria (McKenna, Mies et al. 2005; Seyfert, Mancini et al. 2006; Phung, Saelid et al. 2011). Muscles pertaining the aforementioned qualities were recognized as good models for this study due to a pronounced color intensity.

The *M. semimembranosus* is a large (~5 kg) muscle from cattle located at the ventral surface close to the hip and hind limb (Figure 3). *M. semimembranosus* has large gradients (color, protein and pH) across the muscle. The *M. masseter* is a small pork jaw muscle (~100 g, Figure 3) that can be obtained easily and early *post mortem* from the abattoir, but the muscle is not consumed as steaks. It is, therefore, an efficient muscle for establishing methodological frames. Muscle fibrils are bathed in a liquid called the sarcoplasm which contains lipids, fatty acids and glycogen. For both muscles the glycogen in the sarcoplasm is metabolized to pyruvate and converted to lactic acid in *post mortem* muscles.

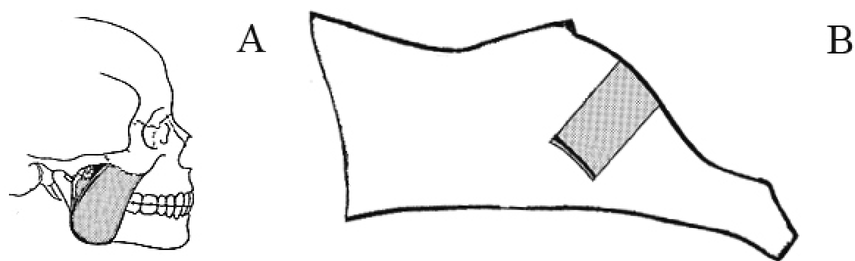

Figure 3. Placement of muscles marked in grey color with *masseter* from human (A) and *semimembranosus* from cattle back half (B).

Porcine liver was used in the preliminary studies for establishing and validation of mitochondrial isolation and respiration protocols. Liver is a dark colored soft tissue containing a high amount of mitochondria.

1.1.2 Biological variation of muscle and mitochondria

Muscle composition and color stability varies from animal to animal (Millar, Wilson et al. 1994) due to variations in genetical (growth rate, hormone etc.), environmental (feed, climate etc.) and metabolical (exercise, sleep etc.) factors. Moreover, the same muscle from different species may have entirely different fibre compositions and eating quality (Kujawska, Sobczak et al. 2009). Biological variation also exists within the same species, breeds and gender (Rikans, Moore et al. 1991; Kolath, Kerley et al. 2006; Ryu and Kim 2006; Hollung, Veiseth et al. 2007; Carstens and Kerley 2009; Phung, Khatri et al. 2012). Meat color stability is highly related to muscle type (Renerre and Labas 1987), myoglobin content (Renerre, Dumont et al. 1996) and mitochondrial content (Bendall and Taylor 1972; O'Keeffe and Hood 1982; Madhavi and Carpenter 1993).

Differences due to fibre types are caused by both genetic and environmental influences, and may occur both within and between animals of the same species (Essen-Gustavsson, Karlstrom et al. 1992). Furthermore, the fibre composition and metabolic profile of a muscle with similar fibre composition may differ between breeds (Essen-Gustavsson and Fjelknermodig 1985; Ruusunen and Puolanne 1997). Even within the same muscle there can be variations as Beecher et al. (1965) reported *M. biceps femoris* to have red muscle with high oxidative activity on the inner part while outer part of the muscle consisted of white muscle with low oxidative activity. The *M. semimembranosus* is a heterogenous muscle due to its large size; the muscle has a *post mortem* pH gradient, temperature gradient and different tenderness characteristics. These qualities are important in regard to e.g. *post mortem* storage since all parts are not chilled at an equal rate; the surface is chilled faster than interior, hence affecting glycolysis and protein denaturation.

Differences in metabolism are mainly due to different mitochondrial activity, amount and population. The mitochondria vary in size, shape, internal structure and metabolism (Kuznetsov, Mayboroda et al. 1998; Rossignol, Gilkerson et al. 2004; Scheffler, 2007). Mitochondrial physiology is diverse and reflects the organ's energy expenditure as they may function merely to produce heat by dissipating the proton gradient (Matthias, Jacobsson et al. 1999). Furthermore, organs and muscles may have several subpopulations of mitochondria depending on the fibre or biochemical gradients (Lanni, Moreno et al. 1996; Koekemoer and Oelofsen 2001; James, Madhani et al. 2002; Riva, Tandler et al. 2005; Kuznetsov, Troppmair et al. 2006). Forner,

Foster et al. (2006) have shown through mass spectrometric analyses that there are a number of proteins which are tissue specific. They compared heart muscle, skeletal muscle and liver mitochondrial proteome and reported that liver associated mitochondria had the highest number of tissue specific proteins.

Differences are also expressed at the biochemical level due to the respective gender. Female rats have been shown to have a lower amount of mitochondria but higher protein content than males. The differences were most apparent in older animals where enzyme activities are less efficient and have a major role in alleviating accumulated ROS and ROS induced damages (Carrillo, Kanai et al. 1992; Rodriguez-Cuenca, Pujol et al. 2002; Sobocanec, Balog et al. 2003; Justo, Frontera et al. 2005; Guevara, Santandreu et al. 2009). Moreover, as demonstrated by Carrillo et. al. (1992) and Sobocanec et. al. (2003), the catalase activity in relation to removing H_2O_2 was upregulated in female rats and mice and accompanied by lower oxidation capacities. Contrary to Renerre and Labas (1987) which demonstrated that gender related properties were insignificant to meat color stability as the muscles with the lowest color stability had the highest oxygen consumption and myoglobin oxidation rates, independent of gender.

1.2 Mitochondria in meat

Mitochondria are responsible for roughly 90% of the energy production in the cell in the form of ATP (Kidd 2005). ATP is produced when oxygen is reduced to H_2O through oxidative phosphorylation. Substrates such as glucose, amino acids and fatty acids are oxidized in the cytosol and imported to the tricarboxylic acid (TCA) cycle in the mitochondrial matrix. Electrons that are extracted from oxidation of TCA substrate are stored in the form of reduced nicotinamide adenine dinucleotide ($NADH_2$) and flavin adenine dinucleotide ($FADH_2$), which in turn are fed into the electron transport system (ETS) generating an electrochemical gradient and production of ATP (Figure 4). In total the mitochondria receive reducing equivalents from four entries, all of which feeds into ubiquinone (also known as the Q-junction). $NADH_2$, which arises predominantly from the TCA cycle, enters the ETS through complex I. $FADH_2$, which arise from succinate oxidation, enters from complex II. β-oxidation of fatty acids (e.g. octanoyl carnithin) and glycerol 3-phosphate oxidation donate electrons to flavoproteins (ETF) at the

inner and outer face of the inner mitochondrial membrane, respectively (Figure 4). The reduced flavoproteins donate their reducing equivalents to the ETS at the Q-junction.

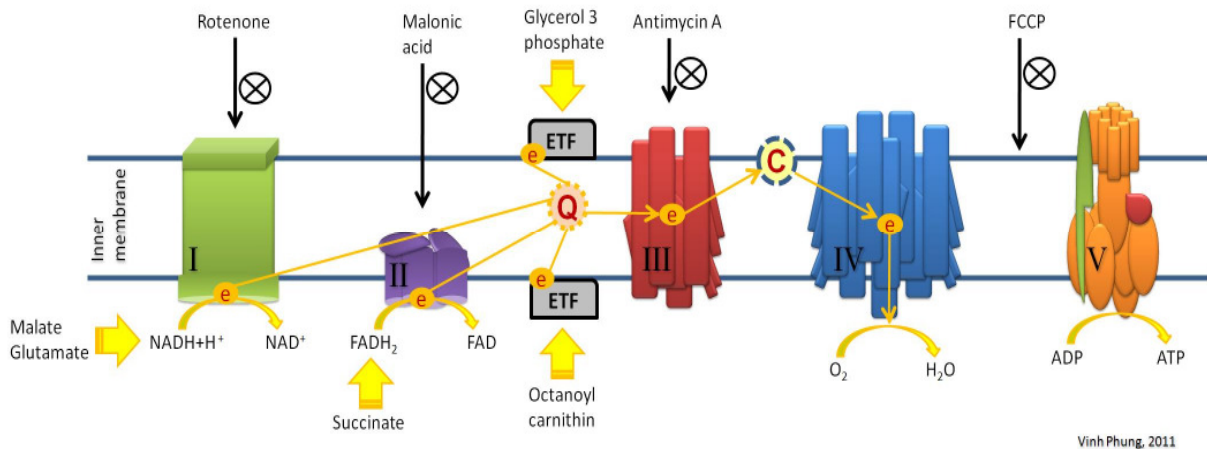

Figure 4. Mitochondrial ETS depicting the different complexes involved in the generation of an electrochemical gradient and ATP production. Roman letters represent mitochondrial complex I, II, III, IV and V. Cross circle represent inhibitors of the ETS. Yellow arrows represent entry of reducing equivalents. ETF; electron transferring flavoproteins, C; cytochrome C, Q; ubiquinone.

Mitochondria from liver tissue are perhaps the most studied due to its ease of handling (soft tissue) and high abundance. Liver mitochondria make up 36-39mg per gram of tissue, as measured by succinate dehydrogenase activity in whole liver homogenates (Katyare and Rajan 1988). Moreover, a single cell may contain up to 2000 mitochondria occupying nearly 25% of the liver cell's volume in humans (Bellomo 2006).

1.2.1 Oxygen delivery to mitochondria

Oxygen delivery to mitochondria occurs in two ways:

1. Myoglobin delivered oxygen
2. Diffusion as dissolved oxygen

Diffusion of oxygen from the lungs into the blood occurs rapidly. Oxygen are then reversibly bonded to hemoglobin and transported to the different organs, where it subsequently dissociates due to the decreased oxygen pressure (Sand, Haug et al. 2001). The oxygen then diffuses from the circulation and into mitochondria in the tissues.

Under heavy physical exercise or hypoxia (similar to *post mortem* muscle), myoglobin mediated oxygen delivery is switched on. Myoglobin functions as an oxygen storage molecule in the body and releases its bound oxygen when the blood and peripheral tissues are oxygen deprived (Maglischo 2003). The oxygen delivery to mitochondria by myoglobin facilitates oxidative phosphorylation, thus maintaining a high level of muscle activity for a longer period of time (Figure 5).

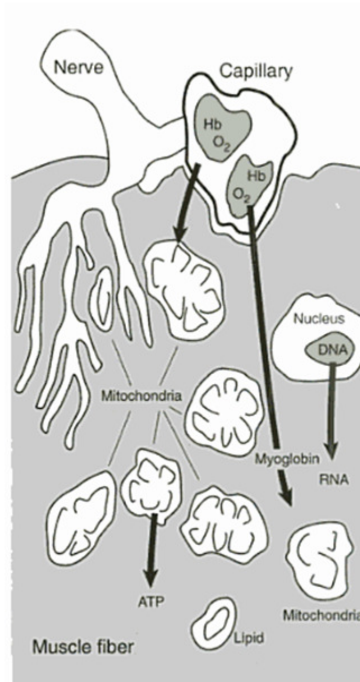

Figure 5. Oxygen delivery to mitochondria with diffusion, hemoglobin and myoglobin. Hb; hemoglobin. Reprinted with permission from E. W. Maglischo, 2003. ***Swimming fastest*** **(Champaign, IL: Human Kinetics), 361.**

However, it is not yet indisputably known if myoglobin has the ability to both store and transport oxygen under physiological conditions (Wittenberg 1970; Wittenberg, Wittenberg et al. 1975; Jurgens, Papadopoulos et al. 2000). Myoglobin-dependent oxygen delivery to mitochondria was shown by Wittenberg et al. (1987) by blocking the oxygen binding ability of myoglobin using carbon monoxide. It is suggested that a physical interaction occurs between mitochondria and myoglobin (Postnikova, Tselikova et al. 2009). Postnikova et al. (2009) proposed a mechanism where myoglobin docks at the inner leaflet of the cell membrane and

absorbs oxygen, after which it migrates and docks to the outer mitochondrial membrane and releases its bound oxygen. Myoglobin is a sarcoplasmic protein and too large to cross the mitochondrial membrane. The electron transfer between mitochondria and myoglobin was suggested to take place between complex III and complex IV (Wittenberg and Wittenberg 1987; Tang, Faustman et al. 2005b). Indeed, results by Postnikova, Tselikova et al. (2009) showed that the rate of oxymyoglobin (OMb) deoxygenation was equal to the rate of oxygen uptake by mitochondria. The contact between mitochondria and myoglobin was suggested to take place at the outer mitochondrial membrane on the cytochrome b_5 site (Livingston, McLachlan et al. 1985; Arihara, Cassens et al. 1995; Postnikova, Tselikova et al. 2009); see also section 1.3.1 regarding myoglobin.

1.2.2 *Post mortem* oxygen consumption

Mitochondrial activity can be characterized by the energy production and oxygen consumption that is mediated by enzymes of the ETS. Like many other enzyme complexes, the mitochondrial activity is highest near physiological pH (around pH 7) and temperature (around 37°C) (Cheah and Cheah 1971; Bendall and Taylor 1972). However, in *post mortem* meat the pH and temperature decline with time due to increased glycolysis and decreased metabolism (Farouk and Swan 1998; Young, Priolo et al. 1999). As a consequence, the oxygen consumption in *post mortem* meat declines with time in porcine (Atkinson and Follett 1973) and bovine muscle (Bendall and Taylor 1972; Atkinson and Follett 1973; Lanari and Cassens 1991; Madhavi and Carpenter 1993).

Having a low oxygen consumption compared to a high myoglobin content can be beneficial to color as myoglobin oxidation may occur at a slower rate than reduction (Atkinson and Follett 1973; O'Keeffe and Hood 1982; Renerre and Labas 1987) and muscles that have a high reducing activity customary also have high color stability (Reddy and Carpenter 1991). The *post mortem* oxygen consumption is dependent on the depth of oxygen penetration into the meat, which in turn is determined by; the partial pressure of oxygen (pO_2), the rate of oxygen penetration, and OCR of the tissue. It was found by O'Keeffe and Hood (1982) that the muscle with the lowest OCR also had the highest oxygen penetration depth after 7 days of storage and 2 hours exposure

to air. This was due to a lower oxygen consumption caused by loss of structural integrity in the mitochondria (Cheah and Cheah 1971; Giddings and Hultin 1974; Tang, Faustman et al. 2005a). Less efficient mitochondrial oxygen consumption ultimately allows oxygen to penetrate further into the muscle as oxygen consumption and oxygen penetration depth are negatively correlated (Millar, Wilson et al. 1994; McKenna, Mies et al. 2005). However, a functional mitochondrial oxygen consumption is crucial for the oxidation of substrates and hence production of reducing equivalents (Sammel, Hunt et al. 2002), which ultimately creates a layer of reduced myoglobin. The color in *post mortem* meat is therefore determined by the rate of myoglobin reduction, oxygenation and depth of oxygen penetration.

1.2.3 Mitochondria, myoglobin and meat color

Myoglobin reduction, oxygenation and oxygen penetration into the meat are topics related to mitochondrial activity. Mitochondria have been reported to be involved in meat color regulation where an actively respiring ETS maintains a dark purple color in the muscle *Longissimus dorsi* (Lawrie 1958; O'Keeffe and Hood 1982). The high respiration rate in this muscle stimulates a lower concentration of OMb as the decreased availability of oxygen would increase the prevalence of DMb (Phung, Bjelanovic et al. 2012). However, when oxygen is present the opposite would occur where a high oxygen consumption would lead to an increased metmyoglobin (MMb) formation (Renerre and Labas 1987; Phung, Bjelanovic et al. 2012). It is therefore reasonable to assume that a high content of mitochondria would enhance the conversion from MMb to deoxymyoglobin (DMb) in low oxygen environment as this is an enzyme dependent step, while conversion from OMb to MMb is enhanced by the presence of oxygen (Figure 7). Indeed, according to Nollet and Boylston (2007) DMb is most stable at low oxygen while OMb is stable at high oxygen levels. However, OMb will be oxidized to MMb (through an intermediary DMb) and remain so if the oxygen level decreases and there are no available source of substrate (Nollet and Boylston 2007).

Using different bovine skeletal muscles to find the localization of MMb reducing enzymes, Arihara, Cassens et al. (1995) reported a potential contact point for MMb reduction to be situated at the mitochondrial outer membrane. It is suggested by the authors that the enzyme

MMb reductase reside transiently on the surface of mitochondria and receive electrons from $NADH_2$, whereby it subsequently reduces MMb (Reddy and Carpenter 1991; Arihara, Cassens et al. 1995). Tang, Faustman et al. (2005b) showed that complex I and II inhibition decreased the reduction of MMb at various degrees, implementing the site of MMb reduction to be located between complex III and IV. Indeed, an inhibition of complex II (succinate dehydrogenase) by malonic acid did not completely inhibit MMb reduction (Arihara, Itoh et al. 1996).

1.2.4 Reactive oxygen species and lipid oxidation

Lipid membranes in cells and mitochondria are important for the taste and look of meat (Melton 1990; Lawlor, Sheehy et al. 2000). Oxidation of lipids decreases the quality of meat, caused by development of rancidity due to the degradation products, changes of color and texture, and nutritional loss of essential fatty acids and vitamins and increases in health risks due to generation of toxic compounds.

Lipid oxidation may be autocatalytic or mediated by free radicals provided by ETS. During ischemia and hypoxia and *post mortem* meat storage, there is an increase in the intracellular accumulation of ROS as the mitochondrial ETS capacity to produce reducing equivalents decreases with time. Mitochondria therefore have a central role in meat color stability and flavor. However, there are not much known about the specific role of mitochondria in these processes, as well as the proteins and mechanisms that are involved. In cut meat, mitochondria enter an ischemic and hypoxic state, and these changes inevitably affect the mitochondrial protein content and morphology. How the mitochondria attempts to cope with deleterious situation and how it affects the quality of meat is not yet known. Moreover, feeding trials with antioxidants has been shown to have a positive effect giving reduced lipid and OMb oxidation (Lawlor, Sheehy et al. 2000). Furthermore, studies have shown that female individuals have an increased content of mitochondrial proteins and enzyme activity over time compared to males, and might therefore be better protected against ROS damage and cellular aging (Rikans, Moore et al. 1991; Carrillo, Kanai et al. 1992; Sobocanec, Balog et al. 2003; Sverko, Sobocanec et al. 2004).

A cause of rancidity in cut meat is the gradual lipid peroxidation of membranes and fatty acids. Mitochondria are important as it is a major site of intracellular ROS which can initiate these reactions, additionally, exogenous oxygen radicals and other factors such as ionizing radiation, heat and pH also influence taste. The lipids that contribute to taste in meat are triglycerides and membrane phospholipids, the latter contains large amounts of unsaturated fatty acids such as arachidonic acid, linolenic acid, docosahexanoic acid and so on (Wood, Richardson et al. 2004; Campo, Nute et al. 2006). When these lipids undergo oxidation byproducts evolve, such as hexanal, pentanal, and 2-pentyl furan which gives the meat a rancid flavor and smell (Wood and Enser 1997; Campo, Nute et al. 2006; Stetzer, Cadwallader et al. 2008).

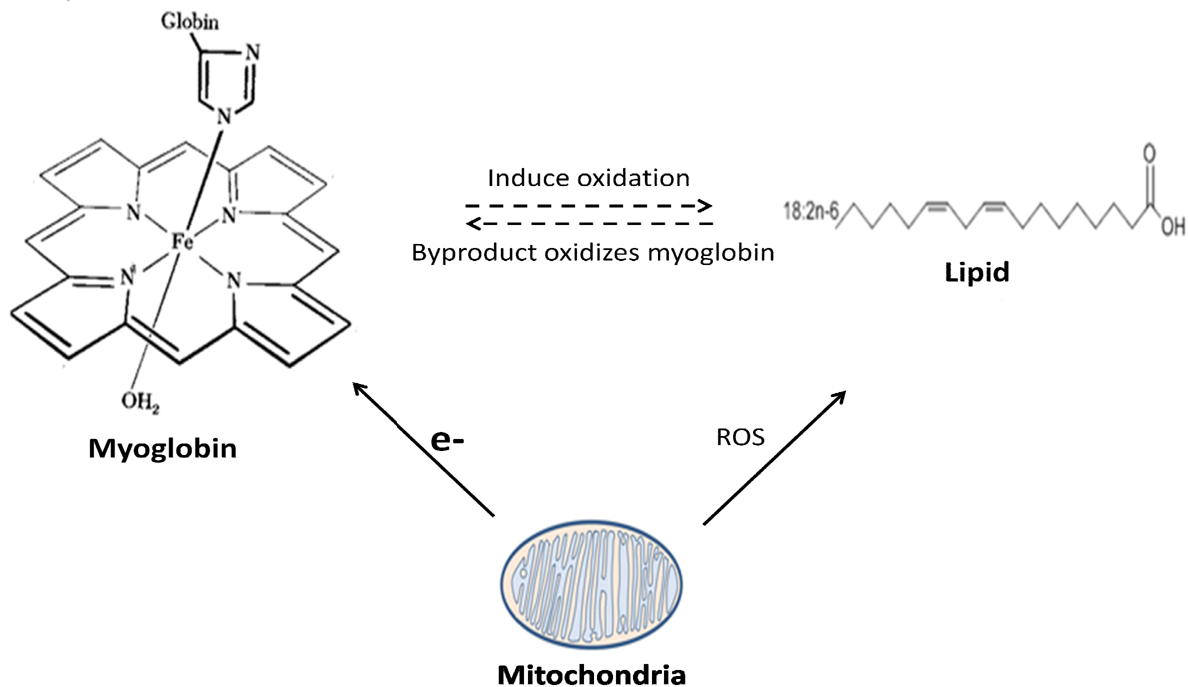

Figure 6. Relationships between mitochondria, myoglobin (MMb) and lipid. Representing myoglobin is the iron containing porphyrin with a bound water molecule and representing lipid is a structure of linoleic acid.

Myoglobin has been demonstrated to also have oxidation capacity and may induce lipid oxidation (Chan, Faustman et al. 1997; Faustman, Liebler et al. 1999; Volden, Bjelanovic et al. 2011). Likewise, byproducts of lipid oxidation are reported to promote oxidation of myoglobin. Indeed, a review by Faustman and Cassens (1990) reports that lipid and myoglobin oxidation are closely related, showing that an increase in one results in a similar increase of the other. Furthermore, the formation of free radicals is self propagating once initiated. The most prominent of these byproducts are 4-hydroxy-2-nonenal which acts by forming direct covalent

attachment to the protein portion of myoglobin (Esterbauer, Schaur et al. 1991; Faustman, Liebler et al. 1999; Suman, Faustman et al. 2007; Volden, Bjelanovic et al. 2011). Feeding cattle with vitamin E led to an increase in muscle α-tocopherol content with a subsequent delay in the post-slaughter discoloration of beef (Faustman, Cassens et al. 1989). By and large, meat color stability and flavor are determined by interrelated processes between lipid oxidation, myoglobin redox reactions, and mitochondrial antioxidative properties (Figure 6).

1.3 Factors affecting meat color stability

The color of meat is dependent on several intrinsic and extrinsic factors. The intrinsic factors are many and can be assigned *e.g.* mitochondrial activity, microbial load, enzyme activity and pH. Some of the extrinsic factors are *e.g.* storage method, packaging method or light exposure. Perhaps the most central factor is myoglobin, which contains the color pigment in meat and is discussed below.

1.3.1 Myoglobin

Myoglobin is the main color pigment in meat. The three dominant states of myoglobin are OMb, DMb and MMb. The different myoglobin states are determined by the oxidative status of its single iron molecule, which is part of a heme porphyrin ring. The iron molecule can be ferrous (Fe^{2+}), ferric (Fe^{3+}) or as an intermediate ferryl radical (Fe^{4+}) as a product of H_2O_2 oxidation (Kanner and Harel 1985). The iron molecule can bind to a ligand such as H_2O, oxygen, carbon monoxide or none, which is characterized as DMb, OMb, carboxymyoglobin or MMb, respectively (Table 2). It is the combination of the reductive state of the iron and its ligand and the total composition of myoglobin in the respective state that determines color in meat in addition to light scattering.

Myoglobin fluctuates between the three dominant states in uncured products, where MMb is the most stable state and changes from this state are slow and require more favorable conditions (Pearson and Gillett 1996; Mancini and Hunt 2005). Conversion from MMb proceeds to DMb, which is transient and combines readily with oxygen to form OMb. Myoglobin remains as DMb under low oxygen environment due to deficiency of oxygen. OMb is, however, fairly stable

under normal atmosphere and does not easily oxidize to MMb. Over time, when endogenous substrates in the meat have been depleted and myoglobin can no longer remain reduced, OMb is converted back to MMb (Figure 7).

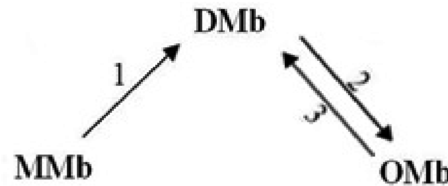

Figure 7. 1. MMb is reduced to DMb. 2. Presence of oxygen converts DMb to OMb. 3. Absence of oxygen allows myoglobin to exist as DMb.

The different states of myoglobin absorb visible light due to its porphyrin ring and iron molecule. DMb has a dark purple/red color and is characterized by a broad peak at 555nm with molar extinction coefficient of 12.9×10^{-3}. OMb has a bright red color and its absorption spectrum yields peaks at 535-545nm and 575-585nm with extinction coefficients equal to 14.6 and 15.1, respectively. The absorption peaks of MMb are located at 505nm and 627nm with extinction coefficients equal to 9.8 and 3.8, and MMb produces a distinctive brown-red color (Table 2).

1.3.2 Pre-slaughter treatment

Pre-slaughter handling affects stress levels inflicted on the animals. Stressful conditions elicit higher muscle temperature combined with lower pH early *post mortem* (Schafer, Rosenvold et al. 2002). Pre-slaughter stress may induce high carcass temperature and low pH due to increased glycogen metabolism (Stoier, Aaslyng et al. 2001; Schafer, Rosenvold et al. 2002; Rosenvold and Andersen 2003; Simela, Webb et al. 2004). These factors in turn decrease color stability (Monin and Sellier 1985; Ledward, Dickinson et al. 1986) and oxygen consumption in meat (Farouk and Swan 1998; Young, Priolo et al. 1999). However, pre-slaughter stress has no influence on color stability in stored meat (Milligan, Ramsey et al. 1998; Rosenvold and Andersen 2003; Phung, Khatri et al. 2012).

1.3.3 Post-slaughter treatment and storage

A quick chilling of the carcass after slaughter (carcass temperature decreases quickly after death) is important in order to slow down the *post mortem* glycolysis and hence interrupt the pH decline in addition to preventing microbial growth and evaporation. However, a low ultimate pH promotes low color stability in *post mortem* meat (Phung, Bjelanovic et al. 2012). Chilling the carcass too quickly has also been reported to decrease tenderness in addition to color stability (Simela, Webb et al. 2004). A slow chilling rate gives a good initial color as oxygen is allowed to penetrate into the tissue and bind to myoglobin. However, a slow chilling may result in early depletion of mitochondrial substrates and after storage the meat may have a low color stability (Farouk and Swan 1998; Young, Priolo et al. 1999).

Packaging is commonly done with high oxygen for retail display of steaks and minced meat. High oxygen packaging (80% O_2 and 20% CO_2) promotes OMb prevalence for as long as up to one week (Taylor and MacDougall 1973). Moreover, high oxygen packaging can reduce product's shelf life due to increased aerobic bacterial growth and lipid and protein oxidation (Jakobsen and Bertelsen 2000; Jeremiah 2001), accompanied by off-odor development and discoloration (Sorheim, Nissen et al. 1999; Grobbel, Dikeman et al. 2008). Packaging in low oxygen using modified atmosphere (containing CO_2 or N_2 or mixed) prolongs product shelf life and color stability (Isdell, Allen et al. 1999). Furthermore, myoglobin in low oxygen atmosphere exists as DMb in packages containing less than 0.1% O_2 and will remain reduced as long as oxygen is excluded (Sorheim, Westad et al. 2009).

Frozen storage is important for preserving meat but compromises color stability. Endogenous water molecules form ice crystals (both intra- and intercellular) upon freezing, and hence mechanically rupture cells, distort tissue texture and dehydrate the meat (Rahelic, Puac et al. 1985; Wheeler, Miller et al. 1990). However, freeze storage preserves enzyme activity and freezing close to physiological pH preserves much better enzyme activity than at low pH (Bodwell, Pearson et al. 1965). Freezing stops the activity of some enzymes (*e.g.* calpain) but some enzyme activity can be resumed after thawing (Dransfield 1994). Furthermore, freezing damage has been reported to increase oxidation in meat (Campo, Nute et al. 2006). Accordingly,

frozen-thawed meat is less color stable than fresh meat (Moore and Young 1991; Jeong, Kim et al. 2011).

1.3.4 Oxygen scavengers

There will always be a certain amount of residual oxygen even when the meat is packaged in low oxygen. The low oxygen packaging may therefore induce protein and lipid oxidations and this is relevant as myoglobin is most pro-oxidative at 1-2% v/v oxygen. Using oxygen scavengers to remove oxygen is an approach that has been adopted by many industries *e.g.* food industry, chemical production and for protection of metal surfaces. Oxygen scavengers in the food industry often come in small iron-containing packages containing powdered iron, salt and moisture. Iron is oxidized to iron oxide in the presence of (residual) oxygen. Oxygen scavengers that are used in conjunction with low oxygen packaging, would therefore extend color stability of meat by reducing the prevalence of MMb and may be a better alternative than addition of chemical compounds into the meat (Isdell, Allen et al. 1999; Beggan, Allen et al. 2006).

1.4 Additives in the meat industry

Additives in the meat industry are widely used from preservation of a product to enhancement of taste. Salts of sodium, potassium or calcium in combination with TCA substrates used in this work (succinate, glutamate, malate, citrate and pyruvate) are approved by the European Food Safety Authority (http://www.efsa.europa.eu/). Food related information regarding the additives employed in this work was found at natural-food-additive.com and chemistry.about.com/od/foodcookingchemistry/a/foodadditives.htm.

Saleh et al. (1968) tested several mitochondrial and glycolytic intermediates based on the idea that their oxidation would reduce MMb. Indeed, addition of specific substrates such as glycerol 3-phosphate or malate leads to increased reducing activity in meat. Oxidation of these substrates by mitochondria generates reducing equivalents that affects the myoglobin redox states. Reducing equivalents such as $NADH_2$ and $FADH_2$ ultimately forms 2.5 and 1.5 ATP, respectively (Nelson and Cox 2005). The functions and effects of each substrate and its location in mitochondrial metabolism are discussed in the following subchapters (Figure 8).

Malate dehydrogenase and the β-oxidation are likely the most fragile enzyme complexes toward *post mortem* inactivation (Bodwell, Pearson et al. 1965). Furthermore, cytochrome c oxidase did not lose activity 24 hours *post mortem* while succinate dehydrogenase activity increased during the same time span (Bodwell, Pearson et al. 1965). As shown by Ogata and Mori (1964), enzyme activities can be ranked from the most to the least stable as follows succinate dehydrogenase > malate dehydrogenase > glutamate dehydrogenase.

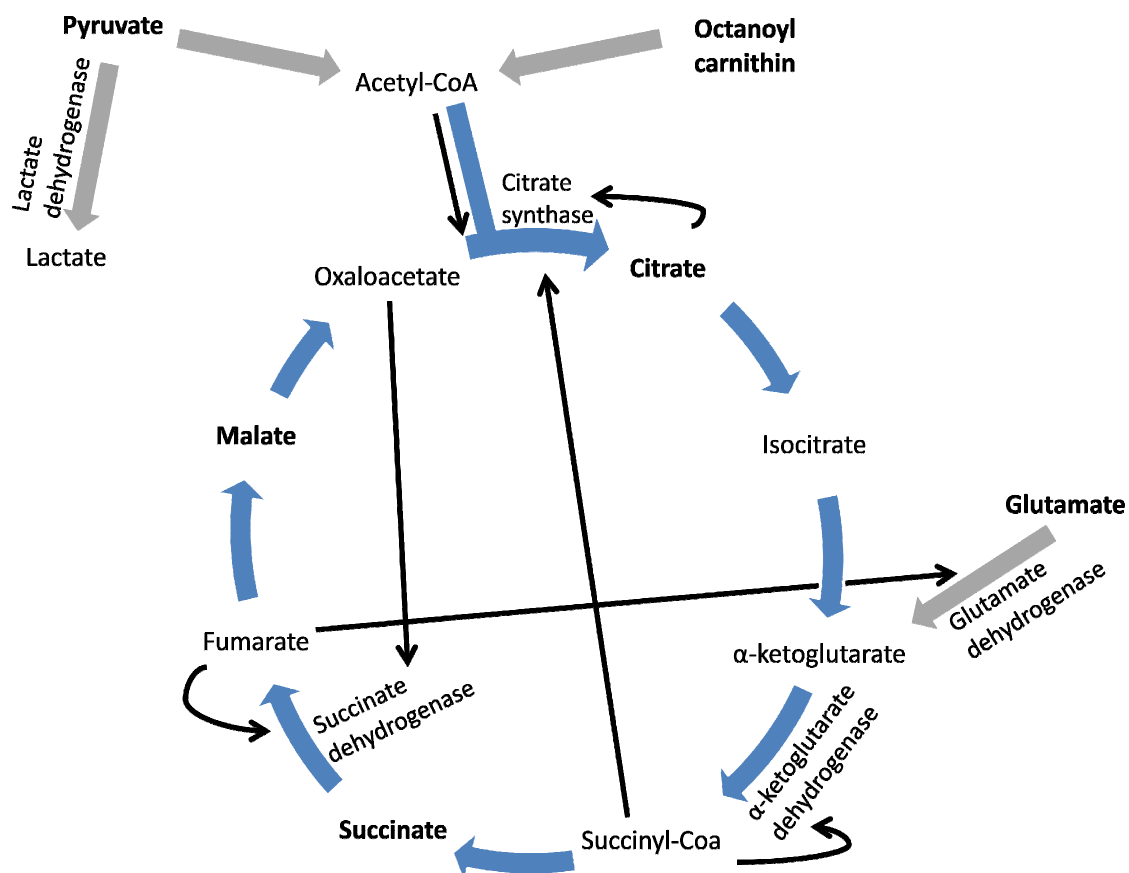

Figure 8. Schematic drawing of the tricarboxylic acid cycle (TCA, blue arrows) which occur in the mitochondrial matrix. Relevant additives are in bold. Grey arrows denote enzyme processes not part of the TCA. Black arrows denote negative feedback that inhibits the particular step of the cycle. Many of the enzymes in the TCA cycle are regulated by concentration dependent negative feedback from substrates, ATP and NADH. These mechanisms are to ensure that excess substrates are not oxidized when the energy content of the cell is abundant. However, regulation by ATP and NADH is not present in our system as it is assumed that the mitochondrial ETS is already uncoupled in meat.

1.4.1 Succinate

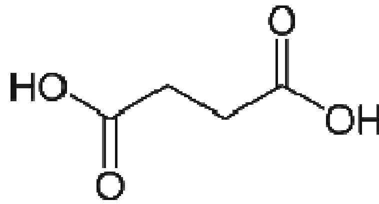

Figure 9. Succinic acid.

Succinate is used either alone or in combination with other flavor-enhancers, such as glutamate. It is an ingredient in hams, sausages and seasoning liquids.

Succinate is oxidized by succinate dehydrogenase to fumarate, with the concomitant reduction of FAD to $FADH_2$. Mitochondrial respiration on succinate alone is higher than on the combination of glutamate and malate (Kuznetsov, Schneeberger et al. 2004). The malate to fumarate equilibrium ratio is 4.1, which means that a large concentration of malate will prevent the hydration of fumarate to malate (Gnaiger, Mendez et al. 2000). The bottleneck created by fumarate will eventually inhibit the oxidation of succinate (Figure 8).

1.4.2 Pyruvate

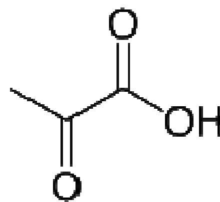

Figure 10. Pyruvic acid.

Pyruvate is a dietary supplement proposed to increase weight loss and fat burn (Stanko, Tietze et al. 1992). It has also been suggested to exert antioxidative effects (O'Donnell-Tormey, Nathan et al. 1987; Borle and Stanko 1996).

Pyruvate is exchanged electroneutrally for OH^- by the pyruvate carrier (Hildyard and Halestrap 2003). Pyruvate becomes decarboxylated to acetyl-CoA (acetyl coenzyme A) by the pyruvate dehydrogenase complex upon transport into the mitochondria. $NADH_2$ is produced in the

process. Condensation of acetyl-CoA and oxaloacetate yields citrate. A substrate combination of pyruvate and malate may therefore produce a complete TCA cycle. Indeed, uncoupled respiration on pyruvate and malate (in the presence of ADP) has been shown to produce 15% of the maximum respiration capacity (Rasmussen and Rasmussen 2000). However, the TCA cycle is self regulating and an excess of citrate will lead to inhibition of citrate synthase and export of citrate to the cytoplasm. In a study by Mancini et al. (2004) it is proposed that *post mortem* injected lactate may be converted to pyruvate by endogenous lactate dehydrogenase, with a concomitant reduction of NAD^+ to $NADH_2$. However, lactate dehydrogenase may also work in reverse (Figure 8) where an excess of pyruvate is likely to shift the equilibrium toward lactate and consume $NADH_2$ (Nelson and Cox 2005).

1.4.3 Malate

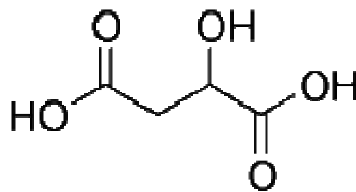

Figure 11. Malic acid.

Malate is used as supplement in food where it enhances fruit flavors. It is also used in combination with citrate to mask undesirable flavors.

Oxidation of malate by mitochondria may generate ATP in both aerobic (Cheeseman and Clark 1988) and anaerobic conditions (McKenna, Tildon et al. 1990). Oxidation of malate by NAD-linked malate dehydrogenase yields oxaloacetate with the concomitant reduction of NAD^+ to NADH. Condensation of oxaloacetate with acetyl-CoA by citrate synthase is responsible for pulling the malate dehydrogenase reaction forward in the TCA cycle. Consequently, oxaloacetate cannot be metabolized in the absence of a source of acetyl-CoA (e.g. pyruvate) and accumulates in the mitochondria as it cannot permeate the membrane (Figure 8). Respiration on malate is therefore expected to be miniscule when endogenous substrates are depleted.

1.4.4 Glutamate

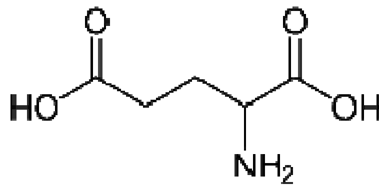

Figure 12. Glutamic acid.

The sodium salt of glutamate, monosodium glutamate, is the most common form of additive employed by the food industry. Glutamate is used for flavor enhancement and provides the umami taste. It is a natural constituent of many fermented food such as cheese and soya sauce.

Respiration supported by glutamate is carried out by glutamate dehydrogenase. Oxidation by glutamate dehydrogenase yields α-ketoglutarate and NADH. However, glutamate dehydrogenase may become inhibited by accumulation of fumarate (Caughey, Smiley et al. 1957). Glutamate may be used in combination with malate in the presence of transaminase. The catalyzed reaction of glutamate with malate by transaminase is reversible and produces α-ketoglutarate and an amino acid (Nelson and Cox 2005). Respiration on glutamate and malate were identical or a little higher than respiration with pyruvate and malate (Rasmussen and Rasmussen 2000). α-ketoglutarate is oxidized by the α-ketoglutarate dehydrogenase complex and yields succinyl-CoA together with NADH, however, the enzyme complex may be inhibited by product accumulation of succinyl-CoA (Figure 8).

1.4.5 Citrate

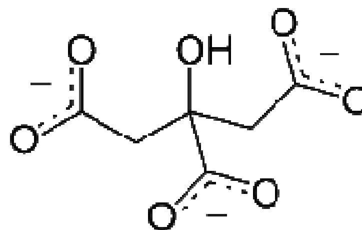

Figure 13. Citrate anion.

Citrate has commonly been used in the meat industry as a buffer and it has also been shown to improve tenderness by raising the pH (Jerez, Calkins et al. 2000; Perversi, Calkins et al. 2002;

Sitz, Matayumpong et al. 2005). However, color stability as measured by L*, a* and b* values did not improve with citrate additions (Jerez, Calkins et al. 2000). An excess of citrate may inhibit the condensation between oxaloacetate and acetyl-CoA. The excess of citrate is used to transport acetyl-CoA from the mitochondria to the cytoplasm where it is used for fatty acid and cholesterol synthesis.

1.4.6 Other additives affecting meat color

Lactate

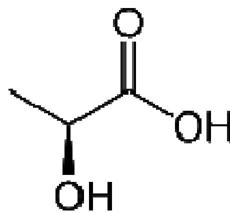

Figure 14. Lactic acid.

Lactate is a product of glycolysis in *post mortem* muscle and anaerobic metabolism. Accumulation of lactic acid decreases the pH and may therefore affect both tenderness and color. Animals that are stressed before slaughter may have a low ultimate pH due to increased anaerobic glycogen metabolism (Schafer, Rosenvold et al. 2002; Rosenvold and Andersen 2003).

Moreover, lactate has been shown to increase OMb and decrease MMb formation at pH 5.6 and 7.4 (Mancini and Ramanathan 2008). Indeed, it was suggested by Mancini et al. (2004) that in *post mortem* metabolism lactate is converted to pyruvate with the concomitant reduction of NAD to NADH, which in turn may reduce MMb. Another study found that enhancing beef with lactate lead to increased NADH concentration and lactate dehydrogenase activity, further supporting the color promoting properties of lactate (Kim, Hunt et al. 2006).

Vitamin E

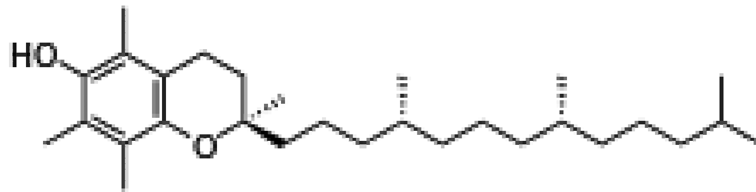

Figure 15. α-tocopherol form of Vitamin E

Vitamin E exists in eight different forms of which α-tocopherol is the most active. Vitamin E cannot be synthesized by the body and have to be supplied through the diet. Vitamin E is recognized as a potent antioxidant and is believed to alleviate the oxidation of lipids and myoglobin (Chan, Faustman et al. 1998; O'Grady, Monahan et al. 1998). Moreover, the effect of vitamin E occurs indirectly as it is proposed to delay the oxidation of OMb (induced by lipid oxidation), and thereby prevent the onset of MMb (Faustman, Chan et al. 1998).

Cattle fed with vitamin E have also demonstrated improvement of beef color (less MMb and higher a* values than controls) and reduced lipid oxidation (Chan, Faustman et al. 1998; O'Grady, Monahan et al. 2001).

1.5 Principals of spectroscopy

When a source of light reaches the surface of the meat, the light becomes modified. The light that enters the meat are scattered, absorbed (myoglobin absorbs the blue and green light) or reflected so that the beef appears red to the observer (Pearson and Gillett 1996).

Absorption, reflection, transmission and transflection

The color that our eyes perceive depends on how the wavelength of light are absorbed by the meat and reflected to the eyes. The absorbed light becomes thermal energy. The reflected light characterizes the color of meat. If the meat absorbs all frequencies of visible light except for the wavelengths associated with red light, then the meat will appear as red. If the meat is (somewhat) transparent, then the light passes through the material and is emitted on the opposite side, the effect is known as transmission.

Measurements of transparent materials would is done in transmission. However, transmission measurement in meat is difficult as the tissue absorbs efficiently light falling on the sample in the visible range. Reflection mode is then more efficient. If the reduction of light energy (also called attenuation) is not too strong, the sample may be subjected to something called the transflection mode. This mode has a reflector placed at the opposite side of the light source. The light source transmits light through the sample to reach the reflector and then reflects back into the sample. The transflectance mode receives signals in two parts. Firstly, some of the reflected energy (by the reflector) passes through the sample again and are detected at the detector somewhat offset to the light source. Secondly, another signal arises from the emitted light that is scattered back by the meat without hitting the reflector.

1.6 Statistical analysis

Multivariate analysis is a study that includes the observation and analysis of more than one statistical variable. The analysis elucidates the cause and effect of variables, how they relate to each other and their relevance to the problem being assessed. Some of the statistical approaches are discussed below.

1.6.1 Statistical methods

Factorial and fractional factorial designs

A full factorial design is a common experimental design with all factors set at two levels. These levels are "high" or "low", "1" or "0" and so on. The experiment takes on possible combinations of these levels across all factors; a full factorial design therefore has 2n number of runs (where n=number of factors). The design allows studying the effect of each factor on the response variable, as well as the effects of interactions between factors on the response variable.

When a large number of factors are involved and therefore the number of possible combinations in a full factorial design would be too time consuming, a fractional factorial design is chosen instead. The fractional factorial design consists of only some of the combinations (usually at least half are omitted)

from the full factorial design, thereby allowing the experimental design to be carried out in a feasible manner.

Mixture designs

A mixture design aims to find the optimal mixture between various dependent components. The sum of the mixture must be 1.0 (or a constant so that re-parameterization is possible) such that the values of each component can be interpreted as proportions of 1.0. The response surface of the mixture is achieved by regression and the components are present as factors in the regression equation. Common approaches to the mixture designs are simplex-lattice and simplex-centroid. These mixture designs evolve over an L-simplex (the pure "corners"). The Simplex Centroid Design has points at the corners, the midpoints of the sides, and the center. A simplex lattice design of degree m consists of m+1 points (0, 0.5 and 1); it gives totally 6 combinations with three "corners". Both designs can be augmented.

Stepwise regression; forward and backward

Stepwise regression using forward selection starts by testing variables one by one and includes them in the model if found to be statistically significant. Backward elimination use the opposite approach as it starts with all variables and continue the removal until it reaches the minimum number of significant variables.

Principal component analysis

Principal component analysis (PCA) is used to transform a set of response variables (Y-matrix) into a smaller number of uncorrelated variables called principal components. The interrelation between different factors can be explored by plotting the principal components and survey for sample patterns, groupings or trends. The first principal component has the highest explained variance, the second principal component is orthogonal to the first and explains the remaining variation, and so forth.

Partial least square regression

Similar to PCA, partial least square regression (PLS regression) is used to find covariance between predicted variables (X-matrix) and response variables (Y-matrix). The PLS model does this by finding the relevant components in the X-matrix that explains the maximum variance in the Y-matrix. In contrast to PCA, partial least square regression (PLS regression) can make predictive models with an

even a larger number of factors that are in addition highly correlated (or even collinear). This step is followed by a regression step where the X-matrix is decomposed and used to predict Y. The regression allows for the prediction modeling of Y.

Three way regression techniques

N-PLS, which is a multi-way regression method, uses three or more modes (Bro 1996). The Y-variable (response) in N-PLS can have multi-way array responses unlike PLS regression which has single matrix responses.

PARAFAC

PARAFAC is a method to decompose a multi-way data array and is especially useful to analyze spectral data. The method unfolds the multi-way array to a matrix and then performs a standard two-way analysis, such as PCA. The decomposed array is sorted into "scores" and "loadings" and describes the data in a more condensed form.

