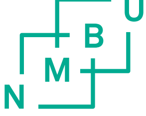

Norwegian University of Life Sciences
Faculty of Chemistry, Biology and Food Science

Philosophiae Doctor (PhD)
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QUALITY CHANGES IN LAMB/ SHEEP AND BEEF MEAT WITH EMPHASIS ON ITS COLOUR AND FLAVOUR

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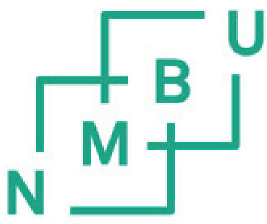
Ås 2017



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Preface

This thesis is for the Philosophiae Doctor (PhD) degree at the Norwegian University of Life Sciences (NMBU) – Faculty of Chemistry, Biotechnology and Food Science (IKBM), Norway. The present work has been carried out under the supervision of Professor Bjørg Egelandsdal (NMBU) and Professor Emeritus Erik Slinde.

The thesis consists of a theoretical introduction, and five scientific papers discussing the importance of variations in colour and colour stability of ground beef (**Paper I** and **Paper II**). The first paper studied a new method of myoglobin quantification and pre-processing of the obtained measurements for myoglobin state quantification. The role of the addition of various mitochondrial substrates to meat is being increasingly recognized, as they can have an important role regarding preservation of the colour stability of ground meat packaged in modified atmosphere (**Paper II**), and might be important in reducing lipid peroxidation in ground meat (**Paper III**).

In the next two papers (**Paper IV** and **manuscript paper V**), the effects of production systems and age on sheep/lamb of different meat qualities were studied. In the final manuscript, various flavour compounds detected by gas chromatography were related to selected sensory attributes of sheep and lamb meat (**manuscript paper V**).

List of errata

	Written	**Should be**
Page VII, line 10	fibers	fibres
Page VII, line 26	Krebs cycle compounds	Krebs cycle substrates
Page VII, line 27	Kubelka- Munk	Kubelka-Munk
Page VIII, line 24	pm	*pm*
Page VIII, line 33	wavelenght	wavelength
Page IX, line 3	fibers	fibres
Page XIII, line 22	forbindelser..	forbindelser.
Page XIII, line 22	Tilslutt	Til slutt
Page 2, line 5, 9	fiber	fibre
Page 2, line 8, 9	fibers	fibres
Page 6, line 14, 15, 16	fibers	fibres
Page 6, line 15	fiber	fibre
Page 7, line 2	$Fe\ ^{2+}$	Fe^{2+}
Page 8, line 29	fibers	fibres
Page 10, line 29	fiber	fibre
Page 14, line 16, 25	fibers	fibres
Page 33, line 1	Mintab	Minitab
Page 46, line 28	75% O_2	75% O_2/ 25% CO_2
Page 49, line 15	α-tokoferolekvivalenter	α-tocopherol equivalentes
Page 49, line 16	α-tocoferol	α-tocopherol
Page 50, line 16	differentation	differentiation
Page 50, line 19	off-flavor	off-flavour
Page 51, line 9	Kathri	Khatri

The doctoral thesis was submitted to the Faculty of Chemistry, Biotechnology and Food Sciences at the Norwegian University of Life Sciences (NMBU, Ås, Norway). The thesis project has been carried out during the time period of 2014-2016 and consists of a theoretical introduction with five scientific papers.

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Dedication

This work is dedicated to my family

and

to my beloved brother, who left us too early.

// Acknowledgments

Creating a Ph. D. thesis requires teamwork and involves many people whom I would like to acknowledge. You will find their names when reading the articles included in this Ph. D. thesis. Anyway, I would like to thank my main Ph.D. supervisor, Professor Bjørg Egelandsdal, and co-supervisor, Erik Slinde, for their supervision, and for being supportive during these years. I also want to thank the members of the "Muscle Food group" for their friendship.

To all of you who have stood by

me in awaiting the end of this work

THANK YOU AGAIN!

Abbreviations

ADP	Adenosine di-phosphate
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
B&H	Bosnia & Herzegovina
BCFA	Branched- chain fatty acids
CDP	Calcium dependent proteases
CSA	Cross-sectional area of fibers
DPPH	2,2-diphenyl-1-picrylhydrazyl / total antioxidant capacity
EMSC	Extended Multiplicative Scatter Correction
ES	Electrical stimulation
ETS	Electron transport system
FA	Fatty acid
FBS	Ferric/ferrous binding substrate
$FADH_2$	Reduced flavine adenine dinucleotide
FAME	Fatty acid methyl esters
FSA	Fetal bovine serum
GSHPx	Selenium-glutathione peroxidase
HB	Hemoglobin
HNE	4-hydroxy-2-nonenal
HPLC	High pressure liquid chromatography
HS-GC/MS	Headspace gas chromatography mass spectrometry
IMF	Intramuscular fat
KCS	Krebs cycle compounds
K/S	Kubelka- Munk ratio
LC/MS	Liquid chromatography mass spectrometry
LO	Lipid oxidation
LTL	*M. longissimus thoracis et lumborum*
MANOVA	Multivariate analysis of variance
MAP	Modified atmospheric pressure
MALDI	Matrix assisted laser desorption/ionization

Mb	Myoglobin
OMb	Oxymyoglobin
MMb	Metmyoglobin
DMb	Deoxymyoglobin
MDA	Malondialdehyde
MDH	Malate dehydrogenase
MN	Montenegro
MRA	Metmyoglobin reducing activity
MSem	*M. semimembranosus*
MSG	Monosodium glutamate
MUFA	Monounsaturated fatty acids
NAD^+	Nicotinamide adenine dinucleotide, oxidative form
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NIR	Near infrared reflectance
NO	Norway
NORA	Nitric oxide reducing ability
NWS	Norwegian White Sheep
OCR	Oxygen consumption rate
PCA	Principal component analysis
PP	Pivska Pramenka
PFP	Peroxide forming potential
PLSR	Partial least square regression analysis
pm	post mortem
PSE	Pale Soft and Exudative
PUFA	Polyunsaturated fatty acid
PV	Peroxide values
PVC	Polyvinyl chloride
RIMF	Resistance to induced met myoglobin formation
RMSECV	Root mean square error of cross validation
SFA	Saturated fatty acid
SOD	Superoxide dismutase
SW	Selected wavelenght
ROS	Reactive oxygen species

TAG	Triacylglycerols, triglycerides
TBARS	Thiobarbituric acid reactive substances
TNF	Total number of fibers
VOC	Volatile compounds
VP	Vacuum packaging
VPr	Vlasicka Pramenka
WB	Western Balkan
WHC	Water holding capacity

Abstract

The term known as meat quality is related to complex biochemical processes and changes that occur in the animal tissue before and after slaughtering. From an analytical point of view, the more factors describing meat quality that have been measured, the more compounds researchers would have liked to assess.