Classical Analysis of variance

Analysis of variance (ANOVA) is a method that decomposes the response's variance into several parts that can be compared against each other for significance testing. Significance testing of an effect is done by comparing the particular effect's response variance to the residual variance (experimental error). If the response variance is larger than the residual variance then the effect is considered as significant. The effect is given as p-value where $p<0.05$ is significant.

Some other ANOVA output results are: sum of squares, degrees of freedom, mean square and F-value.

Multivariate analysis of variance

Multivariate analysis of variance, in comparison to ANOVA, involves comparison of multiple responses against several groups. MANOVA investigate if changes in the groups have significant effects on the responses and what are the interactions among the responses and among the groups. In other words, MANOVA measures the variability in the covariance between responses and groups, and among responses and groups. 50:50 MANOVA is a special approach that eliminates the errors involved through high co linearity of variables in the MANOVA analysis.

2 Objectives

This work was part of a larger study on the role of mitochondria as an antioxidant in *post mortem* muscle. The *post mortem* condition of muscle is set to accumulate oxidative compounds and mitochondria are one of the few (if not the only) organelle that can produce and rejuvenate the pool of reducing equivalents. It is therefore of interest to investigate mitochondria's role in the regulation of meat color.

The objective of this study was to elucidate the mitochondrial qualities that affect the myoglobin redox status. Of special interest were the mitochondrial substrates that may lead to the oxidation or reduction of myoglobin, the relevant enzyme complexes and finally the packaging conditions that best preserve the meat color for retail customers.

It should be pointed out that, to our knowledge, this is the first work that aims at using muscle mitochondria and muscle permeabilized fibres and relate the oxygen consumption properties to colour stability. Previous attempts have either been on heart mitochondria and whole muscles (Tang, Faustman et al. 2005a; Tang, Faustman et al. 2005b). Our objective was also to make a more stringent quantitative approach to the relation between mitochondrial oxygen consumption and colour stability.

3 Methods

The methods employed in this work were chosen as to elucidate mitochondria's role in meat color. Oxygen consumption in meat and its relation to meat color was studied by measuring oxygen consumption by isolated mitochondria and permeabilized tissue. The color of meat was assessed by measuring L*, a*, b* and myoglobin states. Both reflectance (Mancini, Hunt et al. 2003) and absorbance (Tang, Faustman et al. 2004) data were used. The resulting spectra were processed statistically, as described in section 3.4.

3.1 Assessment of L, a* and b* color parameters*

A chroma meter (CR-410, Konica Minolta, sensing inc. B8403706, Japan) measuring CIE L*, a* and b* color space is commonly used to assess meat surface color (CIE 1976; Pauli 1976). The L*, a* and b* represents three visual dimensions where L* denotes brightness range (L* = 0 as black and L* = 100 as white), a* denotes a range from red to green (+a* = red and -a* = green) and b* gives a range from yellow to blue (b* = yellow and -b* = blue). Calibration of the instruments was done with a white ceramic plate (L*= 97.9; a *=0.05; b*=2). Hence, L*, a* and b* are referred to as the tristimulus parameters (Table 1).

Since myoglobin is the major color pigments in meat, L*, a*, and b* represent these three variables.

Table 1. Properties of the tristimulus parameters L*, a* and b* according to CIE (1976).

CIE color	Range	Color
L* (lightness)	0 to 100	black – white
a* (redness)	-128 to 128	red – green
b* (yellowness)	-128 to 128	yellow – blue

3.2 Assessment of myoglobin states

The instrument used for measuring the sample surface was an optical probe from FOSS NIRSystems (Model 6500, 0654-Oslo, transflectance modus with a 40 x 40mm 2 Optiprobe TM system, 23 x 23 mm sensor area). The NIRSystems could measure visible, near infrared reflectance and absorbance (log (1/R), where R is the reflectance) from 400-1100nm.

Using the reflectance and absorbance spectra, myoglobin redox states in meat sample can be measured. Different myoglobin states have different spectra. Myoglobin states can be calculated by using isosbestic points for their spectra (Krzywicki 1979; AMSA 1991). Isosbestic points are the points where either 2 or 3 myoglobin states have the same reflectance/absorbance. All the three states have the same isosbestic point at 525nm in transparent solutions. The wavelength 525 nm is often used to eliminate differences in myoglobin content between different meat samples. Additionally, 473nm is the first isosbestic point for MMb and OMb and 572nm is the isosbestic point for DMb and OMb (Krzywicki 1979), see also Table 2.

Table 2. Myoglobin properties according to (Bowen 1949; Krzywicki 1979; AMSA 1991; Pearson and Gillett 1996; Tang, Faustman et al. 2004).

Myoglobin state	Oxidative state	Ligand	Color	Peak (nm)	Extinction coeff. × 10^{-3}	Isosbestic point (nm)
DMb	Fe^{2+}	None	Purple	555	12.9	572, 525
OMb	Fe^{2+}	O_2	Bright red	535-545 and 575-585	14.6 and 15.1	474, 525 and 572
MMb	Fe^{3+}	Water	Brown	505 and 627	9.8 and 3.7	474 and 525

Quantifying myoglobin states with absorbance measurements have some disadvantages compared with reflectance measurements on the meat surface. Obtained spectra may depend on how deep the measurements were taken *e.g.* the layer of surface myoglobin may vary in regard to oxygen consumption and hence the thickness of the particular myoglobin layer. Another imperfection is the physical characteristics of the meat *e.g.* the inherent structure is not taken into account. On the other hand, knowing the myoglobin states allows the researcher to predict and elucidate color changes that are masked in a*; assessing the ratios of DMb and OMb are also interesting. Additionally, L* (a* and b*) measurements would be compromised in situations where e.g. the meat has a watery surface and hence interferes with the reflectance.

Phung, Khatri et al. (2012) and others (Malley, Yesmin et al. 2002; Saeys, Xing et al. 2005) suggested that transflectance measurements may come out as superior due to their precision. The reflectance mode is also a good alternative due to its brevity and convenience.

3.3 Protocols for mitochondrial isolation and oxygen consumption

Mitochondria function as a natural antioxidant in meat since it removes oxygen, an initiator of oxidation. Oxidation of substrates in the mitochondrial matrix generates reducing equivalents that are fed into the ETS, which ultimately fuels the consumption of oxygen in the production ATP (*in vivo*). As stated by O'Keeffe and Hood (1982) and discussed above; the most important endogenous factor affecting color in muscle is the mitochondrial enzymes' activities and their ability to reduce myoglobin by elimination of oxygen and then release electron to MMb.
Oxygen consumption measurements in this study were conducted on isolated mitochondria and permeabilized tissue. An overview and comparison of the approaches are given in Table 3.

Table 3. Isolation method for mitochondria and permeabilized tissue used for oxygen consumption measurements.

	Isolated mitochondria		Permeabilized cells	
	Liver	**Muscle**	**Liver**	**Muscle**
Starting material	10 g	20 g	10 – 70 mg	10 – 70 mg
Protease	Not used	Trypsin/Nagarse	Not used	Not used
Preparation time	3 hour(s)	4 hour(s)	1 hour(s)	1.5 hour(s)
Yield	High (~2.5mg/ml)	Low (~0.8mg/ml)	Not relevant	Not relevant
Substrate availability	None	None	Low	Low

Isolated mitochondria have the advantage of being a transparent mono-subject system that can be surveyed without much noise and interferences. Isolated mitochondria allowed us to elucidate the functions of the different ETS complexes in *post mortem* meat, such as mechanism and location of electron transfer and robustness of the system in *post mortem* tissue and storage (Tang, Faustman et al. 2005b; Phung, Saelid et al. 2011). The study of mitochondrial function may therefore be best approached through isolated mitochondria. However, isolated mitochondria do not have endogenous substrates, inhibitors, other oxygen consuming components or electron carriers to the same degree as do permeabilized tissue due to a wash-out step (see isolation protocol). Furthermore, the sensitivity of isolated mitochondria towards stimulation by exogenous substrates was stronger in isolated mitochondria compared to permeabilized tissue due to a faster diffusion and lack of robustness (uncoupling) that a permeabilized tissue-system provided (Phung, Saelid et al. 2011). Hence, it was an advantage to

study the effects of mitochondria on meat color by first observing the behavior of isolated mitochondria and relate these to responses in meat by permeabilized tissue.

3.3.1 Mitochondrial isolation

Isolation by sedimentation coefficient

Mitochondrial isolation is a rigorous process and was adopted from methods by Frezza et al. (Frezza, Cipolat et al. 2007), Bhattacharya et al (1991) and Slinde et al. (1973; 1975).

The protocol was estimated for isolation of mitochondria from 20g of muscle tissue or 10g of liver. All buffers, solutions and equipment were pre-chilled at 4°C the night before experiments, otherwise ice was used at all times to keep materials chilled under the experiments. A Sorvall RC5-5C (Thermo Scientific, North Carolina, USA) refrigerated centrifuge was used with the swinging-bucket HB-4 rotor for sedimentation procedures. The rotor speed was verified manually with a tachometer and the time integral was read from the display as rad^{2} sec^{-1}. All chemicals were of analytical grade and purchased from Sigma Chemicals Corp. (St. Louis, MO), except for sucrose (VWR International AS, Norway).

Meat was placed on ice and the experiments carried out in a refrigerated room at 4°C. For pork liver it was important to minimize differences in mitochondrial populations due to the presence of periportal and peripheral liver cells. The slices were therefore consistently cut from the centre of the greatest lobe every time. Likewise, *M. semimembranosus* tissue was isolated from the centre of the muscle and at a similar location each time. Pork *M. masseter* was a small muscle and several muscles (3-4 pieces) were compiled to reach a sufficient amount of tissue for isolation. The tissues were excised and minced in homogenization medium containing 0.25 M sucrose, 1 mM EDTA and 5 mM potassium phosphate at pH 6.0. Skeletal muscle was digested for 30 min with 0.05% trypsin in phosphate buffered saline solution supplemented with 10 mM EDTA (pH 7.0). The tissue mince was then homogenized at 400 rpm in a Teflon-glass Potter Elvehjem homogenizer of 50 mL capacity; the plunger was forced to the bottom three times. The homogenate was transferred to four 50-ml centrifuge tubes, diluted with homogenization medium (R_{min} = 6.2 cm and R_{max} = 14.4 cm), and centrifuged at a time integral of 5.92×10^{7}

rad^2 sec^{-1} (3000 rpm for 10 min). The cell debris was discarded and supernatants combined into one container and homogenization buffer replenished. This dilution was defined as "100% homogenate". The 100% homogenate was centrifuged at 3.7 × 10^8 rad^2 sec^{-1} (7500 rpm for 10 min) according to the formula of for average sedimentation coefficient by (De Duve and Berthet 1953): $\bar{s} = \frac{3.5 \, log_{10} R_{max}/R_{min}}{\int_0^t rpm^2 dt} \, x \, 10^{-13}$

Where R_{min} and R_{max} are defined as the distances from the axis of rotation to the bottom and the surface of the liquid, respectively. S_{avg} is the average sedimentation coefficient and $\int_0^t rpm^2 dt$ is the time integral/minute.

Isolation by kit

In order to collect mitochondrial proteins quickly from the slaughter line and due to instrumental limitations at the abattoir, it was necessary to establish a mitochondrial isolation procedure that was more convenient and swift than isolation by sedimentation coefficient. A total of five commercially available mitochondria isolation kits were investigated (Table 4) and 2 kits tested (Qiagen and Sigma). The isolation kit from Sigma employed proteases (trypsin) to digest skeletal tissue while Qiagen did not. However, the advertised capacities did not match the proposed yields. The kit from Sigma gave the highest amount of mitochondrial proteins with an average of 134.6µg per 0.1g tissue, however, isolation using >0.3g tissue yield less proteins and more cell debris. In comparison to the isolation method using sedimentation coefficient, the yield was 2250µg per 10g skeletal tissue.

Table 4. Mitochondria isolation methods.

Method	Posted capacity	Posted yield	Protease
Sigma kit	10g skeletal muscle	*Not available*	Yes
Pierce kit	0.2g skeletal muscle	*Not available*	Yes
Mitosciences kit	0.5g liver	400µg	No
Qiagen kit	60mg liver/heart	20-80µg	No
IMGENEX kit	10g liver/muscle	230µg	No
Sed.coefficient	10g skeletal muscle	2250µg	Yes

*sed.coefficient: sedimentation coefficient

Marker enzymes

Glutamate dehydrogenase and succinate dehydrogenase were used as marker enzymes for mitochondria since they are localized to the mitochondrial matrix and inner membrane, respectively. Glutamate dehydrogenase activity was determined using kit from Dialab (Wiener Neudorf, Austria). The presence of glutamate dehydrogenase leads to a reduction of NAD to NADH, which has absorption at 340 nm. The marker enzyme succinate dehydrogenase was tested according to Padh (1992). The presence of succinate dehydrogenase was confirmed by adding succinate and nitroblue tetrazolium to the mitochondrial suspension. Nitroblue tetrazolium functions as an artificial electron acceptor which turns blue when succinate becomes oxidized by succinate dehydrogenase.

Determination of protein concentration

Protein concentration determination was carried out using a Qubit protein assay (Invitrogen, Carlsbad, Calif., U.S.A.). The assay is a fluorescence-based quantitation method using 3 internal standards that makes a standard curve for estimation of unknown. Excitations were at 570 to 645nm and emission at 655 to 725nm.

3.3.2 Mitochondrial oxygen consumption

Oxygen consumption measurements were done at ~200 µM O_2 using an Oroboros Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) as described by Gnaiger (2001). The oxygraph delivered all responses in real time. The instrument had 2 chambers each with a volume of 2 ml. Permeabilized tissue was added before closing the chambers. Isolated mitochondria and substrates were added using a syringe through an inlet at the top of the chamber. Adjustment of oxygen level in the experimental chamber was done by H_2O_2 titration through chamber inlets. Measurements were conducted in 20°C as isolated mitochondria quickly tend to deplete added substrates at physiological pH due to a high concentration of mitochondria. Measurements of permeabilized tissue were conducted at 37°C.

The protocol was adopted from Oroboros (Oroboros Instruments, Innsbruck, Austria) and contained chemicals that elicits the desired effect by mitochondria (Phung, Saelid et al. 2011; Phung, Bjelanovic et al. 2012; Phung, Khatri et al. 2012). A small amount of ADP (1µl) was added prior to every run in order to deplete endogenous substrates. An experimental run can be seen in Figure 16, 10 and Table 5 where the numbers indicate the sequence of chemical addition 1; malate, 2; octanoyl carnithin, 3; ADP, 4; glutamate; 5; succinate, 6; FCCP, 7; FCCP, 8; rotenone, 9; malonic acid, 10; antimycin A.

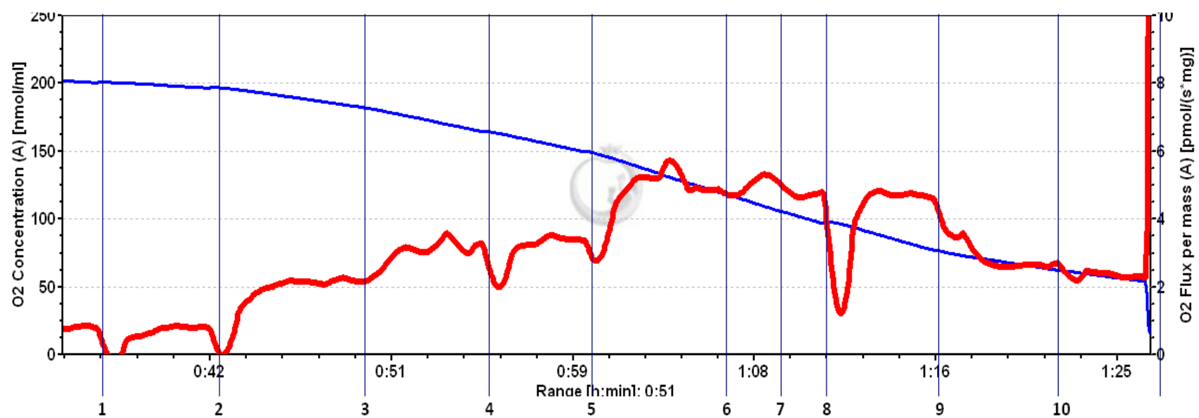

Figure 16. An experimental run from one of the chambers in the Oxygraph. The entire experiment takes 3 hours (sample preparation and background correction) while the experimental run took ~1 hour.

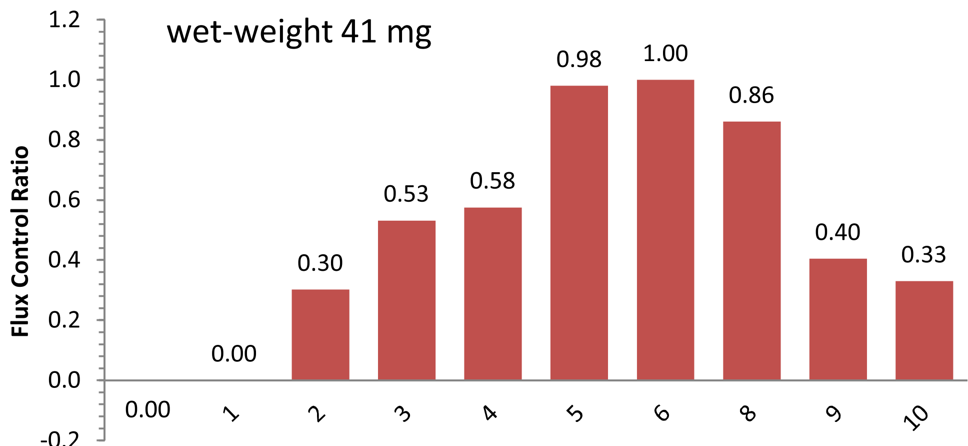

Figure 17. Data from the experimental run represented as ratios of FCCP response.

Table 5. Data from the experimental run were exported to Excel. Second row indicates the effect (numbers 6-9) or the location of effect (numbers 1-5). Tissue; only tissue without added chemicals, betaOx; betaoxidation. Sequence number 7 is omitted due to similar response as sequence number 6.

	2010-09-30 AB-03.DLD			1	2	3	4	5	6	8	9	10
	Averages	**Unit**	tissue	complex I	betaOx	ADP	complex I	complex II	uncouple	inhibit-I	inhibit-II	inhibit-III
	Start time		00:34:35	00:40:55	00:46:27	00:51:37	00:59:09	01:02:34	01:08:20	01:15:44	01:19:38	01:25:11
	Stop time		00:36:58	00:42:25	00:49:22	00:55:08	00:59:59	01:03:18	01:08:42	01:16:16	01:21:33	01:26:07
	O2 Concentration (A)	**nmol/ml**	**201.82**	**197.27**	**186.23**	**170.63**	**151.19**	**136.61**	**109.18**	**78.73**	**66.23**	**55.35**
X	**O2 Flux per mass (A)**	**pmol/(s*mg)**	**0.79**	**0.80**	**2.16**	**3.19**	**3.38**	**5.20**	**5.29**	**4.67**	**2.62**	**2.28**
	Block Temp.	°C	37.00	37.00	37.00	37.00	37.00	37.00	37.00	37.00	37.00	37.00
	Barom. pressure	kPa	99.30	99.30	99.30	99.30	99.30	99.30	99.20	99.20	99.20	99.20
	Peltier power	%	-30.10	-30.35	-30.56	-31.04	-31.10	-31.13	-31.52	-31.90	-32.13	-32.36
	pX Raw Signal (A)	V	0.41	0.45	0.47	0.50	0.49	0.49	0.47	0.46	0.48	0.48
	Slope pX (A)	mpX/s	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	pX Raw Signal (B)	V	0.47	0.49	0.48	0.51	0.48	0.47	0.47	0.48	0.49	0.49
	Slope pX (B)	mpX/s	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	O2 Flux per mass (A)	**mt**	**-0.01**	**0.00**	**1.36**	**2.39**	**2.58**	**4.40**	**4.49**	**3.87**	**1.82**	**1.48**
	Flux Control Ratio	**mt**	**0.00**	**0.00**	**0.30**	**0.53**	**0.58**	**0.98**	**1.00**	**0.86**	**0.40**	**0.33**

3.4 Statistical analysis

Statistical analyses were performed using Minitab (versions 15 and 16 from Minitab Inc., State College, PA, USA), The Unscrambler X 10.1 (CAMO Software, Oslo, Norway) or MATLAB (version 2011a, The MathWorks, Inc., Natick, Massachusetts, USA).

3.4.1 Paper I

The absorbance spectra of the *M. semimembranosus* were transformed into Kubelka-Munk K/S ratios and reflectance using The Unscrambler software. The transformation step is necessary in order to avoid the disturbance of light scattering in the spectroscopic data. Similarly, the data from reflectance and K/S ratio were alternatively treated to extended multiplicative scatter correction (EMSC) for correction. K/S ratios at wavelengths 474nm, 525nm, 572nm and 610nm were used to calculate the percentage of myoglobin using published formula (Krzywicki 1982; AMSA 1991). The myoglobin states that had the highest correlation coefficient (R_c) and lowest root mean square error of cross validation (RMSCEV) were chosen for processing.

Finally, the data were treated to PLS regression with cross validation to find the relationship between spectra (X-data) and myoglobin states (Y-data). Cross validation was included to minimize error in the predictive model generated by PLS, allowing the selection of the number of factors that were most accurate for the model. Therefore, PLS regression combined with cross validation can help pick the specific wavelengths giving lowest error for prediction of myoglobin states. The maximum number of PLS factors was set at 10, and systemic validation had 4 samples (repeated measurements) per segment for each myoglobin state. The result and number of PLS factors are presented in Paper I.

The resulting myoglobin values were normalized in an Excel spreadsheet. Criteria were set as OMb+MMb+DMb=1 as total amount of myoglobin cannot be more than 1. For pure states xMb=1 where x can be any of the myoglobin forms, and the prevalence of any form of myoglobin cannot be less than 0 (xMb=0). R_c and RMSCEV were used as the criteria for comparing myoglobin sample preparation and calculation method.

3.4.2 Paper II

The Minitab was used for calculation of significance of effect from mitochondrial substrates. The approach used was one way ANOVA and Tukey's test for comparison between liver tissue, muscle tissue, and isolated mitochondria from the respective tissues. Finally, General Linear Model was used to find means and standard errors of each effect.

3.4.3 Paper III

Minitab software was used for calculation of one way ANOVA with random effects and Tukey's test for multiple comparisons for significant differences in OCR between additives as a result of animal variation.

The Unscrambler software, principal component analysis (PCA) was used to find tendencies in muscle characteristics (weighting = 1/stand.dev). Minitab software was used to find the relationship between OCR and individual muscle characteristics by linear regression and stepwise regression (forward and backward).

NPLS was used to process data with multiple dimensions. The relationship between animals, changes in color (L*, a*, b* or myoglobin states), time and OCR (or individual muscle characteristics) were organized as a three-way array and processed by NPLS. Hence, the X-block data are three way having a sample mode which were animals, a first variable mode which was time, and a second variable mode which was color (L*, a*, b* or myoglobin states). The Y-block were either controllable variables (such as storage, transport time e.t.c.), uncontrollable variables (such as temperature, pH e.t.c.), 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.

3.4.4 Paper IV

The experimental design contained 8 points (Figure 18), where each point in the triangle represents a factorial design.

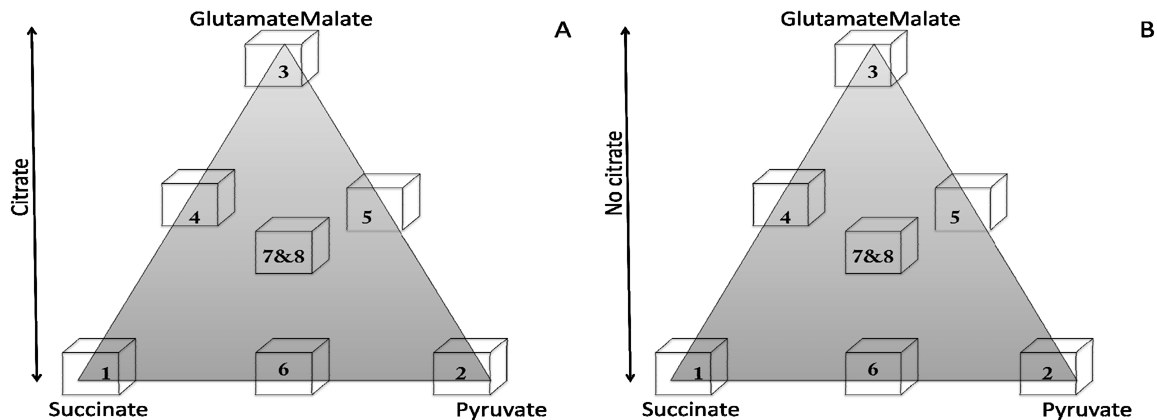

Figure 18. The two triangles are mirror images where A) includes citrate and B) has no citrate. The corners of each triangle represent pure chemicals and the spaces between corners are gradients of either increasing/decreasing concentration of the closes/furthest chemical, respectively. The cubical numbers represent 7 experimental points and 1 replicate. The experiment was repeated 4 times (4 days) with combinations of fat type and animal age (2^2 design).

The original full factorial design (Figure 18) consisted of a 3-component (succinate, glutamate/malate and pyruvate) simplex-lattice mixture design with an additional centre point (8 points), four 2-level variables (glutamate/malate, total Krebs' cycle substrates, citrate and oxygen; 16 points) and two extra whole-day 2-level variables (pork fat, beef fat, young and old animals; 4 points). A full design would therefore require 8*16*4= 512 experiments.

However, by using the fractional factorial approach, the numbers of experiments were reduced to 128 (32 each day). In addition, three extra samples from each corner of the 32-design containing pure treatments of succinate, pyruvate and glutamate-malate and 4 replicates were examined (the pure forms at each corner and at design point 7/8). The total number of samples adds up to 32 + 3 + 4 = 39 each day. Another 2 samples with only water were also added (total 41 samples).

4 Main results and discussions

There are many factors which affect consumer's preference of one meat product over the other. The decision is commonly based on meat color as it is an indication of quality, freshness and shelf life. Meat color stability is affected by several factors, some of them were discussed in section 1.3, and central to them all is the reduction of myoglobin by mitochondria.

The direct link between mitochondria and myoglobin reduction was found in an experiment with isolated mitochondria and MMb (Figure 19). In a solution containing MMb and isolated mitochondria respiring on succinate (complex I was inactivated by rotenone) it was proven that MMb became gradually reduced in a low oxygen environment (Slinde, Phung et al. 2011). The reduction was further enhanced by addition of cytochrome c, which is believed to aid respiration and functions as an electron mediator (Jones and Brewer 2009).

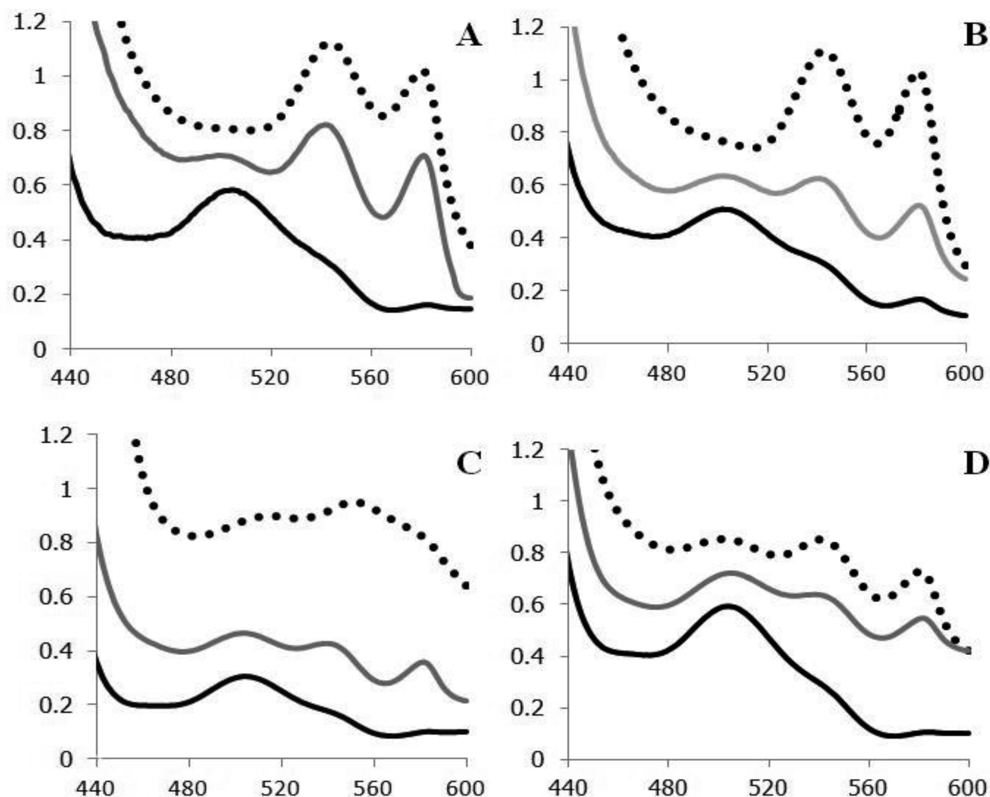

Figure 19. Absorption spectra of MMb (1.8mg/ml) and isolated liver mitochondria (5.5mg/ml) respiring on succinate at three different times after reaching zero oxygen pressure. Liver mitochondria are A and B and muscle (*M. masseter*) mitochondria are C and D. Fresh mitochondria are A and C and frozen stored mitochondria (-20°C) are B and D. Black line signifies absorption at 15min, grey at 3 hours and dotted line at 20 hours.

The experiments depended on being able to measure myoglobin states quite accurately. As an introductory approach experiments were arranged in order to find the most accurate method for measuring myoglobin states (Paper I). Secondly it was our aim to use isolated mitochondria or permeabilized fibres to study oxygen consumption rate to be compared with color stability; a comparison of their oxygen consumption was therefore made (Paper II). In this paper we also studied liver tissue since it is the golden standard tissue in mitochondrial research while using muscle mitochondria especially from mechanically tough tissues is still not very often used.

As meat color can be influenced by many factors, the study was continued by investigating the different variables that may affect color and mitochondria. Variables related to animal, pre-slaughter condition and storage were the focus of Paper III.

The packaging of meat and mitochondrial substrate was the focus of the last study. Packaging of meat in high oxygen is commonly used in the retail market (Carpenter, Cornforth et al. 2001) but the high levels of oxygen promote oxidation in meat. Paper IV therefore compared packaging in high and low oxygen and the possible additives (and mixtures of additives) that may affect meat color stability.

4.1.1 Paper I

This article addressed the current method in calibration modeling of different states of myoglobin. The method of sample preparation for calibration was according to guidelines by the American Meat Science Association (AMSA 1991) that used chemicals to obtain the different myoglobin states (CHEM). Their method was compared to our method which used oxygen partial pressure (OPP). Comparisons were based regarding the methods' ability to separate the three myoglobin states and the lowest number of prediction errors.

A comparison was also made of the method for calculating calibration data. The AMSA guidelines (AMSA 1991) recommended using selected wavelengths (SW) while our method employed all wavelengths (400-1100nm) by using partial least square (PLS) multivariate regression.

Our results show that all spectra that were treated to extended multiplicative signal correction (EMSC) and calculated by PLS using all wavelengths resulted in the lowest prediction errors, regardless of physical sample treatment or transformation by absorbance and Kubelka-Munk. However, physical treatment with the OPP method could only obtain a better prevalence for DMb and OMb, while MMb was best achieved by the CHEM method.

4.1.2 Paper II

The aim of this paper was to establish a method that monitors the oxygen consumption of muscle mitochondria. A comparison between liver and muscle tissue was made. The tissues were used as soon as possible *post mortem* (3 hours). Furthermore, it was important for the color studies to understand whether there was any difference between isolated mitochondria or permeabilized tissue oxygen consumption. Hence, the materials in this manuscript were liver and muscle tissue, as well as isolated mitochondria from both tissues. The oxygen consumption measurement protocol is described in section 3.2.2.

An unidentified oxygen consuming process, ROX, occurred only in the presence of biological material (permeabilized fibres) and continued to consume oxygen even after inhibition of the mitochondrial ETS. It was found that isolated mitochondria yielded much less ROX than permeabilized muscle. Liver tissue performed as expected with insignificant ROX and confirmed the robustness of the methods. Moreover, meat that was frozen stored yields more ROX than fresh meat and isolated mitochondria. Isolated mitochondria was therefore more suited for functional studies as the system was more transparent and had less noise than permeabilized tissue. But these findings also showed that isolated mitochondria should not be used in relation to meat color evaluations as they resemble much less the *in situ* processes that occur in meat. Permeabilized tissue is therefore more relevant as a model system due to its increasing ROX production with storage time, endogenous contents of cytochrome c and the ability to better cope with changes in pH than isolated mitochondria.

4.1.3 Paper III

Having established that permeabilized fibres would be more relevant models for oxygen consumption and since there seemed to be substantial biological variation between the different batches that was used in paper II, we continued with a study that looked at biological variation in oxygen consumption among different animal using fresh muscles obtained from a commercial hot boning slaughter line in an abattoir. Our studies showed a large animal variability, concerning both inter-animal differences and inter-treatment responses.

The acquired muscles were transported to the lab and kept refrigerated and vacuum-packed for 3 weeks. The animals that had high early *post mortem* oxygen consumption also had the strongest oxygen consumption responses after storage. Weighting (1/st.dev) and assigning all variables into one principal component analysis (PCA) plot revealed the importance of oxygen consumption (along PC-1 axis) and animal and muscle characteristics (along PC-2 axis). Two groups of oxygen consuming activities were identified, each related separately to the activity of complex I and complex II. In fresh meat the groups were allocated in the same direction along PC-1, however, after storage most of the complex I activity was lost while complex II was still viable, resulting in the two groups being allocated at opposite directions. It is interesting to note that ROX did not reside together with oxygen consumption variables in either fresh or stored measurements and this could indicate that ROX is not ETS related.

Changes in oxygen consumption after 3 weeks were related to color as L*, a*, b* and myoglobin states by methods established in Paper I. Myoglobin measurements gave superior detection of color changes over time as compared to L*, a* and b* measurements which had low resolution but small standard deviations. The results showed that oxygen consumption was the dominant factor for color changes in meat, 75% of the variation in oxygen consumption reflected 99% of changes in L*, a* and b* and 66% the variation in oxygen consumption reflected 82% of changes in myoglobin states.

Relating animal and muscle characteristics to oxygen consumption revealed that animal weight and age were the most important factors related to changes in color. However, the ultimate pH was identified to have the largest impact on color changes when all variables were weighted

(1/st.dev) for importance. The ultimate pH is known to affect *post mortem* enzyme and mitochondrial activity, hence myoglobin are more susceptible to oxidation at low pH (Goto and Shikama 1974; Ledward, Dickinson et al. 1986).

4.1.4 Paper IV

Having investigated the OCR and animal variables that affected color in the general meat production (Paper III), we proceeded to investigate the ability to use mitochondrial substrates to stabilize color.

Our results showed that reduced myoglobin and a* values in low oxygen packaging increased and stabilized with time, exceeding the color stabilities that were found in high oxygen packaging which decreased from day 1. It is also important to note that as color in low oxygen packaging improved with time, MMb prevalence and b*-values decreased concomitantly. The results indicate that the prevalence of reduced myoglobin was increased by adding mitochondrial substrates to the ground meat, as reported by a lower amount of MMb.

Response surface analysis showed that a mixture composition of glutamate-malate and succinate yielded the highest amount of reduced myoglobin and best a*-values in low oxygen packaging. Having established that the mitochondrial complex II is more long-lived than complex I (Paper II, III and IV), the oxidation of succinate is therefore more influential for color stability than oxidation of glutamate and malate in low oxygen. Pyruvate was most often associated with MMb prevalence and to a smaller degree OMb formation. However, considering the meager occurrence of OMb in low oxygen packaging, pyruvate was therefore a pro-oxidant in our system.

The situation was similar in high oxygen packaging as succinate was shown to promote DMb, glutamate and malate promoted OMb and a*-values and pyruvate promoted MMb formation. Moreover, these results supported the notion that myoglobin was transiently reduced to DMb before becoming OMb (Mancini and Hunt 2005), which is the most stable state under high oxygen environment (Nollet and Boylston 2007), and eventually oxidizes to MMb. However, the

magnitude of each additive combination depended largely on the packaging environment. Low oxygen packaging required nearly equal amounts of glutamate, malate and succinate for the generation of the highest a*-values and DMb, while high oxygen packaging required solely glutamate and malate for obtaining highest a*-values and OMb and succinate were only involved to promote DMb. Citrate addition to glutamate-malate stabilized OMb at the longer storage times.

5 Conclusions Paper I-IV

Diminishing meat color and oxygen consumption is due to mitochondrial decay and depletion of endogenous substrates. Myoglobin reduction by muscle mitochondria has been demonstrated by addition of exogenous substrates. Permeabilized muscle fibres will give another oxygen consumption profile than isolated muscle mitochondria especially if observations are made at low pH. Measurements of color stability of minced beef meat at low pH suggested mitochondrial enzymes to be important. The mitochondrial ETS was still viable, though severely impaired, after 3 weeks of storage and complex II was then responsible for most of the oxygen consumption. Under the right conditions, the mitochondria can sustain redness, suppress brownness and prolong color. Mitochondrial substrates such as glutamate/succinate will quickly establish and maintain DMb as the dominant form in low oxygen packaging. In high oxygen packaging glutamate/citrate can maintain OMb at a high level until day 8.

Exploiting mitochondrial enzymes' activities to stabilize color of meat that has even been chill stored for several weeks should be possible.

6 Future perspectives

The optimal combinations of additives arrived at here for stabilizing specific myoglobin states have not been described earlier (not even in the patent literature). The additives we have identified here represented optimized mixtures for 4 different relatively fresh beef muscles. It is possible that the optimal mixture ratio could be different in other types of muscles and of different age, and storage time.

To be of practical relevance (the additives are all E-numbered and in active use), minimum amounts of additive would be used and preferably used where additions serve multiple purposes like reducing lipid oxidation, increasing tenderness, preserving antioxidants and similar. This should be investigated in the future.

Minimizing additives in high oxygen should be possible through reducing oxygen concentration also at concentrations down to 50-60 % O_2 should be feasible. To what extent our optimal mixture can reduce lipid and protein and antioxidant oxidation in high oxygen packing remains to be seen.

A really small amount of glutamate/ succinate in skin-packed or vacuum packed beef samples should be tested. Also a comparison should be made between adding oxygen absorbers and these additives added to meat.

Finally, although our method for calculating myoglobin states seemed very efficient and gave useful estimates for the states, the methods of preparing the states should be furthered focused at eliminating the frequency (and magnitude) of samples predicted to have states with myoglobin fractions above 1 or below 0. The more close the sums of all states are to 100 %, the better.

7 References

AMSA (1991). *Guidelines for meat color evaluation*. Proc. 44th Recip. Meat Conf., Manhattan, KS.

Animalia (2011). Kjøttets tilstand 2011. Status i norsk kjøtt- og eggproduksjon. A. M. S. Johnsen, O. Nafstad, O. Alvseike and T. A. Ruud. Oslo, Animalia**:** 1-91.

Arihara, K., R. G. Cassens, M. L. Greaser, J. B. Luchansky and P. E. Mozdziak (1995). "Localization of metmyoglobin-reducing enzyme (NADH-cytochrome-b(5) reductase) system components in bovine skeletal-muscle." Meat Sci **39**(2): 205-213.

Arihara, K., M. Itoh and Y. Kondo (1996). "Contribution of the glycolytic pathway to enzymatic metmyoglobin reduction in myocytes." Biochem mol biol int **38**(2): 325-331.

Atkinson, J. L. and M. J. Follett (1973). "Biochemical studies on the discoloration of fresh meat." J. Food Sci. Technol. **8**(1): 51-58.

Beecher, G. R., R. G. Cassens, W. G. Hoekstra and E. J. Briskey (1965). "Red and White Fiber Content and Associated Post-Mortem Properties of 7 Porcine Muscles." J. Food Sci. **30**(6): 969-&.

Beggan, M., P. Allen and F. Butler (2006). "Oxygen scavenger effect on the development of metmyoglobin on beefsteaks during early low-oxygen storage." J Muscle Foods **17**(4): 381-397.

Bellomo, M. (2006). The Stem Cell Divide, Amacom Books.

Bendall, J. R. and A. A. Taylor (1972). "Consumption of oxygen by the muscles of beef animals and related species. II. Consumption of oxygen by post-rigor muscle." J Sci Food Agric **23**(6): 707-719.

Bhattacharya, S. K., J. H. Thakar, P. L. Johnson and D. R. Shanklin (1991). "Isolation of skeletal-muscle mitochondria from hamsters using an ionic medium containing ethylenediarninetetraacetic acid and nagarse." Anal Biochem **192**(2): 344-349.

Bodwell, C. E., A. M. Pearson and R. A. Fennell (1965). "Post-Mortem Changes in Muscle .3. Histochemical Observations in Beef and Pork." J Food Sci **30**(6): 944-&.

Borle, A. B. and R. T. Stanko (1996). "Pyruvate reduces anoxic injury and free radical formation in perfused rat hepatocytes." Am. J. Physiol. **270**(3 Pt 1): G535-540.

Bowen, W. J. (1949). "The absorption spectra and extinction coefficients of myoglobin." J Biol Chem **179**(1): 235-245.

Bro, R. (1996). "Multiway calibration. Multilinear PLS." J Chemometr **10**(1): 47-61.

Campo, M. M., G. R. Nute, S. I. Hughes, M. Enser, J. D. Wood and R. I. Richardson (2006). "Flavour perception of oxidation in beef." Meat Sci **72**(2): 303-311.

Campo, M. M., G. R. Nute, S. I. Hughes, M. Enser, J. D. Wood and R. I. Richardson (2006). "Flavour perception of oxidation in beef." Meat Science **72**(2): 303-311.

Carpenter, C. E., D. P. Cornforth and D. Whittier (2001). "Consumer preferences for beef color and packaging did not affect eating satisfaction." Meat Sci **57**(4): 359-363.

Carrillo, M. C., S. Kanai, Y. Sato and K. Kitani (1992). "Age-related changes in antioxidant enzyme activities are region and organ, as well as sex, selective in the rat." Mech Ageing Dev **65**(2-3): 187-198.

Carstens, G. E. and M. S. Kerley (2009). Biological basis for variation in energetic efficiency of beef cattle. Proceedings of the Beef Improvement Federation 41st Annual Research Symposium, Sacramento, California, USA.

Caughey, W. S., J. D. Smiley and L. Hellerman (1957). "L-Glutamic Acid Dehydrogenase - Structural Requirements for Substrate Competition - Effect of Thyroxine." J Biol Chem **224**(1): 591-607.

Chan, W. K., C. Faustman, J. Velasquez-Pereira, L. R. McDowell and T. R. Batra (1998). "Effects of alpha-tocopherol on metmyoglobin formation and reduction in beef from cattle fed soybean or cottonseed meal diets." J Anim Sci **76**(5): 1421-1426.

Chan, W. K. M., C. Faustman, M. Yin and E. A. Decker (1997). "Lipid oxidation induced by oxymyoglobin and metmyoglobin with involvement of H2O2 and superoxide anion." Meat Sci. **46**(2): 181-190.

Cheah, K. S. and A. M. Cheah (1971). "Post-mortem changes in structure and function of ox muscle mitochondria. 1. Electron microscopic and polarographic investigations." J Bioenerg **2**(2): 85-92.

Cheeseman, A. J. and J. B. Clark (1988). "Influence of the malate-aspartate shuttle on oxidative metabolism in synaptosomes." J. Neurochem. **50**(5): 1559-1565.

CIE (1976). International Commission on Illumination, Colorimetry: Official Recommendations of the International Commision on Illumination. CIE No 15 (E-1.3.1), Bureau Central de la CIE, Paris, France.

De Duve, C. and J. Berthet (1953). "Reproducibility of differential centrifugation experiments in tissue fractionation." Nature **172**(4390): 1142.

Dransfield, E. (1994). "Optimization of Tenderization, Aging and Tenderness." Meat Sci **36**(1-2): 105-121.

Essen-Gustavsson, B. and S. Fjelknermodig (1985). "Skeletal-Muscle Characteristics in Different Breeds of Pigs in Relation to Sensory Properties of Meat." Meat Sci **13**(1): 33-47.

Essen-Gustavsson, B., K. Karlstrom and K. Lundstrom (1992). "Muscle-Fiber Characteristics and Metabolic Response at Slaughter in Pigs of Different Halothane Genotypes and Their Relation to Meat Quality." Meat Sci **31**(1): 1-11.

Esterbauer, H., R. J. Schaur and H. Zollner (1991). "Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes." Free Radic Biol Med **11**(1): 81-128.

Farouk, M. M. and J. E. Swan (1998). "Effect of rigor temperature and frozen storage on functional properties of hot-boned manufacturing beef." Meat Sci **49**(2): 233-247.

Faustman, C. and R. G. Cassens (1990). "The biochemical basis for discoloration in fresh meat: a review." J. Muscle. Foods **1**: 217-243.

Faustman, C., R. G. Cassens, D. M. Schaefer, D. R. Buege, S. N. Williams and K. K. Scheller (1989). "Improvement of pigment and lipid stability in holstein steer beef by dietary supplementation with vitamin-E." J Food Sci **54**(4): 858-862.

Faustman, C., W. K. M. Chan, D. M. Schaefer and A. Havens (1998). Beef color update: The role for vitamin E.

Faustman, C., D. C. Liebler and J. A. Burr (1999). "alpha-tocopherol oxidation in beef and in bovine muscle microsomes." J Agric Food Chem **47**(4): 1396-1399.

Faustman, C., D. C. Liebler, T. D. McClure and Q. Sun (1999). "alpha,beta-unsaturated aldehydes accelerate oxymyoglobin oxidation." J Agric Food Chem **47**(8): 3140-3144.

Forner, F., L. J. Foster, S. Campanaro, G. Valle and M. Mann (2006). "Quantitative proteomic comparison of rat mitochondria from muscle, heart, and liver." Mol Cell Proteomics **5**(4): 608-619.

Frezza, C., S. Cipolat and L. Scorrano (2007). "Organelle isolation: functional mitochondria from mouse liver, muscle and cultured filroblasts." Nature Protocols **2**(2): 287-295.

Giddings, G. G. and H. O. Hultin (1974). "Reduction of ferrimyoglobin in meat." Critical Reviews in Food Technology **5**(2): 143-173.

Gnaiger, E. (2001). Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. Respiration physiology, Elsevier Science Bv.

Gnaiger, E., G. Mendez and S. C. Hand (2000). "High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia." Proc Natl Acad Sci U S A **97**(20): 11080-11085.

Goto, T. and K. Shikama (1974). "Autoxidation of native oxymyoglobin from bovine heart muscle." Arch. Biochem. Biophys. **163**(2): 476-481.

Grobbel, J. P., M. E. Dikeman, M. C. Hunt and G. A. Milliken (2008). "Effects of different packaging atmospheres and injection-enhancement on beef tenderness, sensory attributes, desmin degradation, and display color." J Anim Sci **86**(10): 2697-2710.

Guevara, R., F. M. Santandreu, A. Valle, M. Gianotti, J. Oliver and P. Roca (2009). "Sex-dependent differences in aged rat brain mitochondrial function and oxidative stress." Free Radical Bio **46**(2): 169-175.

Hildyard, J. C. W. and A. P. Halestrap (2003). "Identification of the mitochondrial pyruvate carrier in Saccharomyces cerevisiae." Biochem. J. **374**: 607-611.

Hollung, K., E. Veiseth, T. Froystein, L. Aass, O. Langsrud and K. I. Hildrum (2007). "Variation in the response to manipulation of post-mortem glycolysis in beef muscles by low-voltage electrical stimulation and conditioning temperature." Meat Sci **77**(3): 372-383.