The colour of fresh meat is one of the more important quality characteristics, appreciated at first glance, noted, and accepted or refused by consumers. The colour of the meat and meat products is also a result of complex physical, chemical, and biological *post mortem* (*pm*) processes. Thus, the errors in the production process can firstly be observed as a less optimal and characteristic colour of the product, thereafter followed by undesirable odour and flavour.

In paper I, a method for preparing the different myoglobin (Mb) redox states on a surface of ground beef mixed with fat tissues from either beef or pork is presented. In addition, a principle for the calculation of myoglobin redox states was presented. The oxygen pressure packaging was used to induce oxymyoglobin (OMb) and deoxymyoglobin (DMb) redox states on surfaces of ground beef, while a metmyoglobin (MMb) state was produced by using potassium ferricyanide $K_3[Fe(CN)_6]$. After three storage times, reflectance spectra were measured on surfaces of ground beef, and multivariate regression analyses were performed. The transformations of the spectra were done by using extended multiplicative scatter correction (EMSC). Principal component analysis (PCA) was used to explore and diagnose the relationship between the three different Mb states, while partial least square (PLS) regression was used to find the fundamental relations between two matrices (X and Y) – spectra and Mb states. Validation of the model resulted in, after correction and normalization, prediction errors of about 5% for all the three Mb states.

In paper II, the capacity of mitochondrial substrates in order to improve meat colour stability was studied. The antioxidants nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide ($FADH_2$) presumed formed through the metabolism of added glutamate, malate, succinate, pyruvate, and citrate (up to 0.1 mol/kg) altered the Mb redox forms of ground beef mixed with fat (ground porcine or bovine). The ground beef (*M. semimembranosus*) mixed with fat (14%) was stored in darkness at 4º C for 8 days in aerobic (75% O_2 and 25% CO_2), and for 13 days in anaerobic (60% CO_2 and 40% N_2) conditions.

In aerobic packaging, the highest OMb level occurred with a molar ratio of glutamate-malate to citrate of 3:1. OMb was more prevalent after 6-8 days of storage in the aerobic condition.
In anaerobic packaging, a mixture of succinate and glutamate generated DMb rapidly and it endured this state for 13 days. Pyruvate induced MMb formation, acting as a pro-oxidant. $FADH_2$ presumed formed through oxidation of succinate was most effective at converting MMb to DMb in anaerobic packaging.

In paper III, how different Krebs cycle substrates (KCS) may affect lipid oxidation of packaged ground beef during storage in modified atmosphere packaging (MAP) was explored. The mixtures of succinate, pyruvate, glutamate, malate and citrate combined with two different age and fat types were mixed and packaged in MAP for 13 and 8 days, respectively. The type of fat tissue influenced the development of lipid oxidation. Moreover, KCS such as glutamate, malate, and citrate, increased peroxide forming potential (PFP) and Thiobarbituric acid reactive substances (TBARS) under high oxygen storage conditions.

In Paper IV and manuscript paper V, quality characteristics of sheep/lamb meat from Bosnia and Herzegovina (B&H), Montenegro (MN), and Norway (NO) were identified and compared. Breed, production systems, and age differences were evident for sheep/lamb originated from specific grazing regions. The autochthonous Pramenka breed (Vlasicka and Pivska) was, in general, smaller, with higher fat content, better tenderness (only for B&H), and better colour stability compared to crossbreed Norwegian White sheep that had a better nutritional value. After the identification of quality characteristics, the animal groups were additionally investigated for sensory and flavour characteristics.
The volatile and metabolite profiles of lipid tissue and lean meat were first generally related to sensory attributes. Sensory gamy, grassy, and bitter flavours were related to different compounds. Meats from each sheep/lamb group were also associated with specific compounds. Finally, manuscript paper V discusses characteristic flavour compounds identified in different production systems prevalent in B&H, MN, and NO.

Sammendrag

Begrepet kjøttkvalitet er forbundet med komplekse biokjemiske prosesser og endringer som finner sted i dyremuskler før og etter slakting. Fra et analytisk synspunkt er det slik at, jo flere faktorer som beskriver kjøttkvalitet blir målt, dess mer komplett blir kvaliteten blir presentert.

Fersk kjøttfarge er en av de viktigste kvalitetsegenskapene, verdsatt ved første blikk, notert, akseptert eller avist av forbrukerne. Fargen til kjøtt og kjøttprodukter er også et resultat av komplekse fysiske, kjemiske og biologiske *post mortem* (*pm)* prosesser. En feil i løpet av produksjonsprosessen kan resultere i mangel på optimal og karakteristisk produktfarge, etterfulgt av uønsket lukt og smak.

I artikkel I er en metode for å lage de de tre ulike myoglobin (Mb) -formene i en blanding sammensatt av kjøtt og fett fra enten storfe eller svin brukt for å predikere Mb tilstand. Oksygenpartialtrykk ble brukt til å indusere oxymyoglobin (OMb) og deoxymyoglobin (DMb) redox former på overflater av pakket kjøttdeig. Metmyoglobin (MMb) formen ble produsert ved hjelp av kalium ferricyanid [K_3Fe $(CN)_6$] Etter tre forskjellige lagringstider, ble reflektansspektra målt på kjøttdeig, og multivariat regresjonsanalyse ble utført. Transformasjon av spektrene ble gjort ved hjelp av utvidet multiplikativ spredningskorreksjon (EMSC). Principal komponent analyse (PCA) ble brukt til å utforske og diagnostisere forholdet mellom de tre forskjellig fremstilte myoglobin (Mb) former, mens "partial last square" (PLS) regresjon ble benyttet for å finne forholdet mellom to matriser (X og Y) - spektra og Mb-former. Validering av modellene ga etter korrigering og normalisering, prediksjonsfeil på ca 5% for alle de tre Mb formene.

I artikkel II ble kapasiteten til mitokondrielle substrater for å bevare kjøttfarges undersøkt. Antioksidantene nikotinamid-adenin-dinukleotid (NADH) og flavine-dinukleotid ($FADH_2$) dannet gjennom metabolisme av substratene glutamat, malat, suksinat, pyruvat og sitrat. Disse substratene er blitt tilsatt (opp til 0,1 mol / kg) til kjøttdeig fra *M. semimembranosus* og blandet med svine- eller storfefett. Kjøttdeigen ble lagret i mørke ved fire grader i 8 dager under aerobe (75% O_2 og 25% CO_2), og i 13 dager under anaerobe (60% CO_2 og 40% N_2) betingelser, noe som førte til at Mb former ble endret.

Det høyeste nivå av OMb forekom under aerobe betingelser med et molart forhold mellom glutamat-malat til sitrat på 3:1. OMb var mer hyppig etter 6-8 dagers lagring under aerobe betingelser.

I anaerob emballasje, genererte en blanding av ravsyre og glutamat raskt DMb og denne holdt seg stabil i 13 dager. Pyruvat induserte MMb formasjon, og fungerte som en pro-oksidant. $FADH_2$ dannet gjennom oksidasjon av ravsyre var mest effektiv med hensyn til å konvertere MMb til DMb under anaerobe betingelser.

I artikkel III ble det utforsket hvordan ulike Krebs syklus substrater (KCS) kunne påvirke lipid oksidasjon av kjøttdeig pakket og lagret i MAP. Ravsyre, pyruvat, glutamat, malat og sitrat ble tilsatt kjøtt og fett fra storfe med ulik alder samt svinefett. Blandingene ble pakket i MAP i henholdsvis 8 og 13 dager. Fettype påvirket utviklingen av lipidoksidasjon. Glutamat, malate og sitrat økte potensialet for peroksiddannelse (PFP) samt tiobarbitursyre-reaktive forbindelser (TBARS) når høy oksygen pakking ble benyttet.

I artikkel IV og manuskript V ble kvalitet av sau/lamme kjøtt fra Bosnia-Hercegovina (B&H), Montenegro (MN) og Norge (NO) undersøkt. Produksjonssystemer og aldersforskjeller var signifikant for sau/lam som kom fra forskjellige beiteregioner. Den opprinnelige Pramenka-rasen (Vlasicka og Pivska) besto av mindre dyr, med høyere fettinnhold, bedre mørhet (kun B&H), og bedre fargestabilitet i forhold til den «syntetiske rasen»- norsk hvit sau (NWS). Ernæringsmessig hadde NWS bedre egenskaper. Etter å ha undersøkt kvalitetsegenskaper ble også kjøtt fra dyregruppene undersøkt sensorisk. Flyktige organiske forbindelser samt metabolitter fra henholdsvis fett og magert kjøtt ble først generelt relatert til sensoriske attributter. Sensorisk vilt-, gress- og bittersmak ble relatert til ulike forbindelser. Kjøtt fra saue-/lammegrupper ble også relatert til spesifikke kjemiske forbindelser.. Tilslutt diskuteres hvordan karakteristiske aromakomponenter kan relateres til ulike produksjonssystemer i B&H, MN og NO.

List of papers:

1. Bjelanovic, M., Sørheim, O., Slinde, E., Puolanne, E., Isaksson, T. & B. Egelandsdal (2013). Determination of the myoglobin states in ground beef using non-invasive reflectance spectrometry and multivariate regression analysis. *Meat Science*, *(95),* 451–457.

2. Bjelanovic, M., Egelandsdal, B., Phung, V.T., Langsrud, Ø., Sørheim, O., Hunt, M. & E. Slinde. (2016) Effects of metabolic substrates on myoglobin redox forms in packaged ground beef. *Food Packaging and Shelf Life*, *(8)* 24–32.

3. Yi, G., Grabež, V., Bjelanovic, M., Slinde, E., Olsen, K., Langsrud, O., Phung, V. T.,Haug, A., Oostindjer, M. & B. Egelandsdal (2015) Lipid oxidation in minced beef meat with added Krebs substrates to stabilize colour. *Food Chemistry,* (*187*), 563-571.

4. Bjelanović, M., Grabež, V., Vučić, G., Martinović, A., Lima, L. R., Marković, B., & B. Egelandsdal (2015) Effect of different production systems on carcass and meat quality of sheep and lamb from Western Balkan and Norway. *Journal of Biotechnology in Animal Husbandry, 31*(2), 203-221.

5. Grabež, V., Bjelanović, M., Rohloff, J., Martinović, A., Berg, P., Tomović, V., Rogić, B., & B. Egelandsdal (2017) The relationship between volatile compounds, metabolites and sensory attributes: a case study using lamb and sheep meat. *Manuscript*

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1 Introduction - meat quality

Meat quality is a broad topic. It is related to the properties of the meat itself and to the technological conditions that the meat is subjected to. Major food quality issues imply safety/health, sensory attributes, and sustainability (reduction of valuable nutrients loss) and even animal welfare.
Meat is a nutrient dense food with a composition that varies depending on the type of muscle (complex in terms of structural and biochemical properties), origin of the muscle (ruminants, pigs, poultry), and the preparation process (curing, drying, fermentation or sausage making) (Dikeman & Devine, 2014).

Meat quality perception differs depending on the point of view, but generally, the concept is in constant change and varies between producers, slaughterers, processors, distributors, and consumers. The most challenging quality questions are associated with nutritional value, production systems, breeds, and processing strategies (meat and additives with positive health benefits). Increased attention is also paid to the so-called "ethical quality" involving religious slaughtering and extreme breeding goals. Finally, sustainability debates involving low feed conversion for some animals and the impact on environments of feed digestion for ruminants are of concern to consumers.

The Food and Agriculture Organization of the United Nations (FAO, 2014) refers to meat quality as compositional quality (lean to fat ratio) and palatability factors such as visual appearance, smell, firmness, juiciness, tenderness, and flavour.