Hunt, M. C. and H. Hedrick (1977). "Profile of fiber types and related properties of five bovine muscles." J. Food Science **42**(2): 513-517.

Isdell, E., P. Allen, A. M. Doherty and F. Butler (1999). "Colour stability of six beef muscles stored in a modified atmosphere mother pack system with oxygen scavengers." Int. J. Food Sci. Technol. **34**(1): 71-80.

Jakobsen, M. and G. Bertelsen (2000). "Colour stability and lipid oxidation of fresh beef. Development of a response surface model for predicting the effects of temperature, storage time, and modified atmosphere composition." Meat Sci **54**(1): 49-57.

James, P. E., M. Madhani, W. Roebuck, S. K. Jackson and H. M. Swartz (2002). "Endotoxin-induced liver hypoxia: defective oxygen delivery versus oxygen consumption." Nitric Oxide **6**(1): 18-28.

Jeong, J. Y., G. D. Kim, H. S. Yang and S. T. Joo (2011). "Effect of freeze-thaw cycles on physicochemical properties and color stability of beef semimembranosus muscle." Food Research International **44**(10): 3222-3228.

Jeremiah, L. E. (2001). "Packaging alternatives to deliver fresh meats using short- or long-term distribution." Food Research International **34**(9): 749-772.

Jerez, N., C. R. Calkins and J. Velazco (2000). "Adding value to low-quality beef muscles through glycolytic inhibition in pre-rigor muscle." Nebraska Beef Report: 79-81.

Jones, T. T. and G. J. Brewer (2009). "Critical age-related loss of cofactors of neuron cytochrome C oxidase reversed by estrogen." Exp Neurol **215**(2): 212-219.

Jurgens, K. D., S. Papadopoulos, T. Peters and G. Gros (2000). "Myoglobin: Just an oxygen store or also an oxygen transporter?" News Physiol Sci **15**: 269-274.

Justo, R., M. Frontera, E. Pujol, S. Rodriguez-Cuenca, I. Llado, F. J. Garcia-Palmer, P. Roca and M. Gianotti (2005). "Gender-related differences in morphology and thermogenic capacity of brown adipose tissue mitochondrial subpopulations." Life Sci. **76**(10): 1147-1158.

Kanner, J. and S. Harel (1985). "Initiation of Membranal Lipid-Peroxidation by Activated Metmyoglobin and Methemoglobin." Arch. Biochem. Biophys. **237**(2): 314-321.

Katyare, S. S. and R. R. Rajan (1988). "Enhanced Oxidative-Phosphorylation in Rat-Liver Mitochondria Following Prolonged Invivo Treatment with Imipramine." Brit J Pharmacol **95**(3): 914-922.

Kidd, P. M. (2005). "Neurodegeneration from mitochondrial insufficiency: nutrients, stem cells, growth factors, and prospects for brain rebuilding using integrative management." Altern Med Rev **10**(4): 268-293.

Kim, Y. H., E. Huff-Lonergan, J. G. Sebranek and S. M. Lonergan (2010). "High-oxygen modified atmosphere packaging system induces lipid and myoglobin oxidation and protein polymerization." Meat Sci **85**(4): 759-767.

Kim, Y. H., M. C. Hunt, R. A. Mancini, M. Seyfert, T. M. Loughin, D. H. Kropf and J. S. Smith (2006). "Mechanism for lactate-color stabilization in injection-enhanced beef." J Agric Food Chem **54**(20): 7856-7862.

Klont, R. E., L. Brocks and G. Eikelenboom (1998). "Muscle fibre type and meat quality." Meat Sci **49**: S219-S229.

Koekemoer, T. C. and W. Oelofsen (2001). "Properties of porcine white adipose tissue and liver mitochondrial subpopulations." International Journal of Biochemistry & Cell Biology **33**(9): 889-901.

Kolath, W. H., M. S. Kerley, J. W. Golden and D. H. Keisler (2006). "The relationship between mitochondrial function and residual feed intake in Angus steers." J Anim Sci **84**(4): 861-865.

Krzywicki, K. (1979). "Assessment of relative content of myoglobin, oxymyoglobin and metmyoglobin at the surface of beef meat." Meat Sci. **3**: 1-10.

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

Kujawska, J. Z., M. Sobczak and K. Lachowicz (2009). "Comparison of the texture, rheological propteries and myofibre characteristics of sm (semimembranosus) muscle of selected species of game animals." Polish Journal of Food and Nutrition Sciences **59**(3): 243-246.

Kuznetsov, A. V., O. Mayboroda, D. Kunz, K. Winkler, W. Schubert and W. S. Kunz (1998). "Functional imaging of mitochondria in saponin-permeabilized mice muscle fibers." J Cell Biol **140**(5): 1091-1099.

Kuznetsov, A. V., S. Schneeberger, R. Seiler, G. Brandacher, W. Mark, W. Steurer, V. Saks, Y. Usson, R. Margreiter and E. Gnaiger (2004). "Mitochondrial defects and heterogeneous cytochrome c release after cardiac cold ischemia and reperfusion." Am J Physiol Heart Circ Physiol **286**(5): H1633-1641.

Kuznetsov, A. V., J. Troppmair, R. Sucher, M. Hermann, V. Saks and R. Margreiter (2006). "Mitochondrial subpopulations and heterogeneity revealed by confocal imaging: possible physiological role?" Biochim Biophys Acta **1757**(5-6): 686-691.

Lanari, M. C. and R. G. Cassens (1991). "Mitochondrial activity and beef muscle color stability " J Food Sci **56**(6): 1476-1479.

Lanni, A., M. Moreno, A. Lombardi and F. Goglia (1996). "Biochemical and functional differences in rat liver mitochondrial subpopulations obtained at different gravitational forces." Int J Biochem Cell Biol **28**(3): 337-343.

Lawlor, J. B., P. J. A. Sheehy, J. P. Kerry, D. J. Buckley and P. A. Morrissey (2000). "Measuring oxidative stability of beef muscles obtained from animals supplemented with vitamin E using conventional and derivative spectrophotometry." J Food Sci **65**(7): 1138-1141.

Lawrie, R. A. (1958). "Physiological stress in relation to dark-cutting beef." J Sci Food Agric **9**(11): 721-727.

Ledward, D. A., R. F. Dickinson, V. H. Powell and W. R. Shorthose (1986). "The Color and Color Stability of Beef Longissimus-Dorsi and Semimembranous Muscles after Effective Electrical-Stimulation." Meat Sci **16**(4): 245-265.

Livingston, D. J., S. J. McLachlan, G. N. La Mar and W. D. Brown (1985). "Myoglobin: cytochrome b5 interactions and the kinetic mechanism of metmyoglobin reductase." J Biol Chem **260**(29): 15699-15707.

Madhavi, D. L. and C. E. Carpenter (1993). "Aging and processing affect color, metmyoglboni reductase and oxygen-consumption of beef muscles." J. Food Science **58**(5): 939-942.

Maglischo, E. (2003). Energy metabolism and swimming performance. Swimming fastest. M. Barnard and L. Hambly, Human Kinetics**:** 361-361.

Malley, D. F., L. Yesmin and R. G. Eilers (2002). "Rapid analysis of hog manure and manure-amended soils using near-infrared spectroscopy." Soil Sci Soc Am J **66**(5): 1677-1686.

Mancini, R. A. and M. C. Hunt (2005). "Current research in meat color." Meat Sci. **71**(1): 100-121.

Mancini, R. A., M. C. Hunt and D. H. Kropf (2003). "Reflectance at 610 nanometers estimates oxymyoglobin content on the surface of ground beef." Meat Sci **64**(2): 157-162.

Mancini, R. A., Y. H. Kim, M. C. Hunt and T. E. Lawrence (2004). "How does lactate-enhancement stabilize beef color?" In Proceedings, 50th International Congress of Meat Science Technology: 8-13.

Mancini, R. A. and R. Ramanathan (2008). "Sodium lactate influences myoglobin redox stability in vitro." Meat Sci **78**(4): 529-532.

Matthias, A., A. Jacobsson, B. Cannon and J. Nedergaard (1999). "The bioenergetics of brown fat mitochondria from UCP1-ablated mice. Ucp1 is not involved in fatty acid-induced de-energization ("uncoupling")." J Biol Chem **274**(40): 28150-28160.

McKenna, D. R., P. D. Mies, B. E. Baird, K. D. Pfeiffer, J. W. Ellebracht and J. W. Savell (2005). "Biochemical and physical factors affecting discoloration characteristics of 19 bovine muscles." Meat Sci **70**(4): 665-682.

McKenna, M. C., J. T. Tildon, R. Couto, J. H. Stevenson and F. J. Caprio (1990). "The metabolism of malate by cultured rat brain astrocytes." Neurochem. Res. **15**(12): 1211-1220.

Melton, S. L. (1990). "Effects of feeds on flavor of red meat: a review." J Anim Sci **68**(12): 4421-4435.

Millar, S., R. Wilson, B. W. Moss and D. A. Ledward (1994). "Oxymyoglobin formation in meat and poultry." Meat Sci **36**(3): 397-406.

Milligan, S. D., C. B. Ramsey, M. F. Miller, C. S. Kaster and L. D. Thompson (1998). "Resting of pigs and hot-fat trimming and accelerated chilling of carcasses to improve pork quality." J Anim Sci **76**(1): 74-86.

Monin, G. and P. Sellier (1985). "Pork of low technological quality with a normal rate of muscle pH fall in the immediate post-mortem period - the case of the Hampshire Breed." Meat Sci **13**(1): 49-63.

Moore, V. J. and O. A. Young (1991). "The effects of electrical stimulation, thawing, ageing and packaging on the colour and display life of lamb chops." Meat Sci **30**(2): 131-145.

Nelson, D. L. and M. M. Cox (2005). Lehninger Principles of Biochemistry, WH Freeman.

Nollet, L. M. L. and T. Boylston (2007). Pork color basics. Handbook of meat, poultry and seafood quality. L. M. L. Nollet. Ghent, Blackwell Publishing Professional**:** 719.

O'Donnell-Tormey, J., C. F. Nathan, K. Lanks, C. J. DeBoer and J. de la Harpe (1987). "Secretion of pyruvate. An antioxidant defense of mammalian cells." J. Exp. Med. **165**(2): 500-514.

O'Grady, M. N., F. J. Monahan, J. Bailey, P. Allen, D. J. Buckley and M. G. Keane (1998). "Colour-stabilising effect of muscle vitamin E in minced beef stored in high oxygen packs." Meat Sci **50**(1): 73-80.

O'Grady, M. N., F. J. Monahan and N. P. Brunton (2001). "Oxymyoglobin oxidation and lipid oxidation in bovine muscle - Mechanistic studies." J Food Sci **66**(3): 386-392.

O'Keeffe, M. and D. E. Hood (1982). "Biochemical Factors Influencing Metmyoglobin Formation on Beef from Muscles of Differing Color Stability." Meat Sci **7**(3): 209-228.

Ogata, T. and M. Mori (1964). "Histochemical Study of Oxidative Enzymes in Vertebrate Muscles." J. Histochem. Cytochem. **12**(3): 171-&.

Padh, H. (1992). Organelle isolation and marker enzyme assay. 13th Workshop/Conference of the Association for Biology Laboratory Education (ABLE).

Parker, R. O. (2003). Foods and Food products. Introduction to Food Science. S. L. Simpfenderfer, M. M. Prat, Z. M. Lawrenceet al, Thomson Learning**:** 275-276.

Pauli, H. (1976). "Proposed Extension of Cie Recommendation on Uniform Color Spaces, Color Difference Equations, and Metric Color Terms." J Opt Soc Am **66**(8): 866-867.

Pearson, A. M. and A. T. Gillett (1996). Processed meats. New York, Aspen Publishers Inc.

Perversi, C., C. R. Calkins and J. Velazco (2002). "Use of Sodium Citrate to Enhance Tenderness and Palatability of Pre-Rigor Beef Muscles." Nebraska Beef Report.

Phung, V. T., M. Bjelanovic, O. Langsrud, E. Slinde, O. Sorheim, T. Isaksson and B. Egelandsdal (2012). "Color of ground beef as affected by mitochondrial substrate respiration and exposure to oxygen." Submitted for publication Meat Sci.

Phung, V. T., M. Khatri, E. Slinde, O. Sorheim, K. H. Liland, T. Almoy, K. Saarem and B. Egelandsdal (2012). "Mitochondrial oxygen consumption in permeabilized fibers and its link to colour stability in bovine M. semimembranosus muscle." Submitted for publication Meat Sci.

Phung, V. T., E. Saelid, B. Egelandsdal, J. Volden and E. Slinde (2011). "Oxygen Consumption Rate of Permeabilized Cells and Isolated Mitochondria from Pork M. Masseter and Liver Examined Fresh and After Freeze-Thawing at Different pH Values." J Food Sci **76**(6): 929-936.

Postnikova, G. B., S. V. Tselikova and E. A. Shekhovtsova (2009). "Myoglobin and mitochondria: oxymyoglobin interacts with mitochondrial membrane during deoxygenation." Biochemistry (Mosc) **74**(11): 1211-1218.

Rahelic, S., S. Puac and A. H. Gawwad (1985). "Structure of Beef Longissimus-Dorsi Muscle Frozen at Various Temperatures .1. Histological-Changes in Muscle Frozen at -10-Degrees-C, -22-Degrees-C, -33-Degrees-C, -78-Degrees-C, -115-Degrees-C and -196-Degrees-C." Meat Sci **14**(2): 63-72.

Rasmussen, U. F. and H. N. Rasmussen (2000). "Human quadriceps muscle mitochondria: a functional characterization." Mol. Cell. Biochem. **208**(1-2): 37-44.

Reddy, I. M. and C. E. Carpenter (1991). "Determination of metmyoglobin reductase-activity in bovine skeletal-muscles." J Food Sci **56**(5): 1161-1164.
Renerre, M., F. Dumont and P. Gatellier (1996). "Antioxidant enzyme activities in beef in relation to oxidation of lipid and myoglobin." Meat Sci **43**(2): 111-121.
Renerre, M. and R. Labas (1987). "Biochemical factors influencing metmyoglobin formation in beef muscles." Meat Sci **19**(2): 151-165.
Rikans, L. E., D. R. Moore and C. D. Snowden (1991). "Sex-dependent differences in the effects of aging on antioxidant defense mechanisms of rat liver." Biochim Biophys Acta **1074**(1): 195-200.
Riva, A., B. Tandler, F. Loffredo, E. Vazquez and C. Hoppel (2005). "Structural differences in two biochemically defined populations of cardiac mitochondria." Am J Physiol Heart Circ Physiol **289**(2): H868-872.
Rodriguez-Cuenca, S., E. Pujol, R. Justo, M. Frontera, J. Oliver, M. Gianotti and P. Roca (2002). "Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue." J Biol Chem **277**(45): 42958-42963.
Rosenvold, K. and H. J. Andersen (2003). "The significance of pre-slaughter stress and diet on colour and colour stability of pork." Meat Sci **63**(2): 199-209.
Rossignol, R., R. Gilkerson, R. Aggeler, K. Yamagata, S. J. Remington and R. A. Capaldi (2004). "Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells." Cancer Research **64**(3): 985-993.
Ruusunen, M. and E. Puolanne (1997). "Comparison of histochemical properties of different pig breeds." Meat Sci **45**(1): 119-125.
Ryu, Y. C. and B. C. Kim (2006). "Comparison of histochemical characteristics in various pork groups categorized by postmortem metabolic rate and pork quality." J Anim Sci **84**(4): 894-901.
Saeys, W., J. Xing, J. De Baerdemaeker and H. Ramon (2005). "Comparison of transflectance and reflectance to analyse hog manures." J near Infrared Spec **13**(2): 99-107.
Saleh, B. and B. M. Watts (1968). "Substrates and intermediates in enzymatic reduction of metmeyoglobin in ground beef." J. Food Science **33**(4): 353-&.
Sammel, L. M., M. C. Hunt, D. H. Kropf, K. A. Hachmeister, C. L. Kastner and D. E. Johnson (2002). "Influence of chemical characteristics of beef inside and outside semimembranosus on color traits." J Food Sci **67**(4): 1323-1330.
Sand, O., E. Haug, Ø. V. Sjaastad and K. C. Toverud (2001). Menneskets fysiologi. Oslo, Gyldendal akademisk.
Schafer, A., K. Rosenvold, P. P. Purslow, H. J. Andersen and P. Henckel (2002). "Physiological and structural events postmortem of importance for drip loss in pork." Meat Sci **61**(4): 355-366.
Scheffler, I. E. (2007). Mitochondria, John Wiley & Sons.
Seyfert, M., R. A. Mancini, M. C. Hunt, J. Tang, C. Faustman and M. Garcia (2006). "Color stability, reducing activity, and cytochrome c oxidase activity of five bovine muscles." J Agric Food Chem **54**(23): 8919-8925.
Simela, L., E. C. Webb and L. Frylinck (2004). "Effect of sex, age, and pre-slaughter conditioning on pH, temperature, tenderness and colour of indigenous South African goats." S Afr J Anim Sci **34**: 208-211.
Sitz, M., P. Matayumpong, C. Perversi and C. R. Calkins (2005). "Pre-rigor Water Injection and Post-rigor Sodium Citrate Treatment on Beef Tenderness." Nebraska Beef Report: 90-92.

Slinde, E. and T. Flatmark (1973). "Determination of sedimentation coefficients of subcellular particles of rat liver homogenates. A theoretical and experimental approach." Anal Biochem **56**(2): 324-340.

Slinde, E., E. Morild and T. Flatmark (1975). "A general and rational approach to the optimal recovery of mitochondria by differential centrifugation in homogenous media." Anal Biochem **66**(1): 151-158.

Slinde, E., V. T. Phung and B. Egelandsdal (2011). Conversion of met-myoglobin directly to oxy-myoglobin by mitochondria from pork muscle (M. masseter) and liver. ICOMST 2011, Ghent, Belgium, 57th International Congress of Meat Science and Technology.

Sobocanec, S., T. Balog, V. Sverko and T. Marotti (2003). "Sex-dependent antioxidant enzyme activities and lipid peroxidation in ageing mouse brain." Free Radic Res **37**(7): 743-748.

Sorheim, O., H. Nissen and T. Nesbakken (1999). "The storage life of beef and pork packaged in an atmosphere with low carbon monoxide and high carbon dioxide." Meat Sci **52**(2): 157-164.

Sorheim, O., F. Westad, H. Larsen and O. Alvseike (2009). "Color of ground beef as influenced by raw materials, addition of sodium chloride and low oxygen packaging." Meat Sci. **81**(3): 467-473.

Stanko, R. T., D. L. Tietze and J. E. Arch (1992). "Body composition, energy utilization, and nitrogen metabolism with a 4.25-MJ/d low-energy diet supplemented with pyruvate." Am. J. Clin. Nutr. **56**(4): 630-635.

Stetzer, A. J., K. Cadwallader, T. K. Singh, F. K. McKeith and M. S. Brewer (2008). "Effect of enhancement and ageing on flavor and volatile compounds in various beef muscles." Meat Sci **79**(1): 13-19.

Stoier, S., M. D. Aaslyng, E. V. Olsen and P. Henckel (2001). "The effect of stress during lairage and stunning on muscle metabolism and drip loss in Danish pork." Meat Sci **59**(2): 127-131.

Suman, S. P., C. Faustman, S. L. Stamer and D. C. Liebler (2007). "Proteomics of lipid oxidation-induced oxidation of porcine and bovine oxymyoglobins." Proteomics **7**(4): 628-640.

Sverko, V., S. Sobocanec, T. Balog and T. Marotti (2004). "Age and gender differences in antioxidant enzyme activity: potential relationship to liver carcinogenesis in male mice." Biogerontology **5**(4): 235-242.

Tang, J., C. Faustman and T. A. Hoagland (2004). "Krzywicki revisited: Equations for spectrophotometric determination of myoglobin redox forms in aqueous meat extracts." J Food Sci **69**(9): C717-C720.

Tang, J., C. Faustman, T. A. Hoagland, R. A. Mancini, M. Seyfert and M. C. Hunt (2005a). "Postmortem oxygen consumption by mitochondria and its effects on myoglobin form and stability." J Agric Food Chem **53**(4): 1223-1230.

Tang, J., C. Faustman, R. A. Mancini, M. Seyfert and M. C. Hunt (2005b). "Mitochondrial reduction of metmyoglobin: dependence on the electron transport chain." J Agric Food Chem **53**(13): 5449-5455.

Taylor, A. A. and D. B. MacDougall (1973). "Fresh beef packed in mixtures of oxygen and carbon dioxide." Int. J. Food Sci. Technol. **8**(4): 453-461.

Volden, J., M. Bjelanovic, G. Vogt, E. Slinde, M. Skaugen, B. Nordvi and B. Egelandsdal (2011). "Oxidation progress in an emulsion made from metmyoglobin and different triacylglycerols." Food Chem **128**(4): 854-863.

Wheeler, T. L., R. K. Miller, J. W. Savell and H. R. Cross (1990). "Palatability of Chilled and Frozen Beef Steaks." J Food Sci **55**(2): 301-304.

Wittenberg, B. A. and J. B. Wittenberg (1987). "Myoglobin-Mediated Oxygen Delivery to Mitochondria of Isolated Cardiac Myocytes." Proc. Natl. Acad. Sci. U. S. A. **84**(21): 7503-7507.

Wittenberg, B. A., J. B. Wittenberg and P. R. Caldwell (1975). "Role of myoglobin in the oxygen supply to red skeletal muscle." J Biol Chem **250**(23): 9038-9043.

Wittenberg, J. B. (1970). "Myoglobin-facilitated oxygen diffusion: role of myoglobin in oxygen entry into muscle." Physiol Rev **50**(4): 559-636.

Wood, J. D. and M. Enser (1997). "Factors influencing fatty acids in meat and the role of antioxidants in improving meat quality." Br J Nutr **78 Suppl 1**: S49-60.

Wood, J. D., R. I. Richardson, G. R. Nute, A. V. Fisher, M. M. Campo, E. Kasapidou, P. R. Sheard and M. Enser (2004). Effects of fatty acids on meat quality: a review, Elsevier Sci Ltd.

Young, O. A., A. Priolo, N. J. Simmons and J. West (1999). "Effects of rigor attainment temperature on meat blooming and colour on display." Meat Sci **52**(1): 47-56.

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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 different meat surface areas were measured in reflectance mode (400–1100 nm) for each preparation method. Reflectance spectra were converted to absorbance (A) and then transformed by Kubelka–Munk transformation (K/S) and/or extended multiplicative scatter correction (EMSC). Transformed spectra of prepared pure states were used to make calibration 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 no state was <0 or >1 and the sum of all states equal to 1. Multivariate calibrations (i.e. PLS) outperformed the univariate calibrations (i.e. SW). The OPP method of preparing pure states was clearly best for OMb while the CHEM method was best for preparing MMb on fresh meat surfaces. Both preparation methods needed improvement concerning DMb. 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. This new reflectance protocol has potential for routine meat color measurements.

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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 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 (AMSA) guidelines for meat colour evaluation., 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, & 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,

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& Shaw, 1990; Hunt, Sørheim, & Slinde, 1999). The latter group of packaging methods will be called Oxygen Partial 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 525, 473, 572 and 730 nm. 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 limited by varying light scattering, myoglobin amount, packaging material and instrument wavelength calibration may call for a "softer" mathematical approach. In principle, we question the existence of true isobestic points for reflectance measurements in meat systems where the path of light or the concentration of myoglobin for each sample is not known.

The method of partial least squares regression (PLS) (Martens & Næs, 1989) finds fundamental relations between two matrices (*spectra* and *myoglobin state*). A PLS model will model the multidimensional direction in the *X* (spectra) space that covariate with 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* variables. This also means that the inherent nature of this regression method is to pick the wavelengths of the spectra that will fit and 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 & Stark, 1991) to handle diffuse reflectance spectra is called extended multiplicative scatter correction (EMSC) and is used to remove complicated multiplicative and additive effects, such as those caused by e.g. 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. from the surface of bovine *M. semimembranosus*. 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. Two spectral transforming techniques: the K/S transformation and the EMSC method were also compared. Finally the calculation principles using SW as explained by AMSA (1991) with the multivariate calibration method called PLS regression were compared. 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 samples

Fresh (3 days *post mortem*) vacuum packaged beef *M. semimembranosus* (SM) was obtained from a local slaughter house (Fatland, Oslo, Norway). The muscles were collected and kept on ice or at 4°C until used the next day, i.e. the 4th day *post mortem*. Breed and age group were unknown. Muscles (one side) from three different animals were collected. Each muscle was cut parallel to the fiber direction into 8 steaks/slices (starting from proximal end) of approximately identical size and at least 1.5 cm in thickness. The steaks were cut parallel to the fiber direction since the predictive equation would be used on samples cut parallel to the fiber direction. Each steak was subdivided into as many samples as possible, only ensuring that the samples were of sufficient size for the optical probe to be placed in four positions on the meat surface. In total forty-eight meat samples were randomly allocated for further preparation (OPP and CHEM methods). True replicates were secured through the use of muscles from different animals, cuts from different locations and by repeating the preparation technique (OPP or CHEM) on different samples. The samples prepared from SM covered the anatomical region used to prepare the additional samples (see below). 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 (A1-A6)

Six additional slices from fresh beef *SM* muscles obtained from a local slaughter house (Nortura, Rudshøgda, Norway) were used. These slices were vacuum-packaged and stored for 3 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 for the calibration samples. Immediately after removal (0.5 to 1 min) from the vacuum bags (type polyethylene/polyamide (PE/PA), oxygen transmission rate (OTR) of 30–40 $cm^3\ m^{-2}\ 24\ h^{-1}$ at 23 °C and 75% RH, LogiCon Nordic A/S, Kolding Denmark), the samples were wrapped with low density polyethylene (LDPE) film (OTR 6500–8500 $cm^3\ m^{-2}\ 24\ h^{-1}$ at 23 °C and 75% RH, Toppits-glad, Melitta Group, Klippan, Sweden). The samples were in direct contact with the film only when the probe was placed on the sample for spectroscopic measurements (otherwise the film was not directly in contact and was 2–3 cm above the meat surface). The samples were 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 30:1. The samples were analyzed for changes in myoglobin state on the surface due to air and light (see below) exposure.

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

2.2.1. CHEM method

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

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

2.2.1.2. MMb. The samples were treated with 1% potassium ferricyanide (Merck Eurolab) for 1 min, drained, blotted dry, packaged in LPDE film (Toppits-glad, Melitta group, Sweden) to oxidize at 2–4 °C for 12 h and measured.

2.2.1.3. DMb. The samples were treated with 10% sodium dithionite (BDH, Prolab, Lutterworth, UK) for 2 min, drained, blotted dry and vacuum packaged using the Original Henkelman Vacuum 300 system (<0.8 Torr, type 300II, Hertogenbosch, The Netherlands) in PE/PA bags (Maskegruppen) to reduce for 2 h 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. Samples for OMb and MMb were packaged on a Polimoon 511VG tray sealing machine (Promens, Kopavogur, Iceland). The trays were

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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^3 m^{-2} 24 h^{-1} at 23 °C at 50% RH, respectively.

2.2.2.1. 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 h).

2.2.2.2. 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 air with syringes through self sealing septas (Toray TO 125, Toray Engineering, Osaka, Japan). Concentration of O_2 was measured with a CheckMate 9900 instrument (PBI Dansensor, Ringsted, Denmark). Color measurements were made on different time intervals/days until day 7 (see below).

2.2.2.3. DMb. M. semimembranosus muscle samples were vacuum packaged in PE/PA bags (Maskegruppen) and stored at 2–4 °C for 48 ± 2 h before taking color measurements.

2.3. Color spectrum measurements

2.3.1. Calibration samples

A probe designed to measure large areas for spectroscopic measurements was selected to minimize the influence of fat and larger connective tissues. All the samples were covered with LDPE film (Toppits-glad, Melitta Group) and were kept in HDPE (Dyno 516, SWF Companies, Reedley, USA) black trays. The samples were measured as soon as the surface reached room temperature (17 ± 1 °C). The actual temperature is not important, but the calibration should be built at the same temperature that was used, or will be used, to measure the samples to be predicted (here A1–A6).

FOSS NIRSystems (Model 6500, 0654-Oslo, transflectance modus with a 40 × 40 mm^2 optiprobe™ system, 23 × 23 mm illumination area) was used for the color measurements. Absorbance (A = log (1/R); R is reflection) was calculated between 400 and 1100 nm in 2 nm steps giving 350 spectral variables. As a reference a white ceramic plate (from Minolta measurements: $L^* = 101.01$, $a^* = 1.74$ and $b^* = 5.3$) was used before measurements. Each spectral measurement is an average of 32 scans, and was recorded during 20 s. For each calibration sample, four random measurements from different positions on the surface of a sample were taken (giving 48 × 4 measurements). Finally, for each myoglobin state (DMb, OMb and MMb) and preparation method (CHEM and OPP) there were 32 (1 state × 4 positions × 8 different samples) spectra. Each of the CHEM and the OPP method consisted of 96 (3 states × 4 positions × 8 samples) spectra.

2.3.2. Additional samples (A1–A6)

Two spectroscopic measurements at different positions on the surface per samples were taken at 17 °C (the samples had a surface temperature of 17 °C for less than 5 min) and these were averaged before the myoglobin states were modeled. The measurements of surface color were taken at time 0 and after 0.25, 0.5, 0.75, 1, 1.5, 4, 24, 48, 72, 142 and 167 h. The samples were illuminated in a constant temperature cooler at 4 °C under a Phillips Master TL-D 36 W/830 H9 tube (color code 3000 K) attached to tube-holder Phillips IKC 1/36, 1 × 6 W–K with light intensity of 800 lx between measurement times.

2.4. Processing of spectral data and statistical analysis

The starting matrix for the calculations contained 96 (3 states × 4 locations × 8 samples) × 350 (absorbance at different wavelengths) variables for each preparation method. The Unscrambler software version 9.7 (CAMO software AS, Oslo, Norway) data spreadsheet was used for multivariate calibrations and scatter corrections.

2.4.1. Calibration samples

2.4.1.1. 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 reflectance, in The Unscrambler software version 9.7 (CAMO software AS, Oslo, Norway). As shown in Fig. 1, the data were either processed using EMSC then using SW or processed directly to SW without EMSC. The data were corrected using EMSC in The Unscrambler software version 9.7 (CAMO software AS, Oslo, Norway) using model and subtract options of the software and further processed using SW.

The data at wavelengths 474 nm, 525 nm, 572 nm and 610 nm were saved (Krzywicki, 1979; AMSA, 1991). These data were exported to Microsoft Excel, Version 2007 (Redmond, Washington: Microsoft, Computer software) and were used to calculate the 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 state. Myoglobin states could be calculated using two SW formulas, as shown below (for OMb):

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

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

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 to calculate the myoglobin states. The formulae giving the lowest RMSCEV (root mean square error of cross validation) and the higher R_c (correlation coefficient) were selected.

2.4.1.2. Principal component analysis (PCA). This method (Martens & Næs, 1989) was used to explore the relation between the different pure states. PCA uses an orthogonal transformation to convert the spectra into a set of values of uncorrelated variables called principal components.

2.4.1.3. Partial least square (PLS) regressions. First all the spectral data in absorbance form were run through PLS regression with cross validation (leave one sample out), or systematic validation with the 4 locations from the same sample per segment (leave 4 measurements out) for each myoglobin form. Secondly, the spectral data were treated by using extended multiplicative signal correction (EMSC) (Martens & Stark, 1991). The EMSC, if used, was performed using model and subtract options of the software and then run through PLS regression as described above.

2.4.1.4. Post transformation. This transformation after calculation of each state independently consisted of three steps: (1) $\hat{y} > 1$, corrected to $\hat{y} = 1$; (2) $\hat{y} < 0$, corrected to $\hat{y} = 0$; and (3) $\hat{y}_{OMb} + \hat{y}_{MMb} + \hat{y}_{DMb} = 1$. The sum of the 3 states after steps 1 and 2 will then always be positive and were normalized in the last step proportionally. R_c and RMSECV from each state were used as criteria for best performance and were calculated after the 3 steps above. Data output (scores, myoglobin states, number of principal components) were

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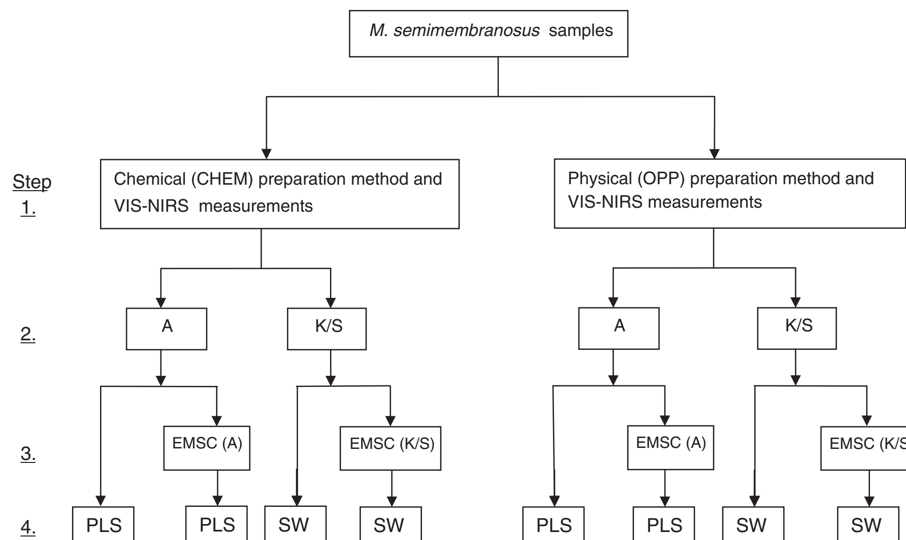

Fig. 1. Flowchart showing the overall physical and statistical treatments done in calibration and chemically treated samples before normalization (for abbreviations see Materials and methods): Step1—physical treatment for sample preparation either by OPP or CHEM method with visual (VIS-NIRS) measurements; 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); i.e. 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 (1991).

post transformed in Microsoft Excel, Version 2007. This step was applied for all data processed using SW or PLS regression.

Details regarding the processing of data are given in Fig. 1 and Table 1. Table 1 also indicates which variables were used to evaluate methods and processes (RMSECV and R_c). RMSECV indicates the error to be expected when a specific calibration model is used for prediction of myoglobin states. The RMSECV values can, in general, be used to define a prediction confidence interval. Assuming, normal distribution and no bias, one can expect with about 95% confidence that a prediction of a state ($\hat{y}$) will be within the interval $\hat{y} \pm 2\text{RMSECV}$.

2.4.2. Additional samples (A1–A6)

After selecting the calibration method with the lowest prediction error, the myoglobin states of the additional samples (A1–A6) were calculated using the predictive model. The OPP method of sample preparation was compared with that used in 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 measurements (from different positions of a sample) at each time for the A1–A6 samples, the predicted outcome of the spectra was averaged.

Finally, the data of additional samples were normalized and post transformed in a similar way as for calibration and chemically treated samples.

3. Results and discussion

3.1. Comparison of spectra and PCA plots from OPP and CHEM methods

Spectra in Fig. 2a and c show the distinct spectral patterns of the three prepared myoglobin states using the two preparation methods (OPP and CHEM). The EMSC adjusted the replicates for both preparation methods; i.e. after EMSC the replicates of the three myoglobin states showed less deviation (Fig. 2b and d). The difference between the two preparation methods of states also became less apparent after EMSC. This result is in agreement with the purpose of this transformation method (Gallagher, Blake, & Gassman, 2005). The CHEM method gave less precise replicates for OMb and DMb states, and the spectra were overlapping at most wavelengths (Fig. 2b) between 500 and 900 nm whereas in Fig. 2d, there are substantial differences ≥ 700 nm. The guidelines of the CHEM method also inform the user that maintaining a pure DMb state in the meat surface is difficult (AMSA, 1991). The DMb on the surface will rapidly pick up oxygen and become transformed into OMb. Thus the intended pure DMb will be a mixture of DMb and OMb and its spectra become more similar to that of OMb.

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). The most accurate predictions for each myoglobin state are underlined.

Myoglobin states	Treatments			RMSECV	No. PLS factors	R_c
	Physical[a]	Spectra processing[b]	Calc. method (formula used)[c]			
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

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

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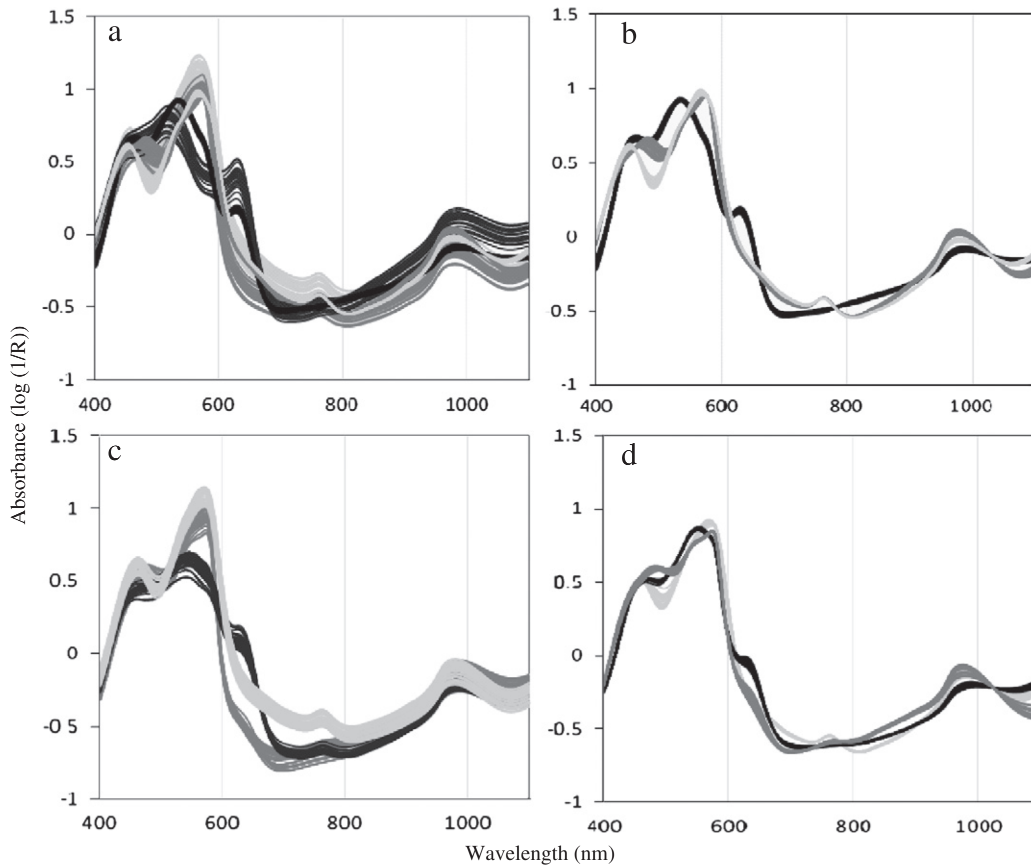

Fig. 2. The absorbance (A = log(1/R), where R is the reflectance) spectra of the three different myoglobin states of *M. semimembranosus*; a) without and b) with extended multiplicative scatter correction (EMSC), using the CHEM method of redox form preparation, c) without and d) with EMSC using the OPP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

Fig. 3 shows how the percentages of myoglobin redox forms differed when calculated using the CHEM (K/S) SW method and the OPP EMSC (A) PLS method. Fig. 3a (CHEM method with K/S spectroscopic transformation) shows that all three myoglobin states were not clearly separated from each other. OMb and in particular the DMb state were neither well reproduced nor well separated in the PCA plot. However, the three different states of myoglobin were well separated by the OPP method with EMSC of spectra into three clusters, indicating that each state had spectra that made them unique (Fig. 3b).

3.2. Regression comparisons

3.2.1. CHEM versus OPP

As shown in Table 1, modeling data from 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. 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

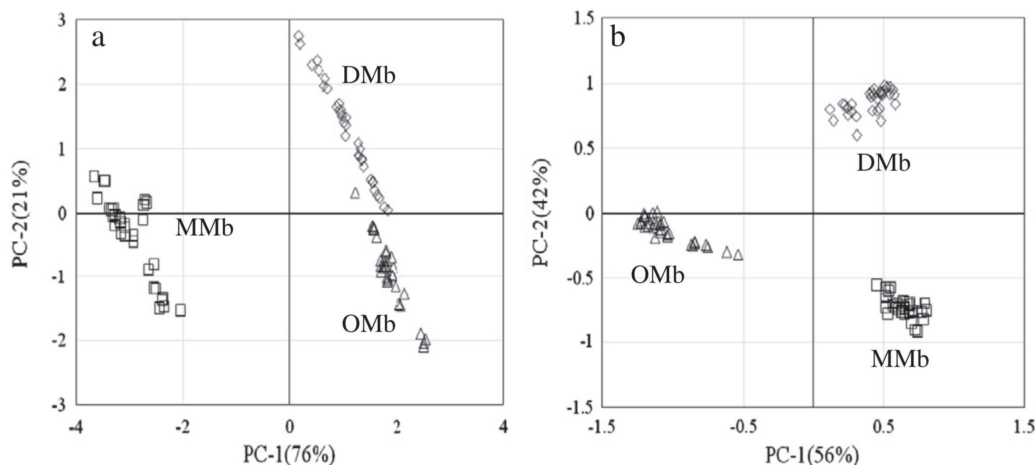

Fig. 3. Principal component analysis of spectra of experimentally prepared pure myoglobin states. Explained variance of the two components was 97% (a) and 98%. (b). PC-1 is principal component one and PC-2 is principle component two; a) CHEM (chemically induced myoglobin states) treatments and K/S spectroscopic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

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MMb, respectively. The AMSA guidelines (AMSA, 1991) using the CHEM (K/S) SW method do not indicate an expected error for any state. Ledward (1970) determined MMb of muscle using CHEM (K/S) SW and reported an error between (±)0.05 and 0.07 comparable to our findings for the CHEM method. The CHEM method thus seemed better than the OPP method for obtaining MMb. This may suggest that it is difficult to prepare pure MMb by selecting headspace volume and oxygen concentration.

Table 1 indicates that OMb was not very accurately determined either. The OPP method was a better preparation option than the CHEM method 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.

DMb was not precisely determined using any method. The AMSA guidelines caution about the difficulties that will be experienced for the DMb state. Ledward (1970) indicated an error (±0.05) for DMb. This is contradictory to the large error reported here for DMb. It appears that Ledward (1970) kept the sample under nitrogen gas after dithionite reduction and until spectral measurements. Ledward (1970) also used 20% dithionite of unknown incubation length for reduction of myoglobin. The result also indicated that both preparation methods used for DMb needed improvement. In the present case, the repackaging with oxygen permeable film was a step that introduced oxygenation. It would be better to prepare DMb without repackaging.

3.2.2. SW versus whole spectra with PLS regression

Including all wavelengths largely eliminated the difference between the two preparation methods (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. Using the complete spectrum apparently partially counteracted the consequence of having an imprecise method (OPP or CHEM) for preparing a pure myoglobin state. It is important to stress that the lower error obtained using PLS regression makes it more relevant to actually predict DMb and OMb on meat samples. The AMSA Guideline preparation method (AMSA, 1991) for MMb using CHEM (ferricyanide) still seemed preferable to using OPP (low oxygen packaging).

3.2.3. 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 EMSC(A) was included 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 (Table 1). However, the spectra from CHEM preparation with revised transformation using EMSC and PLS regression instead of K/S and SW gave a slightly better result (from 0.041 to 0.029; Table 1) only for MMb; but the difference may not be significant. This result also suggested that the improvement upon adding a scatter correction when only a few specific spectral wavelengths have been selected was small.

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. The OPP method now gave the same low RMSECV for all forms. As pointed out above, 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 $\pm 2\times 0.18$ (DMb); $\pm 2\times 0.16$ (OMb) or $\pm 2\times 0.08$ (MMb) obtained using transformation to K/S values and restricting the analysis to specific wavelengths. Despite the fact that the OPP method gave good predictions for DMb, it should be mentioned that the time allocated for myoglobin reduction (48 h) may not always be optimal. When the complete spectrum was used, scatter correction was a useful transformation before calculation of states.

3.3. Illustration of the predictability of OMb for two models

The true OMb class, i.e. the experimentally attempted pure OMb state, was defined as 1. The true OMb class was defined as zero when either pure DMb or MMb was modeled. Each state is thereafter predicted with an error reflecting the accuracy obtained when the state was prepared experimentally and the scattering properties of the meat sample. Possibly other phenomena may influence the prediction accuracy of each state. But the present approach gives predictive models where each myoglobin state was predicted independent of the two others. Thus each state will be predicted with an error where values <0 and >1 were mathematical possibilities. The predicted states for DMb, OMb, and MMb were therefore recalculated so their sum added up to one. This was done after correcting data for physically impossible states (e.g. negative values). Fig. 4a gives the relationship between the prepared fraction of the OMb state and the correspondingly predicted value using the CHEM (K/S) SW method for calculating states. The data shows higher deviation, compared to the OPP EMSC(A) PLS method (Fig. 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, $(0\pm 3\times\cdot 0.16)$ away from the true value.

DMb was however, the state predicted (using OPP EMSC(A) PLS) with the most samples >105%, or as a state with the most samples <95%, for an assumingly pure state. Minimum and maximum deviations from 1 were 0.79–1.10 (79–110%) for DMb, respectively.