Some *ante-mortem* factors are nutrition, age, gender, live weight (Argüello, Castro, Capote, & Solomon, 2005), stress, genotype, transportation, lairage time, season of the year of slaughtering, and the environmental conditions' influence on meat quality (Küchenmeister, Kuhn, & Ender, 2005).

Freezing/storage (Muela, Sandüo, Campo, Medel, & Beltran, 2012), aging (Campo, Sañduo, Panea, Alberti, & Santolaria, 1999), type of conservation (Medel, et al., 2002), electrical stimulation that increases pH decline and anaerobic glycolysis (Mombeni, Mombeini, Figueiredo, Siqueira, & Dias, 2013) are factors of importance *post mortem* (*pm*) for meat quality.

Variation in meat quality is also dependent on muscle fibre type, fibre areas, and capillary density of specific muscles that influence *ante mortem (am)* and *post mortem* biochemical processes (Klont, Brocks, & Eikelenboom, 1998). In adult skeletal muscle, there are different types of muscle fibre groups: slow-oxidative or type I, fast oxido-glycolytic or type IIA, and fast glycolytic IIX and IIB fibres (Schiaffino & Reggiani, 1996). Fiber type composition effects muscle metabolic properties differently (Ozawa, et al., 2000; Lee, et al., 2015), due to different enzyme activity patterns (Pette & Spamer, 1987). Major factors that represent muscle mass and meat quality, are morphological traits of fibers, like total number of fibers (TNF) cross-sectional area of fibers (CSA) (Joo, Kim, Hwang, & Ryu, 2013) and their length. Muscle fiber type is an important factor in colour stability, tenderness, water holding capacity, and edible quality of beef meat (Klont, Brocks, & Eikelenboom, 1998).

Marcinkowska-Lesiaka, et al. (2015) pointed out that meat quality is the main criterion of food product evaluation, where storage has a direct impact on quality changes. Some of the main causes of quality changes are microbiological breakdown, lipid oxidation, and discolouration (Šuput, Lazić, Lević, Pezo, Tomović, & Hromiš, 2013; Marcinkowska-Lesiaka, et al., 2015). On final shelf life date of fresh meat, the quality is determined by factors such as: unacceptable aroma, appearance, and colour, and unacceptable microbial counts (Antoniewski, Barringer, Knipe, & Zerby, 2007; Pietrasik, Dhanda, Shand, & Pegg, 2006).

Growth of microorganisms and their ability to cause spoilage of, for example, vacuum-packed lamb meat is dependent on many factors, such as: type and initial concentration of spoilage bacteria, meat pH, water activity, availability of substrates, oxygen availability, storage time, and temperature of the packaged product (Mills, Donnison, & Brightwell, 2014).

2 Objectives

The five papers have the following specific objectives:

1. The prediction of myoglobin (Mb) states in stored ground beef samples with fat and water added, and evaluation of the robustness of the prediction.
2. Investigate how Krebs cycle substrates (KCS) and their combinations at various concentrations stabilize Mb redox states of ground beef in both aerobic and anaerobic packaging conditions.
3. Explore how KCS prevent or accelerate lipid oxidation during storage in ground beef packaged in MAP.
4. The influence of the different production systems, covering age and breed variation on standard meat quality measurements; and additional investigations of vitamin E, protein, dry matter, water and haem content, TBARS, PV, DPPH, cooking loss, conformation score, fat content, and fatty acid composition of sheep and lamb meat.
5. The impact of the different volatiles and metabolites on commonly used sensory attributes often associated with unwanted flavour of sheep and lamb. Use the flavour compounds from different metabolic pathways to discuss correlation with sensory attributes using a flavoromics approach.

There is not much discussion in the literature related to the accuracy of predicted Mb states in ground beef systems. The focus was on making a local calibration model for the evaluation of the colour stability in ground beef during storage in different MAP systems.
The experimental work was initiated to determine the capacity of KCS combinations to stabilize Mb redox states of ground beef in MAP. The research carried out covered a greater complexity than previously reported when adding KCS at different levels to ground bovine meat, mixed with either ground porcine or bovine fat. This was done to identify optimal blends for improving meat colour stability in MAP.

KCS substrates can, in principle, act both as pro-oxidants and anti-oxidants and thereby affect lipid oxidation in different MAP packaging. The experimental work was carried out to categorize KCS with respect to undesirable colour development and lipid oxidation.

The indigenous Pramenka sheep breed undoubtedly constitutes a valuable breed in Balkan countries, but little is described about this animal's genetic resource in literature. The experimental work was initiated to determine the physical and chemical characteristics of the Western Balkans (WB) Pramenka sheep (PS) as raised in the Balkans. It was, for the sake of having some sort of meat sample that was well known in Norway, compared with the characteristics of the Norwegian White Sheep (NWS) crossbreed dominating the market in Norway. However, even the quality of the meat from the Norwegian White Sheep (NWS) is not well explored.

In addition, volatiles and metabolites characterising lamb/sheep meat flavour from the different animal groups, as well as the sensory properties of meat from these animals, were investigated. This work is especially important to the Balkans because the WB Pramenka sheep has never been investigated with respect to volatiles and metabolites as well as sensory properties. Finally, the quality of WB Pramenka meat has never been compared to the meat quality of other sheep breeds that have been selected, at least not as a temporary end point for the national breeding program in Norway.

3 Theoretical background

Animal food is almost irreplaceable because of its nutritional values. Some of its important nutrients are proteins, fats, fatty acids, vitamins, minerals etc. The myoglobin (Mb) and haemoglobin (Hb) iron in food of animal origin is more easily absorbed than iron from plant based foods.

The physical and chemical processes that take place in the muscle after an animal's death are very important for the conversion of muscle to meat. The conversion process starts with the slaughter of the animal and bleeding. During the early *pm* hours, a critically important interaction occurs between pH and temperature that affects the meat's water holding capacity (WHC), meat colour, tenderness, juiciness, firmness, extent of muscle shortening, protein denaturation and susceptibility to bacterial growth (Brown, 2015). The interruption of blood circulation leads to loss of oxygen and nutrition transfer to the muscle, and, consequently, the muscle cells shift their energy production from aerobic biochemical pathways to anaerobic metabolism. The level of adenosine triphosphate (ATP) still remains low in *pm* and cannot sustain normal muscle-energy needs (Savell, Muelle, & Baird, 2005). Besides irreversible muscle contractions, lactic acid is formed and the muscle pH decreases from about 7.0 to 5.5-5.8 in ideal conditions, which is a desirable pH for shelf life of meat. If the pH decline is rapid and reaches 5.5-5.8 and muscle temperature is still high (above 36 ºC), the meat may become PSE (Pale Soft and Exudative). PSE occurs in pork, but may also affect beef, lamb, and poultry. The increased acidity of *pm* muscle is caused by the accumulation of lactic acid, which is formed by anaerobic glycolysis where glycogen degrades to lactic acid. Change in pH during conversion of muscle to meat is very important because it affects so many physical, chemical, and sensory traits of meat (Brown, 2015). Thicker fat layers may prevent cold shortening during chilling, fast enzyme inactivation, and contribute to tenderization of the muscle (Savell, Muelle, & Baird, 2005).

3.1 Meat colour

Colour perception is one of the most important physical traits because once colour is defined as unacceptable, all other sensory attributes lose their importance to consumers (Bekhit & Faustman, 2005; Mancini & Hunt, 2005). The colour of meat is a complex topic which involves

animal genetics, conditions *am* and *pm*, basic muscle chemistry, and many factors related to meat processing, packaging, distribution, storage, display and final preparation to consumption (Goñi, Beriain, Indurain, & Insausti, 2007; Mancini & Hunt, 2005).

Post mortem (*pm*) competition for oxygen, primarily between Mb and mitochondria is a mechanism associated with meat colour intensity and stability. Mitochondrial activity affects colour stability and intensity trough:
a) oxygen consumption, a process that results in dark coloured muscle
b) mitochondria-mediated metmyoglobin reduction (Ramanathan, Mancini, & Naveena, 2010), a processes that limits surface discolouration (Ledward D. A., 1992).

In living cells, Mb and mitochondria are interrelated and Mb serves as an oxygen reservoir and oxygen transporter for mitochondria. In addition, mitochondria are important subcellular organelles involved in energy metabolism (Tang, Faustman, Hoagland, Mancini, Seyfert, & Hunt, 2005a). The distribution of the mitochondria in skeletal muscles is uneven, and slow-contracting muscle fibers possess a higher mitochondria concentration compared to fast-contracting fibers (McCormick, 1994). Myoglobin is found in all muscle fiber types, but is present in greater concentrations in type I muscle fibers (Wittenberg & Wittenberg, 2003).

Variation in the activity of isocitrate dehydrogenase is a relatively simple way of comparing the oxidative capacity of the muscles between different animals. This enzyme is pivotal in the oxygen-dependent citric acid cycle of mitochondria, which are greater and more abundant in oxidative myofibres (Hoppeler, 1985). Oxidative capacity of muscle alters due to a range of factors such as increased selection for muscle weight, resulting in an increase in type IIX glycolytic myofibres (Greenwood, Harden, & Hopkins, 2014). An increased animal maturity associates with a greater amount of type I oxidative myofibres (White, McGavin, & Smith, 1978) and selection for high muscling sire also influence oxidative capacity of muscle (Calnan, Jacob, Pethick, & Gardner, 2014).

Meat colour is also determined by the amount of Mb, a globular single chain protein located in sarcoplasm (Suman S. P., Faustman, Stamer, & Liebler, 2007).
Myoglobin is a cytoplasmic hemoprotein composed of 153 amino acids that typically exists in three redox forms: purple- deoxymyoglobin (DMb), red- oxymyoglobin (OMb), and brownish - metmyoglobin (MMb). During storage time, the meat colour surface changes upon exposure to

oxygen, primarily from DMb to OMb and then to MMb (Calnan, Jacob, Pethick, & Gardner, 2014). The iron in DMb and OMb is in the reduced state (ferrous Fe^{2+}), while MMb comes as a result of an oxidation of the ferrous (Fe^{2+}) Mb to ferric (Fe^{3+}) state (Figure 1).

The OMb state is associated with desired colour in fresh meat, where its iron is still in the ferrous state, but where the distal histidine interacts with bound oxygen, both Mb's structure and stability alter. The thickness of the OMb layer and depth of oxygen penetration in the muscle tissue can all be affected by high temperature, low pH, very low oxygen partial pressure (OPP), and low MMb reducing ability, resulting with MMb formation (Sørheim, Westad, Larsen, & Alvseike, 2008; Mancini & Hunt, 2005).

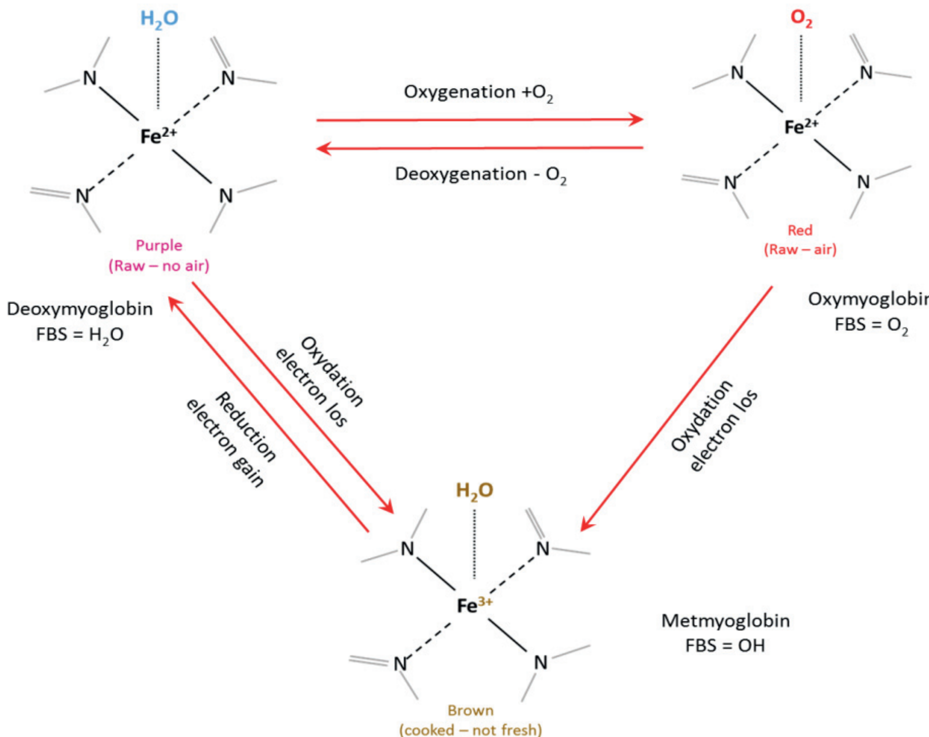

Figure 1: Different Mb redox states and their interconversion. The figure is adapted from (Suman, Faustman, Stamer, & Liebler, 2007)