3.4. Prediction of myoglobin states in additional samples (A1–A6) using CHEM and OPP methods

The model with lowest prediction error (OPP EMSC(A) PLS) was used here to demonstrate the predictability of states on additional samples (A1–A6) (can also be called a test set). A comparison with the predictions from CHEM (K/S) SW model (Fig. 5) was made. Fig. 5a and b shows, 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 (Fig. 5a and b for one sample). However, during the first observation hours the CHEM (K/S) SW model predicted that the sample contained 20–30% MMb (Fig. 5a), while the OPP EMSC(A) PLS model suggested that the MMb content was 5% (Fig. 5b). This relatively large absolute difference observed for one sample (Fig. 5a and b) regarding MMb fraction still remained using all six samples (A1–A6; Fig. 5c and d), but the relative changes were fairly similar. The reason for using both one and then a six sample set (A1–A6) was to demonstrate the robustness of the prediction. The difference observed 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 (Fig. 5b and d) 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. Fig. 5b also demonstrated why it is difficult to measure DMb if repackaging is done; since changes in myoglobin state took place within minutes. There were some resemblances regarding changes in OMb with time for the two methods, while the changes in DMb with time

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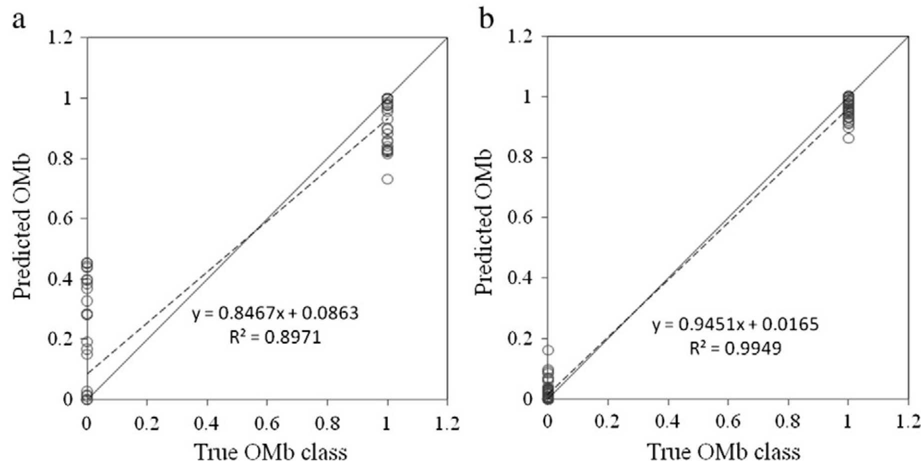

Fig. 4. Corrected predicted oxymyoglobin (OMb) versus true (as prepared) OMb state; a) CHEM (K/S) SW data for OMb (correlation coefficient $R_c = 0.947$); b) OPP (EMSC(A) PLS) for OMb ($R_c = 0.997$). The diagonal line illustrates the target line (x = y). The dotted line is the fitted regression line.

were completely different. The fact that the prediction obtained from the OPP EMSC(A) PLS seemed in accordance with expectations concerning myoglobin behavior 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 Fig. 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. Large absolute magnitudes of the regression coefficients would be regarded as important with respect to identifying the states; e.g. like the wavelength region 584–588 nm for MMb (Fig. 6) and 590–596 nm for DMb. However, it should be mentioned that exact allocation of peaks is not possible if the spectra of different species overlaps. The above wavelength region was not selected in the method CHEM K/S SW. This figure 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, the minimum wavelengths that would be picked for prediction of states from the present dataset may not 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 the EMSC(A) PLS approach using all

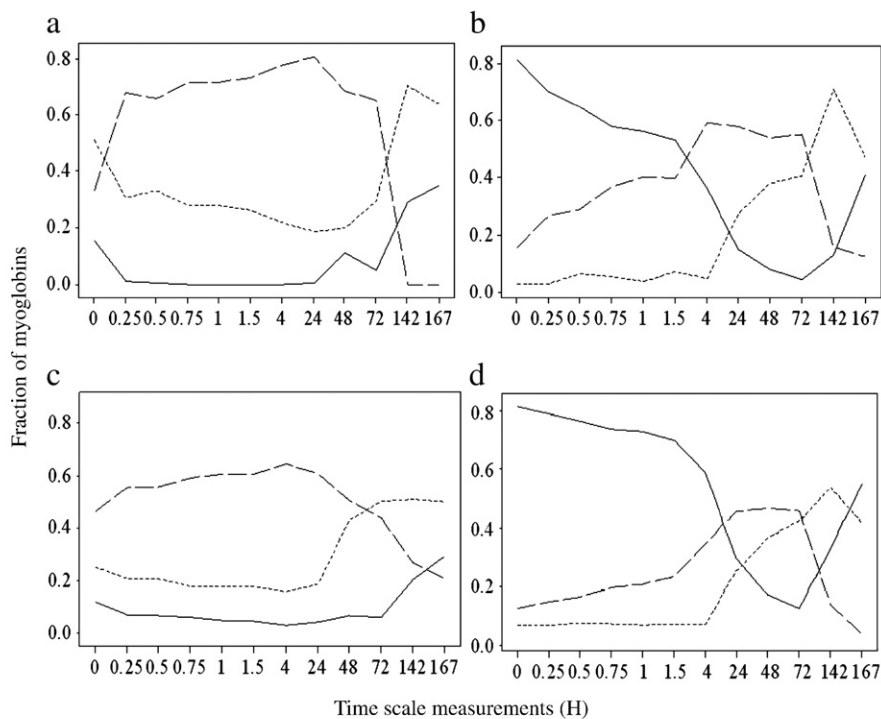

Fig. 5. Predicted fractions (0–1) of myoglobin states (—— deoxy myoglobin, - - - - oxymyoglobin and ········ metmyoglobin) versus storage time in oxygen permeable (low density polyethylene) film. At each time point, reflectance spectra were measured. Myoglobin fractions were calculated from spectra using 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 (A1) used; b) OPP preparation method with extensive multiplicative scattering on the absorbance spectra (called EMSC(A)) before Partial least square (PLS) regression, i.e. OPP EMSC(A) PLS, one sample (A1) used; c) prepared as in 5a, average of 6 (A1–A6) samples; d) prepared as in 5b, average of 6 (A1–A6) samples.

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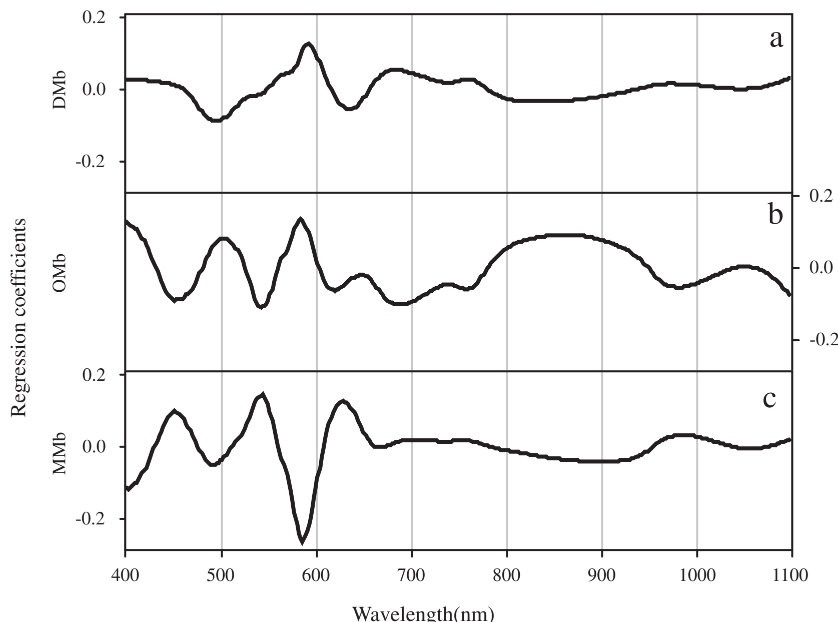

Fig. 6. Regression coefficients used in the predictive equations for myoglobin states at different wavelengths. The preparation and calculation method was OPP EMSC(A) PLS (for abbreviations see Materials and methods). The number of PLS factors used in the regression model was 2, 3 and 3 for deoxymyoglobin, oxymyoglobin and metmyoglobin, respectively.

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 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 recommended. In addition, the calculation principle also seemed to give relevant prediction errors for DMb despite the fact that preparing pure DMb is difficult. This seemed possible by using the complete reflectance spectrum instead of a few wavelengths. The general recommendation for obtaining good predictions of all three myoglobin states is 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. EMSC of the absorbance data gave lower prediction error compared to only using the Kubelka–Munk (K/S) transformation. Finally, the use of all wavelengths (400–1100 nm) and a multivariate regression method (PLS regression), gave clearly lower prediction errors compared to the use of the selected wavelengths. No single wavelengths could be identified from the PLS regression coefficient that are unique for the three states of myoglobin. It seemed that the several regions in the whole spectra from 400 to 1100 nm contributed to the predictive ability for the three states of myoglobin. Based on this study and the principles presented, this color measurement methodology should be considered by anyone interested in documenting the dynamics of meat pigment color changes.

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References

American Meat Science Association (AMSA) guidelines for meat colour evaluation. (1991). Hunt, M. C., Acton, J. C., Benedict, R. C., Calkins, C. R., Cornforth, D. P., Jeremiah, L. E., Olson, D. G., Salm, C. P., Savell, J. W., & Shivas, S. D. Proceedings 44th Reciprocal Meat Conference. Appendix pp. 1–17, Savoy, IL, USA.

Bekhit, A. E. D., & Faustman, C. (2005). Metmyoglobin reducing activity. *Meat Science*, *71*, 407–439.

CIE (Commission Internationale de l'E'clairage) (1976). Recommendation on uniform color space-color difference equations, psychometric color terms. *Suppl. 2 to CIE, Publication No. 15 (E-1.3.1) 1971/(TC-1-3), Paris, France*.

Feldhusen, F., Warnatz, A., Erdmann, R., & Wenzel, S. (1995). Influence of storage time on parameters of color stability of beef. *Meat Science*, *40*, 235–243.

Francis, F. J., & Clydesdale, F. M. (1975). *Food colorimetry: Theory and applications.* Westport, Connecticut, USA: The AVI Publishing Company, Inc.

Gallagher, N. B., Blake, T. A., & Gassman, P. L. (2005). Application of extended inverse scatter correction to mid-infrared reflectance spectra of soil. *Journal of Chemometrics*, *19*, 271–281.

Hopkins, D. L., Khliji, S., van de Ven, R., Lamb, T. A., & Lanza, M. (2010). Relationship between consumer ranking of lamb colour and objective measures of colour. *Meat Science*, *85*, 224–229.

Hunt, M. C., Sørheim, O., & Slinde, E. (1999). Colour and heat denaturation of myoglobin formed in ground beef. *Journal of Food Science*, *64*, 847–851.

Krzywicki, K. (1979). Assessment of relative content of myoglobin, oxymyoglobin and metmyoglobin at the surface of beef. *Meat Science*, *3*, 1–10.

Krzywicki, K. (1982). The determination of heme pigment in meat. *Meat Science*, *7*, 29–36.

Kubelka, P. (1948). New contributions to the optics of intensely light-scattering materials part 1. *Journal of the Optical Society of America*, *38*, 448–457.

Ledward, D. A. (1970). Metmyoglobin formation in beef stored in carbon dioxide enriched and oxygen depleted atmospheres. *Journal of Food Science*, *35*, 33–37.

Mancini, R. A., Hunt, M. C., & Kropf, D. H. (2003). Reflectance at 610 nanometers estimates oxymyoglobin content on the surface of ground beef. *Meat Science*, *64*, 157–162.

Martens, H., & Næs, T. (1989). *Multivariate calibration.* Chichester, United Kingdom: John Wiley and Sons.

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Martens, H., & Stark, E. (1991). Extended multiplicative signal correction and spectral interference subtraction: New pretransformation methods for near infrared spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, *9*, 625–635.

McKenna, D. R., Mies, P. D., Baird, B. E., Pfeiffer, K. D., Ellebracht, J. W., & Savell, J. W. (2005). Biochemical and physical factors affecting discoloration characteristics of 19 bovine muscles. *Meat Science*, *70*, 665–682.

Renerre, M., & Labas, R. (1987). Biochemical factors influencing metmyoglobin formation in beef muscles. *Meat Science*, *19*, 151–165.

Tapp, W. N., Yancey, J. W. S., & Apple, J. K. (2011). How is the instrumental color of meat measured? *Meat Science*, *89*, 1–5.

Taylor, A. A., Down, N. F., & Shaw, B. G. (1990). A comparison of modified atmosphere and vacuum skin packing for the storage of red meats. *International Journal of Food Science and Technology*, *25*, 98–109.

Wilson, G. D., Ginger, I. D., Schweigert, B. S., & Aunan, W. J. (1959). A study of the variations of myoglobin. *Journal of Animal Science*, *18*, 1080–1086.

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Paper II

Oxygen Consumption Rate of Permeabilized Cells and Isolated Mitochondria from Pork *M. Masseter* and Liver Examined Fresh and after Freeze-Thawing at Different pH Values

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C: Food Chemistry

Abstract: The oxygen consumption rate (OCR) of 2 types of permeabilized tissues and their corresponding isolated mitochondria from porcine *M. masseter* and liver, resulting in 4 systems, was studied at different pH values (5.0 to 7.1) using fresh samples and samples frozen directly in liquid nitrogen (N_2) or air-frozen at −20°C. A protocol with the additive sequence rotenone–succinate–ADP (adenosine diphosphate)–cytochrome c–FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was used to study respiration changes. The OCR of liver respiring on succinate (OCR_S) was higher than that of muscle tissue. pH had a larger effect on OCR_S than freeze-thawing. Low pH was associated with reduced OCR_S. The OCR_S of isolated muscle mitochondria appeared to be an underestimated relative to the OCR_S of permeabilized muscle cells. Increasing pH, following prior subjection to pH 5.0, showed partial reversibility of the OCR_S. The freeze-thaw cycle increased the OCR_S when muscle systems were frozen and examined above pH 6.0; this effect was less apparent for liver tissue. A response to cytochrome c addition, indicating a defective outer mitochondrial membrane, was observed for all 4 systems. The response was, however, lowest for permeabilized cells. The ADP/FCCP additive pair indicated partial coupling for isolated liver and muscle mitochondria. These additives gave weak responses for the permeabilized liver cells while the OCR seemed to be inhibited for permeabilized muscle fibers when ADP/FCCP was added.

Keywords: antioxidant, color, cytochrome c, meat, succinate

Practical Application: The mitochondrial state is believed to be important for myoglobin reduction, development of flavor, and possibly other meat qualities. By monitoring the oxygen consumption in mitochondria and meat we can better understand and control such processes following freezing and thawing.

Introduction

The *postmortem* oxygen consumption rate (OCR) of muscle has been studied for many decades (for example, Bendall and Taylor 1972). The OCR, as supported by endogenous muscle substrates, plays an important role in keeping myoglobin in a reduced state, leading to immediate blooming when exposed to oxygen after the packaging is opened. Mitochondria play a pivotal role in the *postmortem* color stability of muscle (Bendall and Taylor 1972; Okeeffe and Hood 1982; Renerre and Labas 1987; Madhavi and Carpenter 1993; McKenna and others 2005). It is generally accepted that succinate is an important component for maintaining myoglobin in a reduced state, as it donates electrons to the electron transport system (ETS) (Tang and others 2005a). The current theory suggests an electron transfer event between complexes III and IV prior to the final reduction of myoglobin on the mitochondrial outer membrane (Tang and others 2005a). A balance between electron transfer to reduce metmyoglobin at the outer membrane and to reduce oxygen at complex IV (cytochrome c oxidase) is assumed to be established, but is not fully explained. Cytochrome c could carry electrons between complexes III and IV and is also proposed as being indirectly involved in reducing myoglobin by providing electrons to an outer membrane protein (Wu and others 1972; Tang and others 2005a).

Ramanathan and others (2010) and Tang and others (2005a) showed that metmyoglobin reduction may occur through oxidation following the addition of lactate with a concomitant donation of electrons by NADH to complex I, or through succinate via FADH at complex II in the ETS. In addition to the importance of oxygen removal for color stability, endogenous removal of oxygen is important for preventing lipid oxidation (Tang and others 2005c) and off-flavor development, as well as for maintaining enzymes in an active state (Kim and others 2010).

Using beef heart mitochondria, Tang and others showed that the state IV OCR (low ADP and high substrate level) was reduced at lower pH values (Tang and others 2005b) and after multiple

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freeze-thaw cycles (Tang and others 2006). The mitochondria were studied at different pH values following 3 freeze-thaw cycles. However, liver tissue, and in particular muscle tissue, are more stable sources of food raw material than heart muscle.

The aim of the study was: (1) to examine the OCR of liver and muscle tissues at pH values between 5.0 and 7.0 using 2 freezing protocols (rapid and slow freezing) on perfused muscle and liver cells and on isolated mitochondria. Only the OCR of complex II was measured after succinate stimulation (hereafter called OCR_S) and the addition of rotenone, an inhibitor of NADH dehydrogenase (complex I); (2) to determine the response of cells and mitochondria using cytochrome c addition to determine outer mitochondrial membrane intactness; (3) to test for variation in the OCR caused by coupled respiration using the ADP/FCCP pair. FCCP functions as an uncoupler of the ATP synthase and allows the ETS to run at maximum speed when substrates are present; (4) to study the effect of ingredient addition and pH on oxygen consumption.

Materials and Methods

Liver, muscle, and chemicals

Porcine liver and *M. masseter* muscle from Norwegian Landrace were put on ice at a local abattoir within 1 h of slaughter. This muscle can be obtained early *postmortem*, which is important in order to get highly functional mitochondria. The samples were obtained from 32 animals in total. Mitochondria were isolated from 2 to 4 pieces of masseter in each case. Oxygen consumption measurements were carried out with 2 replicates. All chemicals were of analytical grade and purchased from Sigma Chemicals Corp. (St. Louis, Mo., U.S.A.).

Fibertyping

The muscle was fiber-typed using a succinate dehydrogenase assay (Kelly 2010), which showed that it contained mainly type I fibers (intensely stained) and to a lesser extent type II A and B (62 %, 30 %, and 8%, respectively).

pH

pH was measured with a Beckman pH meter (Beckman Instruments Inc., Brea, Calif., U.S.A.) equipped with a Mettler Toledo Inlab 427 electrode (Mettler-Toledo Gmbh, Urdorf, Switzerland), calibrated at the sample temperature. The pH dropped below 6.2 after 4 to 5 h for *M. masseter* and its ultimate pH was 6.09 ± 0.18 (mean and standard deviation). The ultimate pH for liver was 6.17 ± 0.21 (mean and standard deviation).

Tissue permeabilization

Muscle tissue fibers were separated (within 3 h *postmortem*) in 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 (Saks and others 1998; Lassnig and others 2008). Liver tissue was treated similarly to muscle tissue, but without the rigorous separation of fibers. Muscle fibers with an average length of approximately 7 mm and with a diameter of approximately 0.5 mm were dissected. Both tissues were permeabilized in the same relaxing solution containing 0.052 mg/mL saponin for 30 min. Samples were subsequently washed for 10 min at 4 °C with shaking in the culture medium (see OCR measurements below).

Isolation of mitochondria

Liver. Slices of liver were cut (3 h *postmortem*) from the centre of the greatest lobe in order to minimize differences in mitochondrial population due to the presence of periportal and peripheral cells. A 10-g piece of liver was excised and minced in homogenization medium (0.25 M sucrose, 1mM EDTA, and 5.0 mM potassium phosphate adjusted to pH 6.0). The minced tissue was homogenized at 400 rpm in a glass/teflon Potter Elvehjem and the homogenate was centrifuged to determine the sedimentation coefficients using glutamate dehydrogenase (EC 1.4.1.3) as a mitochondrial marker enzyme (glutamate dehydrogenase kit, Dialab, Wiener Neudorf, Austria). The presence of glutamate dehydrogenase was assayed using the conversion of NADH to NAD by monitoring ΔA_{340nm}/min in the linear range.

Mitochondria were isolated using time integrals of 5.92×10^7 rad^2/sec (3000 rpm, 10 min) and 3.7×10^8 rad^2/sec (7500 rpm, 10 min) in a HB-4 rotor at 4 °C in a Sorvall RC5–5C centrifuge (Thermo Scientific, Asheville, N.C., U.S.A.) where R_{min} and R_{max} were 6.2 cm and 14.4 cm, respectively (Slinde and others 1975). Calculations were based on the formula below (De Duve and Berthet 1953; Slinde and others 1975; Slinde and Flatmark 1973):

$$S_{avg} = \frac{3.5 \log_{10} R_{max}/R_{min}}{\int_0^t rpm^2\, dt} \times 10^{-13}$$

where S_{avg} is the average sedimentation coefficient, R_{min} and R_{max} are the distances from the axis of rotation to the surface and bottom of the fluid column, and $\int_0^t rpm^2\, dt$ is the time integral per minute.

Prior to the 2nd centrifugation the mitochondrial pellets were resuspended in a small glass/teflon Potter Elvehjem homogenizer and the volume was adjusted to the 6.2 cm mark of the centrifuge tube. Resedimentation was performed twice.

Muscle. Similar conditions as for liver mitochondria were applied to muscle. From 3 pieces (200 g) of *M. masseter* a total of 20 g of connective tissue free samples was obtained. After mincing, the tissue was digested for 30 min (Bhattacharya and others 1991) with 0.05% trypsin in phosphate buffered saline solution supplemented with 10 mM EDTA (pH 7.0). Isolations using nagarse (subtilisin) and without proteolytic enzymes were also conducted but the yields were not satisfactory (results not shown). Tissue, connective tissue, and cell debris were removed by medical gauze filtering. The filtrate was centrifuged as described above (for example, isolation of mitochondria).

Protein concentration

Protein concentrations were measured by fluorescence-based quantitation using a Qubit® fluorometer (Invitrogen, Carlsbad, Calif., U.S.A.). The method uses excitation at 570 to 645nm, emission at 655 to 725 nm, and 3 internal standards make up the standard curve for estimation of unknowns; all according to the manufacturer's descriptions.

Freeze-thawing of samples

Mitochondrial pellets and tissues were either frozen at −20 °C for 24 h or flash frozen in liquid nitrogen and subsequently stored at −80 °C for 48 h. Isolated mitochondria were frozen as pellets. The OCR of isolated mitochondria was measured using 0.60 to 3.44 mg protein/mL. Small pieces (10 to 70 mg of wet weight tissue) of *M. masseter* tissue were isolated from the center of a specific sample for OCR measurements. Frozen mitochondrial

pellets were quickly thawed in a water bath at 37 °C and then resuspended in isolation buffer at 4 °C.

OCR measurements

High-resolution respirometry was carried out with Oroboros Oxygraph-2K instruments (Oroboros Instruments, Innsbruck, Austria) as described by Gnaiger (2001). The OCR of isolated mitochondria was measured at 20 °C and approximately 200 μM O_2. OCR measurements were reported as (pmol O_2/sec)/mg protein. To ascertain the OCR of permeabilized fibers at 37 °C, the oxygen concentrations in the chambers (2 mL) were increased by addition of H_2O_2 and catalase until an O_2 concentration of approximately 300μM was reached. The reduction in H_2O_2 was regarded as instant. The intention was to measure at 4°C, but as it was difficult to measure with sufficient accuracy at that temperature due to low responses, a higher temperature (37 °C) was chosen.

The OCR measurement was carried out by blocking complex I with rotenone (Table 1). Oxidative phosphorylation was stimulated by the addition of ADP; mitochondrial membrane intactness was investigated by adding porcine heart cytochrome c (Gnaiger 2008) and finally uncoupling was measured by FCCP addition. 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 culture medium was adjusted to pH 7.1, 6.5, 6.0, 5.5, and 5.0 prior to measurements. To lower the pH, 10 mM KH_2PO_4 or 5.0 M KOH was added.

Statistical analysis

The experiment consisted of 264 OCR measurements (on average 4.4 replicates): 5 pH levels, 3 storage states (fresh/−20 °C/−80 °C), and 4 categories (liver tissue/muscle tissue/mitochondria from the 2 sources). In each of the replicates, a few measurements were removed as outliers. One-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons were used to test significant differences between the 4 categories following ADP, cytochrome c, and FCCP addition. The General Linear Model procedure of Minitab (version 15 and 16 from Minitab Inc., State College, Pa., U.S.A.) was used for statistical calculations. Means are shown with standard errors of the mean (SEM) bars in graphs while errors are also explained in the table text.

Results and Discussion

Animal-to-animal variation

Liver and muscle tissue from 32 pigs were used for these experiments. The pigs were divided into 14 batches where muscle tissue from 1 or 2 animals was used each time while liver from one pig was sufficient for each experiment. Animal differences could be tested for at pH 7.1, since this pH was used for batch control. Animal differences were significant ($P < 0.05$, one-way ANOVA) for permeabilized tissues and mitochondrial isolations. Animal differences were particularly large for isolated mitochondria compared to permeabilized tissues (results not shown), which could also reflect the heterogeneity of the mitochondrial population. Animal number, however, was not a design variable in the experiment, and has therefore not been used in the statistical calculations below. However, the practical implication is that we have not computed interaction effects in the statistical models, although our data suggest that the effect of freezing was more severe for some pH values than others (see below). Kolath and others (2006) and Richardson and Herd (2004) have indicated that animal variations in oxygen consumption may (amongst other factors) be due to differences in hydrogen peroxide production, feed, size, activity, fat content, sex, age, heredity, as well as other factors such as preslaughter stress and tissue heterogeneity. Since animal variations were found to be large, it would be relevant to examine this in future studies.

OCR_S of permeabilized liver and muscle tissue

pH affected OCR_S more than freezing when succinate was added to fibers where complex I had been inhibited by rotenone (Figure 1). OCR_S was higher for pH > 6.0 than for pH values of below 5.0 and 5.5.

Permeabilized frozen-thawed muscle had a higher ($P = 0.005$) OCR_S than fresh, unfrozen muscle in the pH range 6.0 to 7.1. The OCR_S at pH 5.0 and 5.5 of muscle frozen at −20 °C was lower ($P < 0.05$) than that of fresh muscle; this was not the case for samples above pH 6.0. Thus, the OCR_S of permeabilized fibers was sensitive to freezing in the pH region relevant to meat raw materials and products that is, below pH 6 (Figure 1). The fact that the OCR_S was below its maximum value, as observed for fresh muscle systems with pH ≥ 6.0, was interpreted

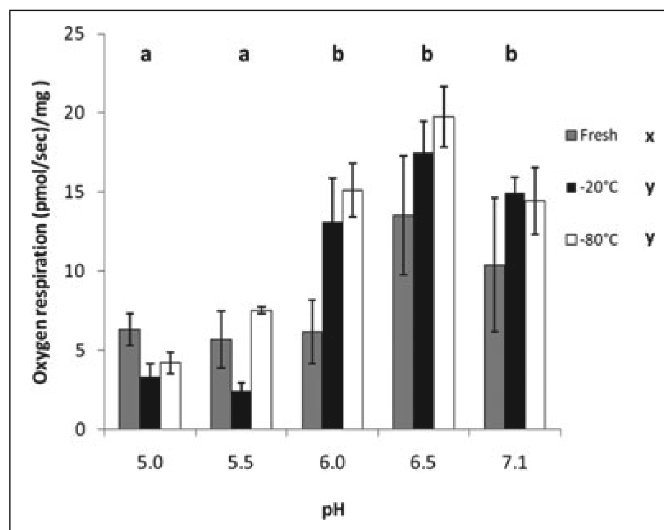

Figure 1–OCR_S at 37 °C of permeabilized muscle tissue (per mg wet weight) respiring on succinate. Different letters in the columns (state) and rows (pH) indicate significant ($P < 0.05$) differences in main effects.

Table 1–Abbreviations used for the protocol.

	Addition sequence				
Symbol	**1**	**2**	**3**	**4**	**5**
OCR_s	0.25 μM rotenone	5 mM succinate	–	–	–
OCR_{ADP}	0.25 μM rotenone	5 mM succinate	5 μM ADP	–	–
OCR_C	0.25 μM rotenone	5 mM succinate	5 μM ADP	10 μM cytochrome c	–
OCR_{FCCP}	0.25 μM rotenone	5 mM succinate	5 μM ADP	10μM cytochrome c	0.5 to 1 μM FCCP

as showing that the system was intact and the mitochondria were coupled in the permeabilized muscle tissues. Loss of coupling would thus be beneficial for meat quality, as the OCR_S would increase.

The average OCR_S for permeabilized liver cells (Figure 2) was higher ($P < 0.002$) than for permeabilized muscle cells, except at pH 5.0 (Figure 1 and 2). The higher rate of respiration for soft tissues, such as liver cells, compared to hard muscle tissues, is presumed to be partly caused by slower oxygen diffusion in permeabilized muscle than in a liver cell homogenate. This would explain the lower respiration rate for the muscle system. For liver the effect of pH on the OCR_S was larger ($P < 0.007$) than the effect of freeze-thaw cycles, and a substantial slowdown in the OCR_S was observed at pH 5.5 and 5.0. Freezing liver tissue at −20 °C was consistently detrimental to the OCR_S, except at pH 7.1 (Figure 2).

OCR_S of liver and muscle mitochondria

pH was also a more important ($P < 0.006$) factor of variation than freeze-thaw cycle for the OCR_S of isolated muscle mitochondria. The OCR_S at pH 5.5 and 5.0 was low, and lower in the pH range 6 to 7.1 (Figure 3). The difference between fresh mitochondria and the mitochondria frozen at −80 °C and then thawed was not significant at pH 5.0 to 5.5 or at pH 6.0 to 7.1, but a significant main effect was found for freezing at −20 °C (Figure 3). Muscle mitochondria frozen at −20 °C at pH 6.5 and 7.1 tended ($P = 0.068$) toward a higher OCR_S relative to the unfrozen system, in the same way as observed for permeabilized muscle cells (Figure 1). The OCR_S of frozen mitochondria dropped to the same level as the OCR_S of fresh mitochondria at pH 6.0 and further below that of fresh mitochondria at pH ≤ 6.0 (Figure 3), which is in agreement with the results for frozen permeabilized muscle tissue. No effect of freeze-thawing was observed by Tang and others (2006) using bovine heart mitochondria (with substrate added) that had undergone 3 freeze-thaw cycles, either at pH 7.2 or at 5.5. This may be due to differences in experimental approach or also to animal variations such as species difference and tissue type. The OCR_S of mitochondria stored at −80 °C was consistently closer to the OCR_S of fresh mitochondria than it was to that of mitochondria frozen at −20 °C and thawed at pH 6.0 (Figure 3), although there was still a difference.

Liver mitochondria generally had a higher OCR_S than muscle mitochondria ($P = 0.007$), but both systems were sensitive to freezing and low pH values (Figure 4). Changes in pH affected OCR_S more than freezing. At pH 5.5, the OCR_S was lower than the OCR_S at pH 7.1.

However, freezing to −20 °C did not increase the OCR_S at pH 6.5 and 7.1 as observed for muscle mitochondria. Furthermore, fresh liver mitochondria and those flash frozen with N_2 and subsequently stored at −80 °C had a higher ($P = 0.046$) and more pH-resistant OCR_S than those frozen at −20 °C (except at pH 5.0). This indicates that not all observations made for liver can be transferred to muscle.

Sedimentation coefficients, obtained using the methodology of Slinde and Flatmark (1973) and glutamate dehydrogenase as the mitochondrial marker enzyme, suggested that mitochondria in

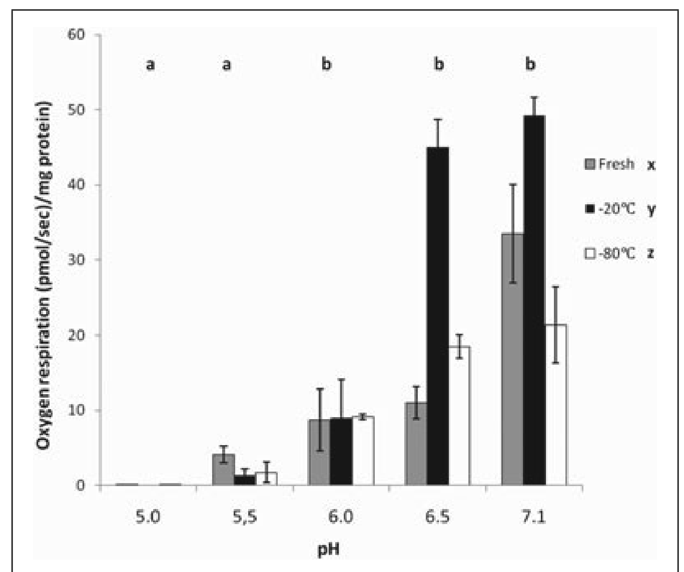

Figure 3–OCR_S for isolated muscle mitochondria respiring at 20 °C on succinate. Different letters in the columns (state) and rows (pH) indicate significant ($P < 0.05$) differences in main effects.

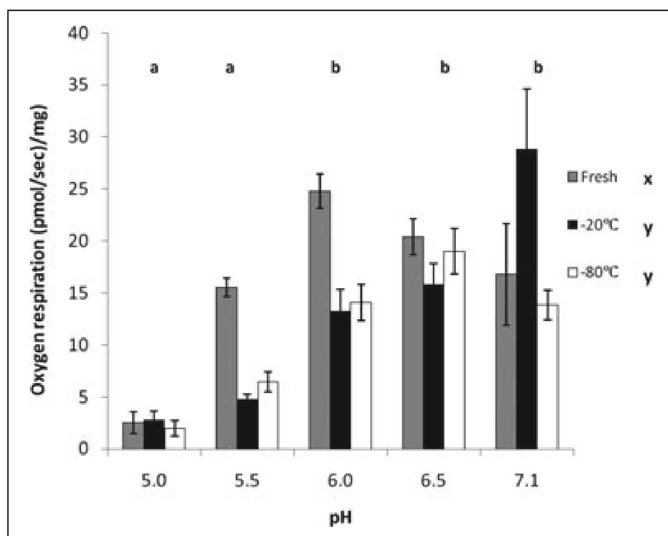

Figure 2–OCR_S at 37 °C for permeabilized liver tissue (per mg wet weight) respiring on succinate. Different letters in the columns (state) and rows (pH) indicate significant ($P < 0.05$) differences in main effects.

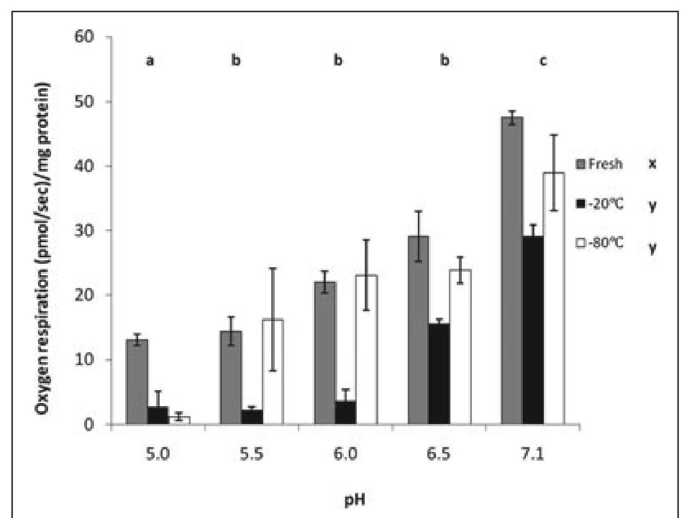

Figure 4–OCR_S for isolated liver mitochondria respiring at 20 °C on succinate. Different letters in the columns (state) and rows (pH) indicate significant ($P < 0.05$) differences in main effects.

isotonic sucrose medium gradually swelled with decreasing pH (Figure 5). Maximum swelling was observed at pH 5.0 and 4.5. At low pH, the mitochondria became unstable and the error in S_{avg} increased considerably. Below pH 5 cellular organelles swelled and changed shape (Novelli and others 1962; Wrigglesworth and Packer 1970). The formation of mitochondrial porin channels occurs more efficiently at lower pH (Shimizu and others 1999) and the opening of the channels allowed protons and ions to freely equilibrate across the inner membrane. The average sedimentation coefficient for a particle is dependent on the density and volume of the particle, in addition to other factors that remained constant in this work (De Duve and Berthet 1953). As a result, the subsequent swelling changed the volume and density of mitochondria, and the sedimentation coefficients of the organelle increased. Isolated mitochondria are known to swell in most media but can nonetheless be fully capable of metabolizing substrates, reducing oxygen, and producing ATP. The low OCR_S measured at low pH values may thus only partly reflect the presence of swollen, damaged, and uncoupled mitochondria (Figure 5). It might be assumed that mitochondria in permeabilized muscle are better preserved against swelling than when isolated.

Response in OCR of permeabilized cells and isolated mitochondria to cytochrome c addition

The OCR protocol (rotenone–succinate–ADP–cytochrome c–FCCP) was primarily selected to elucidate the effect of cytochrome c additions and coupling. Cells treated with detergents, such as saponin or digitonin, have been shown to lose myoglobin and cytosolic components but retain mitochondrial cytochromes and matrix enzymes (Altschuld and others 1985; Kunz and others 1993; Saks and others 1993). The mitochondrial membrane is then not permeable to cytochrome c, and any oxygen consumption following cytochrome c addition therefore reflects lack of intactness of the outer mitochondrial membrane (Saks and others 1993; Kay and others 1997). Our protocol is interpretable at physiological pH (Gnaiger and Kuznetsov 2002), but since the pH of *postmortem* muscle can be as low as 5.3 and for some products even lower (for example, some dry-fermented sausages), the protocol was also used in the pH range 5.0 to 7.1.

The OCR_C/OCR_{ADP} ratio clearly differed ($P = 0.003$) between permeabilized cells (Table 2) and isolated mitochondria (Table 3), being higher in isolated mitochondria. Our results thus suggested that the outer membrane is relatively more intact in

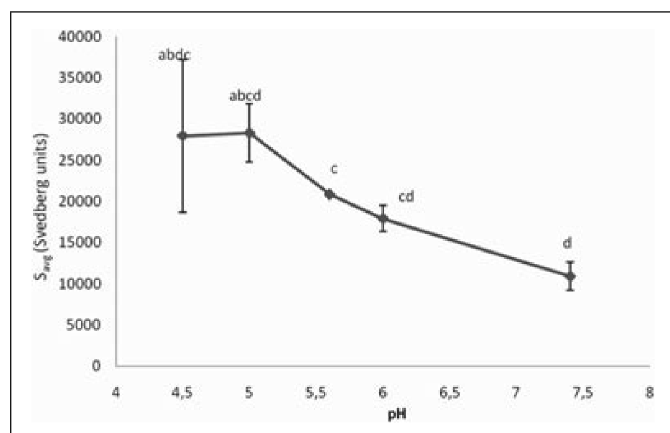

Figure 5–S_{avg} of isolated liver mitochondria at various pH values. Centrifugations were carried out in buffers with different pH values at 4 °C. Error bars indicate standard deviations.

Table 2–OCR of permeabilized liver and muscle: the oxygen consumption of each step (i+1) of the protocol was divided by the OCR of the previous step (i). Main effects are presented as means with their corresponding standard errors of the mean (SEM). Tissue OCR_{i+1}/OCR_i when respiring on ADP, cytochrome c and uncoupled by FCCP is shown in rows. OCR related to changes in pH and state is shown in columns with the respective *P*-value. Table 1 explains the symbols.

		pH*			Effect	State			Effect	R^2(%),
Tissue	OCR ratio	5.0	6.0	7.1	(*P*-value)	Fresh	Frozen −20 °C	Frozen N_2	(*P*-value)	RSD**
Muscle	OCR_{ADP}/OCR_S	$0.90^a \pm 0.02$	$0.99^b \pm 0.02$	$0.90^a \pm 0.03$	0.020	$0.93^x \pm 0.02$	$0.89^x \pm 0.04$	$0.85^x \pm 0.05$	0.21	33.8;0.09
	OCR_C/OCR_{ADP}	$1.22^a \pm 0.07$	$1.14^a \pm 0.06$	$1.16^a \pm 0.06$	0.92	$1.08^x \pm 0.07$	$1.18^x \pm 0.04$	$1.23^x \pm 0.07$	0.14	16.8;0.27
	OCR_{FCCP}/OCR_C	$0.94^a \pm 0.04$	$0.94^a \pm 0.03$	$0.77^b \pm 0.04$	<0.001	$0.90^x \pm 0.03$	$0.84^x \pm 0.04$	$0.91^x \pm 0.02$	0.26	47.6;0.12
Liver	OCR_{ADP}/OCR_S	$0.84^a \pm 0.07$***	$1.09^b \pm 0.03$	$0.98^c \pm 0.02$	<0.001	$1.10^x \pm 0.07$	$0.99^y \pm 0.02$	$0.95^y \pm 0.02$	<0.001	35.2;0.16
	OCR_C/OCR_{ADP}	$1.13^a \pm 0.09$	$1.25^a \pm 0.03$	$1.21^a \pm 0.03$	0.005****	$1.10^x \pm 0.03$	$1.26^y \pm 0.03$	$1.28^y \pm 0.04$	0.030	31.0;0.19
	OCR_{FCCP}/OCR_C	$1.00^a \pm 0.03$	$1.11^b \pm 0.03$	$1.04^a \pm 0.02$	0.009	$1.12^x \pm 0.03$	$1.07^{x,y} \pm 0.02$	$1.01^y \pm 0.02$	0.008	34.5;0.11

*Five pH levels were used in the statistical model.
**R^2(%) = coefficient of determination.
***one extreme measurement removed.
****the difference was at pH 6.5.
RSD = residual standard deviation as obtained from the GLM model (Minitab); Superscripts indicate significant differences ($P < 0.05$) to be read row by row.

Table 3–OCR of mitochondria from liver and muscle: the OCR of each step (i+1) of the protocol was divided by the OCR of the previous step (i). The data are presented as means with the corresponding standard errors of the mean (SEM). Tissue OCR_{i+1}/OCR_i when respiring on ADP, cytochrome c and uncoupled by FCCP is shown in rows. The OCR related to changes in pH and state is shown in columns with the respective *P*-value. Table 1 explains the symbols.

Tissue	OCR ratio	pH*			Effect (*P*-value)	State			Effect (*P*-value)	R^2(%), RSD**
		5.0	6.0	7.1		Fresh	Frozen −20 °C	Frozen LN		
Muscle	OCR_{ADP}/OCR_S	$1.02^a \pm 0.02$	$4.44^b \pm 1.18$	$3.39^b \pm 0.67$	0.008	$5.09^a \pm 0.80$	$2.76^b \pm 0.50$	$1.19^b \pm 0.05$	< 0.001	48.9;2.1
	OCR_C/OCR_{ADP}	2.17 ± 0.66***	1.38 ± 0.05	1.73 ± 0.12	< 0.001	$1.57^x \pm 0.40$	$1.30^x \pm 0.15$	$1.27^x \pm 0.15$	0.39	35.2;0.92
	OCR_{FCCP}/OCR_C	$0.84^a \pm 0.12$***	$1.06^a \pm 0.03$	$1.25^a \pm 0.05$	< 0.001	$7.34^x \pm 2.53$	$5.02^x \pm 2.53$	$6.44^x \pm 2.44$	0.96	78.2.5;6.0
Liver	OCR_{ADP}/OCR_S	$1.22^a \pm 0.23$	$1.16^a \pm 0.09$	$1.30^a \pm 0.13$	0.83	$1.68^x \pm 0.11$	$1.03^y \pm 0.11$	$1.13^y \pm 0.12$	0.002	42.0;1.5
	OCR_C/OCR_{ADP}	$2.79^a \pm 1.06$***	$1.21^a \pm 0.37$	$3.12^a \pm 0.22$	< 0.001	$1.44^x \pm 0.28$	$2.29^x \pm 0.65$	$1.81^x \pm 0.37$	0.23	54.7;1.5
	OCR_{FCCP}/OCR_C	$1.48^a \pm 0.47$***	$1.24^a \pm 0.08$	$0.81^a \pm 0.11$	< 0.001	$11.1^x \pm 4.8$	$5.38^x \pm 2.14$	$6.51^x \pm 2.41$	0.054	87.0;5.8

*Five pH levels were used in the statistical model.
**R^2(%) = coefficient of determination.
*** the difference was at pH 5.5.
RSD = residual standard deviation as obtained from the GLM model (Minitab).

muscle than in isolated muscle mitochondria. Isolation of mitochondria, in particular with the use of proteases (Wilson 1987; Bhattacharya and others 1991; Frezza and others 2007) could induce a leaky outer mitochondrial membrane, allowing both cytochrome c (12 kDa) and myokinases (MW approximately 21 kDa) to be released. Cytochrome c is believed to function as an electron shuttle both for oxygen consumption in the ETS and metmyoglobin reduction (Jacobs and Sanadi 1960; Tang and others 2005a). Moreover, the damage to the outer mitochondrial membrane may actually affect the suggested electron shuttle mechanism for cytochrome c to cytochrome b5 of the outer membrane, thereby influencing the reduction mechanism of metmyoglobin (Tang and others 2005a).

pH was the dominant cause of variation in the OCR_C/OCR_{ADP} ratio for all systems except for permeabilized muscle fibers, which showed no significant response to cytochrome c addition (Table 2). The OCR_C $/OCR_{ADP}$ ratio at pH 7.1 was, however, greater than one (1.16; Table 2), and with a tendency ($P = 0.14$) for a higher average with freezing (Table 2). The OCR_C/OCR_{ADP} ratio of permeabilized liver tissues also increased (1.21; Table 2) when cytochrome c was added at pH 7.1. The OCR_C $/OCR_{ADP}$ ratio of permeabilized liver fibers was pH and state dependent (Table 2). Frozen samples had the larger response to cytochrome c addition (Table 2). Including an interaction term between pH and state gave a much higher R^2 (not shown) and this may suggest that the response to cytochrome c following freezing depended on pH. The response of permeabilized liver cells of different states (fresh and frozen) to cytochrome c was always > 1, suggesting damage to the outer membrane.

Response in the OCR of permeabilized cells and isolated mitochondria to ADP and FCCP addition

If the mitochondria were coupled and had a largely intact ETS, a response to ADP (at pH 7.1) would be expected. However, if cytochrome c was depleted, for example, due to membrane damage, it would be impossible to obtain a full response to ADP (and succinate). As ADP was added prior to cytochrome c, our protocol may therefore not have determined the maximum OCR as a response to ADP addition.

Isolated muscle mitochondria responded more ($P < 0.05$, one-way ANOVA) to ADP than isolated liver mitochondria. The difference in OCR_{ADP}/OCR_S between isolated liver mitochondria (Table 3) and permeabilized liver cells (Table 2) was significant ($P < 0.05$), but much smaller than the difference in OCR_{ADP}/OCR_S between isolated muscle mitochondria and permeabilized muscle cells. ADP addition to permeabilized muscle tissue actually appeared to inhibit respiration (Table 2). For fresh permeabilized muscle tissues at pH 6.5, the OCR_{ADP}/OCR_S ratio was in fact 1.01 (not shown), but otherwise a ratio lower than 1.0 was measured. The OCR_{ADP}/OCR_S ratio found for permeabilized muscle fibers was surprising and means that an alternative enzymatic reaction to ATP synthase may use ADP. Phosphotransferases are a group of enzymes that metabolize and facilitate the interconversion of ATP to ADP. These enzymes consist of myokinase, creatine kinase, and nucleoside diphosphate kinase and these enzymes reside in the intermitochondrial space. Myokinases carry out the following reaction in skeletal muscle (Kalckar 1942, 1943):

$$2ADP \leftrightarrow ATP + AMP$$

Consequently, when permeabilized muscle fibers were overstimulated by exogenous ADP, the equilibrium may have favored

conversion toward ATP and AMP by myokinase as opposed to conversion to ATP at complex V. In comparison to isolated muscle mitochondria, the OCR in tissue is much lower when respiring on ADP. This could indicate a significant loss of myokinase (and other proteins such as for example, cytochrome c) from the mitochondria intermembrane space during isolation. Indeed, myokinase has been reported to be readily lost and become allocated to the supernatant of mitochondrial pellets (Dallam 1955; Siekevitz and Watson 1956; Baumgarten and others 1983). This issue was also addressed by Wiseman and others (1996) where detergent-treated mitochondria and muscle fibers were compared to their respective untreated controls. The results concur with ours that samples treated with detergents had a much lower sensitivity to ADP than untreated samples. Additionally, Kuznetsov and others (1998) showed that the diffusion of oxygen is restricted in permeabilized tissue in comparison to isolated mitochondria, and the oxygen consumption increased upon treatment of the permeabilized tissue with collagenase. Hence, ADP diffusion is severely retarded in cells due to a low permeability of the outer mitochondrial membrane to ADP (Saks and others 1995). Fibers generally have lower oxygen sensitivity than isolated mitochondria due to oxygen diffusion limitations (Bygrave and Lehninge 1967; Kuznetsov and others 1998; Gnaiger 2003).

OCR_{ADP}/OCR_S varied with pH (Table 2 and 3), although this was not significant for liver mitochondria. Also, the state (fresh or frozen) influenced the response to ADP, although this was not significant for permeabilized muscle cells. Interestingly, the explainable variances (R^2; Table 2 and 3) for permeabilized cells were dominated by their sensitivity to changes in pH, while for isolated mitochondria, and particularly for liver, the state (fresh or frozen) was a relatively more important variable, with a significantly lower OCR_{ADP}/OCR_S ratio being obtained after freezing. The trend was also for lower OCR_{ADP}/OCR_S ratios at lower pHs for isolated mitochondria, as the minimum value measured for OCR_{ADP}/OCR_S at pH 7.1 was almost 9 times higher than the minimum value measured at pH 5.0 (results not shown).