During oxidation, electron loss leads to the transformation of Fe^{2+} to Fe^{3+}. DMb turns to OMb when exposed to oxygen. The redox conversion of OMb to DMb is described by Mancini &

Hunt (2005) as an indirect, two-step process. The OMb firstly appears to convert MMb as the muscle consumes oxygen, consequently, a low oxygen partial pressure forms and autoxidizes the haem iron, which produces MMb. Depending on the muscle's reducing capacity and the meat temperature, MMb can then be converted to DMb. To get predominant DMb on the meat surface, the packaging atmosphere needs to contain less than 0.1% O_2 (Sørheim, Westad, Larsen, & Alvseike, 2008). Mancini & Hunt (2005) also emphasized that absence of oxygen in MAP packaging allows for the formation of the surface DMb.

3.2 Flavour

One of the most important single factor for meat acceptability is flavour (Theunissen, Kouwenhoven, & Blauw, 1979). It is often dependent of consumer's personal perception of flavour. In the flavour profile, words used to describe nuances of flavour are umami, sour, rancid, metallic, bitter, sweet etc.

Several *am* and *pm* factors like animal diet, breed, gender, age, aging, and cooking conditions are related to flavour development of cooked meat (Khan, Jo, & Tariq, 2015; Melton, 1990). Crocker (1948) and Bender & Ballance (1961) emphasized that interactions between volatile compounds, nonvolatile compounds (free amino acids, peptides, reducing sugars, vitamins, and nucleotides), and lipids via Strecker degradation, Maillard reactions, thermal processing, and/or oxidation, develop the overall flavour of beef. A grain-based type of diet is associated with greater accumulation of branched-chain fatty acids (BCFA), some aldehydes, and lactones in meat, while meat fat from grazing animals contains high levels of phenols, terpenes, indoles and sulphur compounds. In ruminants, precursors or aroma compounds can also originate from ruminal microorganisms or by a direct transfer from feeds (Vasta & Priolo, 2006).

Diet affects carcass conformation, the amount of total fat, intramuscular fat (IMF), and its fatty acid composition in the meat. This has a direct effect on meat texture and juiciness as well as flavour, and its release during eating (Watkins, Frank, Singh, Young, & Warner, 2013; Roberto Germano Costa, de Araújo Filho, de Sousa, Neto, Madruga, & Fraga, 2010). An increased proportion of type I fibers, (rich in phospholipids) in cattle and lambs is associated with improved juiciness and flavour of cooked meat (Maltin, Warkup, Matthews, Grant, Porter, & Delday, 1997; Valin, Touraille, Vigneron, & Ashmore, 1982; Kriketos, et al., 1996). However,

the increased rancid taste of meat is associated with the high content of PUFA in phospholipids (Wood J. , et al., 2008).

Diet, together with the digestive system of the animal and biosynthetic process within the animal influences FA composition as well (Woods & Fearon, 2009). The amount of IMF is affected by animal breed, slaughter weight (Park, et al., 2002), growth rate (Smith, Kawachi, Choi, Choi, Wu, & Sawyer, 2009), and feeding strategy (Du, Yinb, & Zhu, 2010). According to Hwang, Kim, Jeong, Hur & Joo (2010), IMF differs among species and increases with age. IMF deposition is highly heritable and increases with the body fat of the animal and is negatively correlated with white muscle fibre and positively correlated with red muscle fibre (Hwang, Kim, Jeong, Hur, & Joo, 2010).

The composition of the meat with respect to the fat content will also affect the release of flavour compounds. Wood et al. (2008) argue that fatty acid composition either from adipose tissue or muscles is generally similar, but adipose tissue has higher content of FA compared to muscle. There are high levels of PUFA in pig adipose tissue and muscle including the long chain (C20-C22) PUFA. In sheep adipose tissue, the full range of PUFA are also found, and also in muscle phospholipids, where cattle "conserve" their long chain PUFA.

Apart from its naturally associated flavour to specific type of meat, the fat can also contribute to other flavours related to the animal diet. Certain aromatic compounds derived from lipid oxidation can again interact with other volatile compounds (Mottram, 1998), and impart characteristic odour or flavour components to the meat (Nollet, 2012). Any changes in diet that affect the final protein or antioxidant status of muscle derived from sheep can affect the final flavour characteristics. The variation in meat flavour is also associated with pasture differences. Diets with white clover, lucerne, phalaris, and rape (*Brassica*) lead to an unacceptable meat flavour. The metabolites obtained through diet or metabolized by animals deposited in the muscle or adipose tissue contribute to meat flavour (Watkins, Frank, Singh, Young, & Warner, 2013). Sheep meat flavour is described as sweaty, oily, acidic, acrid, urinary, faecal, barnyard, and sharp (Wong, Nixon, & Johnson, 1975). Faecal flavour related to skatole is, at least in some production units, a real challenge. Pastoral sheep meat flavour, defined as animal, grassy, sheep-like, barnyard, gamey, milky, and faecal, involves, in addition to skatole, indole and phenols (Priolo, et al., 2004; Young, Lane, Priolo, & Fraser, 2003). Despite the issue with perceived negative flavours of sheep meat, few markers of these off-flavours are agreed upon beyond

skatole/ indole/ phenols and the branched chain fatty acids (BCFA) such as 4-methyloctanoic acid, 4-methylnonanoic acid, and 4-ethyloctanoic acid. The latter compounds are formed in the rumen. The flavour notes of 4-methylnonanoic acid are dairy/cheese and fatty and it is actually recommended as an additive to increase lamb flavour, while 4-ethyloctanoic acid has the characteristic of lamb/ mutton fat flavour with low odour threshold of 1.8 μg/ kg. 4-methyloctanoic acid is similar to 4-ethyloctanoic acid regarding odour thresholds (The God Scents Company). It is worth noting that these compounds were all related to the dislike of smell/odour and seemed not possible to relate to taste/flavour (Watkins, Frank, Singh, Young, & Warner, 2013). The literature is still limited regarding flavour compounds.