Short-term FCCP treatment (Terada 1981) should stimulate respiration due to the facilitation of proton translocation across the inner membrane of the mitochondria. Isolated mitochondria had strong responses (that is, increased respiration) to FCCP, while the responses were lower for permeabilized fibers (Table 2). Although not significantly different from permeabilized liver fibers, the response of permeabilized muscle fibers to FCCP addition had to be interpreted as a tendency for inhibition of respiration (Table 2). Despite the low response to ADP/FCCP for permeabilized muscle cells, we still believe that the fresh tissue was at least partly coupled due to the effect of freezing on the OCR_S.

Tsou and others (1969) and Maclenna and others (1966) indicated that cytochrome c depleted mitochondria become more tightly coupled to oxidative phosphorylation in the presence of exogenous cytochrome c. Cytochrome c addition to cytochrome c-deficient mitochondria saturated with ADP therefore stimulated the OCR, as observed in our mitochondrial system.

The fact that fresh or frozen meats examined for their OCR through permeabilization provided less response to additives than isolated mitochondria could be relevant to our understanding of the OCR of mitochondria and the reduction of metmyoglobin and other redox processes affecting meat quality. Succinate and cytochrome c induced a good response in respiration, however, as previously discussed, respiration on ADP was constrained by tissue complexity and possibly also by the fact that *M. masseter* is a muscle rich in connective tissue. Therefore, the response pattern of permeabilized tissue to substrates is assumed to be similar but stronger under more ideal conditions, compared to what has been observed here.

Table 4–Relative OCR of permeabilized fresh muscle tissue subjected to pH 5.0 at 37 °C for various incubation times following OCR_i measured at pH 6.3. Succinate (OCR_S) and ADP (OCR_{ADP}) were defined relative to cytochrome c respiration (OCR_C) at the different incubation times.

	Time (h)		
OCR_i^n	0	1	5
OCR_S	76 ± 1%	51 ± 4%	38 ± 4%
OCR_{ADP}	86 ± 1%	65 ± 2%	49 ± 5%
OCR_C	100%	100%	100%

n = 2.

Industrial meat processes and oxygen consumption

Elevating the pH level is frequently done in the meat industry by adding conjugate bases (for example, phosphates) in order to improve water-binding capacity. The reversibility of the mitochondrial ETS after exposure to pH 5.0 followed by an increase in pH to 6.3 was therefore tested.

Low oxygen consumption prevailed at pH 5.0 (Figure 1 and 3). However, by increasing the alkalinity in the OCR_S chambers, after 1st adjusting pH to 5.0, the ETS responded to the substrates added. However, the resulting OCR_S was 5 times smaller (results not shown) than the OCR of samples without initial exposure to pH 5.0. After 5 h at 37 °C and pH 5.0 muscle tissues titrated to pH 6.3 had about 40% of the activity of the OCR_S of the tissue with minimum exposure to pH 5.0. Table 4 also suggests a shift in the reversibility of complexes. Meat stored at low temperatures and pH 5.0 should retain OCR_S capacity for a longer period at chill temperatures. Novelli and others (1962) and Wrigglesworth and Packer (1970) showed mitochondrial pH reversibility with respect to swelling and protein structure between pH 5.0 and pH 9.0. Here, we have shown reversible functionality of the ETS.

Oxygen consumption of meat is relevant to cold storage, where protein and in particular myoglobin and lipid oxidation should be prevented.

Conclusions

The OCR_S of permeabilized tissues and mitochondria from pork liver was higher than that of *M. masseter*. pH (range 5.0 to 7.1) affected OCR_S more than one freeze-thaw cycle in all 4 systems (permeabilized muscle and liver tissue and their corresponding mitochondrial isolates). Combining low pH with freeze-thawing reduced the OCR_S most, particularly for mitochondria. Using mitochondria as a model system may lead to underestimated of muscle OCR_S at low pHs. The OCR_S of permeabilized muscle cells can recover from exposure to pH 5.0 within certain time frames. All 4 systems responded to cytochrome c, indicating mitochondrial outer membrane defects. However, the isolated mitochondria responded more than permeabilized tissues. Furthermore, pH also affected the OCR more than freeze-thawing when cytochrome c, ADP, and FCCP were added. However, ADP and FCCP additions gave lower responses in permeabilized tissues than in mitochondria.

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References

Altschuld RA, Wenger WC, Lamka KG, Kindig OR, Capen CC, Mizuhira V, Vanderheide RS, Brierley GP. 1985. Structural and functional-properties of adult-rat heart myocytes lysed with digitionin. J Biol Chem 260(26):4325–34.

Baumgarten E, Brand MD, Pozzan T. 1983. Mechanism of activation of pyruvate dehydrogenase by mitogens in pig lymphocytes. Biochem J 216(2):359–67.

Bendall JR, Taylor AA. 1972. Consumption of oxygen by the muscles of beef animals and related species. II. Consumption of oxygen by post-rigor muscle. J Sci Food Agric 23(6):707–19.

Bhattacharya SK, Thakar JH, Johnson PL, Shanklin DR. 1991. Isolation of skeletal-muscle mitochondria from hamsters using an ionic medium containing ethylenediarninetetraacetic acid and nagarse. Anal Biochem 192(2):344–9.

Bygrave FL, Lehninge Al. 1967. Affinity of mitochondrial oxidative phosphorylation mechanisms for phosphate and adenosine diphosphate. Proc Natl Acad Sci U S A 57(5):1409–15.

Dallam RD. 1955. Studies on the chemical composition of nuclei and cytoplasmic granules. Arch Biochem 54(1):24–37.

De Duve C, Berthet J. 1953. Reproducibility of differential centrifugation experiments in tissue fractionation. Nature 172(4390):1142.

Frezza C, Cipolat S, Scorrano L. 2007. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured filroblasts. Nat Protoc 2(2):287–95.

Gnaiger E. 2001. Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. Elsevier Sci Bv 128:277–97.

Gnaiger E. 2003. Oxygen conformance of cellular respiration – A perspective of mitochondrial physiology. In: Roach RC, Wagner PD, Hackett PH, editors. Hypoxia: through the lifecycle. New York: Kluwer Academic/Plenum Publishers. p 39–55.

Gnaiger E. 2008. Polarographic oxygen sensors, the oxygraph, and high-resolution respirometry to assess mitochondrial function. In: Dykens J, Will Y, editors. Drug-induced mitochondrial dysfunction. Innsbruck, Austria: John Wiley & Sons, Inc. p 327–52.

Gnaiger E, Kuznetsov AV. 2002. Mitochondrial respiration at low levels of oxygen and cytochrome c. Biochem Soc Trans 30(2):252–8.

Jacobs EE, Sanadi DR. 1960. Reversible removal of cytochrome-c from mitochondria. J Biol Chem 235(2):531–4.

Kalckar HM. 1942. The enzymatic action of myokinase. J Biol Chem 143:299–300.

Kalckar HM. 1943. The role of myokinase in transphosphorylations. J Biol Chem 148:127–37.

Kay L, Rossi A, Saks V. 1997. Detection of early ischemic damage by analysis of mitochondrial function in skinned fibers. Mol Cell Biochem 174(1–2):79–85.

Kelly DP. 2010. Kelly Lab. Washington: Washington University School of Medicine.

Kim YH, Huff-Lonergan E, Sebranek JG, Lonergan SM. 2010. High-oxygen modified atmosphere packaging system induces lipid and myoglobin oxidation and protein polymerization. Meat Sci 85(4):759–67.

Kolath WH, Kerley MS, Golden JW, Keisler DH. 2006. The relationship between mitochondrial function and residual feed intake in Angus steers. J Anim Sci 84(4):861–5.

Kunz WS, Kuznetsov AV, Schulze W, Eichhorn K, Schild L, Striggow F, Bohnensack R, Neuhof S, Grasshoff H, Neumann HW, Gellerich FN. 1993. Functional characterization of mitochondrial oxidative phosphorylation in saponin-skinned human muscle fibers. Biochim Biophys Acta 1144(1):46–53.

Kuznetsov AV, Lassnig B, Margreiter R, Gnaiger E. 1998. Diffusion limitation of oxygen versus ADP in permeabilized muscle fibers. In: Larsson C, Pählman I-L, Gustafsson L, editors. BioThermoKinetics in the post genomic era. Göteborg: Chalmers Reproservice p 273–6.

Lassnig B, Stadlmann S, Rieger G, Haffner B, Lemieux H, Gnaiger E. 2008. 2.3.C. Selected media and chemicals for respirometry with mitochondria and permeabilized cells. MiPNet Protocols Innsbruck 12:2–8.

Maclenna Dh, Lenaz G, Szarkows L. 1966. Studies on mechanism of oxidative phosphorylation. 9. Effect of cytochrome c on energy-linked processes. J Biol Chem 241(22):5251–9.

Madhavi DL, Carpenter CE. 1993. Aging and processing affect color, metmyoglboni reductase and oxygen-consumption of beef muscles. J Food Sci 58(5):939–45.

McKenna DR, Mies PD, Baird BE, Pfeiffer KD, Ellebracht JW, Savell JW. 2005. Biochemical and physical factors affecting discoloration characteristics of 19 bovine muscles. Meat Sci 70(4):665–82.

Novelli A, Nanni G, Baldini I. 1962. pH as a controlling factor of shape and function of mitochondria. Experientia 18(12):558–9.

Okeeffe M, Hood DE. 1982. Biochemical factors influencing metmyoglobin formation on beef from muscles of differing color stability. Meat Sci 7(3):209–28.

Ramanathan R, Mancini RA, Maheswarappa NB. 2010. Effects of lactate on bovine heart mitochondria-mediated metmyoglobin reduction. J Agric Food Chem 58(9):5724–9.

Renerre M, Labas R. 1987. Biochemical factors influencing metmyoglobin formation in beef muscles. Meat Sci 19(2):151–65.

Richardson EC, Herd RM. 2004. Biological basis for variation in residual feed intake in beef cattle. 2. Synthesis of results following divergent selection. Aust J Exp Agric 44(4–5):431–40.

Saks V, Belikova Y, Vasilyeva E, Kuznetsov A, Fontaine E, Keriel C, Leverve X. 1995. Correlation between degree of rupture of outer mitochondrial-membrane and changes of kinetics of regulation of respiration by ADP in permeabilized heart and liver-cells. Biochem Biophys Res Commun 208(3):919–26.

Saks VA, Vasil'eva E, Belikova Yu O, Kuznetsov AV, Lyapina S, Petrova L, Perov NA. 1993. Retarded diffusion of ADP in cardiomyocytes: possible role of mitochondrial outer membrane and creatine kinase in cellular regulation of oxidative phosphorylation. Biochim Biophys Acta 1144(2):134–48.

Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS. 1998. Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo. Mol Cell Biochem 184(1–2):81–100.

Shimizu S, Narita M, Tsujimoto Y. 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399(6735):483–7.

Siekevitz P, Watson ML. 1956. Cytochemical studies of mitochondria. II. Enzymes associated with a mitochondrial membrane fraction. J Biophys Biochem Cytol 2(6):653–69.

Slinde E, Flatmark T. 1973. Determination of sedimentation coefficients of subcellular particles of rat liver homogenates. A theoretical and experimental approach. Anal Biochem 56(2):324–40.

Slinde E, Morild E, Flatmark T. 1975. A general and rational approach to the optimal recovery of mitochondria by differential centrifugation in homogenous media. Anal Biochem 66(1):151–8.

Tang J, Faustman C, Hoagland TA, Mancini RA, Seyfert M, Hunt MC. 2005a. Postmortem oxygen consumption by mitochondria and its effects on myoglobin form and stability. J Agric Food Chem 53(4):1223–30.

Tang J, Faustman C, Hoagland TA, Mancini RA, Seyfert M, Hunt MC. 2005b. Interactions between mitochondrial lipid oxidation and oxymyoglobin oxidation and the effects of vitamin E. J Agric Food Chem 53(15):6073–9.

Tang J, Faustman C, Mancini RA, Seyfert M, Hunt MC. 2005c. Mitochondrial reduction of metmyoglobin: dependence on the electron transport chain. J Agric Food Chem 53(13):5449–55.

Tang J, Faustman C, Mancini RA, Seyfert M, Hunt MC. 2006. The effects of freeze-thaw and sonication on mitochondrial oxygen consumption, electron transport chain-linked metmyoglobin reduction, lipid oxidation, and oxymyoglobin oxidation. Meat Sci 74(3):510–5.

Terada H. 1981. The interaction of highly active uncouplers with mitochondria. Biochim Biophys Acta 639(3–4):225–42.

Tsou CS, van Dam K. 1969. Dependence of the efficiency of uncouplers on the respiratory rate. Biochim Biophys Acta 172(1):174–6.

Wilson EJ. 1987. Should nagarse be used during the isolation of brain mitochondria? Neurochem Res 12(9):831–4.

Wiseman RW, Wenger WC, Lamka KG, Kindig OR, Capen CC, Mizuhira V, Vander Heide RS, Brierley GP. 1996. Why is the sensitivity of mitochondria to ADP over tenfold lower in permeabilized fibers than in vivo. Amsterdam: Biothermokinetics Press. p 124–7.

Wrigglesworth JM, Packer L. 1970. pH-dependent changes in mitochondrial membrane structure. J Bioenerg 1(1):33–43.

Wu CS, Duffy P, Brown WD. 1972. Interaction of myoglobin and cytochrome C. J Biol Chem 247(6):1899–903.

Paper III

*Manuscript
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1 Intended for Meat Sci.

2 **Mitochondrial oxygen consumption in permeabilized fibers and its link to**
3 **colour changes in bovine *M. semimembranosus* muscle**

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16

17 **Abstract**

18 Animal and muscle characteristics were recorded for 41 cattle. The oxygen consumption rate
19 (OCR) of *M. semimembranosus* was measured 4 hours *post mortem* (PM4) and after 3 weeks in a
20 vacuum pack at 4°C. Colour change measurements were performed following the 3 weeks using
21 reflectance spectra (400–1100nm) and the colour coordinates L*, a* and b*, with the samples
22 being packaged in oxygen permeable film and stored at 4°C for 167 hours.

23 Significant individual animal differences in OCR at PM4 were found for mitochondrial
24 complexes I and II. OCR of complex I declined with increased temperature and time PM, while
25 residual oxygen-consuming side-reactions (ROX) did not. OCR of stored muscles was
26 dominated by complex II respiration. A three-way regression between number of samples, colour
27 variables collected upon air exposure and OCR of 3 weeks old fibres revealed a positive
28 relationship between OCR and complex II activity and also between OCR and OCR_{ROX}. The
29 presence of complex I and β-oxidation activities increased metmyoglobin formation.

30 **Keywords:** oxygen consumption rate, colour changes, chill storage, mitochondrial complexes

31

32

33

34 **1. Introduction**

35 Meat colour is important to consumers' priorities. Colour in meat is determined largely by
36 myoglobin that exists in the three dominant states of deoxymyoglobin (DMb), oxymyoglobin
37 (OMb) and metmyoglobin (MMb). These states are interconverted depending on oxygen
38 availability, antioxidant availability and enzyme activity. According to McKenna, Mies, Baird,
39 Pfeiffer, Ellebracht and Savell (2005), colour changes are affected by the same factors. Different
40 muscles have myoglobin's heme iron in the ferrous state for different periods of time. Capacity
41 for oxidation and reduction of myoglobin is dependent on the animal age and weight. Increased
42 age and weight will lead to an accumulation of myoglobin and darker colour (Sookhareea,
43 Taylor, Woodford, Dryden, & Shorthose, 1995). *M semimembranosus* is a large muscle with
44 intermediate colour changes (Hood, 1980; McKenna, et al., 2005; O'Keeffe & Hood, 1982).
45 Meat with the highest oxygen consumption rate (OCR) often has the poorest colour changes
46 (Renerre & Labas, 1987).

47 Meat colour is influenced by mitochondrial oxygen consumption and enzymatic-reducing ability
48 (Arihara, Cassens, Greaser, Luchansky, & Mozdziak, 1995; Livingston, McLachlan, La Mar, &
49 Brown, 1985; O'Keeffe & Hood, 1982). A deficiency in nutrient and oxygen delivery occurs in
50 *post mortem* meat due to the cessation of blood flow. This results in deterioration of the
51 mitochondrial electron transport system (ETS) and an increase in reactive oxygen species (ROS)
52 (Lenaz, 2001). Mouse NADH-ubiquinone oxidoreductase (complex I) and fatty acid oxidation
53 were recently reported as the first enzyme complexes in the ETS to be inactivated *post mortem*
54 (Barksdale, et al., 2010; Werner, Natter, & Wicke, 2010). OCR in meat is determined by the rate
55 at which mitochondria utilize some Krebs cycle substrates for consuming oxygen in the
56 production of ATP.

57 The slaughter process starts with transportation from farm to abattoir, and insufficient lairage
58 time before slaughter may cause pre-slaughter stress leading to increased anaerobic glycogen
59 metabolism and hence higher carcass temperatures early *post mortem* with a concomitant
60 accelerated or earlier fall in muscle pH (Rosenvold & Andersen, 2003; Schafer, Rosenvold,
61 Purslow, Andersen, & Henckel, 2002; Simela, Webb, & Frylinck, 2004; Stoier, Aaslyng, Olsen,
62 & Henckel, 2001). The resulting ultimate pH resides around 5.6. A low meat pH has been
63 reported to decrease colour changes in beef and pork (Ledward, Dickinson, Powell, & Shorthose,
64 1986; Monin & Sellier, 1985) but may increase changes in other species such as goat (Simela, et
65 al., 2004). Farouk and Swan (1998) and Young (1999) reported that a high rigor temperature
66 decreased the endogenous OCR and colour changes, while increasing the immediate redness in
67 beef. Early *post mortem* studies on slaughter procedure, colour changes and mitochondrial
68 changes are limited, as are studies that show quantitatively the direct impact on meat color. This
69 is also the case for research that shows quantitatively the direct impact of mitochondrial
70 complexes on meat colour.

71 The aim of this work was to: 1) measure the response in muscle OCR to the administration of
72 chemicals known to affect different components of the mitochondria; 2) measure the changes in
73 OCR with time of chilled storage; 3) elucidate how the activities of the different complexes of
74 mitochondria contribute to colour changes when meat is stored; 4) understand the importance of
75 different slaughterhouse and related variables on OCR ; 5) relate OCR quantitatively to changes
76 in myoglobin states and L*, a*, b* values collected over time.

77 **2. Materials and methods**

78 **2.1.** *Meat samples*

79 Bovine *M. semimembranosus* muscles from 41 animals were collected from a slaughter line
80 (Nortura SA, Rudshøgda, Norway). The intention was to collect samples from animals
81 representing the types that normally arrive at this slaughterhouse. Samples from thirty
82 Norwegian Red Cattle (NRF), 2 Simmental, 1 Limousine, 1 Angus, 3 Charolais and 4 Hereford
83 animals were collected. Due to the strong dominance of Norwegian Red Cattle at Norwegian
84 slaughterhouses, we have not split our analysis into breeds but kept animals as individual
85 specimens. The animals were low voltage stimulated 15-20 minutes after death at 85 V, duration
86 35 sec, pulse duration 5 ms and pulse pause 65 ms (Carometec A/S, Herlev, Denmark), and were
87 then hot-boned approx. 2 hours *post mortem*. There were 17 young males, 7 mature cows, 2 male
88 castrates, 4 heifers, 8 young cows and 3 mature bulls. Two slices of muscle were obtained from
89 each animal at the cutting line and approx. 3.5 hours *post mortem*. These were internal slices
90 taken at the proximal end of the muscle for all carcasses. Each slice had a thickness around
91 1.5cm and weighted between 170-250g.

92

93 *2.2. Data describing each individual animal*

94 <u>Time variables</u>**:** Transportation time from farm to abattoir (ToT); Lairage time at abattoir before
95 death (ToD); Hot-boning/cutting time after death (ToC); Time from death to initial OCR
96 measurements at ~4hr (ToDO).

97 pH-measurements: pH of *M. semimembranosus* were recorded by inserting the probe (Portamess
98 913, Knick, Berlin, Germany) once the meat was available at the slaughter line (pH4; 3.5-4 hours
99 *post mortem*), after 8 h (pH8; 8 hours *post mortem*) and after 24 h (pH24; 24 hours *post mortem*).

100 Temperature: A slice of beef with the shape of a steak (1.5 cm thickness and 170-250g) was
101 excised from the proximal end of the *M. semimembranosus*. The temperature (Ebro TLC 1598,
102 Ebro Electronic GmbH & Co, Ingolstadt, Germany) of the *M. semimembranosus* was recorded
103 on the meat surface immediately after cutting the section to be used for subsequent analysis
104 (called Temp). A slice was vacuum-packed in polyamide bags, (oxygen transmission rate 30-40
105 $cm^3/m^2/24$ h/atm at 23 °C and 0 % RH, LogiCon Nordic A/S, Kolding, Denmark) and transferred
106 to storage at 10°C (~16 hours) and subsequently to 4°C for 3 weeks' storage.

107

108 *2.3. Analytical measurements*

109 Colour measurements: After three weeks of storage, the slices of *M. semimembranosus* of each
110 animal were taken from the vacuum package and packaged in a tray (20.5 cm by 14.8 cm black
111 amorphous polyethylene terephthalate boxes with 3.5 cm height) covered with oxygen-
112 permeable film with O_2 transmission rate of 6500-8500 $cm^3m^{-2}24h^{-1}$ at 23 ºC and 75 % relative
113 humidity (Toppits-glad, Melitta Group, Helsingborg, Sweden) during the colour measurement
114 period. During this period the test samples were stored under a fluorescent lamp (Phillips Master
115 TL-D 36W/830 H9, Phillips, Oslo, Norway) with a light intensity of 800 lx. The colour of all
116 samples were measured through the oxygen-permeable film with a Konica Minolta Chroma
117 meter CR 410 (Konica Minolta Sensing Inc., Osaka, Japan) (3 replicates) giving L*, a*, b*

118 (lightness, redness and yellowness, respectively) values (CIE, 1976) and visible and near infrared
119 reflectance (400–1100nm, 2 measurements on each slice) with FOSS NIRSystems (Model 6500,
120 Oslo, Norway) in transflectance mode with an interactance probe making use of fibre optics
121 (40x40mm^2 optiprobe ™ system). Absorbance (A= log(1/R); R is reflectance) was measured
122 between 400–1100nm in 2nm steps giving 350 spectral variables. The instrument was calibrated
123 using a ceramic plate as a reference before all measurements. Each spectral measurement is an
124 average of 32 scans and takes about 20 secs. The measurements were taken at the following
125 times: 0, 15, 30, 45, 60, 90 and 240 minutes and at day 1, 2 and 7. The samples were stored at
126 4°C between measurements and exposed to 17°C during measurements (Würth Infrared
127 thermometer, type 08536007, Würth UK Ltd., Kent, United Kingdom). Percentages of MMb,
128 OMb and DMb were calculated according to (Khatri, et al., 2012) with a method that uses a
129 Partial Least Square (PLS) prediction model obtained from reflectance spectra (wavelengths
130 400–1100nm) of meat samples having dominantly one fraction of myoglobin.

131 Tissue permeabilisation: The muscle fibers were permeabilized prior to OCR measurements. The
132 fibres were separated in a relaxing solution containing 15 mM phosphocreatine, 10 mM Ca-
133 EGTA (0.1 mM free calcium), 20 mM imidazole, 20 mM taurine, 6.6 mM $MgCl_2$, 50 mM K-2-
134 (*N*-morpholino) ethanesulfonic acid, 0.5 mM dithiothreitol and 5.8 mM ATP adjusted to pH 7.1.
135 Muscle fibres were separated to an approximate length of 5.0 mm with a diameter of approx. 1.0
136 mm. The tissue was permeabilised in the same relaxing solution containing 0.052 mg/ml saponin
137 for 30 mins. The samples were subsequently washed for 10 min. at 4°C with shaking in the
138 respiration medium (Sperl, et al., 1997).

139 Oxygen consumption rate (OCR): OCR measurements were carried out ~4 hours *post mortem*
140 and after 3 weeks' chilled storage. All chemicals were of analytical grade and purchased from
141 Sigma Chemicals Corp. (St. Louis, MO), except for pyruvate (Applichem, VWR International
142 AS, Norway). High-resolution respirometry was carried out with Oroboros Oxygraph-2K
143 instruments (Oroboros Instruments, Innsbruck, Austria) as described by Gnaiger (2001). The
144 OCR of permeabilised tissue was measured at 20°C and ~200 µM O_2. The closed chambers had a
145 volume of 2.1 ml and results are reported as (pmol O_2/sec)/mg protein. Injection of substrates
146 was done with a Hamilton syringe of 10µl–50µl.

147 The OCR measurements were carried out on permeabilized muscle fibers by stimulating
148 mitochondrial enzyme complexes in sequence. Adding the chemicals is possible as the responses
149 are additive and incremental, so that no response is masked by the chemical ahead. A similar
150 protocol has been run by Scheibye-Knudsen (2009). Complex I respiration was initiated by
151 malate and glutamate, followed by testing β-oxidation using octanoylcarnithin. β-oxidation
152 donates electrons to an electron transferring flavoprotein (ETF) on the mitochondrial inner
153 membrane (Table 1). Oxidative phosphorylation was stimulated by the addition of ADP.
154 Complex II respiration was tested by succinate. Uncoupling was measured by FCCP addition. At
155 this point, rotenone addition blocked complex I and revealed the maximum capacity of complex
156 II. Inhibition of complex II was achieved by adding malonic acid. Finally, inhibition of complex
157 III was by antimycin A. Background oxygen consumption after complete inhibition of the ETS
158 using antimycin A (Gnaiger, 2008) was recognized as residual oxygen-consuming side reactions
159 (ROX). A schematic representation of the functional sites of the substrates is shown in Figure 1
160 and a run of the oxygen consumption measurement in Figure 2. OCR_{Max} is the maximum OCR of
161 the ETS without background adjustment (not subtracted for OCR_{Rox}) and after uncoupling with

162 FCCP. Background adjustment was done for all OCR responses except OCR_{Max}. The respiration
163 medium consisted of 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-methanesulfonate, 20 mM taurine,
164 10 mM KH_2PO_4 (Calbiochem, Darmstadt, Germany), 20 mM HEPES, 110 mM sucrose (Alfa
165 Aesar, Karlsruhe, Germany) and 1.0 g/l BSA (Pesta & Gnaiger, 2010). The respiration medium
166 had pH 7.1.

167 **2.4 *Statistical analysis***

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

173 A principal component analysis (PCA, The Unscrambler X 10.1; CAMO Software, Oslo,
174 Norway) was first carried out with all variables and served as an explorative screening method
175 for identifying both animal and OCR variables that were highly correlated before a stepwise
176 regression was performed. Weighting (1/stand.dev) of all variables was used. A subset of
177 variables was selected based on clustering from the PCA score plot for subsequent use in
178 stepwise regression. The principal component analysis used full cross validation (leave one out)
179 as a method to identify the correct number of principal components (for more details see Martens
180 and Næs, 1989). However, the first component represents more explained variance than the
181 second components and this one again more explained variance than the third component.

182 The relationships between OCR and individual animal and muscle characteristics were obtained
183 through stepwise regression (forward and backward) for identification of significant relationships

184 (Minitab, version 16 from Minitab Inc., State College, PA, USA). The approach served the
185 purpose of identifying significance levels for groups of variables.

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

188 Through MATLAB (version 2011a, The MathWorks, Inc., Natick, Massachusetts, USA) the
189 relationships between changes over time in colour variables L*, a*, b* and myoglobin states and
190 OCR or individual muscle variables were examined using N-way partial least squares (NPLS)
191 (Bro, 1996). NPLS is a multi-way generalization of partial least squares (PLS) (Martens & Naes,
192 1989). Most variants of PLS work on X data in two modes (samples x variables) organized as a
193 matrix. NPLS can handle three or more modes (samples x variables x variables x ...) organized as
194 a three-way (or more) array. The response (Y) in PLS regression is either a single vector or a
195 matrix of responses. In NPLS this can also be a multi-way array of responses. Our X data is
196 three-way with a sample mode (animals), an observation time mode and the colour (a*, b*) or
197 myoglobin state modes. The Y data were either the controllable plus uncontrollable animal and
198 muscle characteristics (storage times, transport times, temperature, pH, ...) or OCR
199 measurements. The X data are sequentially decomposed into orthogonal sets of score and loading
200 vectors maximizing the covariance between the X data and the responses (Y). The loading
201 vectors have unit lengths and show how much weight is given to each variable in each mode for
202 the current component. Since the scale of the Y data differs, these were weighted according to
203 1/st.dev. This makes the variables easier to compare, but can hide the differences in variation.
204 The NPLS method was validated using leave-one-out cross-validation. Stability of the time
205 points and OCRs are indicated by confidence intervals in the figures. Sample 37 was removed as
206 an outlier in analysis of a* and b*.

207

208 3. Results and Discussion

209

210 *3.1 Variation in OCR among fresh and chill-stored muscles of individual animals*

211

212 Animal and muscle characteristics varied widely (Table 2) e.g. time of transport from farm to
213 abattoir (30min – 7 hours), weight (136 – 516kg) and colour characteristics (Table 3) e.g. a*
214 values (7.51 – 17.0) and DMb (0.00 – 1.00). Table 4 shows that OCR in both stored and fresh
215 meat varied significantly between individual animals and between the different substrates used
216 (except for the malonic acid effect on stored samples which was not significant). Of all oxygen-
217 consuming reactions between animals, OCR_{ROX} was the one that increased after storage for some
218 animals (see Table 4 where ΔOCR was significantly different among animals) and occurred only
219 when biological material was present. Furthermore, mean OCR_{ROX} between groups of fresh and
220 stored muscles was not significantly different, indicating an oxygen consuming activity that was
221 perhaps independent from the mitochondrial ETS (Table 4).

222 The enzyme complex most vulnerable to *post mortem* inactivation was complex I of the ETS;
223 OCR_{Glu} was reduced by 61% after 3 weeks, while OCR_{Suc} was only reduced by 13% (Table 4).
224 Werner, Natter and Wicke (2010) reported similar results for pigs where complex I activity
225 increased immediately *post mortem* and then fell dramatically after 12 hours, while other ETS
226 complexes such as complex IV remained constant *ante* and *post mortem* (24 hours before and 12
227 hours after slaughter). The oxidation of succinate by succinate dehydrogenase (complex II) was
228 the strongest and most stable enzyme activity in mitochondria with aging (Table 4). Uncoupling
229 of the ETS by FCCP showed that succinate respiration in many cases represented max OCR
230 capacity of the ETS, thus ATP synthesis was already uncoupled in *post mortem* meat (Table 4).

231 We observed a small increase in OCR after rotenone addition in both fresh and stored muscle.
232 This may be due to electrons from succinate oxidation being prevented from reverse electron
233 transport (RET) to complex I, subsequently shifting to a forward flow to complex IV. RET
234 occurs in fresh meat and for some samples, to an even greater degree in stored meat after
235 rotenone inhibition (0–2.36 pmol/sec/mg, Table 4). Complex I inactivation and RET from
236 complex II to complex I are known to stimulate production of ROS (Capel, et al., 2005;
237 Kushnareva, Murphy, & Andreyev, 2002; Pitkanen & Robinson, 1996), which in turn may lead
238 to increased MMb formation and lipid oxidation (Tang, et al., 2005). Blocking of complex II by
239 malonic acid confirms that oxygen consumption was largely determined by complex II (Table 4).
240 This is especially apparent in stored meat where complex I and β-oxidation were largely
241 inactivated, and thus blocking of complex II resulted in an almost complete inhibition of the ETS
242 (Table 4).

243 Table 4 shows that the average overall activity of the ETS in stored meat was 22% (OCR_{Max})
244 lower than in fresh meat. The reduction in OCR_{Suc} appeared smaller (Table 4), but altogether the
245 reduction in OCR_{Rot} and OCR_{FCCP} suggested that a reduction of around 20% in max OCR could
246 be expected with storage. Furthermore, after 3 weeks the respiration activity was strongly
247 reduced in malate and octanoylcarnithin activity, and so complex II was the main electron-
248 contributing site of the ETS. As much as 90% of OCR can be inhibited by the complex II
249 inhibitor malonic acid in 3 week-aged meat compared to 72% in fresh meat.

250

251 *3.2.Relationship between OCR and individual animal and muscle characteristics*
252
253 The PCA plot in Figure 3 shows how the various variables collected at the slaughterhouse related
254 to each other and to OCR measured on fresh permeabilised muscle fibres and after 3 weeks'

255 chilled storage of the muscle. An impression of inter-correlations between variables are shown;
256 variables that group closely together are most strongly correlated (Fig. 3). OCR_{Glu} and OCR_{ADP}
257 clustered together after ~4 hours and after 3 weeks' storage (Fig. 3). OCR_{Glu} changed from being
258 positively related to OCR_{Suc} in fresh muscle to being negatively related in stored muscle
259 ($p<0.001$, see also Fig. 3). Hence, early and late *post mortem* related oxygen consumption was
260 different.

261 Tables 5 give relationships between OCR and variables selected as significant in the stepwise
262 regression. Animal and muscle characteristics not listed in Tables 5 did not reveal any significant
263 relationships with OCR. The table indicates that temperature, ToDO and the pH 8 were
264 parameters with substantial variations at the slaughter line. pH significantly affected OCR_{Rot}
265 early *post mortem* but had no significant relevance for OCR in meat that had been chill-stored for
266 3 weeks.

267

268 Fresh muscle

269 OCR related most strongly to temperature and weight (Table 5). The OCR_{Max} ($p<0.05$ for Temp.)
270 and OCR_{Suc}, OCR_{FCCP} and OCR_{Rot} all showed a positive increase in OCR with increased weight
271 and a reduction (except OCR_{FCCP}) with increased temperature. Weight and surface temperature
272 were correlated ($r =0.32$; $P=0.04$). But surface temperature related more strongly to OCR than
273 did carcass weights.

274 Animal and muscle characteristics (temperature, pH and times) / weight and age: The
275 temperature profile of the *M. semimembranosus* was the most critical variable for maintaining
276 good oxygen consumption (Table 5). Although the effect of ToDO was significantly related to

277 OCR_{Glu}, the surface temperature was associated with much more variation in OCR_{Glu} than ToDO
278 in the early *post mortem* data due to its larger variation.

279 High temperature due to high carcass weight and thick fat insulation will result in a slower
280 chilling rate. However, large carcasses *per se* did not reduce OCR_{Glu} and OCR_{Suc}. Similarly, high
281 carcass weights stimulated higher respiration on succinate (0.0156; Table 5) and, to a smaller
282 extent, higher respiration on glutamate (0.0105; Table 5).

283

284 Stored muscle

285 The relationships between ToDO (time of OCR measurements after death) and age were the
286 strongest for OCR in 3 weeks chill-stored muscle. The OCR_{Max} and OCR_{Suc}, OCR_{FCCP} and
287 OCR_{Rot} all showed a positive increase with increased age and a reduction with ToDO (Table 5).

288 Complex I appeared related to the time of transport (Table 5). The influence of ToT reflected the
289 only relationship between a stress variable and OCR, and long transport times reduced OCR_{Glu}.
290 OCR_{ROX} had, on the other hand, a positive trend with ToT. OCR_{OC} decreased with age and
291 weight (Table 5).

292 Animal and muscle characteristics: The effect of ToDO on OCR_{Suc} suggested that rapid chilling
293 *early post mortem* may be beneficial for higher oxygen consumption at a later stage and hence
294 affect colour changes positively. This could actually be contrary to conditions that would support
295 tenderization.

296 The inactivation of ATP synthesis and complex I with animal age (Table 5) could be due to an
297 accumulation of mitochondria respiring on complex II rather than complex I, which is more

298 robust against age, oxygen limitation and freeze storage (Galkin, Abramov, Frakich, Duchen, &
299 Moncada, 2009; Jones & Brewer, 2010; Phung, Saelid, Egelandsdal, Volden, & Slinde, 2011;
300 Vitorica, Cano, Satrustegui, & Machado, 1981). Mitochondria in older specimens are believed to
301 have higher OCR in order to compensate for a decreased efficiency in ATP production (Jones &
302 Brewer, 2010).

303 The origin of OCR_{ROX} is not known. The actual magnitude for OCR_{ROX} indicated that this effect
304 could be important in aged meat. Further investigations are needed.

305

306 *3.3.Colour changes in M. Semimembranosus exposed to air*
307

308 Figure 4A shows that a* increased slightly and then decreased after 4 hours. Similarly, the DMb
309 form decreased more rapidly after 1.5 hours (Fig. 4B). L* values were omitted from the NPLS
310 analysis below as it changed little with time.

311 The NIR spectrophotometer provided more details regarding the colour transitions in meat than
312 L*, a* and b*. The general trend was that the DMb fraction gradually declined with time and
313 transformed temporarily into OMb before becoming oxidized to MMb (Fig. 4B). Figure 4B
314 shows that the DMb form remained until 1.5 hours and then decreased rapidly; similar to the
315 increase in a* values. The MMb fraction increased from 1.5 hours (Fig. 4B). This trend was in
316 agreement with Arihara (1995) and others (Hagler, Coppes, & Herman, 1979; Livingston, et al.,
317 1985), where a transition from DMb to MMb may occur rapidly with an OMb intermediate. It
318 was apparent that the reduction in DMb on average preceded changes in a* (Figs. 4A and B).
319 Reduced myoglobin reached a minimum after 3 days (72 hours) and for a* and b* after 6 days
320 (144 hours). This proves that assessing colour by measuring myoglobin forms will detect and

321 predict changes earlier and with more detailed information than when measuring a* or b*.
322 However, the method used for of calculation of myoglobin states is more cumbersome, but in the
323 future calibration transfer between samples may be possible. On the other hand, L*, a* and b*
324 data have a better reproducibility due to its simple and quick approach for colour assessment,
325 even though the method does not have the fidelity of myoglobin state quantification.

326

327 *3.4 Relationship between OCR and colour changes*

328 In comparison to the mean changes given in Figure 4, the individual animal's changes in L*, a*,
329 b* and myoglobin states with time in an oxygen permeable film have been related to OCR
330 measured from the time the meat was exposed to oxygen by using NPLS regression (Figure 5
331 and 6). Both Figure 5 and 6 are scaled as relative contributions and consist of several variables
332 (e.g. time, OCR values and animal/muscle characteristics). The squared sum of all the relative
333 contributions is one. The relative contributions are similar to regression coefficients and give the
334 influence of the different measurements. Figure 5A shows progress in components 1 and 2 of the
335 N-PLS regression with storage time in air (X-block data), and Figure 5B shows the relative
336 importance of the different substrates in the OCR protocol (Y-block) regarding explaining
337 changes in a* (~NPLS component 1) and b* (~NPLS component 2) (X-block). The first NPLS
338 component (Fig. 5A) described 99.37% of the colour (a*) variation and this variation could be
339 explained by 62.67% of the variation in OCR induced by chemicals (*i.e.* of the variation in the
340 OCR protocol, Fig. 5B). Redness was accordingly the single most important variable for
341 describing the variation in colour over time. The first component (Fig. 5A, a*) rose slightly up
342 to four hours, then it dropped more strongly up to 144 hours. The changes in b* values were

343 much less (NPLS component 2; 0.15% explained variance of X-block; Fig. 5A). The second
344 component described only 0.15% of the colour variation (Fig. 5A) but was explained by 12.1%
345 of the variation in OCR observed using additives (Fig. 5B). This component had a more distinct
346 rise (in b* level) until 4 hours and also a slow decline until 144 hours (Fig. 5A). In comparison,
347 McKenna et al. (2005) found few changes in b* values in *M .semimembranonsus* after 1 day's
348 display. We show here that b* values increased substantially until peaking between 1.5–4 hours,
349 followed by a concomitant decrease in a* values (Fig. 5A). Figures 5A and B thus supported that
350 totally 99.55% of the variation in a* and b* was explained by totally 75% of the variation in
351 OCR. This demonstrated that the colour changes in muscle exposed to air were largely explained
352 by the ability of the ETS to remove oxygen. The twocomponents changed with time in a similar
353 way to the mean colour variables a* and b* given in Fig. 4A. Component 1 (Y-block) of Figure
354 5B was explained mainly by the contribution of ROX and secondly by maximum OCR and the
355 complex II group (succinate,FCCP, Rotenone, and Max), while β-oxidation and complex I group
356 (Mal., O.C., ADP and Glu) contributed negatively to a* (Fig. 5A). Moreover, the contribution
357 from ROX decreased since the relative contribution of complex II to color was larger in
358 component 2 (Fig. 5B) than in component 1 (of Y-block). The OCR profile of Figure 5D had a
359 similar relationship to the myoglobin states, where ROX and complex II OCR preserved
360 DMb/OMb (and a*) whereas complex I with β-oxidation did the opposite.

361 Two NPLS components were also calculated from reflectance measurements (Fig. 5C); a third
362 smaller NPLS component could possibly be related to light scattering. The two first NPLS
363 components both consisted of combinations of pure myoglobin states. This is a result of the
364 requirement for orthogonality of components in the calculation and if no pure state is orthogonal
365 to the other, the states are combined in one component. Our component 1 in X-block (65.55%

366 explained variance) nevertheless described predominantly DMb, while component 2 in X-block
367 (16.51% explained variance) described largely OMb but converted gradually to MMb after 48
368 hours. These results indicate that OCR explained 65.55% of the change in DMb prevalence and
369 only 16.51% of the change in OMb and MMb (Fig. 5C). As the components were not related to
370 pure myoglobin states the interpretation is more challenging. Figure 5C shows the average
371 change of DMb (comp. 1) and OMb/MMb (comp. 2) with time and the importance of the
372 different substrates in the OCR protocol regarding explained changes in the myoglobin states
373 (Fig. 5D).

374 Since component 1 (Figure 5C) was dominated by DMb, it made sense that component 1
375 declined as reported in Fig. 4B. Component 2 was a combination of OMb and MMb (loading
376 weight 0.71 and 0.37 for MMb and OMb, respectively) but represented more of MMb than of
377 OMb as this form was more important than the oxyform in the regression model (Fig. 5C and
378 5D). The first NPLS component of X-block described 65.44% of the myoglobin forms and
379 66.28% of the variation in the OCR profiles of the Y-block. With regard to the second
380 component, 16.51% was explained by variation in myoglobin forms and 6.74% by the variation
381 in OCR as a response to additives (Y-block). Altogether 75% of the total variation in OCR
382 explained 82% of the total variation in myoglobin states. The apparently weaker relationship
383 between myoglobin states and variation in OCR than between the variables a*, b* and variation
384 in OCR was at least partly due to the fact that myoglobin states cannot be accurately predicted
385 (Khatri, et al., 2012).

386 Figures 5B and 5D show that the correlated group of variables OCR_{Suc}, OCR_{FCCP}, OCR_{Rot} and
387 OCR_{Max} (see Fig. 3) was important in explaining changes in a*, b* and myoglobin states. Total
388 OCR_{Max} was ranked with high nominal importance (Fig. 5). This variable and its related

389 variables all pointed to the importance of complex II for removing oxygen. The relationship with
390 OCR_{Suc} should be expected since complex II was the only complex with substantial activity *post*
391 *mortem* (except complex IV, which did not have a substrate). OCR by ROX was small in regard
392 to absolute values, however, the importance of ROX became apparent in Figures 5B and 5D
393 where it was the largest contributor to color changes (a* and reduced myoglobin).

394 Our OCR measurements were only intended for ranking activities of the complexes/enzymes and
395 not for quantitatively representing data for the activities in the sample since the ultimate pH
396 varied in meat. However, there was not much variation in OCR_{Suc} between pH 5.5 and 6.0 using
397 permeabilised pork fibres (Phung, et al., 2011). Furthermore, the actual value for OCR_{Suc}
398 between pH 5.5–6.0 was 50–60% of the value at pH 7.1 (Phung, et al., 2011). The data also
399 suggested that although there are only low amounts of ETS substrates after 3 weeks of chilled
400 storage, there are still some substrates supporting ETS through complex II or other complexes, at
401 least for a few hours after atmosphere exposure.

402 It appeared that high OCR_{Suc} promoted the presence of reduced myoglobin, while any activity in
403 the OCR_{Glu} and OCR_{OC} group did the opposite. Thus complex I activity would be regarded as a
404 colour destabiliser. In Figures 5B and 5D the substrates of OCR_{Glu} , OCR_{ADP} and OCR_{OC} are
405 presented as factors that have a relatively large negative weight for colour. However, the small
406 absolute values of these OCR after three weeks (Table 4) make them less influential than
407 OCR_{Suc}. Figure 5 shows variables with opposing effects (above or below zero in relative
408 contribution) on colour changes. The variation in magnitudes of OCR would clearly indicate the
409 importance as: OCR_{Suc} and related variables > OCR_{ROX} >> OCR_{Glu} and related variables.

410 Finally, high OCR_{ROX} maintained DMb for a longer period of time (Fig. 5B and 5D). OCR_{ROX}
411 was on average a small contributor to OCR (Table 4). However, it was of importance as OCR_{ROX}
412 remained with storage time and the substrates that were used. More importantly, OCR_{ROX}
413 continued to consume oxygen even when the mitochondrial ETS had ceased to function due to
414 inhibitors. Results showed that OCR_{ROX} only occurred when biological materials were present
415 and not in isolated mitochondria (results not shown). OCR_{ROX} may therefore gradually begin to
416 play a more important role as meat ages when mitochondria stop functioning and internal
417 substrates are depleted.

418
419 *3.5 Relationship between individual animal and muscle characteristics and colour changes*
420
421 The NPLS regression method was also used to relate animal and muscle characteristics to the 3
422 directions: a*, b*-time-animal number (Fig. 6A) or the three directions: % myoglobin state-time-
423 carcass number (Fig. 6B) respectively. The data in Fig. 6A and 6B used weighted (1/st.dev)
424 animal and muscle characteristics.

425 Unweighted animal and muscle characteristics suggested that weight and age affected colour
426 changes (not shown) in accordance with Table 5 that identified these variables as important for
427 OCR. However, when weighting was introduced (Fig. 6A and 6B), pH24 (ultimate *post mortem*
428 pH) was identified as important for colour changes. Components 1 and 2 together described
429 99.5% of the variation in a* and b* (X-block) and this could be related to 98.9% of the variation
430 in animal and muscle characteristics (Y-block, Fig 6A, weighted animal and muscle
431 characteristics). For myoglobin states, components 1 and 2 explained 74.6% of the variation (X-
432 block) and this could be related to 99.8% (components 1 and 2) of the variation in animal and
433 muscle characteristics (Fig. 6B). pH24 (ultimate pH) thus accounted for high a* values and DMb

434 states (the larger components) under weighted conditions. Ultimate pH is therefore important for
435 slaughter animals that have less variation in for example weight and age than the current group
436 of animals.

437 According to Hood (1980) and McKenna et al. (2005), colour changes are barely affected by pH.
438 However, colour changes are severely affected by OCR in muscle. This means that OCR
439 measurements at the specific pH of the muscle should have given an even stronger relationship
440 between OCR and colour changes than those reported in Fig. 5. pH plays a role in colour
441 changes and can also affect mitochondrial activity and protein denaturation (Joo, Kauffman,
442 Kim, & Park, 1999).

443 Higher surface temperature early *post mortem* affected colour changes (Fig. 6).The temperature
444 effect was not identified as the most important effect independent of regression method used
445 (Fig. 6). This is in agreement with Table 5. Quick cooling is important for colour and OCR
446 through maintaining complex II activity as suggested above. However, it may not be important to
447 keep an active complex I, thus optimal chilling could be important for optimal colour changes.
448 The colour changes after 3 weeks possibly reflected both the slaughterhouse environment (pH24)
449 and intrinsic variables of each carcass such as weight.