Among consumers the lamb/sheep meat is less popular due to its specific flavour and the high melting point of its lipids (Shahidia, Rubinb, D'Souzac, Teranishid, & Butterye, 1986). According to Young, Reid, & Scales (1993) a high average pH value in the meat adversely affects flavour and aroma. Ageing time, which results in the gradual breakdown of the myofibrillar protein structure of meat, may influence the development of flavour through the generation of peptides and amino acids (Wood, et al., 1999). However, Martinez-Cerezo, et al. (2005a) found that ageing time did not affect odour significantly and showed that only breed influenced fat flavour intensity.

3.3 Texture

The texture usually refers to hardness and the structure of meat, and is, among others, associated with *pm* changes in the meat (Shiranita, Miyajima, & Takiyama, 1998). However, some scientists associate texture with tenderness as the mechanical behavior of food, while the others associate it with a psychological response to physical-chemical stimuli caused by chewing (Harries, Rhodes, & Chrystall, 1972).

The texture of the meat is often closely related to age, species, gender, breed and nutritional status of the animals (Szczesniak, 2002). On the other hand, it is directly associated with the size of muscle fiber and the amount of connective tissue, and is affected by the quantity of intramuscular fat (IMF) and, in particular, sarcomere length (Kemp, Sensky, Bardsley, Buttery,

& Parr, 2010). Thus, Joo, Kim, Hwang and Ryu (2013) argued that the coarse, undesirable texture on the transversely cut surface of meat is related with the prevalence of relatively large muscle bundles.

In meat, *pm* proteolytic degradation by endogenous proteolytic enzymes is associated with meat tenderness (Koohmaraie, 1988). Kemp, Sensky, Bardsley, Buttery & Parr (2010) also underlined that the extent of proteolysis of key proteins within muscle fibres is a significant determinant of ultimate tenderness. Numerous proteolytic enzymes found in skeletal muscle play a major role in *pm* tenderization of meat. The calpains are believed to play a major role in the tenderization process of meat from warm-blooded animals (Lonergan, Zhang, & Lonergan, 2010), while cathepsins are more related to tenderization of fish muscle (Warriss, 2010; Koohmaraie, 1996). There is considerable evidence that calpains have been identified as calcium dependent proteases (CDP) in *pm* proteolysis and that they play an important role in meat tenderness (Koohmaraie, 1992). m-and μ-calpain known as the two main calpains can be activated by high or low concentrations of Ca^{2+} ions, respectively (Warriss, 2010).

Kemp, Sensky, Bardsley, Buttery & Parr (2010) also observed that an important role in meat *pm* proteolysis is played by calpain and calpain-specific inhibitors that play a vital role in meat tenderization and act as a marker for meat quality. According to these authors, other novel proteolytic systems, such as caspases, may contribute to *pm* proteolysis and meat tenderization, but this was not well examined. The proteolytic enzymes calpains are activated by Ca- ions and are inhibited by the endogenous calpastatins which reduce proteolysis in muscles (Warriss, 2010). Calpastatin levels are quite variable across several animal-related elements. If the calpastatin levels increase, the *pm* tenderization of meat occurs due to lower proteolysis activity of calpain (Kerth, 2013).

According to Olivera, Bambicha, Laporte & Coll (2013) storage period, temperature and packaging conditions are associated with the extent of texture and colour variations of raw beef. However, the effects of aerobic packaging conditions and the highest storage temperature result in decreased redness, while tenderness of raw beef meat increases during storage times in aerobic and vacuum packaging.

3.4 Sensory evaluation of meat

The accepted and endorsed definition of sensory evaluation has been defined as a scientific method used to evoke, measure, analyse, and interpret those responses to products as perceived through the senses of sight, smell, touch, taste, and hearing (Hildegarde & Harry, 1998). Quality of meat and meat products is estimated by sensory and laboratory (objective, analytical) tests. In order to evaluate the quality of meat, a good knowledge of the individual characteristics of meat, such as colour, smell, taste, and consistency is necessary (Bejerholm & Aaslyng, 2004).

It is known that the sensory quality of the meat may be changed after heating and depends to a large extent on the treatment method and the temperature reached (Heymann, Herdrick, Karrasch, Eggeman, & Ellersieck, 1990; Wood, Nute, Fursey, & Cuthbertson, 1995). The surface and core temperature of the heated meat and the means of the heat transfer (contact, air or steam) has a great influence on the sensory properties (Knipe & Rust, 2010). An increase in a temperature above 110°C accelerates the Maillard reaction (important for the formation of flavouring and colouring agents). An increase of core temperature in the meat influences mostly the juiciness, and then the taste, colour, aroma, and tenderness of meat (Wood, Nute, Fursey, & Cuthbertson, 1995). Selecting proper techniques for thermal processing of fresh meat can affect all sensory quality parameters (Bejerholm & Aaslyng, 2004).
Methodology of sensory assessment involves a number of different types of tests that can be used for analysis, depending on what is desired as the final result (Lawless & Heymann, 2010).
There are international standards that are dedicated to sensory evaluations, consisting of basic standards.
- ISO 6658:2017, Sensory analysis - Methodology - General guidance (ISO - International Organization for Standardization)
- ISO 5492:2008, Sensory analysis - Vocabulary (ISO - International Organization for Standardization)
- ISO 8586:2012, Sensory analysis - General guidance for the selection, training and monitoring of selected and expert assessors (ISO - International Organization for Standardization)
- ISO 8589:2007, Sensory analysis - General guidance for the design of test rooms (ISO - International Organization for Standardization)
- ISO 11037:2011, Sensory analysis - Guidelines for sensory assessment of the colour of products (ISO - International Organization for Standardization)

- IEC 60050-845 CIE 17:1987, International electro technical vocabulary -Chapter 845: Lighting International lighting vocabulary (IEC - International Electrotechnical Commission).