450

451 **Conclusion**

452 The enzyme activities of complex I and β-oxidation are involved in the early muscle-to-meat
453 transition after slaughter. Our results show that these systems are not dominant in determining
454 colour changes. Early *post mortem* treatment, such as slow chilling, reduced the activity level of
455 complex I. After 3 weeks of chilled storage 61% reduction in complex I activity was found. At

456 this time complex II activity related predominantly to colour changes as shown by measuring
457 changes in L*, a*, and b* values and myoglobin states. Carcass weight, age and ultimate pH
458 affected colour changes, but ultimate pH was not a significant factor for OCR. After 3 weeks
459 chill storage OCR could be related to animal age and carcass weight and to early *post mortem*
460 cooling profile. A hitherto unknown factor, OCR_{ROX}, was important for the oxygen consumption
461 and color changes in meat and was the only factor which did not decrease with storage of meat.

462
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472

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474 Arihara, K., Cassens, R. G., Greaser, M. L., Luchansky, J. B., & Mozdziak, P. E. (1995).
475 Localization of metmyoglobin-reducing enzyme (NADH-cytochrome-b(5) reductase)
476 system components in bovine skeletal-muscle. *Meat Sci, 39*(2), 205-213.
477 Barksdale, K. A., Perez-Costas, E., Gandy, J. C., Melendez-Ferro, M., Roberts, R. C., & Bijur,
478 G. N. (2010). Mitochondrial viability in mouse and human postmortem brain. *The FASEB*
479 *journal, 24*(9), 3590-3599.
480 Bro, R. (1996). Multi-way calibration. Multi-linear PLS. *J. Chemom., 10*(1), 47-62.
481 Capel, F., Rimbert, V., Lioger, D., Diot, A., Rousset, P., Mirand, P. P., Boirie, Y., Morio, B., &
482 Mosoni, L. (2005). Due to reverse electron transfer, mitochondrial H2O2 release
483 increases with age in human vastus lateralis muscle although oxidative capacity is
484 preserved. *Mech Ageing Dev, 126*(4), 505-511.
485 CIE. (1976). *Recommendations on uniform color spaces-color difference equations* (Vol.
486 Supplement No. 2 to CIE). Paris, France: Commission Internationale de l'Eclairage.
487 Farouk, M. M., & Swan, J. E. (1998). Effect of rigor temperature and frozen storage on
488 functional properties of hot-boned manufacturing beef. *Meat Sci, 49*(2), 233-247.
489 Galkin, A., Abramov, A. Y., Frakich, N., Duchen, M. R., & Moncada, S. (2009). Lack of oxygen
490 deactivates mitochondrial complex I: implications for ischemic injury? *J Biol Chem,*
491 *284*(52), 36055-36061.
492 Gnaiger, E. (2001). Bioenergetics at low oxygen: dependence of respiration and phosphorylation
493 on oxygen and adenosine diphosphate supply. In (Vol. 128, pp. 277-297): Elsevier
494 Science Bv.
495 Gnaiger, E. (2008). Polarographic oxygen sensors, the oxygraph, and high-resolution
496 respirometry to assess mitochondrial function. In J. Dykens & Y. Will (Eds.), *Drug-*
497 *Induced Mitochondrial Dysfunction* (pp. 327-352). Innsbruck: John Wiley & Sons, Inc.
498 Hagler, L., Coppes, R. I., Jr., & Herman, R. H. (1979). Metmyoglobin reductase. Identification
499 and purification of a reduced nicotinamide adenine dinucleotide-dependent enzyme from
500 bovine heart which reduces metmyoglobin. *J Biol Chem, 254*(14), 6505-6514.
501 Hood, D. E. (1980). Factors Affecting the Rate of Metmyoglobin Accumulation in Pre-Packaged
502 Beef. *Meat Sci, 4*(4), 247-265.
503 Jones, T. T., & Brewer, G. J. (2010). Age-related deficiencies in complex I endogenous substrate
504 availability and reserve capacity of complex IV in cortical neuron electron transport.
505 *Biochim Biophys Acta, 1797*(2), 167-176.
506 Joo, S. T., Kauffman, R. G., Kim, B. C., & Park, G. B. (1999). The relationship of sarcoplasmic
507 and myofibrillar protein solubility to colour and water-holding capacity in porcine
508 longissimus muscle. *Meat Sci, 52*(3), 291-297.
509 Khatri, M., Phung, V. T., Isaksson, T., Sorheim, O., Slinde, E., & Egelandsdal, B. (2012). A
510 comparison of two sample preparation methods and two methods for calculating different
511 myoglobin states in beef M. semimembranosus surfaces. *Meat Sci*.
512 Kushnareva, Y., Murphy, A. N., & Andreyev, A. (2002). Complex I-mediated reactive oxygen
513 species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state.
514 *Biochem J, 368*(Pt 2), 545-553.
515 Ledward, D. A., Dickinson, R. F., Powell, V. H., & Shorthose, W. R. (1986). The Color and
516 Color Stability of Beef Longissimus-Dorsi and Semimembranous Muscles after Effective
517 Electrical-Stimulation. *Meat Sci, 16*(4), 245-265.
518 Lenaz, G. (2001). The mitochondrial production of reactive oxygen species: mechanisms and
519 implications in human pathology. *IUBMB Life, 52*(3-5), 159-164.

520 Livingston, D. J., McLachlan, S. J., La Mar, G. N., & Brown, W. D. (1985). Myoglobin:
521 cytochrome b5 interactions and the kinetic mechanism of metmyoglobin reductase. *J Biol*
522 *Chem, 260*(29), 15699-15707.
523 Lumeng, L., Bremer, J., & Davis, E. J. (1976). Suppression of the mitochondrial oxidation of (-)-
524 palmitylcarnitine by the malate-aspartate and alpha-glycerophosphate shuttles. *J Biol*
525 *Chem, 251*(2), 277-284.
526 Martens, H., & Naes, T. (1989). *Multivariate calibration*.
527 McKenna, D. R., Mies, P. D., Baird, B. E., Pfeiffer, K. D., Ellebracht, J. W., & Savell, J. W.
528 (2005). Biochemical and physical factors affecting discoloration characteristics of 19
529 bovine muscles. *Meat Sci, 70*(4), 665-682.
530 Monin, G., & Sellier, P. (1985). Pork of low technological quality with a normal rate of muscle
531 pH fall in the immediate post-mortem period - the case of the Hampshire Breed. *Meat*
532 *Sci, 13*(1), 49-63.
533 Nowak, G., Clifton, G. L., & Bakajsova, D. (2008). Succinate ameliorates energy deficits and
534 prevents dysfunction of complex I in injured renal proximal tubular cells. *J Pharmacol*
535 *Exp Ther, 324*(3), 1155-1162.
536 O'Keeffe, M., & Hood, D. E. (1982). Biochemical Factors Influencing Metmyoglobin Formation
537 on Beef from Muscles of Differing Color Changes. *Meat Sci, 7*(3), 209-228.
538 Pesta, D., & Gnaiger, E. (2010). High-resolution respirometry. OXPHOS protocols for human
539 cell cultures and permeabilized fibres from small biopsies of human muscle.
540 *Mitochondrial bioenergetics: methods and protocols*.
541 Phung, V. T., Saelid, E., Egelandsdal, B., Volden, J., & Slinde, E. (2011). Oxygen Consumption
542 Rate of Permeabilized Cells and Isolated Mitochondria from Pork M. Masseter and Liver
543 Examined Fresh and After Freeze-Thawing at Different pH Values. *J Food Sci, 76*(6),
544 929-936.
545 Pitkanen, S., & Robinson, B. H. (1996). Mitochondrial complex I deficiency leads to increased
546 production of superoxide radicals and induction of superoxide dismutase. *J Clin Invest,*
547 *98*(2), 345-351.
548 Renerre, M., & Labas, R. (1987). Biochemical factors influencing metmyoglobin formation in
549 beef muscles. *Meat Sci, 19*(2), 151-165.
550 Rosenvold, K., & Andersen, H. J. (2003). The significance of pre-slaughter stress and diet on
551 colour and colour changes of pork. *Meat Sci, 63*(2), 199-209.
552 Schafer, A., Rosenvold, K., Purslow, P. P., Andersen, H. J., & Henckel, P. (2002). Physiological
553 and structural events postmortem of importance for drip loss in pork. *Meat Sci, 61*(4),
554 355-366.
555 Scheibye-Knudsen, M., & Quistorff, B. (2009). Regulation of mitochondrial respiration by
556 inorganic phosphate; comparing permeabilized muscle fibers and isolated mitochondria
557 prepared from type-1 and type-2 rat skeletal muscle. *Eur J Appl Physiol, 105*(2), 279-
558 287.
559 Scholes, T. A., & Hinkle, P. C. (1984). Energetics of ATP-driven reverse electron transfer from
560 cytochrome c to fumarate and from succinate to NAD in submitochondrial particles.
561 *Biochemistry-US, 23*(14), 3341-3345.
562 Simela, L., Webb, E. C., & Frylinck, L. (2004). Effect of sex, age, and pre-slaughter
563 conditioning on pH, temperature, tenderness and colour of indigenous South African
564 goats. *South African Journal of Animal Science, 34*, 208-211.

565 Sookhareea, R., Taylor, D., Woodford, K., Dryden, G., & Shorthose, R. (1995). The effect of
566 slaughter age and sex type on meat quality of Javan ruse (*Cervus timoriensis*) male deer.
567 In *CSIRO Meat Industry Research Conference* (Vol. 38, pp. 17-20). Australia: CSIRO
568 Publishing.
569 Sperl, W., Skladal, D., Gnaiger, E., Wyss, M., Mayr, U., Hager, J., & Gellerich, F. N. (1997).
570 High resolution respirometry of permeabilized skeletal muscle fibers in the diagnosis of
571 neuromuscular disorders. *Mol Cell Biochem, 174*(1-2), 71-78.
572 St-Pierre, J., Buckingham, J. A., Roebuck, S. J., & Brand, M. D. (2002). Topology of superoxide
573 production from different sites in the mitochondrial electron transport chain. *The journal*
574 *of biological chemistry, 277*(47), 44784-44790.
575 Stoier, S., Aaslyng, M. D., Olsen, E. V., & Henckel, P. (2001). The effect of stress during lairage
576 and stunning on muscle metabolism and drip loss in Danish pork. *Meat Sci, 59*(2), 127-
577 131.
578 Tang, J., Faustman, C., Hoagland, T. A., Mancini, R. A., Seyfert, M., & Hunt, M. C. (2005).
579 Interactions between mitochondrial lipid oxidation and oxymyoglobin oxidation and the
580 effects of vitamin E. *J Agric Food Chem, 53*(15), 6073-6079.
581 Vitorica, J., Cano, J., Satrustegui, J., & Machado, A. (1981). Comparison between
582 developmental and senescent changes in enzyme activities linked to energy metabolism
583 in rat heart. *Mech Ageing Dev, 16*(2), 105-116.
584 Werner, C., Natter, R., & Wicke, M. (2010). Changes of the activities of glycolytic and oxidative
585 enzymes before and after slaughter in the longissimus muscle of Pietrain and Duroc pigs
586 and a Duroc-Pietrain crossbreed. *J Anim Sci, 88*(12), 4016-4025.
587 Young, O. A., Priolo, A., Simmons, N. J., & West, J. (1999). Effects of rigor attainment
588 temperature on meat blooming and colour on display. *Meat Sci, 52*(1), 47-56.

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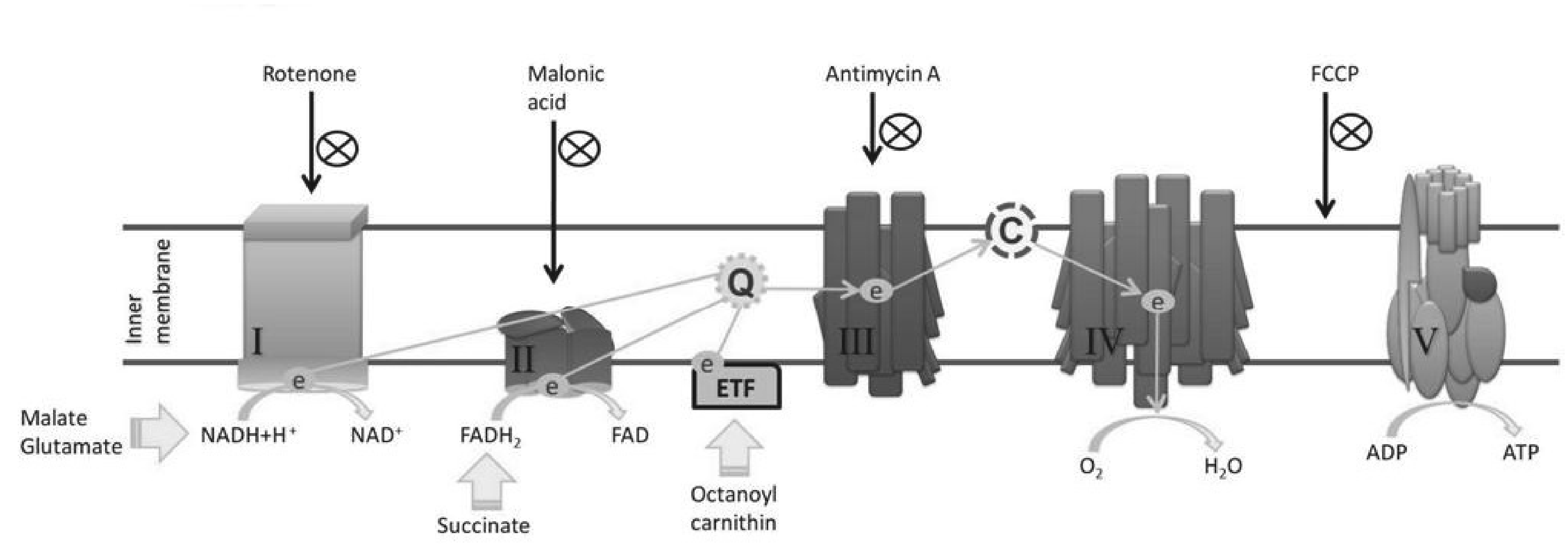
Figure



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2 Fig. 1.

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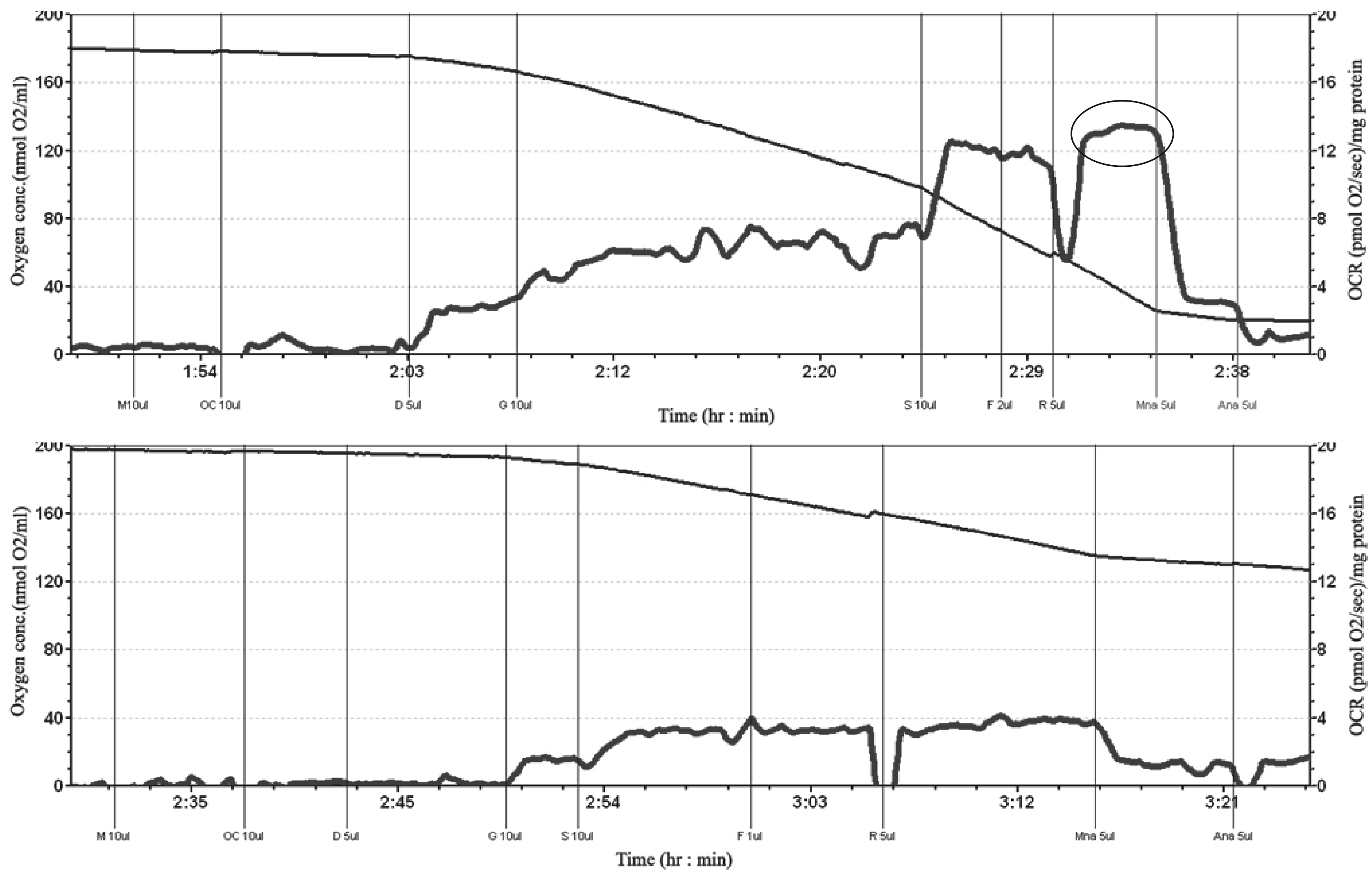
Figure



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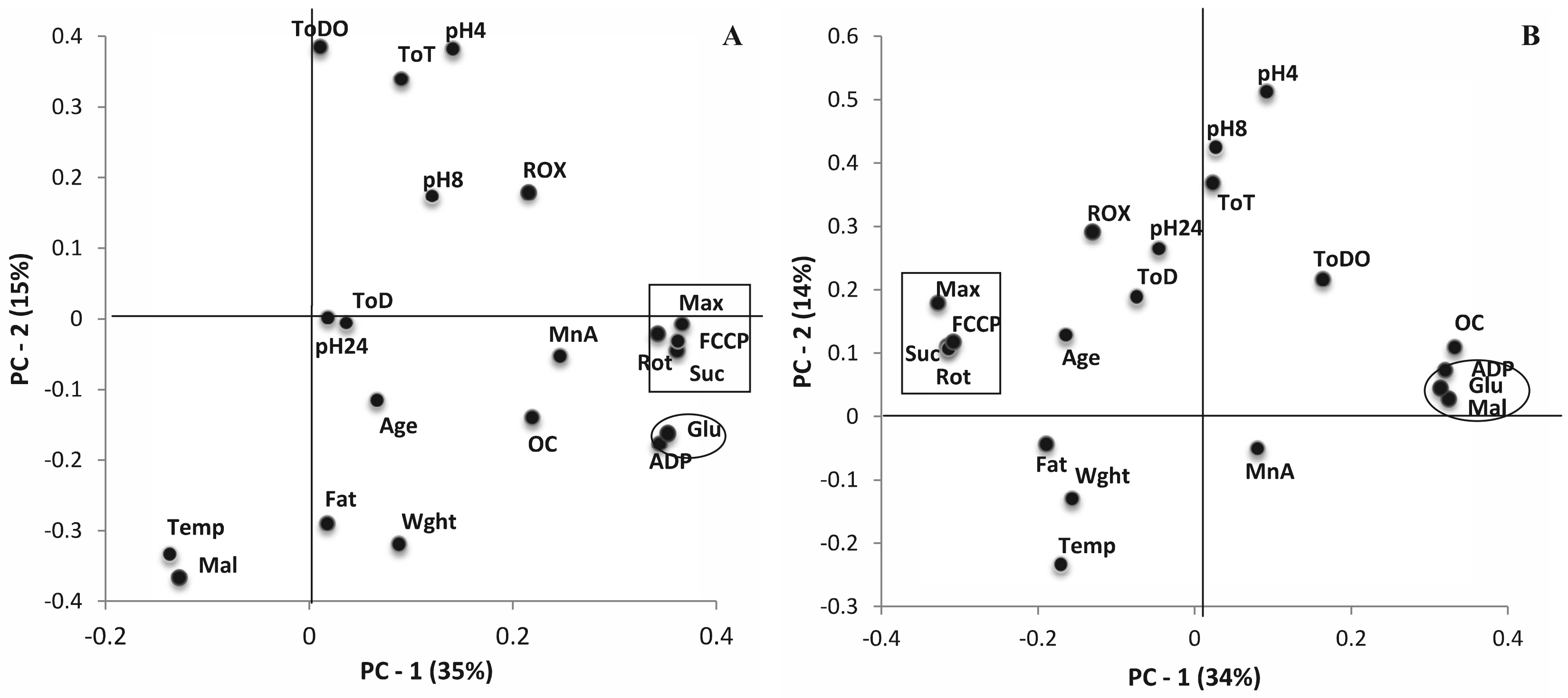

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2 **Fig. 3.**

Figure

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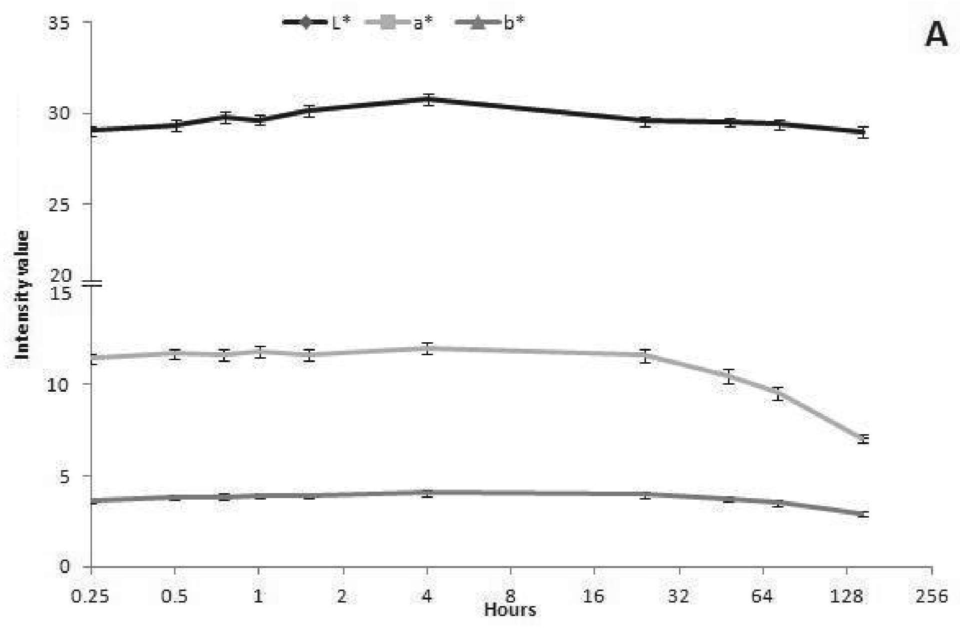

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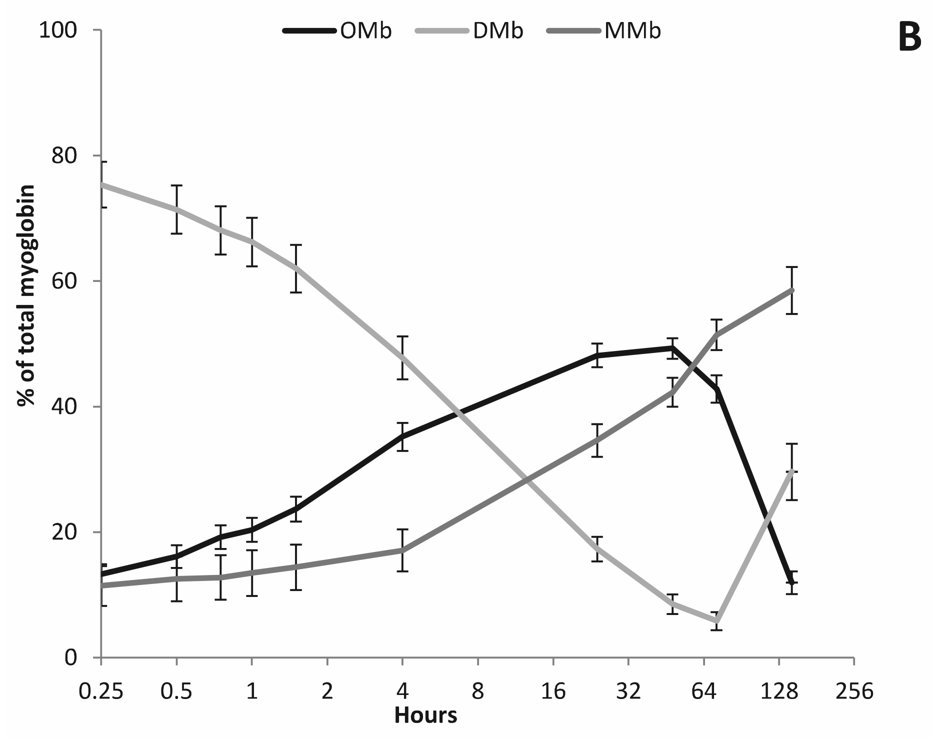

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4 **Fig. 4.**

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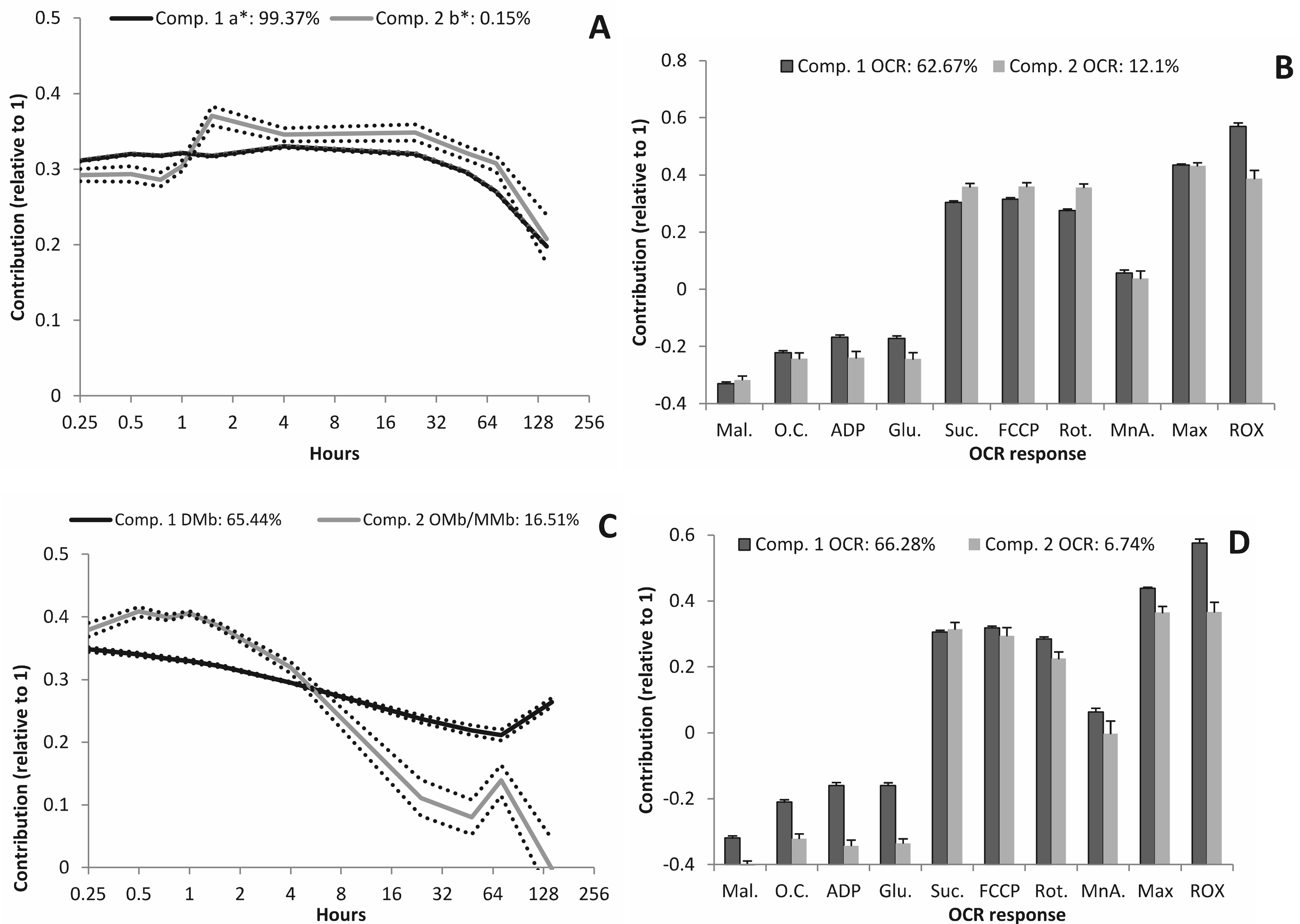

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3 **Fig. 5.**

Figure

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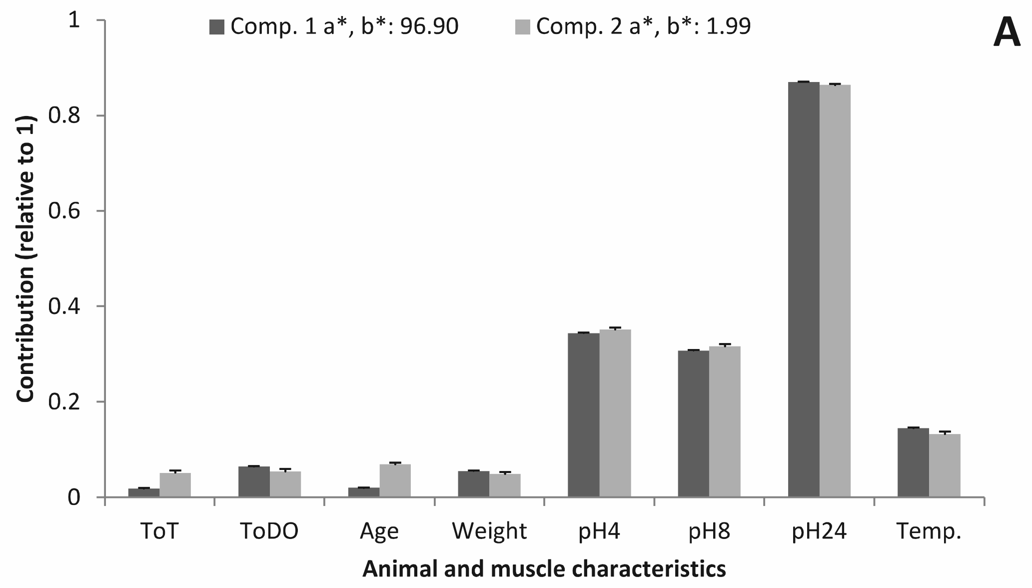

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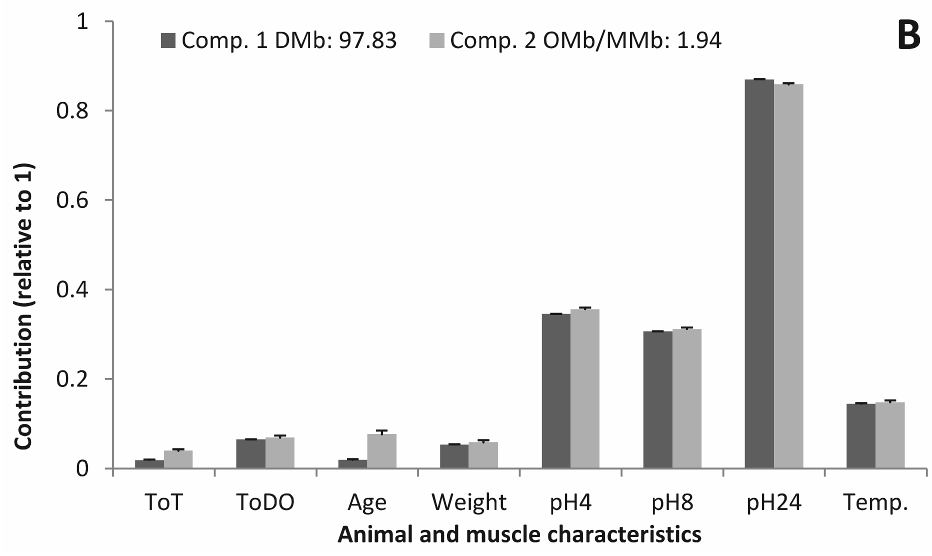

4
5
6 **Fig. 6.**

Figure

Figure text

Figure 1.

Overview of the electron transport system 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.

Figure 2.

Real-time measurements of oxygen consumption of permeabilized *M. semimembranosus* fibers. The panels show a run of the OCR protocol for one sample of fresh (A) and one sample of stored muscle (B). The thin line represents oxygen concentration (left y-axis) and the thick line is oxygen consumption rate (OCR, right y-axis). The X-axis denotes running time from start (instrument switched on) to end of the experiment where each action is indicated with a vertical line and a number. Numbers at the bottom indicate substrate addition sequence where 1: malate, 2: octanoylcarnithin, 3: ADP, 4: glutamate, 5: succinate, 6: FCCP, 7: rotenone, 8: malonic acid, 9: antimycin A. Frequency of chemical addition is dependent on the time it takes for the OCR to reach stability. The oval indicates a stable period for estimation of OCR after addition of rotenone.

Figure 3.

Score plot from principal component analysis (PCA) using weighted (1/st.dev.) animal variables and OCR 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 responses that were the most stable *post mortem*; the circle denotes responses that were least stable *post mortem*. The first two principal components (PC) shown gave explained variation ~50%.

Figure 4.

Time (abscissa) from exposure to air (hours) are given on a multiplicative scale. Average patterns of change with time for (A) L*, a* and b* where L* is scaled between 20-35 and a*, b* are between 0-15, and (B) show myoglobin states in % total myoglobin. Samples were vacuum-packed and chill-stored at 4°C for three weeks and re-packaged in oxygen permeable film when

measurements started. For further details see materials and methods section. Sample 37 was removed as an outlier in analysis of a* and b*. Error bars indicate standard errors.

Figure 5. Results of NPLS regressions with color variables in X block and OCR variables in Y block: color and OCR were measured on *M. semimembranosus* exposed to oxygen permeable film after 3 weeks of storage in oxygen impermeable film. Panels on the left side (A and C) show the time development as samples are exposed to air (log^2 scale), while panels on the right side (B and D) show the relative contributions of each OCR responses in the color development. Dotted lines and error bars give the standard errors while the displayed percentages are the explained variances of the X and Y block per component. The data in the X blocks have dimensions {a*/b* x time x animal} in the upper Panels (A and B), and {% myoglobin x time x animal} in the lower Panels (C and D). In the upper model the major colour change is seen in a* (comp. 1 is dominated by a* while comp. 2 is dominated by b*). For the lower model the major change is seen in DMb (comp.1 is dominated by DMb while comp. 2 is dominated by (OMb/MMb).

Figure 6. Results of two NPLS regressions with color variables in the X block (panel A and B) and animal and muscle characteristics in Y block: color changes were measured in oxygen permeable film on *M. semimembranosus* after 3 weeks of storage in oxygen impermeable film. Panel A was obtained with the matrix (a*/b* x time x animal) in X block and Panel B with the matrix (% myoglobin x time x animal) in X-block. Both panels show relative contributions of standardized (1/st.dev.) animal and muscle characteristics of the Y block to data in X block. Error bars give the standard errors. The colour components of the model were similar to the ones in Figure 5A and 5C.

.

1 **Table 1**
2 Abbreviations and final concentrations of substrates and inhibitors used during OCR measurements. Measurements were in respiration chambers with 2.1ml
3 volumes at 37°C adjusted to pH 7.1. (See also Fig 1 and 2)

Seq.	Chemical	Symbol	Function	Location	Titration volume [µL]	Final conc. [mM]
1	Malate	OCR_{Mal}	Substrate	Complex I	10	4.00
2	Octanoyl carnithine	OCR_{OC}	β-oxidation	ETF*	10	0.50
3	ADP	OCR_{ADP}	Oxidative phosphorylation	Complex V	5	1.25
4	Glutamate	OCR_{Glu}	Substrate	Complex I	10	10.0
5	Succinate	OCR_{Suc}	Substrate	Complex II	10	5.00
6	FCCP	OCR_{FCCP}	Uncoupler	Complex V	1-3 (incremental)	$0.5\text{-}1.5 \times 10^{-3}$
7	Rotenone	OCR_{Rot}	Inhibitor	Complex I	5	2.5×10^{-3}
8	Malonic acid	OCR_{Mna}	Inhibitor	Complex II	5	5 .00
9	Antimycin A	OCR_{Rox}	Inhibitor	Complex III	5	12.5×10^{-3}

4 *ETF: electron transferring flavoprotein.

Table

Table 2

Individual animal and muscle characteristics describing the 41 samples collected on the slaughter line.

Abbreviation	Definition	Mean±SD	Min-max
ToD (hr)	Time of arrival at abattoir until death	5.52±3.40	0.42-24.23
ToC (hr)	Time of arrival at abattoir until cut	23.08±7.00	1.50-25.23
ToT (hr)	Time of transport from farm to abattoir	2.58±1.50	0.30-7.00
ToDO (hr)	Time from death until OCR measurement	4.22±0.14	3.00-6.39
pH4	pH 4 hours *post mortem*	6.38±0.26	5.82-6.91
pH8	pH 8 hours *post mortem*	6.11±0.28	5.53-6.66
pH24	pH 24 hours *post mortem*	5.55±0.09	5.41-5.79
Temp. surface (^{o}C)	Meat surface temperature immediately after cut	31.47±3.07	24.80-40.50
Age (months)	Animal age	34.44±23.13	14.00-116.00
Weight (kg)	Animal weight	285.2±74.80	136.40-516.30

Table 3

Individual p-values (between animal differences) between samples measured for L*, a*, b* and myoglobin states. Values are based on measurements 15min after package opening.

Variable	**L***	**a***	**b***	**DMb (%)**	**OMb (%)**	**MMb (%)**
$\hat{\sigma}_A^2$	1.85	3.10	0.73	0.054	0.010	0.041
$\hat{\sigma}_e^2$	2.36	0.71	0.12	0.0006	0.0004	0.0002
Min-Max	25.06-34.51	7.51-17.00	1.72-6.22	0.00-1.00	0.00-0.40	0.00-0.93
p-value	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

$\hat{\sigma}_A^2$: estimated variance due to animals; $\hat{\sigma}_e^2$: estimated variance due to noise

1
Table 4

2 OCR measurement in fresh and stored samples of *M. semimembranosus* fibres following addition of substrates or inhibitors. Fresh samples were taken from the
3 slaughter line and measured immediately (~4 hours after death). Stored samples were allowed to mature for three weeks at 4°C and were then measured. All
4 variables except for OCR_{Max} and OCR_{ROX} are mitochondrial substrates or inhibitors.

	Variable	**OCR_{Mal}**	**OCR_{OC}**	**OCR_{ADP}**	**OCR_{Glu}**	**OCR_{Suc}**	**OCR_{FCCP}**	**OCR_{Rot}**	**OCR_{Mna}**	**OCR_{Max}**	**OCR_{ROX}**
Fresh	Mean OCR ± st. dev (α)	0.32±0.78	1.34±0.97	2.78±1.83	2.72±1.88	4.50±2.91	4.41±2.83	4.25±3.24	1.04±0.08	5.50±3.02	1.09±0.37
	Var $\hat{\sigma}_A^2 : \hat{\sigma}_e^2$(*)	0.53[a] : 0.08	0.58 : 0.31	2.50 : 0.90	2.80 : 0.77	7.01 : 1.57	6.48 : 1.59	8.95 : 1.64	0.36 : 0.12	7.47 : 1.72	0.10 : 0.05
	Min-Max (pmol O_2/sec)/mg protein	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
Stored	Mean OCR ± st. dev (α)	0.01±0.02	0.06±0.04	0.09±0.03	0.08±0.03	2.56±1.82	2.30±1.57	2.49±1.87	0.05±0.03	3.38±1.67	1.07±0.44
	Var $\hat{\sigma}_A^2 : \hat{\sigma}_e^2$	0.16 : 0.10	0.23 : 0.07	0.31 : 0.09	0.24 : 0.09	2.89 : 0.46	2.19 : 0.30	3.31 : 0.23	0.01 : 0.05	2.50 : 0.34	0.12 : 0.07
	Min-Max (pmol O_2/sec)/mg protein	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
ΔOCR	% ΔOCR**	(-3 %)	(-51%)	(-59%)	(-61%)	(-13%)	(-20%)	(-11%)	(-72%)	(-22%)	(+0.02%)
	Var $\hat{\sigma}_A^2 : \hat{\sigma}_e^2$	0.18 : 0.18	0.54 : 0.42	2.21 : 0.98	2.81 : 0.84	10.46 : 1.61	9.10 : 1.55	13.63 : 1.46	0.39 : 0.13	9.78 : 1.68	0.10 : 0.11

5 Var = variance; (*): $\hat{\sigma}_A^2$: estimated variance due to animals; $\hat{\sigma}_e^2$: estimated variance due to noise; Differences between OCR of fresh and stored muscles were
6 all significant ($p < 0.001$), except for OCR_{ROX}.α: (pmol O_2/sec)/mg protein; st. dev: standard deviation; O.C.: octanoylcarnitine; Mna: malonic acid; OCR_{ROX}:
7 oxygen-consuming side reactions; OCR_{Max}: max OCR including ROX background. **Percentage reduction in absolute value. [a] All animal effects were
8 significant ($p \leq 0.001$) except for the Mna response of stored muscle ($p = 0.27$).

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10

1 **Table 5**

2 Estimated regression coefficients between different OCR(pmol O_2/sec)/mg protein) measured approx. 4 hrs[a] and 3 weeks[b] *post mortem* individual animal and
3 muscle characteristics of 41 samples (stepwise regression). Remaining characteristics that are not listed below, were not significant.

OCR reponse	Weight ($\alpha \times kg)^x$	Age (α × months)	Temp. surface ($\alpha \times {}^oC$)	ToT (α × time)	ToDO (α × time)	pH8 (α × pH)	% expl. var.
$^aOCR_{Mal}$	n.s.	n.s.	0.084*	n.s.	n.s.	n.s.	42.4
$^bOCR_{Mal}$	-0.0028**	-0.0061*	n.s.	n.s.	n.s.	n.s.	43.7
$^aOCR_{OC}$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	21.2
$^bOCR_{OC}$	-0.0025*	-0.0059*	n.s.	n.s.	n.s.	n.s.	48.8
$^aOCR_{ADP}$	0.0097**	n.s.	-0.245**	n.s.	-0.59*	n.s.	44.0
$^bOCR_{ADP}$	-0.0028	-0.0085*	n.s.	n.s.	0.24**	n.s.	46.7
$^aOCR_{Glu}$	0.0105**	n.s.	-0.284**	n.s.	-0.58*	n.s.	47.6
$^bOCR_{Glu}$	n.s.	-0.007*	n.s.	-0.098*	n.s.	n.s.	44.4
$^aOCR_{Suc}$	0.0156**	n.s.	-0.39**	n.s.	n.s.	n.s.	52.4
$^bOCR_{Suc}$	n.s.	0.029**	n.s.	n.s.	-0.74**	n.s.	51.0
$^aOCR_{FCCP}$	0.0149**	n.s.	0.37**	n.s.	n.s.	n.s.	52.9
$^bOCR_{FCCP}$	n.s.	0.019*	n.s.	n.s.	-0.69**	n.s.	48.2
$^aOCR_{Rot}$	0.019**	n.s.	-0.43**	n.s.	n.s.	3.8*	53.9
$^bOCR_{Rot}$	n.s.	0.026*	n.s.	n.s.	-0.77**	n.s.	50.1
$^aOCR_{Mna}$	n.s.	n.s.	-0.09**	n.s.	n.s.	n.s.	35.3
$^bOCR_{Mna}$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	21.1
$^aOCR_{Max}$	0.0162**	n.s.	-0.42**	n.s.	n.s.	n.s.	54.6
$^bOCR_{Max}$	n.s.	0.024*	n.s.	n.s.	-0.59*	n.s.	54.8
$^aOCR_{ROX}$	0.00142*	n.s.	-0.043**	n.s.	n.s.	n.s.	42.0
$^bOCR_{ROX}$	0.0017*	n.s.	n.s.	0.094**	n.s.	n.s.	47.2

4 [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. Temp.surface: meat surface
5 temperature immediately after cut, ToT: time of transport from farm to abattoir, ToDO: time of death until OCR measurement, pH8: pH 8 hours after death, expl.
6 var: explained variance.
7

Paper IV

*Manuscript
Click here to view linked References

1 **Color of ground beef as affected by mitochondrial substrate respiration and exposure to**
2 **oxygen**

3 Phung[a,]*, V. T., Bjelanovic, M., Langsrud[c], O., Slinde[a,b], E., Sørheim[d], O., Isaksson[a], T., Egelandsdal[a],
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23

24 **Abstract**

25 Bovine *M. semimembranosus* of different age groups comminuted with porcine or bovine fat
26 were added an array of mitochondrial substrates (glutamate, malate, succinate, pyruvate and
27 citrate) giving 156 samples. A pure deoxymyoglobin state was maintained for 13 days in low
28 oxygen packaging by a mixture of succinate combined with glutamate and/or malate (molar
29 ratio 1:1). In high oxygen atmosphere the highest predicted level of oxymyoglobin (98%) was
30 obtained by glutamate-malate to citrate equal to 3:1 after 3 days of packaging (total amounts
31 0.05 or 0.1 mol/kg). Pyruvate did not improve color relative to controls; the compound was a
32 prooxidant at both oxygen levels. No significant difference between the concentrations 0.05
33 mol/kg or 0.1 mol/kg of additives on color was found. Complex II of the mitochondrial
34 electron transport system worked best in conversion of metmyoglobin to deoxymyoglobin in
35 low oxygen packaging. In high oxygen the mechanism may also involve complex I.

36

37 **Introduction**

38 The consumer prefers red meat as it looks better and is associated with fresh rather than
39 stored meat. An attractive red color in meat is difficult to maintain in *post mortem* muscle
40 independent of the composition of head space gases. The shelf life of meat during display is
41 often limited by defects in color.

42 Muscle is purple under anaerobic conditions due to a high content of deoxymyoglobin
43 (DMb). Myoglobin in meat turns transiently red upon exposure to oxygen and becomes
44 oxymyoglobin (OMb). Finally, the muscle converts to a brown color as a result of oxidation
45 and metmyoglobin (MMb) formation by ageing. Processing, storage condition (Phung,
46 Saelid, Egelandsdal, Volden, & Slinde, 2011) and post-storage treatment also affects the
47 color of meat depending on the intactness of the myoglobin reducing systems such as
48 mitochondria and MMb reductase activity (MRA) (Ledward, 1985; Madhavi & Carpenter,
49 1993; Slinde, Phung, & Egelandsdal, 2011). A high oxygen content in packages may
50 increases the formation of MMb (Sorheim, Westad, Larsen, & Alvseike, 2009).

51 Packaging in high oxygen atmosphere may give an initial red, but unstable color and yields
52 undesirable effects on taste and lipid oxidation, tenderness and bacterial growth. As reported
53 by Taylor and MacDougall (1973) a starting concentration of 80% O_2 can maintain sufficient
54 red color of fresh steaks for one week. Prolonged storage needs, however, anaerobic
55 conditions to avoid oxidation. For ground beef, due to disruption of tissue, a large surface
56 area and incorporation of oxygen, results in a reduced color stability compared to steaks.
57 Removal of oxygen (Tewari, Jayas, & Holley, 1999) can be done with either CO_2, N_2 or a
58 combination of both and will yield a better color stability and extended shelf life. However,
59 packaging in low oxygen may still result in abundant surface MMb caused by residual O_2.
60 Ground beef with a purple color consisting predominantly of DMb could be obtained by
61 storage in atmospheres with less than 0.1 % O_2 for more than 48 hours (Sorheim, et al.,
62 2009). It is therefore appropriate to reduce residual oxygen quickly and completely in order
63 to avoid accumulation of MMb, and create a MMb reducing environment. Another approach
64 for removing oxygen, especially in situations where the packaging film is oxygen permeable,
65 is the use of oxygen scavenger sachets. The sachets are often iron-based oxygen absorbers,
66 which become oxidized in the presence of oxygen and are sealed inside the meat package
67 (Beggan, Allen, & Butler, 2006; Isdell, Allen, Doherty, & Butler, 1999).

68 Absence of oxygen prevents discoloration, but does not activate formation of the attractive
69 bright red color of OMb. Myoglobin needs to be maintained in a reduced state, and
70 mitochondria function both as a reductant and oxygen scavenger (Slinde, et al., 2011). It has
71 been assumed that myoglobin is reduced at the mitochondrial outer membrane where
72 electrons are accepted (Arihara, Cassens, Greaser, Luchansky, & Mozdziak, 1995; Hagler,
73 Coppes, & Herman, 1979; Livingston, McLachlan, La Mar, & Brown, 1985) through
74 diffusion (Wittenberg, 1970; Wittenberg & Wittenberg, 2007), or through a hitherto unknown
75 carrier. For this reason, additives that are oxidized naturally by mitochondria were tested
76 (Phung, et al., 2011). These additives have been shown to increase the rate of MMb reduction
77 through production of reduced nicotinamide adenine dinucleotide (NADH) and flavin
78 adenine dinucleotide (FADH) (Saleh & Watts, 1968). The additives are oxidized by the
79 electron transport system (ETS) at complex I and complex II at the inner mitochondrial
80 membrane, respectively.

81 Glutamate and malate are two additives that are commonly used as flavor enhancers. In the
82 current system, glutamate and malate were used to generate NADH. Succinate has been
83 reported to decrease lipid oxidation (Puntel, et al., 2007) and MMb formation (Tang,
84 Faustman, Mancini, Seyfert, & Hunt, 2005). Pyruvate and citrate have also been tested since
85 they occur naturally as products in glycolysis and tricarboxylic acid (TCA) cycle. Pyruvate
86 was shown by Mancini, Ramanathan and Dady (2011) to reduce MMb, while citrate is
87 commonly used as a buffer and metal ion chelator, especially for iron, to prevent enzymatic
88 oxidation and color degradation. Furthermore, citrate when used in combination with
89 ascorbate was shown to be an effective antioxidant and color protectant (Lund, Hviid, &
90 Skibsted, 2007).

91 The aim of this study was to investigate the effect on color of ground beef after stimulation of
92 the mitochondrial respiration using succinate, glutamate, malate, puruvate and citrate in the
93 presence or absence of oxygen. Color was measured as L*, a*, b* values and myoglobin
94 states. The concentration and combination of additives were investigated together with
95 packaging in low and high oxygen in search for combinations of additives that could utilize
96 mitochondria to maintain myoglobin either as DMb or OMb, and keep these states longer
97 than a reference with no additives.

98

99

100 ## 2. Materials and methods

101 ### 2.1. *Chemicals*

102 The following chemicals were purchased from Alfa Aesar GmbH & Co KG (Karlsruhe,
103 Germany): Sodium salts of succinate hexa-hydrate, succinic acid, and pyruvic acid. These
104 chemicals were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany):
105 Sodium salts of pyruvate, glutamic acid, malic acid and DL-malic acid disodium salts.
106 Sodium salts of hydrogen glutamate monohydrate were purchased from VWR International
107 BVBA (Leuven, Belgium). Sodium salts of citric acid monohydrate and trisodium citrate
108 were purchased from Merck KGaA (Darmstadt, Germany). All chemicals were of analytical
109 grade.
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111 ### *2.2 Animal tissues*

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Beef *M. semimembranosus* (M.S.) and beef fat tissue were collected four days *post mortem* (Fatland A/S, Oslo, Norway). The packaging day is defined as day zero, however, the tissues were 4 days *post mortem* on packing day. A piece of meat from proximal end was cut from each muscle in order to measure oxygen consumption, pH and surface color. Fat tissue from pigs fed rapeseed and vitamin E to enhance the content of polyunsaturated fatty acids were from HKScan (Ruokatalo, Finland). The fat tissue was transported vacuum-packaged and frozen. Samples of minced fat tissue were removed for fatty acid analysis.

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2.3. *Preparation of ground beef*

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The beef muscles were cut into pieces of approximately 10 x 5 cm and mixed to 14% w/w fat by using the fat tissues. Beef fat and pork fat tissues were ground twice with either raw beef from four young (16-19 months) or four old (46-81 months) cattle. The meat had pH of 5.65 and was ground with a Seydelmann ME-130 (Seydelmann, Stuttgart, Germany) grinder through a plate with 3 mm openings. The ground meat was blended manually with solutions (all at pH 5.8) with various TCA and glycolytic ingredients (see below).

127 The experiment was designed using four combinations of raw materials: young beef and
128 bovine fat (experiment 1); young beef and pork fat (experiment 2); old beef and bovine fat
129 (experiment 3); old beef and pork fat (experiment 4).

130

131 *2.4. Modified atmosphere packaging (MAP) and storage*

132 The ground beef was packaged in black amorphous polyethylene terephthalate (APET) trays
133 that were sealed with ethylene vinyl alcohol (EVOH) top films. The trays and films (Wipak
134 Multipet and Wipak Biaxer, both Wipak, Nastola, Finland) had oxygen transmission rates of
135 7 and 5 $cm^3/m^2/24$ h at 23 °C at 50 % relative humidity. Packaging was carried out within 1
136 hour after grinding. The samples were stored in darkness at 4°C for 8 days in a high-oxygen
137 atmosphere (75% oxygen and 25% CO_2) and for 13 days in a low-oxygen atmosphere (60%
138 CO_2 and 40% N_2). These food grade gas mixtures were supplied by AGA (Oslo, Norway).

139 A tray sealing machine was used for the packaging (Promens 511VG, Kristiansand, Norway).
140 Measurements of the CO_2 and O_2 contents were carried out with a CheckMate 9900 (PBI
141 Dansensor, Ringsted, Denmark) by inserting a needle through self-adhesive and self-sealing
142 rubber septas (Toray TO 125, Toray Engineering, Osaka, Japan). Gas measurements were
143 performed at days 0 (packaging day) and 13 for low oxygen packages and days 0, 1, 3, 6 and
144 8 for high oxygen packages. The gas to meat ratio was approximately 2:1.

145

146 *2.3 Preparation of solutions*

147

148 The solutions were prepared using succinate, pyruvate, glutamate, malate and citrate in
149 different combinations, either as the pure sodium-based/acid based chemicals or as mixtures
150 of 2, 3, 4 and 5 chemicals of varying concentrations. To maintain pH at 5.8 the solutions were
151 prepared by mixing the acid form and the corresponding sodium salt of these chemicals.

152 40 g of 0.1 M or 0.05 M solutions were added to ground beef-fat mixture (360g) to a total
153 weight of 400g. The solution was quickly and properly stirred into the minced meat and the
154 blended system was subsequently packaged. The final diluted concentration was either 0.1 or
155 0.05 mol/kg. Two reference samples with 40 ml of distilled water added to 360 gram of

156
157 ground beef were also prepared each of the four production day, *i.e*. altogether 8 references were prepared. The samples were packaged in either low or high oxygen atmosphere.

158

159
2.6. Fatty acid composition

160 The fatty acid composition of the selected fat tissues was determined as methyl esters,
161 analyzed by GC-MS (7890A GC, Agilent Technologies, Palo Alto, USA). Transesterification
162 of lipids to fatty acid methyl esters (FAME) were performed, as described by Devle et al.
163 (2009) with minor modifications. Briefly 0.01 g fat was dissolved in 2.0 ml hexane and added
164 1.5 ml of 3.33 mg/ml sodium methanolate solution, placed on a shaker for 30 min, before the
165 samples were left to settle for 10 min. Two hundred µl of the top layer were transferred to a
166 new vial. Fatty acid analysis was performed by autoinjection of 1 µL of each sample at a split
167 ratio of 80/1, constant flow mode, velocity 20.4 cm/sec. The data were collected as area
168 percentages. Two replicates were taken.

169 To identify FAMEs, their retention times were compared to those of a known 37-component
170 standard FAME mix, and the mass spectra were compared with spectra available from the
171 NIST (National Institute of Standards and Technology) database.

172

173 ### 2*.7. Oxygraph measurements*

174 Measurements of oxygen consumption rates were carried out at pH 7.1 on permeabilized
175 muscle fibers. The fibers were removed from the meat and were 3-5mm in length (Phung, et
176 al., 2011). Oxygen consumption rate (OCR) measurements were done at day 0 (i.e. four days
177 *post mortem*) and on the last day of the experiment. Chemicals were always added in the
178 following sequence and the final concentrations were 4.0 mM malate, 5.0 mM pyruvate, 10.0
179 mM glutamate, 1.25 mM ADP, 0.50 mM octanoylcarnitine, 5.0 mM succinate, 0.5-1.5 µM
180 carbonyl cyanide-p-triflouromethoxy-phenylhydrazone (FCCP), 2.5 µM rotenone, 5.0 mM
181 malonic acid and 2.5 µM antimycin A. High-resolution respirometry was carried out with
182 Oroboros Oxygraph-2K instruments (Oroboros Instruments, Innsbruck, Austria) as described
183 by Gnaiger (2001). Background oxygen consumption after antimycin A inhibition (Gnaiger,
184 2008) was recognized as residual oxygen consuming side reactions (ROX).

185 Four measurements were made on each batch. One measurement on the packaging day failed,
186 however, and was linearly extrapolated back to zero time from consecutive measurements.
187 Complex I activity was calculated as the summation of responses elicited by the
188 corresponding substrates (glutamate, malate and pyruvate). Complex II activity was measured
189 as the response toward succinate. The response toward β-oxidation was mediated by both
190 malate and octanoylcarnitine (Lumeng, Bremer, & Davis, 1976). Inhibition of the ETS was
191 carried out in sequence and started with rotenone (inhibition of complex I), malonic acid
192 (inhibition of complex II) and antimycin A (inhibition of complex III).

193

194 *2.8. Instrumental color analysis (L*, a*, b*)*

195 The color of the ground meat samples was measured with a Konica Minolta Chroma Meter
196 CR 410 (Konica Minolta Sensing Inc., Osaka, Japan) using the glass light projection tube CR
197 A 33e with wide illumination area (61mm) and illuminant D65. The instrument was
198 calibrated each day by measuring a white ceramic plate (L*= 97.9; a *=0.05; b*=2). All
199 color measurements were made with 3 replicates (CIE, 1976).

200

201 *2.9. Reflectance spectra*

202

203 Samples were scanned, 400 – 100nm, with a Foss NIRSystems OptiProbe ™ 6500 Analyzer
204 (Foss NIRSystems Inc., Maryland, USA) without opening of the package. The package was
205 turned upside-down before measurements so that the meat would fall on the packaging film.
206 The samples were scanned with three random placements of the probe on the package surface
207 in order to increase the scanning area and reduce the measurement errors. Thirty -two scans
208 for both reference and samples were acquired and averaged on each measured area. All
209 measurements were performed at room temperature (approx. 20°C) according to (Khatri, et
210 al., 2012). These spectra were used to predict OMb, MMb and DMb according to a principle
211 that has been reported by (Khatri, et al., 2012). It should be noted that a new calibration using
212 comminuted meat with 10 % w/w added water was established (Bjelanovic, M., personal
213 communication).

214 Color of the samples packaged in high O_2 was measured after 0, 1, 3, 6 and 8, days of storage
215 from packaging day, while low O_2 samples were measured at 0 to 13 days of storage using
216 Minolta and reflectance measurements.

217

218 *2.10. Measurements of myoglobin content*

219 Slices of ground beef were excised in frozen state and thawed in 40 mM pH 6.8 sodium
220 phosphate buffer with a weight to volume (gram to milliliter) ratio of 1:10 and mixed using a
221 food processor (HR 1364 600W, Philips, Netherland). After centrifugation the supernatant
222 was measured (440 – 650 nm) with a Shimadzu UV-1800 spectrophotometer (Shimadzu
223 Europa GmbH, Duisburg, Germany). The total myoglobin concentration was calculated
224 according to Faustman and Phillips (2001).

225

226 *2.11. Experimental design and statistical analysis*

227 The experimental design is visualized in Fig. 1. A 2^2 design (animal age and type of fat
228 tissue) was split over 4 experimental days (Fig. 1, Table 1 and 2). This factorial design was
229 originally planned as 4 separate days with the possible intention of not carrying out all four
230 days/ corners of the design. To each corner of this 2^2 design was added a 3 component
231 (succinate, pyruvate and glutamate-malate additions; 0, 50 and 100 %) simplex lattice
232 mixture design with 2 additional centre experiments as shown in Fig. 1 and Table 1 & 2.
233 Each of these eight experimental points had 4-factors (glutamate-malate ratio, total added
234 mixture levels, citrate concentrations, oxygen concentrations) at 2-levels (Fig. 1); *i.e.* a 2^4
235 design. It will be realized below that despite oxygen being a design variable when the
236 experiment was planned, the nature of the data required the observations to be split into low
237 and high oxygen for modeling.
238 The eight experimental points of the mixture design were, upon fractionation of the design
239 (see below), then considered to be the corners of a cube. To do this, the 8-point mixture
240 design was recorded as a 2^3experiment by introducing the three fictitious mixture variables as
241 factorial design variables.
242

243 **Figure 1**

244 **Table 1**

245 **Table 2**

246

247 *Reduction of the design*: The design was regarded as a 2^9 design (512 samples). In order to
248 increase the feasibility of the design, a quarter fraction of the 2^9 design with 128 experiments
249 (32 each day, 4 days total) was constructed by utilizing the theory of fractional factorial for
250 two-level designs. The actual design was chosen from standard designs by considering three
251 aspects; 1) an optimal confounding pattern for the fictitious mixture variables, 2) Good
252 individual designs at each single day, 3) alternative models based on the original variables
253 should behave well according to classical optimal design criteria (D-optimality)(Berger &
254 Wong, 2009). To optimize confounding the mixture points differed for each quarter fraction.
255 The 128 samples represented a balanced design. However, 7 extra samples were added every
256 day. Four replicates (corners and center) were distributed manually in a way that minimized
257 the imbalance caused by adding such replicates. Finally, 3 corner samples consisting of
258 minces with additions of pure glutamate, pure malate or pure citrate solutions were prepared.
259 In addition 2 samples were produced only with water added after each day. The detailed
260 design is sample for sample available as supplementary material.

261 The statistical analysis was performed according to the approach described by Langsrud et al
262 (2007) and implemented in the 50-50 MANOVA software
263 (http://www.langsrud.com/stat/program.htm). This program handles mixture designs as
264 generalized categorical variables (generalized ANOVA) and Egelandsdal et al (2005) is an
265 example of a food science application. When using this method one can always formulate
266 equivalent models that use the individual mixture variables as ordinary (one degree of
267 freedom) regression variables. Then one arbitrary mixture variable has to be omitted (slack
268 variable). Important outputs from the analysis are significance results (p-values), explained
269 variances (based on sums of squares) and adjusted mean values (or mean predictions).
270 Adjusted mean values can be calculated over the whole mixture region and the result can be
271 presented as surfaces.

272 The analysis was based on a model (responses were: a*, b*, myoglobin states) that included
273 the terms given in Table 3. Where “mixture” was the linear effect of the 3-
274 component mixture design in the variables succinate, pyruvate and glutamate-malate,
275 “mixture ^{2}“ was the quadratic effect of the 3-component mixture design, “mixture × age” was

276 the second order interaction term between the 3-component mixture design and age and so on
277 (Table 3). This relatively complicated model was chosen to ensure that the model is useful for
278 all response variables. Since there were as many as 40 degrees of freedom for error and since
279 the analysis were based on a hierarchical approach further model reduction was not needed.

280 **Table 3**

281

282 **3. Results**

283

284 *3.1. Fatty acid composition of fat tissue*

285 *M. semimembranosus* from old (46-81 months) animals had nominally 29% more myoglobin
286 than the young (16-19 months) animals. The difference was, however, not statistically
287 significant since the two muscles from the young group differed much. The fatty acid
288 composition of beef and pork fat tissue differed (Table 4). Porcine fat tissue contained 10
289 times more polyunsaturated fatty acids (PUFA) plus more monounsaturated fatty acids
290 (MUFA) than the beef fat tissue, which in turn was richer in saturated fatty acids (SFA)
291 (Table 4).

292

293 **Table 4**

294

295 *3.2.Oxygen consumption rate (OCR) of the muscles used*

296

297 As shown in Table 5 the mitochondrial substrates that stimulated complex I, were glutamate,
298 malate and pyruvate. Oxidation of succinate led to stimulation of complex II via production
299 of $FADH_2$. β-oxidation of octanoylcarnitine produced reducing equivalents that stimulated
300 both complexes I and II. Complex I was subsequently inactivated by rotenone, and complex
301 II by malonic acid. Complete inhibition of oxygen consumption by the ETS was achieved
302 using antimycin A to inactivate complex III, with ROX remaining as the sole oxygen
303 consuming reaction at this point. For all muscle systems, the mitochondrial respiration
304 (measured as oxygen consumption rate) in the presence of succinate (complex II) had higher

305 activity than respiration in the presence of glutamate-malate (complex I, Table 5). For young
306 animals the response to β-oxidation (octanoylcarnitine) was 60-67 % of the response of
307 complex II response while the response to β-oxidation for old animals was only 10-21 % of
308 the response, to complex II (Table 5). These differences were significant ($p<0.003$).The
309 muscle raw material showed the expected dominant activity of complex II (Phung, et al.,
310 2012; Phung, et al., 2011). The mean (both ages averaged) nominal reduction for complex I
311 activity was 48 % over 13 days of chill storage. The mean, nominal reduction (both ages
312 averaged) in β-oxidation was 58 % over the same 13 days. The mean nominal value for
313 respiration on complex II and I was higher for young than for old animals. Phung et al. (2012;
314 2011) has indicated significant batch variations between muscles for respiration rate on
315 complex I and II. Large standard errors (Table 5) were obtained since the two muscles for the
316 young animals were quite different. However, all four muscle samples showed the expected
317 reduction in OCR with time and the larger oxygen consumption on complex II.

318 **Table 5**

319

320

321 3.3. *Mean change in L*, a*, and b* with time*

322 The overall redness (a*) in ground beef changed the most during the experiments and high
323 oxygen packaging yielded more redness than low oxygen packaging (Fig. 2A and B).
324 However, a* values in high oxygen was unstable and decreased steadily from start (Fig. 2B)
325 while the redness in low oxygen packaging had an increase from day 1 until day 6 and
326 stabilized at a plateau (Fig. 2A). Moreover, the ground beef was most yellow (b*) when
327 packaged in high compared to low oxygen (Fig. 2A and B). Surface darkening occurred due
328 to oxidation of myoglobin and occurred faster in high than in low oxygen packaging (Fig. 2A
329 and B).

330 Figure 2 shows a breakpoint on L* at day 6 and day 3 for low and high oxygen packaging,
331 respectively. At these breakpoints smell was detected due to bacterial growth (not shown),
332 which occurs earlier in high than in low oxygen packaging. Furthermore, according to
333 oxygen concentration measurements there was a sharp drop at day 6 in high oxygen packages
334 supporting the notion of bacterial growth (results not shown).

335 **Figure 2.**

336

337 *3.4. Changes in a*, and b* promoted by the mixture*

338

339 **Figure 3.**

340

341 The design variable mix containing succinate, pyruvate and/or glutamate-malate, influenced
342 color variable a* the most (explained variance = 54.1% in low oxygen) at day 3. The
343 response surface to different combinations of succinate, pyruvate and glutamate-malate can
344 be seen at each respective corner in Figure 3. As L* values did not change much and had
345 little influence on color (Fig. 2), a* and b* changes were the focus when packaged in low
346 (Fig. 3A and C) and high oxygen (Fig. 3B and D). Pyruvate did not improve redness (a*) at
347 any packaging condition (Fig. 3A and B). Moreover, pyruvate even seemed to decrease a*
348 and increase b* values in low oxygen packages (Fig. 3A and C). The response surface in low
349 oxygen packaging at day 3 shows succinate with glutamate-malate to have the highest a*
350 values while pyruvate had a negative effect on a* (Fig. 3A). Pyruvate promoted increased
351 yellowness (increasing b*) and had the opposite effect to succinate (Fig. 3C). It was apparent
352 that high oxygen lead to both high a* and b* values for glutamate-malate (Fig. 3B and D).
353 The direction of a* changed when the meat was packaged under high oxygen and revealed a
354 gradual shift from high a* values with glutamate-malate and pyruvate at day 0 to glutamate-
355 malate and succinate at day 8 (results not shown). In high oxygen packaging high b* values
356 was largely located at the glutamate-malate corner throughout the experiment (Fig. 3D).

357 Actual concentration of the chemical mixture (any composition) was not significant for
358 maintaining color ($p>0.05$). However, glutamate lead to nominally higher a* values and
359 malate to nominally higher b* values when the meat was packaged in low oxygen (not
360 shown).

361

362 3.5. *Mean change in myoglobin states with time*

363 **Figure 4.**

364

365 The change in the average percentage distribution of the different states of myoglobin with
366 time in low and high oxygen atmosphere is shown in Figure 4A and B, respectively. The
367 prevalence of OMb and MMb decreased over time in low oxygen packaging; OMb became
368 nearly depleted at day 6 (Fig. 4A). There was a peak in MMb at day 1 (in agreement with a*,
369 Fig. 3A), indicating that myoglobin converted from OMB to MMB to DMB in agreement
370 with (Mancini & Hunt, 2005). There were little change after day 6, indicating that the oxygen
371 in the packages became quickly depleted within the first 5-6 days (Fig. 4A). The prevalence
372 of DMb had the opposite trend to MMb and OMb and increased from start until day 10 when
373 it leveled off (Fig. 4A). On the other hand, under high oxygen environment there were almost
374 no DMb accumulation (<2%, Fig. 4B). OMb was high, but decreased throughout the
375 experimental time with a concomitant increase of MMb (Fig. 4B), indicating a direct turnover
376 from ferrous myoglobin with bound oxygen to ferric myoglobin or that a transition through
377 DMb was not rate limiting. More importantly, it was observed that the prevalence of MMb
378 increased together with a decreasing OMb when packaged in high oxygen (Fig. 4B), but the
379 MMb content did not increase in low oxygen packages (Fig. 4A).

380

381 *3.6. Changes in DMb, OMb and MMb promoted by the mixture*

382

383 The combination of succinate and glutamate-malate was largely responsible for accumulation
384 of DMB in low oxygen packaging (Fig. 5A). The response surface to DMb, OMb and MMb
385 did not change slope throughout the experimental time of 13 days (not shown) in low oxygen.
386 Moreover, pyruvate increased OMb and MMb formation (Fig. 5C and E). Under high oxygen
387 conditions, DMb generation was strongly supported by succinate plus a smaller concentration
388 of pyruvate (Fig. 5B). Glutamate-malate favored OMb formation, while pyruvate favored
389 MMb in high oxygen (Fig. 5D and F). The ability of glutamate-malate to maintain OMb
390 changed little with time (results not shown). At day 3 (and later days) pyruvate was a color
391 destabilizer under high oxygen conditions (Fig. 5F).

392

393 **Figure 5.**

394

395 *3.7. The effect of factorial (2-level) design variables on colour*

396

397 Adjusted mean effects of age, animal fat, citrate and oxygen on L*, a* and b* are shown in
398 Table 6, where age (after the mix factor) had the largest effect on L* values at all time
399 measurements in both low and high oxygen packaging. Young animals had the highest L*, a*
400 and b* at all times at both oxygen concentrations (Table 6). Fat tissue from pigs resulted in
401 larger L* and a* than beef fat at almost all time points at both oxygen concentrations.

402 The effect of citrate was small but differed between packaging in low and high oxygen. Most
403 important was the development of color after day 1 where a* started to increase until day 8
404 and stabilized in low oxygen packaging (Table 6).

405 The effect of age on myoglobin state was not significant ($P>0.05$), except for day 1 where
406 young animals had the highest prevalence of MMb and old animals had most DMb (Table 7).
407 In low oxygen packaging, young animals promoted the prevalence of MMb (Table 7).
408 Porcine fat gave the highest prevalence of DMb in all measurements and bovine fat the
409 highest of OMb in low oxygen measurements (Table 7). Furthermore, bovine fat had the
410 highest number of MMb from day 8 to 10 in low oxygen while porcine fat was shown to be
411 involved in MMb generation in high oxygen at day 6 and 8, presumably due to fatty acid
412 oxidation.

413 Addition of citrate in low oxygen packaging increased OMb and MMb while the DMb was
414 reduced. The MMb content was at its highest at day 1 (citrate added; Table 7). Under high
415 oxygen conditions, citrate led to nominal higher values for OMb (and nominally lower values
416 for MMb), but the effect was not significant (Table 7).

417

418 *3.8. Mixtures giving maximum and minimum in color variables*

419 Combinations that were found to give maximum and minimum in a* and b* values and
420 myoglobin states for the two packaging methods are shown in Table 8. Values are from day 3
421 and predictions were made on samples containing citrate and no citrate (Table 8).

422 The max a* values (9.7 ± 0.3) in low oxygen packaging was obtained with 41% succinate and
423 59% glutamate-malate (no citrate) and max b* (4.0 ± 0.1) was obtained with 78% pyruvate

424 and 22% glutamate-malate (Table 8 and Fig. 3).Samples in high oxygen packaging had the
425 highest a* and b* values with citrate and glutamate-malate of 13.3±0.2 and 4.9±0.1,
426 respectively (Table 8 and Fig. 3B and D).

427 The conditions that resulted in the highest relative amount of DMb in low oxygen packaging,
428 contained similar combinations of additives as those that yielded maximum a* values.
429 Moreover, low oxygen packaging combinations that were shown to induce high b* values,
430 were also promoting OMb and MMb (Table 8 and Fig. 5). Prevalence of DMb under high
431 oxygen atmosphere was mainly influenced by succinate and to a lesser extent by glutamate-
432 malate, corresponding to 70-80% and 20-30%, respectively (Table 8). The generation of high
433 OMb fractions was maintained solely by glutamate-malate, regardless of citrate
434 concentrations (Table 8). The situation was rather different for MMb accumulation as
435 pyruvate was shown to promote MMb in the absence of citrate, while increasing citrate lead
436 to increased participation of succinate (Table 8). Conversely, conditions that typically
437 resulted in minimum a*, b* or myoglobin values, were inverse to those that yielded the
438 maximum values.

439

440 *3.9. The effect of the mixture compared to adding only water*

441 Compared to the average changes in b* in Fig. 2A, it was apparent that b* declined more
442 rapidly with the mixture. Low b* was obtained with succinate and glutamate-malate in low
443 oxygen packaging. The lowest b* in Table 6 (2.3±0.1) was lower than the lowest b* in Table
444 9 (3.1±1.2). Thus there was a valid reason to think that these ingredients in low oxygen
445 packaging reduced the system quickly and provided more DMb. The situation in high oxygen
446 was less convincing. On day 3 the high oxygen system with water had a* equal to 12.8, while
447 the highest a* value obtained then was 13.3 (Table 8 and 9).

448 Compared to the average changes in DMb in Fig. 4A, it was apparent that DMb increased
449 more rapidly and attained a higher value when only water was added. However, if we
450 compared with the optimal mixture of succinate and glutamate-malate in low oxygen
451 packaging at day 3, the content of DMb was much higher (Table 8 and 9) than in water.

452 OMb in the sample added water in high oxygen packaging was lower than the average
453 sample with additives (Figure 4B and Table 9). The highest OMb (with glutamate-malate) at
454 day 3 in Table 8 (1.0±0.0) was higher than the highest OMb in Table 9 (80±6) Thus both in

455 low and high oxygen packaging the desired state was reached and stabilized earlier with
456 additives.

457 **Table 9.**

458

459 **Discussion**

460 Mitochondrial oxygen consumption has previously been linked to color stability as it reduces
461 the myoglobin oxidative state from MMb to DMb (Phung, et al., 2012; Slinde, et al., 2011).
462 In the present study we have packaged ground beef in low and high oxygen atmosphere, with
463 the addition of a variety of mitochondrial substrates and concentrations to increase the
464 prevalence of reduced myoglobin.

465 *The mixture*

466 Low –oxygen conditions*:* It will be most important to keep DMb at maximum value for this
467 atmosphere. The mixture of succinate and glutamate-malate was the single most important
468 variable affecting DMb and a* values (Table 8). Within the course of the experiment (13 days
469 in low oxygen packaging), DMb formation was stabilized by glutamate/succinate (days > 3
470 days are not shown). As recently reported (Phung, et al., 2012; Phung, et al., 2011), complex
471 I was rapidly reduced in activity early *post mortem* meat, while complex II were upregulated
472 to compensate for the loss of activity (Jones & Brewer, 2010). Also for the material used
473 here, there was a nominal reduction in complex I activity with time. Furthermore,
474 measurements of myoglobin states show a strong response surface toward DMb when a
475 mixture of succinate and glutamate-malate was used. In human skeletal muscles *in vitro* the
476 combination of succinate and glutamate-malate has been shown to consume the most oxygen
477 (Rasmussen & Rasmussen, 2000). Conditions with low oxygen atmosphere have been shown
478 to retain the highest prevalence of DMb (Nollet & Boylston, 2007; Sorheim, et al., 2009), but
479 the speed of DMb formation will increase with addition of succinate and glutamate-malate
480 additions.

481 High –oxygen conditions: It will be most important to keep OMb at maximum value for this
482 atmosphere. Maximum OMb was mediated mainly by glutamate-malate and citrate. It
483 therefore seems that mitochondrial complex I best sustained the prevalence of OMb. Even
484 though the magnitude was 0.0 for DMb, succinate was still important and relevant for DMb

485 in high oxygen, similar to its role in low oxygen packaging. This may suggest that it is acting
486 as a mediator between MMb and OMb. Furthermore, the large effect of succinate (being 70%
487 and 80% of the mixture, with and without citrate) in high oxygen, as compared to low oxygen
488 atmosphere (being 50% of the mixture) indicate that the mitochondrial reduction of
489 myoglobin occurring through complex II was stronger in high than low oxygen packaging
490 (Table 8). These results indicate that succinate metabolism was influenced by the
491 concentration of oxygen and the activity could become upregulated in high oxygen
492 environment. The accumulation of MMb therefore exceeds the mitochondrial capacity of
493 reducing myoglobin in this atmosphere and the meat color turns brown. Glutamate-malate
494 additions slowed down the accumulation of MMb.

495 Pyruvate provoked MMb formation in both low and high oxygen packaging. Pyruvate is a
496 product of glycolysis and can become converted to acetyl-coenzyme A and crosses the
497 mitochondrial membrane into the TCA cycle provided sufficient coenzyme A(CoA) is
498 available. Ramanthan and group (2010) showed that pyruvate may improve meat color
499 significantly at pH 5.6 and 7.4. However, our results (Phung, et al., 2011) showed reduced
500 activity at pH 5.5 for complex I. Oxidation of pyruvate was also reported by (Messer,
501 Jackman, & Willis, 2004) to contribute little (2.1%) to oxidative phosphorylation. In order
502 for pyruvate to function as an electron source in the TCA cycle it needs to be applied together
503 with malate that condenses to oxaloacetate (it still requires that sufficient CoA is present) to
504 occur. Under anaerobic conditions as in meat, pyruvate seems metabolized outside the
505 mitochondria to lactate via lactate dehydrogenase. In our system, pyruvate may therefore
506 have contributed to decreased pH and hence interfered with the reductive system and thereby
507 indirectly generate MMb. Additionally, pyruvate was tested in our system but did not induce
508 oxygen consumption (results not shown).

509 In general, the conditions that gave most DMb in the low oxygen system contained mainly
510 mixtures of succinate and glutamate-malate, while conditions promoting OMb and MMb
511 contained pyruvate and glutamate-malate. However, pyruvate no longer promoted OMb
512 formation in high oxygen packaging (at least not after day one). Glutamate-malate was the
513 most important component to regulate OMb and succinate for DMb formation in high oxygen
514 packaging. Glutamate-malate is known to produce reducing equivalents (NADH) but also
515 ROS formation from complex I (Adam-Vizi & Chinopoulos, 2006). Further experiments
516 could elucidate if ROS is produced here without deteriorating effects on color.

517 Glutamate-malate ratio and the level of mixture: The tendency (not significant though) for
518 glutamate to favour higher a* could be due to the fact that in intact systems glutamate
519 generates more reducing equivalents than malate. Malate alone doesn't support oxygen
520 consumption as oxaloacetate cannot be metabolized if acetyl-CoA (or CoA) is absent.
521 However, glutamate can be oxidized, when acetyl-CoA is absent, by glutamate
522 dehydrogenase, which ultimately leads to the production of two reducing equivalents.

523 It is also possible that saturation of the mitochondrial reductive system has been reached.
524 This would indicate that relatively small concentrations of glutamate-malate are sufficient for
525 the effects observed here. Since we had separate models colour variables at high and low
526 oxygen packaging, it could be concluded that even the higher levels of glutamate-malate and
527 citrate could not retain more than 79% OMb (adjusted mean value) after 8 days of incubation
528 in high oxygen packaging. This was, however, a sample at the higher level of glutamate-
529 malate and citrate. Thus it cannot completely be ruled out that substrate could be limiting and
530 more detailed investigations should be carried out using higher concentrations than 0.1mol/kg
531 in high oxygen in order to verify saturation. On the other hand this higher than adding only
532 water (50%).

533

534 Citrate: This component was previously shown to work synergistically with ascorbate
535 (Jaswir, Man, & Kitts, 2000) and could promote color and protect meat from lipid oxidation
536 (Lund, et al., 2007). Citrate can act as an antioxidant by chelating minerals. Our day 3 results
537 in high oxygen suggested increased stability of OMb with citrate. Moreover, the influence of
538 citrate in maintaining OMb increased dramatically at day 8 in high oxygen packaging relative
539 to the importance of the mixture. These results show that citrate may have an important role
540 in meat color that cannot be deciphered at this time.

541 Age and fat tissue: The variables were included to study effect of the additives on lipid
542 degradation in a separate study. L* in particular was affected by age, presumably partly due
543 to the nominally higher myoglobin content. Old animals were shown to have the darkest meat
544 color (low L* and high DMb) both in low and high oxygen packaging. Furthermore, young
545 animals were found to promote MMb and to a lesser extent OMb. This can be due to a higher
546 accumulation of endogenous antioxidants of old animals since they are extensively fed for
547 longer periods in Norway. Meat color stability was reported to be inversely related to oxygen
548 consumption as myoglobin more frequently became oxidized by a high mitochondrial activity

549 (O'Keeffe & Hood, 1982; Renerre & Labas, 1987). Having a low ratio between mitochondrial
550 activities to myoglobin content may therefore be beneficial in old animals to keep myoglobin
551 in a reduced state. Fat type had the least effect on color in our design.

552

553 **Conclusion**

554 Additives have been shown to remove residual oxygen and reduce myoglobin through
555 mitochondria. For high oxygen storage of ground meat we found that glutamate-malate
556 preserved OMb the most and that citrate became increasingly important with storage time. In
557 low oxygen atmosphere, glutamate-malate plus succinate quickly induced a pure DMb state,
558 while pyruvate promoted MMb formation. Increasing the level of additives above 0.05 mol/
559 kg had no significant effect on color variables.

560

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568 **References**

569 Adam-Vizi, V., & Chinopoulos, C. (2006). Bioenergetics and the formation of mitochondrial reactive
570 oxygen species. *Trends Pharmacol. Sci. , 27*(12), 639-645.
571 Arihara, K., Cassens, R. G., Greaser, M. L., Luchansky, J. B., & Mozdziak, P. E. (1995). Localization of
572 metmyoglobin-reducing enzyme (NADH-cytochrome-b(5) reductase) system components in
573 bovine skeletal-muscle. *Meat Sci, 39*(2), 205-213.
574 Beggan, M., Allen, P., & Butler, F. (2006). Oxygen scavenger effect on the development of
575 metmyoglobin on beefsteaks during early low-oxygen storage. *J Muscle Foods, 17*(4), 381-
576 397.
577 Berger, M., & Wong, W. K. (2009). Designs for Simple Linear Regression. In *An Introduction to*
578 *Optimal Designs for Social and Biomedical Research*. Chichester, UK: John Wiley & Sons, Ltd.
579 CIE. (1976). *Recommendations on uniform color spaces-color difference equations* (Vol. Supplement
580 No. 2 to CIE). Paris, France: Commission Internationale de l'Eclairage.
581 Devle, H., Rukke, E. O., Naess-Andresen, C. F., & Ekeberg, D. (2009). A GC-magnetic sector MS
582 method for identification and quantification of fatty acids in ewe milk by different
583 acquisition modes. *J Sep Sci, 32*(21), 3738-3745.
584 Egelandsdal, B., Dingstad, G., Togersen, G., Lundby, F., & Langsrud, O. (2005). Autofluorescence
585 quantifies collagen in sausage batters with a large variation in myoglobin content. *Meat Sci,*
586 *69*(1), 35-46.
587 Faustman, C., & Phillips, A. (2001). Measurement of discoloration in fresh meat. In *Current protocols*
588 *in food analytical chemistry*: John Wiley & Sons, Inc.
589 Gnaiger, E. (2001). Bioenergetics at low oxygen: dependence of respiration and phosphorylation on
590 oxygen and adenosine diphosphate supply. In (Vol. 128, pp. 277-297): Elsevier Science Bv.
591 Gnaiger, E. (2008). Polarographic oxygen sensors, the oxygraph, and high-resolution respirometry to
592 assess mitochondrial function. In J. Dykens & Y. Will (Eds.), *Drug-Induced Mitochondrial*
593 *Dysfunction* (pp. 327-352). Innsbruck: John Wiley & Sons, Inc.
594 Hagler, L., Coppes, R. I., Jr., & Herman, R. H. (1979). Metmyoglobin reductase. Identification and
595 purification of a reduced nicotinamide adenine dinucleotide-dependent enzyme from bovine
596 heart which reduces metmyoglobin. *J Biol Chem, 254*(14), 6505-6514.
597 Isdell, E., Allen, P., Doherty, A. M., & Butler, F. (1999). Colour stability of six beef muscles stored in a
598 modified atmosphere mother pack system with oxygen scavengers. *Int. J. Food Sci. Technol.,*
599 *34*(1), 71-80.
600 Jaswir, I., Man, Y. B. C., & Kitts, D. D. (2000). Synergistic effects of rosemary, sage, and citric acid on
601 fatty acid retention of palm olein during deep-fat frying. *J. Am. Oil Chem. Soc., 77*(5), 527-
602 533.
603 Jones, T. T., & Brewer, G. J. (2010). Age-related deficiencies in complex I endogenous substrate
604 availability and reserve capacity of complex IV in cortical neuron electron transport. *Biochim*
605 *Biophys Acta, 1797*(2), 167-176.

606 Khatri, M., Phung, V. T., Isaksson, T., Sorheim, O., Slinde, E., & Egelandsdal, B. (2012). A comparison
607 of two sample preparation methods and two methods for calculating different myoglobin
608 states in beef M. semimembranosus surfaces. *Meat Sci*.
609 Langsrud, Ø., Jørgensen, K., Ofstad, R., & Næs, T. (2007). Analyzing Designed Experiments with
610 Multiple Responses, Journal of Applied Statistics. *J. Appl. Statist.*(34), 1275-1296.
611 Ledward, D. A. (1985). Post-slaughter influences on the formation of metymoglobin in beef muscles
612 *Meat Sci, 15*(3), 149 - 171.
613 Livingston, D. J., McLachlan, S. J., La Mar, G. N., & Brown, W. D. (1985). Myoglobin: cytochrome b5
614 interactions and the kinetic mechanism of metmyoglobin reductase. *J Biol Chem, 260*(29),
615 15699-15707.
616 Lumeng, L., Bremer, J., & Davis, E. J. (1976). Suppression of the mitochondrial oxidation of (-)-
617 palmitylcarnitine by the malate-aspartate and alpha-glycerophosphate shuttles. *J Biol Chem,*
618 *251*(2), 277-284.
619 Lund, M. N., Hviid, M. S., & Skibsted, L. H. (2007). The combined effect of antioxidants and modified
620 atmosphere packaging on protein and lipid oxidation in beef patties during chill storage.
621 *Meat Sci., 76*(2), 226-233.
622 Madhavi, D. L., & Carpenter, C. E. (1993). Aging and processing affect color, metmyoglboni reductase
623 and oxygen-consumption of beef muscles. *J. Food Science, 58*(5), 939-942.
624 Mancini, R. A., & Hunt, M. C. (2005). Current research in meat color. *Meat Sci., 71*(1), 100-121.
625 Mancini, R. A., Ramanathan, R., & Dady, G. A. (2011). Effects of pyruvate, succinate, and lactate
626 enhancement on beef longissimus raw color. *Meat Sci., 88*(3), 424-428.
627 Messer, J. I., Jackman, M. R., & Willis, W. T. (2004). Pyruvate and citric acid cycle carbon
628 requirements in isolated skeletal muscle mitochondria. *Am J Physiol Cell Physiol, 286*(3),
629 C565-572.
630 Nollet, L. M. L., & Boylston, T. (2007). Pork color basics. In L. M. L. Nollet (Ed.), *Handbook of meat,*
631 *poultry and seafood quality* (pp. 719). Ghent: Blackwell Publishing Professional.
632 O'Keeffe, M., & Hood, D. E. (1982). Biochemical Factors Influencing Metmyoglobin Formation on
633 Beef from Muscles of Differing Color Stability. *Meat Sci, 7*(3), 209-228.
634 Phung, V. T., Khatri, M., Slinde, E., Sorheim, O., Liland, K. H., Almoy, T., Saarem, K., & Egelandsdal, B.
635 (2012). Mitochondrial oxygen consumption in permeabilized fibers and its link to colour
636 stability in bovine M. semimembranosus muscle. *Submitted for publication Meat Sci.*
637 Phung, V. T., Saelid, E., Egelandsdal, B., Volden, J., & Slinde, E. (2011). Oxygen Consumption Rate of
638 Permeabilized Cells and Isolated Mitochondria from Pork M. Masseter and Liver Examined
639 Fresh and After Freeze-Thawing at Different pH Values. *J Food Sci, 76*(6), 929-936.
640 Puntel, R. L., Roos, D. H., Grotto, D., Garcia, S. C., Nogueira, C. W., & Rocha, J. B. (2007). Antioxidant
641 properties of Krebs cycle intermediates against malonate pro-oxidant activity in vitro: a
642 comparative study using the colorimetric method and HPLC analysis to determine
643 malondialdehyde in rat brain homogenates. *Life Sciences, 81*(1), 51-62.
644 Ramanathan, R., & Mancini, R. A. (2010). Effects of pyruvate on bovine heart mitochondria-mediated
645 metmyoglobin reduction. *Meat Sci., 86*(3), 738-741.
646 Rasmussen, U. F., & Rasmussen, H. N. (2000). Human quadriceps muscle mitochondria: a functional
647 characterization. *Molecular and Cellular Biochemistry, 208*(1-2), 37-44.
648 Renerre, M., & Labas, R. (1987). Biochemical factors influencing metmyoglobin formation in beef
649 muscles. *Meat Sci, 19*(2), 151-165.
650 Saleh, B., & Watts, B. M. (1968). Sustrates and intermediates in enzymatic reduction of
651 metmyoglobin in ground beef. *J. Food Science, 33*(4), 353-&.
652 Slinde, E., Phung, V. T., & Egelandsdal, B. (2011). Conversion of met-myoglobin directly to oxy-
653 myoglobin by mitochondria from pork muscle (M. masseter) and liver. In *ICOMST 2011* (Vol.
654 57th). Ghent, Belgium: 57th International Congress of Meat Science and Technology.
655 Sorheim, O., Westad, F., Larsen, H., & Alvseike, O. (2009). Color of ground beef as influenced by raw
656 materials, addition of sodium chloride and low oxygen packaging. *Meat Sci., 81*(3), 467-473.

657 Tang, J., Faustman, C., Mancini, R. A., Seyfert, M., & Hunt, M. C. (2005). Mitochondrial reduction of
658 metmyoglobin: dependence on the electron transport chain. *J Agric Food Chem, 53*(13),
659 5449-5455.
660 Taylor, A. A., & MacDougall, D. B. (1973). Fresh beef packed in mixtures of oxygen and carbon
661 dioxide. *Int. J. Food Sci. Technol., 8*(4), 453-461.
662 Tewari, G., Jayas, D. S., & Holley, R. A. (1999). Centralized packaging of retail meat cuts: A review. *J.*
663 *Food Prot., 62*(4), 418-425.
664 Wittenberg, J. B. (1970). Myoglobin-facilitated oxygen diffusion: role of myoglobin in oxygen entry
665 into muscle. *Physiol Rev, 50*(4), 559-636.
666 Wittenberg, J. B., & Wittenberg, B. A. (2007). Myoglobin-enhanced oxygen delivery to isolated
667 cardiac mitochondria. *J Exp Biol, 210*(Pt 12), 2082-2090.

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Figure

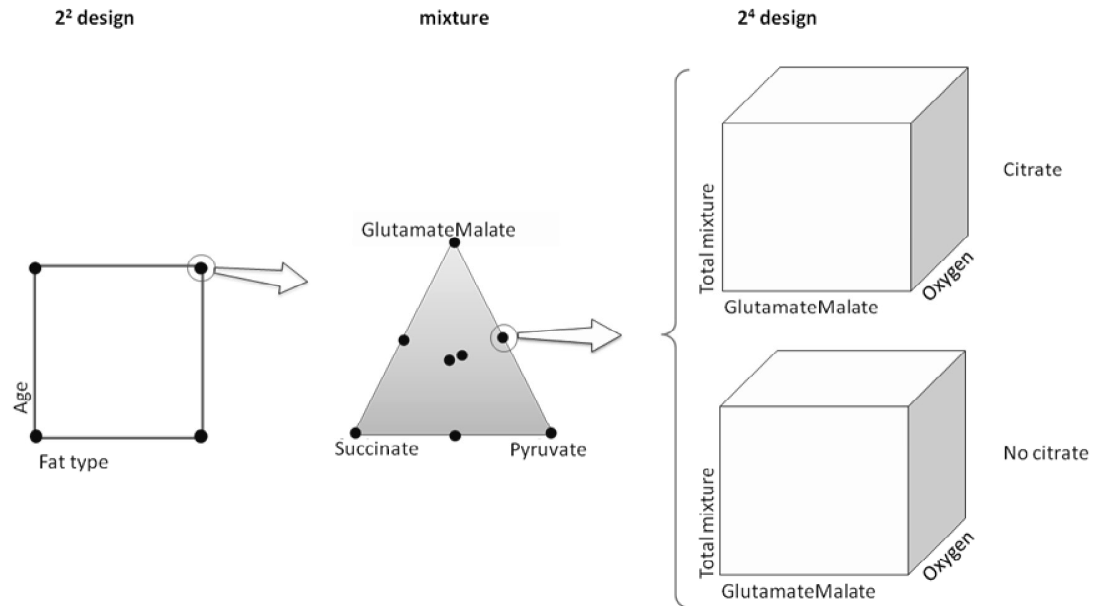
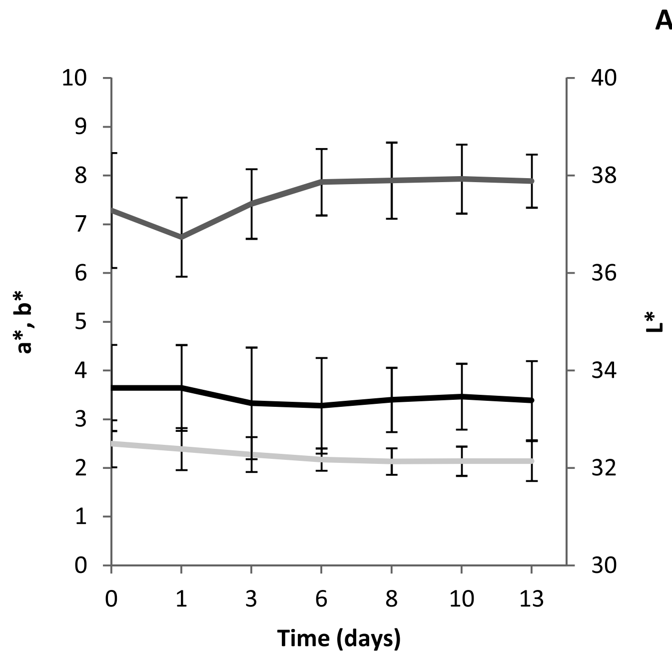


Figure 1



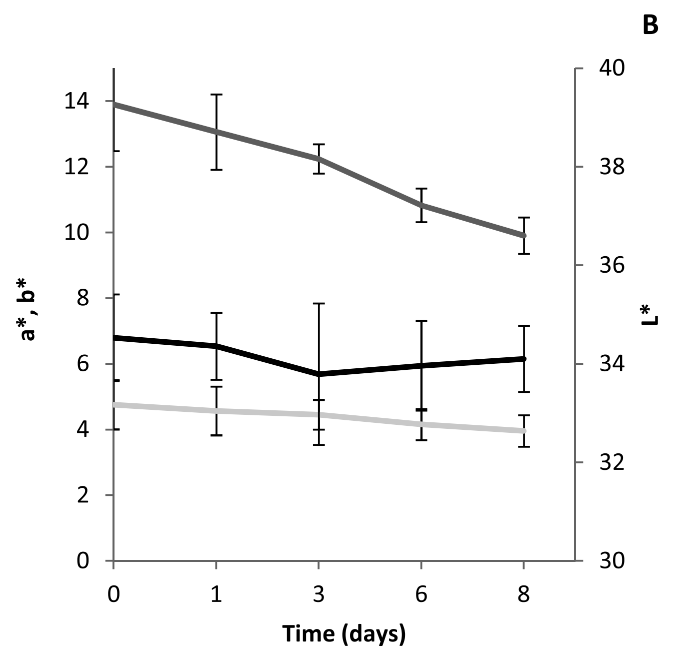


Figure 2.

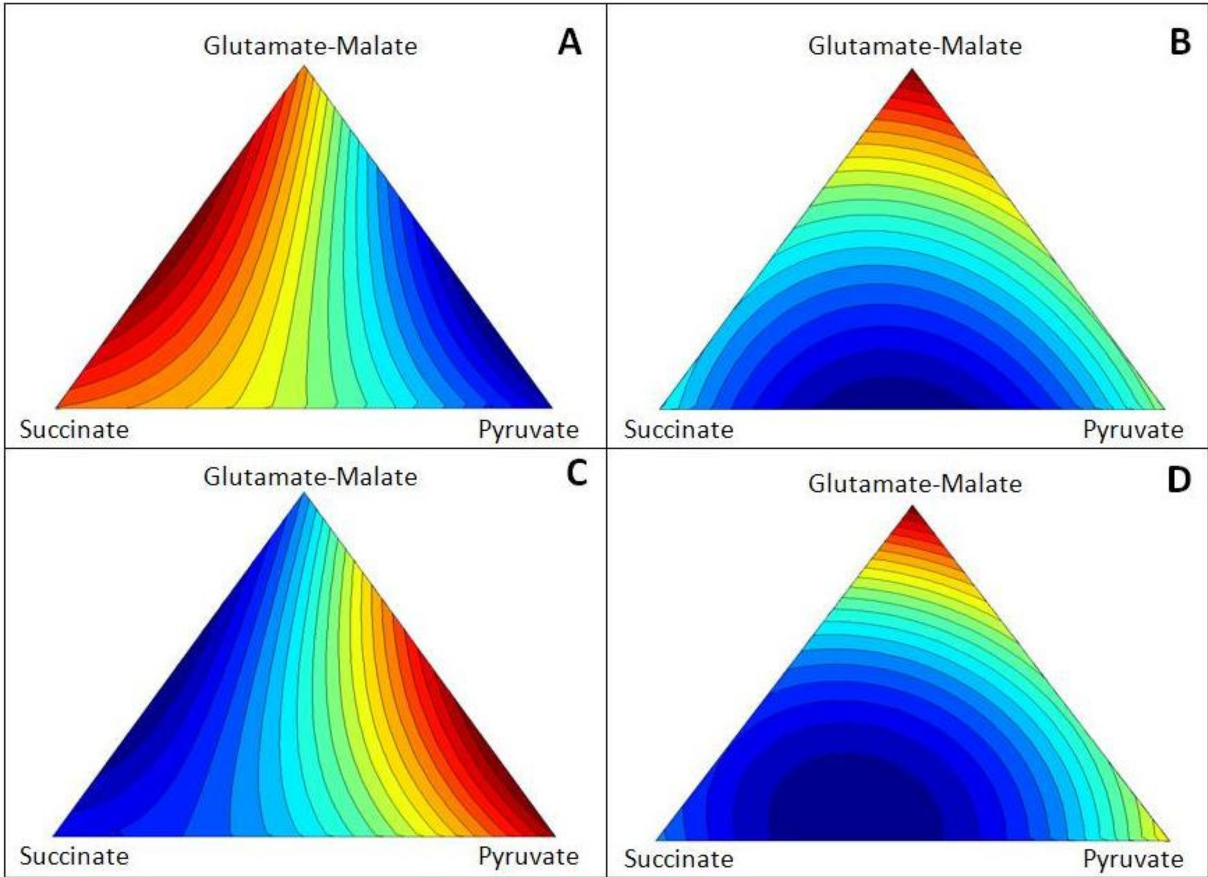
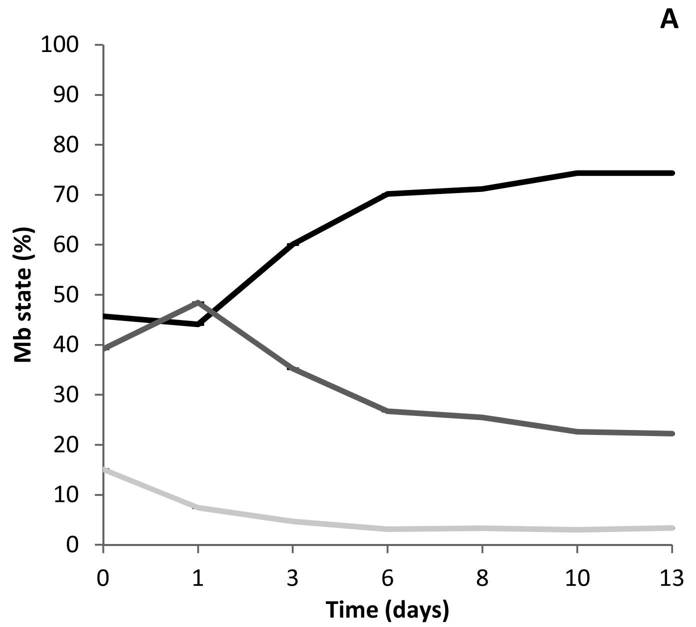


Figure 3.



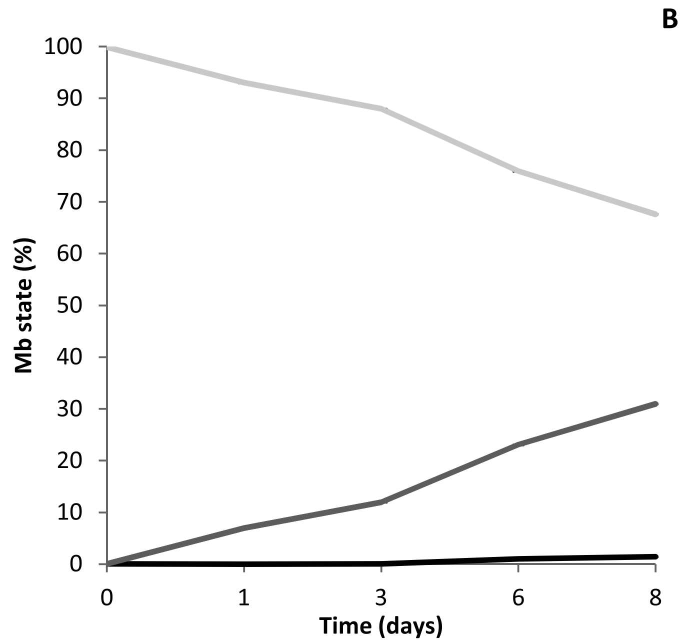


Figure 4.

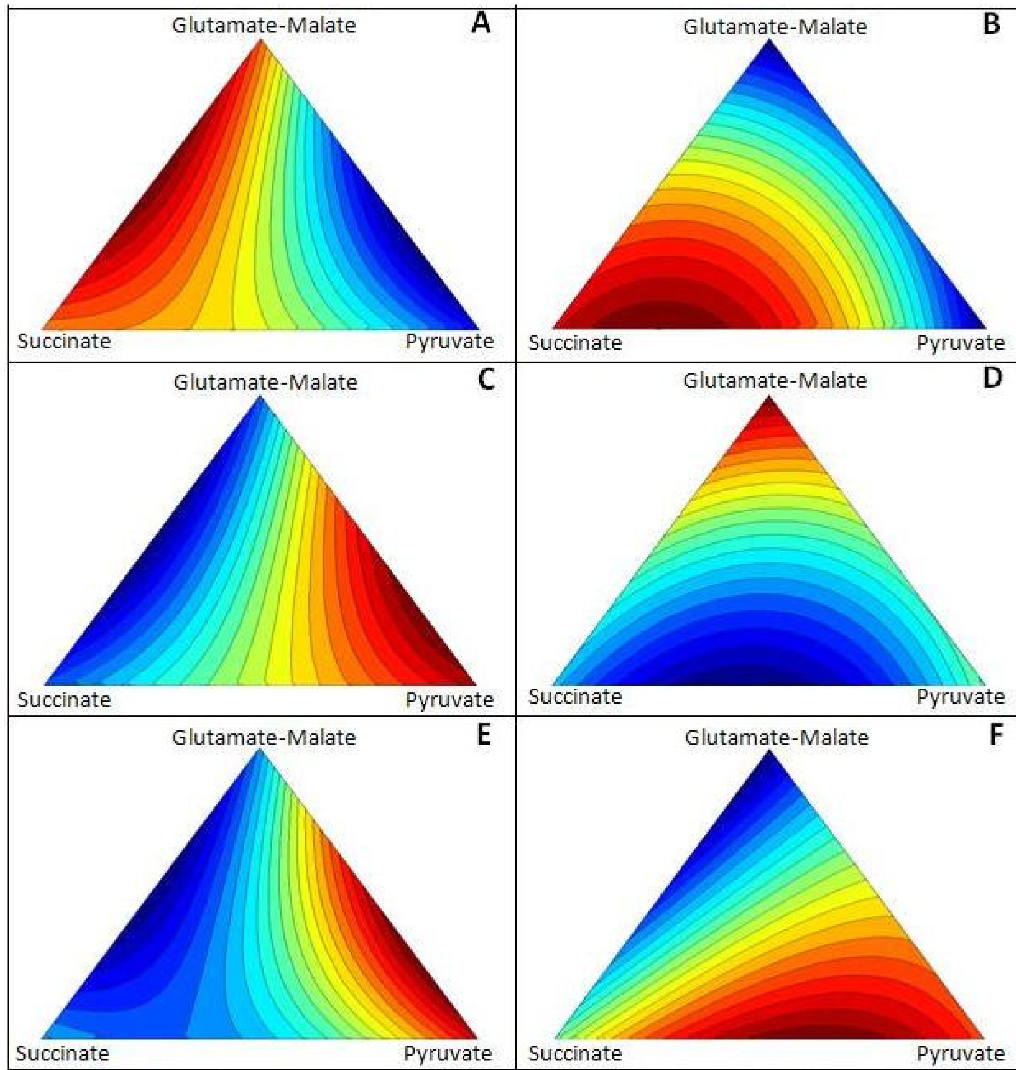


Figure 5.

Figure

Figure 1. The design used was a 2^2 factorial design in age and fat type. To each point was added an 8-point simplex centroid mixture design (7 experimental points and 1 replicate, see triangle) and thereafter a 2^4 design illustrated by two cubes. Arrows indicate that one experimental point is described by the next design. Each corner of the 2^2 design was measured on one day. For further details see Materials and methods.

Figure 2. Average L*, a*, b*values (156 samples) versus time for low (A) and high oxygen (B) packaging. Colors black, dark grey and grey are L*, a* and b*, respectively, where a* and b* related to ordinate is on the left side and L* relate to the ordinate on right side of each figure. Error bars are standard deviations.

Figure 3. Model fit (adjusted means) of a* and b* color measurements as a response to treatment with different additives; each corner represents a brine with one additive. a* is represented in A and B, and b* is represented in C and D. Samples were measured at day 3 of packaging. Samples packed in low oxygen is located in A and C, and samples in high oxygen are located in B and D. Color codes are a gradient from red to blue, where red is the highest response and blue is the lowest response for both variables.
The lines and colours represent a smooth transition from lowest to highest value; the max and min values; the max-min is different for each panel: A) 5.0-9.5;B)11.4-13-3;C)1.2-3-7;D)4.2-4.9. The data are not normalized.

Figure 4. Percentage distribution of DMb, OMb and MMb versus time for low (A) and high (B) oxygen packaging. Colors black, dark grey and grey are DMb, MMb and OMb, respectively. The ordinate shows the relative amount of each myoglobin state at the respective day and error bars are standard deviations. The error bars are too small to be visible.

Figure 5. Model fitted not normalized myoglobin measurements as a response to treatment with different additives, each corner represents brine with a sole additive. DMb is represented in A and B, OMb is represented in C and D, and MMb is represented in E and F. Samples were measured at day 3 of experiment. Samples packed in low oxygen are located in A, C and E, and samples in high oxygen are located in B, D and F. Color codes are a gradient from red to blue, where red is the highest response and blue is the lowest response for all three states. The lines represent a smooth transition from lowest to highest value; the max and min values: for A, C, E the range is from 0.03 to1.03; -0.06 to0.84; -0.02 to 0.09 respectively; for B, D,F the range is from -0.1 to -0.06; 0.79 to 0.94; 0.08 to 0.16, respectively. The data are not normalized.

Table 1. The 8-point simplex centroid mixture design (Figure 1) with the respective chemicals and mixture at each experimental point.

Experimental point	Succinate (percentage)	Pyruvate (percentage)	Glutamate-malate (percentage)
1	100	0	0
2	0	100	0
3	0	0	100
4	0	50	50
5	50	0	50
6	50	50	0
7	33	33	33
8	33	33	33

Table 2. The 2^6 design with the respective chemicals and levels.

Effect	Level 1	Level 2
Mixture comp.(mol/kg)	0.05	0.1
Citrate (mol/kg)	0	0.025
Glutamate-malate level	25:75	75:25
Fat type	Pork	Beef
Age	Young	Old
Oxygen (% v/v)	~0	75

Table 3. The model terms used for analyzing the design described in Figure 1. Two separate models were constructed for high and low oxygen.

First order terms		Second order terms		Third order terms	
Effect	DF	Effect	DF	Effect	DF
Fat type	1	Mixture comp×Fat type	2	Mixture comp×Age×Fat type	2
Age	1	Mixture comp×Age	2	Mixture comp×Fat type×Citrate	2
Citrate	1	Mixture comp×Citrate	2	Age×Fat type×Citrate	2
Total mixture comp	1	Age×Fat type	1	Total mixture comp×ratio malate/glutamate	1
Ratio malate/glutamate	1	Age×Citrate	1	Mixture comp×Age×Fat type	2
Mixture comp	2	Fat type×Citrate	1	Mixture comp× Mixture comp*Citrate	3
		Mixture comp× Mixture comp	3		

DF: degrees of freedom

Table 4. Fatty acid compositions in pork and beef fat

	SFA %	**MUFA %**	**PUFA %**
Pork fat (1)*	26.6±0.2	50.0±0.0	22.0±0.1
Beef fat (2)*	48.6±3.1	42.8±1.8	2.0±0.4

*Remaining fatty acids were not identified.
SFA; saturated fatty acid,
MUFA; monounsaturated fatty acid,
PUFA; polyunsaturated fatty acid.

Table 5. Mean oxygen consumption from permeabilized tissue from *M . semimembranosus* from young and old animals before minced and mixed with fat. Four measurements were made at packing day (day 0) and 13 days later.

		OCR (pmol O_2/sec)/mg protein) – mean ± std. error.					
Animal	**Days**	**Complex I**[a]	**β-oxidation**	**Complex II (unc.)**[b]	**Inhib. I**[c]	**Inhib. II**[d]	**ROX**[e]
Young	0	9.0±5.2	13.8±4.8	20.9±6.1	19.2±5.7	2.8±1.4	11.3±2.6
	13	7.0±2.7	8.0±3.3	13.5±5.3	11.2±3.8	0.3±0.1	4.3±1.2
Old	0	6.2±1.0	3.5±0.4	16.7±3.5	15.2±4.7	0.5±0.6	10.3±0.2
	13	0.9±0.5	0.9±0.5	8.6±1.6	8.6±0.9	-0.7±0.1	10.3±1.1

[a]Complex I response was after stimulation by glutamate-malate and pyruvate. [b] Complex II response was in sequence after complex I and initiated by succinate and uncoupled by FCCP. ADP was present after Complex I stimulation. [c] Inhib. I: complex I inhibited by rotenone, [d]Inhib. II: complex II inhibited by malonic acid, [e]ROX: oxygen consuming side reaction after complete inhibition of ETS with antimycin A.

Table 6. Adjusted (according to model in Table 3) means with standard errors at high and low levels of age, fat and citrate on the development of of L*, a* and b* values with storage time.

			Low O_2						High O_2			
Effect	**Type**	**Variable**	**Day 1**	**Day 3**	**Day 6**	**Day 8**	**Day 10**	**Day 13**	**Day 1**	**Day 3**	**Day 6**	**Day 8**
Age	Young	L*	34.2±0.1	34.0±0.1	33.9±0.1	33.8±0.1	33.8±0.1	34.0±0.1	35.0±0.1	34.7±0.1	34.7±0.1	34.6±0.1
		a*	7.4±0.1	8.0±0.1	8.5±0.1	8.5±0.1	8.6±0.1	8.4±0.2	13.6±0.1	12.4±0.1	11.1±0.1	10.3±0.1
		b*	2.7±0.1	2.5±0.1	2.3±0.0	2.3±0.0	2.4±0.0	2.4±0.1	5.1±0.1	4.8±0.1	4.5±0.1	4.3±0.0
	Old	L*	33.1±0.1	32.7±0.1	32.8±0.1	33.0±0.1	33.2±0.1	32.9±0.1	33.8±0.1	32.8±0.1	33.3±0.1	33.6±0.1
		a*	6.1±0.1	6.8±0.1	7.3±0.1	7.4±0.1	7.4±0.1	7.5±0.2	12.4±0.1	12.1±0.1	10.5±0.1	9.5±0.1
		b*	2.1±0.1	2.0±0.1	2.0±0.0	1.9±0.0	1.9±0.0	1.9±0.1	0.1±0.1	0.1±0.1	0.06±0.1	0.0±0.0
Fat	Pork	L*	34.1±0.1	33.9±0.1	33.9±0.1	33.8±0.1	33.9±0.1	33.7±0.1	34.4±0.1*	34.4±0.1	34.4±0.1	34.4±0.1
		a*	7.1±0.1	7.7±0.1	8.8±0.1	8.3±0.1	8.3±0.1	8.2±0.1	13.2±0.1	12.2±0.1*	11.0±0.1	10.0±0.1*
		b*	2.4±0.1*	2.3±0.1*	2.2±0.0*	2.2±0.0*	2.2±0.0*	2.3±0.1	4.7±0.1	4.5±0.1*	4.3±0.1	4.1±0.0
	Cattle	L*	33.2±0.1	32.8±0.1	32.8±0.1	33.1±0.1	33.1±0.1	33.1±0.1	34.4±0.1*	33.1±0.1	33.7±0.1	33.9±0.1
		a*	6.4±0.1	7.17±0.1	7.6±0.1	7.5±0.1	7.6±0.1	7.7±0.2	12.8±0.1	12.3±0.1*	10.6±0.1	9.8±0.1*
		b*	2.4±0.1*	2.3±0.1*	2.1±0.0*	2.1±0.0*	2.1±0.0*	2.0±0.1	4.4±0.1	4.4±0.1*	4.0±0.1	3.8±0.0
Citrate	Low	L*	33.8±0.3	33.71±0.3	33.4±0.2*	33.5±0.2*	33.6±0.2*	33.6±0.3*	34.5±0.2	33.9±0.3*	34.8±0.3	34.3±0.2
		a*	6.9±0.3*	7.7±0.2	8.2±0.2	8.3±0.2	8.3±0.2	8.3±0.3	13.0±0.2*	12.17±0.2*	10.6±0.2	9.6±0.2
		b*	2.5±0.3	2.3±0.1*	2.2±0.1*	2.2±0.1*	2.2±0.1	2.2±0.2*	4.6±0.1*	4.5±0.1*	4.20±0.2*	4.0±0.1*
	High	L*	33.5±0.3	33.0±0.3	33.3±0.2*	33.4±0.2*	33.4±0.2*	33.2±0.3*	34.2±0.3	33.6±0.3*	33.8±0.3	33.9±0.3
		a*	6.6±+.3*	7.1±0.2	7.5±0.2	7.5±0.2	7.6±0.2	7.6±0.4	13.0±0.2*	12.3±0.2*	11.0±0.3	10.3±0.2
		b*	2.3±0.2	2.3±0.1*	2.2±0.1*	2.1±0.1*	2.1±0.1	2.1±0.2*	4.5±0.1*	4.4±0.1*	4.1±0.2*	4.0±0.1*

*The difference between high and low level of effects at specified times was not significant (P>0.05)

Table 7. Adjusted (according to model in Table 3) means with standard errors at high and low levels of age, fat and citrate on the development of DMb, OMb, and MMb percentages (normalized data) with storage time. Values are in % and not normalized**.**

			Low O_2						High O_2			
Effect	**Type**	**Mode**	**Day 1**	**Day 3**	**Day 6**	**Day 8**	**Day 10**	**Day 13**	**Day 1**	**Day 3**	**Day 6**	**Day 8**
Age	Young	DMb	40.6±3.2	60.2±3.3*	71.8±3.1*	72.1±3.2*	73.9±3.1*	70.8±4.1*	0.0±0.0	0.0±0.0	0.4±0.3	0.2±0.2
		OMb	8.3±0.4	4.7±0.4*	2.8±0.4*	2.7±0.4	3.2±0.3*	3.8±0.5	91.3±0.3	86.6±0.6	76.2±0.7*	66.3±0.7*
		MMb	51.2±2.9	35.1±3.0*	25.4±2.9*	25.3±2.8*	23.0±2.8*	25.4±3.7*	8.7±0.3	13.4±0.6	23.4±0.6*	33.6±0.7
	Old	DMb	49.3±3.2	60.2±*3.3	68.9±3.1*	71.0±3.2*	74.5±3.1*	78.1±4.1*	0.0±0.0	0.1±0.0	1.4±0.3	2.5±0.2
		OMb	6.5±0.4	4.4±0.4*	3.2±0.4*	3.6±0.4	2.7±0.3*	2.5±0.5	94.3±0.3	88.4±0.6	75.4±0.7*	69.0±0.7*
		MMb	44.3±2.9	35.3±3.0*	27.9±2.9*	25.4±2.8*	22.8±2.8*	19.4±3.7*	5.7±0.3	11.6±0.6	23.2±0.6*	28.5±0.7
Fat	Pork	DMb	48.9±3.2*	65.8±3.2	74.7±3.0	76.7±3.1	79.5±3.0	76.0±4.0*	0.0±0.0	0.0±0.0*	0.8±0.3	1.3±0.2
		OMb	6.0±0.4	2.6±0.4	1.4±0.4	1.9±0.4	1.6±0.3	1.9±0.5	91.0±0.3*	88.2±0.6	73.7±0.7*	66.4±0.7
		MMb	45.1±2.9*	31.6±3.0*	24.0±2.9*	21.4±2.8	18.9±2.8	22.1±3.7*	9.0±0.3	11.8±0.6*	25.5±0.6	32.3±0.7
	Cattle	DMb	40.7±3.3*	54.1±3.3	65.6±3.2	65.8±3.2	68.4±3.2	72.9±4.2*	0.0±0.0	0.1±0.0*	1.0±0.3	1.4±0.2
		OMb	8.8±0.4	6.7±0.4	4.8±0.4	4.6±0.4	4.4±0.3	4.4±0.5	94.5±0.3*	86.8±0.6	77.8±0.7*	68.8±0.7
		MMb	50.5±3.0*	39.2±3.1*	29.6±2.9*	29.6±2.9	27.2±2.9	22.7±3.8*	5.5±0.3	13.1±0.6*	21.2±0.6	29.8±0.7
Citrate	Low	DMb	47.6±3.1*	64.6±3.2*	75.3±3.0	76.0±3.1	78.2±3.0	76.5±4.0*	0.0±0.0*	0.1±0.0	1.3±0.3	2.0±0.2
		OMb	7.0±*0.4	3.8±0.4	2.3±0.4	2.3±0.4	2.2±0.3	2.5±0.5*	92.3±0.3	85.7±0.6	72.8±0.7	63.1±0.7
		MMb	45.4±2.9*	31.7±2.9*	22.3±2.8	21.7±2.8*	19.7±2.8*	21.0±3.6*	7.7±0.3	14.2±0.6	25.9±0.6	34.9±0.7
	High	DMb	42.1±3.3*	55.5±3.3*	64.9±3.1	66.6±3.1	69.9±3.0	72.3±4.1*	0.0±0.0*	0.0±0.0	0.5±0.3	0.6±0.2
		OMb	7.8±0.4*	5.5±0.4	3.7±0.4	4.1±0.4	3.8±0.3	3.8±0.5*	93.4±0.6	89.5±0.6	79.1±0.7	72.7±0.8
		MMb	50.2±3.0*	39.1±3.1*	31.4±3.0	29.3±2.9*	26.3±2.9*	23.9±3.8*	6.6±0.3	10.5±0.6	20.4±0.6	26.7±0.7

*The difference between high and low levels of effects at specified times were not significant ($P>0.05$)

Table 8. Combinations of substrates that yield the maximum (max) or minimum (min) responses with standard deviations for a*, b* and myoglobin states (not normalized). Results are shown as % of each addition of the respective chemicals (total amounts either 0.05 mol/ kg or 0.1 mol/kg).Predications were based on unadjusted values (Table 3) of the mixture component effect at day 3. Low and high oxygen packaging and citrate level are shown.

		Low oxygen				High oxygen			
Variable	**Citrate**	**Succinate**	**Pyruvate**	**Glu-Mal**	**Magnitude of variable**	**Succinate**	**Pyruvate**	**Glu-Mal***	**Magnitude of variable**
a*	Low	40	0	60	9.7 ± 0.2^{c}	0	0	100	13.4 ± 0.2^{c}
	High**	60	0	40	9.2 ± 02^{c}	0	0	100	13.2 ± 0.3^{c}
	Low	0	80	20	4.8 ± 0.2^{b}	60	40	0	11.4 ± 0.2^{b}
	High	0	80	20	5.3 ± 0.2^{b}	50	50	0	11.4 ± 0.2^{b}
b*	Low	0	80	20	4.0 ± 0.1^{c}	0	0	100	5.0 ± 0.1^{c}
	High	0	80	20	3.5 ± 0.1^{c}	0	0	100	4.8 ± 0.2^{c}
	Low	70	0	30	1.2 ± 0.1^{b}	60	20	20	4.2 ± 0.1^{b}
	High	50	0	50	1.2 ± 0.1^{b}	50	50	0	4.1 ± 0.1^{b}
DMb[a]	Low	50	0	50	105.0 ± 9.0^{c}	80	0	20	-5.0 ± 1.0^{c}
	High	50	0	50	100.0 ± 9.0^{c}	70	30	0	-6.0 ± 1.0^{c}
	Low	0	70	30	-1.0 ± 8.0^{b}	0	100	0	-10.0 ± 1.0^{b}
	High	0	80	20	6.0 ± 7.0^{b}	0	0	100	-12.0 ± 0.0^{b}
OMb[a]	Low	0	80	20	9.0 ± 1.0^{c}	0	0	100	91.0 ± 2.0^{c}
	High	0	80	20	1.0 ± 1.0^{c}	0	0	100	98.0 ± 2.0^{c}
	Low	40	0	60	-2.0 ± 1.0^{b}	60	40	0	77.0 ± 2.0^{b}
	High	60	0	40	-2.0 ± 1.0^{b}	60	40	0	81.0 ± 2.0^{b}
MMb[a]	Low	0	70	30	87.0 ± 8.0^{c}	0	90	10	17.0 ± 1.0^{c}
	High	0	80	20	81.0 ± 7.0^{c}	50	50	0	15.0 ± 2.0^{c}
	Low	50	0	50	-8.0 ± 9.0^{b}	0	0	100	9.0 ± 2.0^{b}
	High	50	0	50	-3.0 ± 0.0^{b}	20	0	80	7.0 ± 2.0^{b}

*Glu-Mal = glumate-malate. Minimum and maximum obtained color values are designated as [b] and [c], respectively. When citrate is present (high) it is 25 % so total amounts so the other additives should be multiplied by 0.75.

Table 9. Control samples showing values for L*, a*, b* and myoglobin states. The ground beef were added only water and varied in oxygen level (low and high O_2), age (young and old) and fat type (beef and pork fat). Two samples were made for each combination with 3 measurements on each sample, resulting in a total of 8 control samples. Mean and standard deviations are shown (not normalized data for myoglobin percentages)

	Low O_2						**High O_2**			
	Day 1	**Day 3**	**Day 6**	**Day 8**	**Day 10**	**Day 13**	**Day 1**	**Day 3**	**Day 6**	**Day 8**
L*	35.5±0.4	35.2±0.4	34.4±0.4	34.5±0.8	34.5±0.5	34.3±0.9	36.4±1.1	36.5±1.1	36.6±1.1	35.7±1.6
a*	12.0±1.3	8.6±2.4	10.1±1.3	10.3±1.2	10.3±0.7	10.4±0.7	14.7±1.1	12.8±0.9	11.3±0.7	10.7±0.9
b*	4.6±0.8	3.1±1.2	2.4±0.3	2.4±0.4	2.4±0.1	2.4±0.2	5.9±0.2	5.5±0.2	5.2±0.3	5.3±0.2
DMb	25.0±17.0	65.0±38.0	88.0±19.0	67.0±31.0	94.0±7.0	97.0±6.0	-17.0±8.0	-4.0±6.0	0.0±12.0	6.0±7.0
OMb	5.0±3.0	-1.0±3.0	-6.0±5.0	0.0±6.0	-2.0±2.0	-6.0±6.0	102.0±8.0	80.0±6.0	65.0±12.0	50.0±7.0
MMb	67.0±17.0	30.0±7.0	10.0±10.0	22.0±22.0	3.0±1.0	4.0±2.0	10.0±10.0	13.0±5.0	24.0±15.0	30.0±4.0

Supplementary data to the manuscript:

Color of ground beef as affected by mitochondrial substrate respiration and exposure to oxygen

by Vinh , T. P. et al

Supplementary Table 1- Composition of Krebs cycle ingredients used for each sample. Each combination of fat tissue and age was measured on the same day, while the production and measuring order of the Krebs cycle addidtives were randomized.

No	O_2 (v/v%)	Succinate (fraction)	Pyruvate (fraction)	Glutamate (fraction)	Malate (fraction)	Citrate (mol/kg)	Pork/beef fat tissue	Age	Day	Glutamate-malate level	Glutamate-malate (fraction)	Total mixture level (mol/kg)
1	0	0	0	0.225	0.0575	0.3	Beef	Young	1	0.25	0.9	0.1
2	0	0	0.45	0.3375	0.1125	0.3	Beef	Young	1	0.75	0.45	0.1
3	0	0.05	0	0	0	0	Beef	Young	1	0	0	0.05
4	0	0.225	0.225	0	0	0.15	Beef	Young	1	0	0	0.05
5	0	0.2	0.2	0.15	0.05	0	Beef	Young	1	0.75	0.2	0.05
6	0	0.05	0.05	0	0	0	Beef	Young	1	0	0	0.1
7	0	0.3	0.3	0.075	0.225	0.3	Beef	Young	1	0.25	0.3	0.1
8	0	0.225	0	0.16875	0.05625	0.15	Beef	Young	1	0.75	0.225	0.05
9	0	0	0.45	0	0	0.15	Beef	Young	1	0	0	0.05
10	0	0	0.3	0.075	0.225	0	Beef	Young	1	0.25	0.3	0.05
11	0	0.3	0.3	0.075	0.225	0.3	Beef	Young	1	0.25	0.3	0.1
12	0	0.05	0	0	0	0	Beef	Young	1	0	0	0.05
13	0	0	0.1	0	0	0	Beef	Young	1	0	0	0.1

14	0	0.4	0.4	0.3	0.1	0	Beef	Young	1	0.75	0.4	0.1
15	0	0.9	0	0	0	0.3	Beef	Young	1	0	0	0.1
16	0	0.15	0.15	0.0375	0.1125	0.15	Beef	Young	1	0.25	0.15	0.05
17	0	0	0	0	0	0.05	Beef	Young	1	0	0	0.05
18	0	0.05	0	0.15	0.45	0	Beef	Young	1	0.25	0.05	0.1
19	0	0	0	0.45	0.15	0	Beef	Young	1	0.75	0.05	0.05
20	75	0.45	0	0	0	0.15	Beef	Young	1	0	0	0.05
21	75	0	0	0.3375	0.1125	0.15	Beef	Young	1	0.75	0.45	0.05
22	75	0	0.05	0	0	0	Beef	Young	1	0	0	0.05
23	75	0	0	0.3	0.9	0	Beef	Young	1	0.25	0.1	0.1
24	75	0.4	0.4	0.1	0.3	0	Beef	Young	1	0.25	0.4	0.1
25	75	0.45	0.45	0	0	0.3	Beef	Young	1	0	0	0.1
26	75	0.3	0.3	0	0	0	Beef	Young	1	0	0	0.05
27	75	0.15	0.15	0.1125	0.0375	0.15	Beef	Young	1	0.75	0.15	0.05
28	75	0.1	0	0	0	0	Beef	Young	1	0	0	0.1
29	75	0.45	0	0.1125	0.3375	0.3	Beef	Young	1	0.25	0.45	0.1
30	75	0.3	0.3	0.225	0.075	0.3	Beef	Young	1	0.75	0.3	0.1
31	75	0.3	0	0.225	0.075	0	Beef	Young	1	0.75	0.3	0.05
32	75	0	0.9	0	0	0.3	Beef	Young	1	0	0	0.1
33	75	0	0	0.05	0	0	Beef	Young	1	1	0.05	0.05
34	75	0	0.225	0.05625	0.16875	0.15	Beef	Young	1	0.25	0.225	0.05
35	75	0	0	0	0.05	0	Beef	Young	1	0	0.05	0.05
36	75	0	0.05	0.45	0.15	0	Beef	Young	1	0.75	0.05	0.1
37	75	0	0.05	0	0	0	Beef	Young	1	0	0	0.05
38	75	0	0	0.3	0.9	0	Beef	Young	1	0.25	0.1	0.1
39	75	0.2	0.2	0.05	0.15	0	Beef	Young	1	0.25	0.2	0.05
40	0	0	0	0.1	0	0	Pork	Young	2	1	0.1	0.1
41	0	0.05	0.05	0	0	0	Pork	Young	2	0	0	0.1

42	0	0.05	0	0.45	0.15	0	Pork	Young	2	0.75	0.05	0.1
43	0	0.05	0	0	0	0	Pork	Young	2	0	0	0.05
44	0	0.3	0.3	0.225	0.075	0.3	Pork	Young	2	0.75	0.3	0.1
45	0	0	0.45	0	0	0.15	Pork	Young	2	0	0	0.05
46	0	0	0.45	0.1125	0.3375	0.3	Pork	Young	2	0.25	0.45	0.1
47	0	0.9	0	0	0	0.3	Pork	Young	2	0	0	0.1
48	0	0.4	0.4	0.1	0.3	0	Pork	Young	2	0.25	0.4	0.1
49	0	0	0	0	0.1	0	Pork	Young	2	0	0.1	0.1
50	0	0	0	0.15	0.45	0	Pork	Young	2	0.25	0.05	0.05
51	0	0.225	0.225	0	0	0.15	Pork	Young	2	0	0	0.05
52	0	0.15	0.15	0.1125	0.0375	0.15	Pork	Young	2	0.75	0.15	0.05
53	0	0.2	0.2	0.05	0.15	0	Pork	Young	2	0.25	0.2	0.05
54	0	0	0.1	0	0	0	Pork	Young	2	0	0	0.1
55	0	0.225	0	0.05625	0.16875	0.15	Pork	Young	2	0.25	0.225	0.05
56	0	0	0.1	0	0	0	Pork	Young	2	0	0	0.1
57	0	0	0.3	0.225	0.075	0	Pork	Young	2	0.75	0.3	0.05
58	0	0	0	0.0575	0.225	0.3	Pork	Young	2	0.75	0.9	0.1
59	0	0.15	0.15	0.1125	0.0375	0.15	Pork	Young	2	0.75	0.15	0.05
60	75	0.2	0.2	0.15	0.05	0	Pork	Young	2	0.75	0.2	0.05
61	75	0.4	0.4	0.3	0.1	0	Pork	Young	2	0.75	0.4	0.1
62	75	0	0	0.1125	0.3375	0.15	Pork	Young	2	0.25	0.45	0.05
63	75	0.1	0	0	0	0	Pork	Young	2	0	0	0.1
64	75	0	0.225	0.16875	0.05625	0.15	Pork	Young	2	0.75	0.225	0.05
65	75	0	0	0	0	0.1	Pork	Young	2	0	0	0.1
66	75	0.45	0	0.3375	0.1125	0.3	Pork	Young	2	0.75	0.45	0.1
67	75	0.15	0.15	0.0375	0.1125	0.15	Pork	Young	2	0.25	0.15	0.05
68	75	0	0.9	0	0	0.3	Pork	Young	2	0	0	0.1
69	75	0	0	0.9	0.3	0	Pork	Young	2	0.75	0.1	0.1

70	75	0	0.05	0.15	0.45	0	Pork	Young	2	0.25	0.05	0.1
71	75	0.3	0.3	0	0	0	Pork	Young	2	0	0	0.05
72	75	0	0	0.9	0.3	0	Pork	Young	2	0.75	0.1	0.1
73	75	0.45	0.45	0	0	0.3	Pork	Young	2	0	0	0.1
74	75	0	0.05	0	0	0	Pork	Young	2	0	0	0.05
75	75	0.3	0.3	0.075	0.225	0.3	Pork	Young	2	0.25	0.3	0.1
76	75	0.1	0	0	0	0	Pork	Young	2	0	0	0.1
77	75	0.3	0	0.075	0.225	0	Pork	Young	2	0.25	0.3	0.05
78	75	0.45	0	0	0	0.15	Pork	Young	2	0	0	0.05
79	0	0	0	0	0.05	0	Beef	Old	3	0	0.05	0.05
80	0	0.45	0	0.1125	0.3375	0.3	Beef	Old	3	0.25	0.45	0.1
81	0	0.45	0	0	0	0.15	Beef	Old	3	0	0	0.05
82	0	0	0	0.3	0.9	0	Beef	Old	3	0.25	0.1	0.1
83	0	0.3	0.3	0	0	0	Beef	Old	3	0	0	0.05
84	0	0.1	0	0	0	0	Beef	Old	3	0	0	0.1
85	0	0.15	0.15	0.1125	0.0375	0.15	Beef	Old	3	0.75	0.15	0.05
86	0	0.3	0.3	0.225	0.075	0.3	Beef	Old	3	0.75	0.3	0.1
87	0	0	0.225	0.05625	0.16875	0.15	Beef	Old	3	0.25	0.225	0.05
88	0	0.45	0.45	0	0	0.3	Beef	Old	3	0	0	0.1
89	0	0	0	0	0	0.1	Beef	Old	3	0	0	0.1
90	0	0	0	0.3	0.9	0	Beef	Old	3	0.25	0.1	0.1
91	0	0	0	0.05	0	0	Beef	Old	3	1	0.05	0.05
92	0	0.2	0.2	0.05	0.15	0	Beef	Old	3	0.25	0.2	0.05
93	0	0	0.9	0	0	0.3	Beef	Old	3	0	0	0.1
94	0	0	0.05	0	0	0	Beef	Old	3	0	0	0.05
95	0	0.4	0.4	0.1	0.3	0	Beef	Old	3	0.25	0.4	0.1
96	0	0	0.05	0.45	0.15	0	Beef	Old	3	0.75	0.05	0.1
97	0	0	0	0.3375	0.1125	0.15	Beef	Old	3	0.75	0.45	0.05

98	0	0.3	0	0.225	0.075	0	Beef	Old	3	0.75	0.3	0.05
99	75	0.05	0	0	0	0	Beef	Old	3	0	0	0.05
100	75	0.05	0.05	0	0	0	Beef	Old	3	0	0	0.1
101	75	0	0.3	0.075	0.225	0	Beef	Old	3	0.25	0.3	0.05
102	75	0.4	0.4	0.3	0.1	0	Beef	Old	3	0.75	0.4	0.1
103	75	0	0.1	0	0	0	Beef	Old	3	0	0	0.1
104	75	0.15	0.15	0.0375	0.1125	0.15	Beef	Old	3	0.25	0.15	0.05
105	75	0	0.45	0	0	0.15	Beef	Old	3	0	0	0.05
106	75	0.05	0	0	0	0	Beef	Old	3	0	0	0.05
107	75	0.225	0.225	0	0	0.15	Beef	Old	3	0	0	0.05
108	75	0.9	0	0	0	0.3	Beef	Old	3	0	0	0.1
109	75	0.05	0	0.15	0.45	0	Beef	Old	3	0.25	0.05	0.1
110	75	0.15	0.15	0.0375	0.1125	0.15	Beef	Old	3	0.25	0.15	0.05
111	75	0	0.45	0.3375	0.1125	0.3	Beef	Old	3	0.75	0.45	0.1
112	75	0	0.1	0	0	0	Beef	Old	3	0	0	0.1
113	75	0.225	0	0.16875	0.05625	0.15	Beef	Old	3	0.75	0.225	0.05
114	75	0	0	0.45	0.15	0	Beef	Old	3	0.75	0.05	0.05
115	75	0	0	0.225	0.0575	0.3	Beef	Old	3	0.25	0.9	0.1
116	75	0.2	0.2	0.15	0.05	0	Beef	Old	3	0.75	0.2	0.05
117	75	0.3	0.3	0.075	0.225	0.3	Beef	Old	3	0.25	0.3	0.1
118	0	0	0.225	0.16875	0.05625	0.15	Pork	Old	4	0.75	0.225	0.05
119	0	0	0.9	0	0	0.3	Pork	Old	4	0	0	0.1
120	0	0.3	0	0.075	0.225	0	Pork	Old	4	0.25	0.3	0.05
121	0	0.2	0.2	0.15	0.05	0	Pork	Old	4	0.75	0.2	0.05
122	0	0	0	0.9	0.3	0	Pork	Old	4	0.75	0.1	0.1
123	0	0	0	0.9	0.3	0	Pork	Old	4	0.75	0.1	0.1
124	0	0.45	0.45	0	0	0.3	Pork	Old	4	0	0	0.1
125	0	0.3	0.3	0	0	0	Pork	Old	4	0	0	0.05

126	0	0.45	0	0.3375	0.1125	0.3	Pork	Old	4	0.75	0.45	0.1
127	0	0	0	0.1125	0.3375	0.15	Pork	Old	4	0.25	0.45	0.05
128	0	0	0.05	0.15	0.45	0	Pork	Old	4	0.25	0.05	0.1
129	0	0.45	0	0	0	0.15	Pork	Old	4	0	0	0.05
130	0	0.1	0	0	0	0	Pork	Old	4	0	0	0.1
131	0	0.4	0.4	0.3	0.1	0	Pork	Old	4	0.75	0.4	0.1
132	0	0	0.05	0	0	0	Pork	Old	4	0	0	0.05
133	0	0.1	0	0	0	0	Pork	Old	4	0	0	0.1
134	0	0.15	0.15	0.0375	0.1125	0.15	Pork	Old	4	0.25	0.15	0.05
135	0	0.3	0.3	0.075	0.225	0.3	Pork	Old	4	0.25	0.3	0.1
136	0	0	0.05	0	0	0	Pork	Old	4	0	0	0.05
137	75	0	0	0.1	0	0	Pork	Old	4	1	0.1	0.1
138	75	0.3	0.3	0.225	0.075	0.3	Pork	Old	4	0.75	0.3	0.1
139	75	0.05	0.05	0	0	0	Pork	Old	4	0	0	0.1
140	75	0	0	0.15	0.45	0	Pork	Old	4	0.25	0.05	0.05
141	75	0.05	0	0	0	0	Pork	Old	4	0	0	0.05
142	75	0	0.45	0.1125	0.3375	0.3	Pork	Old	4	0.25	0.45	0.1
143	75	0	0	0	0.1	0	Pork	Old	4	0	0.1	0.1
144	75	0.9	0	0	0	0.3	Pork	Old	4	0	0	0.1
145	75	0.4	0.4	0.1	0.3	0	Pork	Old	4	0.25	0.4	0.1
146	75	0.05	0	0.45	0.15	0	Pork	Old	4	0.75	0.05	0.1
147	75	0	0	0.0575	0.225	0.3	Pork	Old	4	0.75	0.9	0.1
148	75	0	0	0	0	0.05	Pork	Old	4	0	0	0.05
149	75	0	0.3	0.225	0.075	0	Pork	Old	4	0.75	0.3	0.05
150	75	0.2	0.2	0.05	0.15	0	Pork	Old	4	0.25	0.2	0.05
151	75	0.15	0.15	0.1125	0.0375	0.15	Pork	Old	4	0.75	0.15	0.05
152	75	0	0.45	0	0	0.15	Pork	Old	4	0	0	0.05
153	75	0.225	0.225	0	0	0.15	Pork	Old	4	0	0	0.05

154	75	0.3	0.3	0.225	0.075	0.3	Pork	Old	4	0.75	0.3	0.1
155	75	0.225	0	0.05625	0.16875	0.15	Pork	Old	4	0.25	0.225	0.05
156	75	0	0.1	0	0	0	Pork	Old	4	0	0	0.1
157	75	0	0	0	0	0	Pork	Old	4	0	0	0
158	75	0	0	0	0	0	Pork	Young	2	0	0	0
159	75	0	0	0	0	0	Beef	Old	3	0	0	0
160	75	0	0	0	0	0	Beef	Young	1	0	0	0
161	0	0	0	0	0	0	Pork	Old	4	0	0	0
162	0	0	0	0	0	0	Pork	Young	2	0	0	0
163	0	0	0	0	0	0	Beef	Old	3	0	0	0
164	0	0	0	0	0	0	Beef	Young	1	0	0	0

157-164 reference samples with only water added (fat type and age varied)