In addition to these, there are standards that describe in detail the different tests used for sensory assessment such as ISO 8587: 2006; Sensory Analysis –Methodology -Rankink, ISO 10399: 2004; Sensory Analysis –Methodology –Duo -trio test, etc. (ISO - International Organization for Standardization). The tests can be divided into discriminatory and descriptive, analytical tests and acceptance tests (*i.e.,* affective tests).

Sensory profiling of sheep and lamb, as stated above, is associated with many different attributes such as: mutton, gamy, wet wool, pastoral, grassy, fecal, bitter, barn-yard, metallic, acidic, sweet, and rancid. It is not so clear how these attributes relate to each other and how many truly independent flavours / smells there are.

3.5 Myoglobin and meat quality

The colour of meat is strongly related to Mb content in the muscles. Mb is a pigmented protein, and is responsible for the colour of meat before, during and after thermal treatment. The more Mb in the muscle cells, the redder or darker is the meat.

3.5.1 Myoglobin concentration

While hemoglobin serves as the oxygen transporter, Mb (Figure 2) serves as a depot for oxygen in the muscles. Mb content is uniformly distributed within individual muscles and varies with regard to type of muscle, species, gender, breed and age of animals (Lawrie, 1998). In addition, the Mb content in muscle is affected by factors such as exercise and feed of the animals, as well as genetic and environmental factors (Joo, Kim, Hwang, & Ryu, 2013). Beef and lamb have more Mb in their leg muscles than pigs and chicken (Terence, 1982). In beef, 70% of iron was associated with the hemeproteins (Hb and Mb), while less than 30% of chicken iron was in this form (Hazell, 1982).

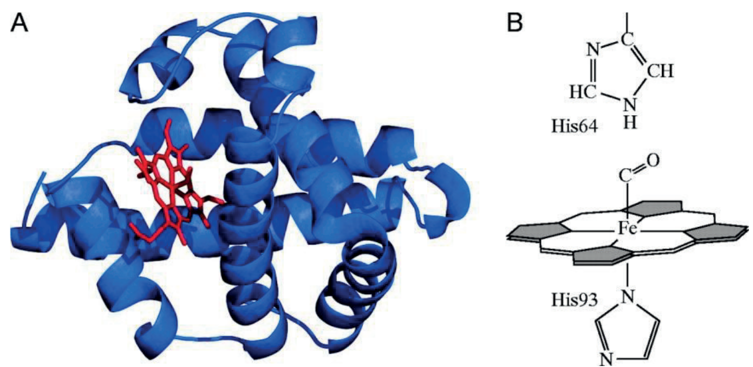

Figure 2: Myoglobin consists of a backbone and haem-binding domain. (A) X-ray crystallography of the myoglobin. The backbone of myoglobin consists of eight α-helices (blue) that wrap around a central pocket containing a haem group (red), which is capable of binding various ligands including oxygen, carbon monoxide and nitric oxide. (B) The protoheme group is bracketed or stabilized by histidine residues above (His64) and below (His93). The figure is adapted from (Ordway & Garry, 2004)

By comparing 19 different bovine muscles, (McKenna, Mies, Baird, Pfeiffer, Ellebracht, & Savell, 2005) found that the muscles had different colour stability. Those with high colour stability also had highest MMb reducing activity (MRA). OMb with a red colour is a most desirable meat colour for fresh meats. Therefore, it is important to maintain the meat colour stability in OMb form.

White (glycolytic) muscles contain less Mb, compared to red (oxidative) muscles (Seideman, Cross, Smith, & Durland, 1984). Muscles such as *M. semimembranosous,* with higher proportions of oxidative myofibres, are darker and more red, initially with higher Mb and iron concentrations. In addition, muscles with a higher proportion of β-red fibers will have higher concentrations of mitochondria. Kirchofer, Calkins & Gwartney (2002) emphasised that intact mitochondria compete for oxygen uptake with Mb. The result is a potentially larger flux in muscle colour, which reduce the depth of the OMb layer, and produces darker muscle appearance.

After slicing for display, oxidative muscles are more prone to rapid discolouration compared to glycolytic muscle types such as *M. semitendinosus* (O'Keeffe & Hood, 1982; Renerre & Labas, 1987). Variation between animals in oxidative capacity of muscles could be related to the genetic selection of modern breeds, which necessarily leads to an increased meat yield/muscling that has been shown to increase the expression of type IIB glycolytic fibers (Wegner, Albrecht, Fiedler,

Teuscher, Papstein, & Ender, 2000), and, consequently less tender meat (Karlsson, Enfält, Essén-Gustavsson, Lundström, Rydhmer, & Stern, 1993) depending of its connective tissue. Even within the same muscle *e.g. M. biceps femoris,* variations in oxidative capacity between the inner part (red muscle with higher oxidative capacity), and outer part of the muscle (red muscle with low oxidative capacity) exist (Beecher, Cassens, & Hoekstra, 1965). An inevitable outcome of changing the myofibre composition is a change in meat colour stability. Meat from older animals is darker due to the increased Mb concentration with animals' age (Lanari M. C., Brewster, Yang, & Tume, 2002).

3.5.2 Oxygen consumption

The most important factors which affect fresh meat colour stability are the rate of oxygen consumption (O'Keeffe & Hood, 1982), the reducing capacity of the meat (Ledward, 1985), temperature, and the composition of the gaseous environment in the package (MacDougall, 1982). Oxygen consumption is a characteristics property of meat where a series of reactions, mainly involving the Krebs cycle enzymes, scavenge oxygen in meat. Such a reaction is responsible for deoxygenation of OMb and a further decrease of oxygen concentration to zero, allowing the reduction of MMb to DMb (AMSA , 2014).

These reactions are carried out in mitochondria, which are important subcellular organelles uniquely poised to play a key role in neuronal cell survival or death because they are regulators of both energy metabolism and cell death pathways (Moreiraa, Carvalho, Zhu, Smith, & Perry, 2010). Approximately 90% of energy production in the cell is in the form of ATP (Kidd, 2005). ATP is produced when oxygen is reduced to H_2O through oxidative phosphorylation. In addition, Mb's role in muscle tissues comprises oxygen transport to mitochondria in cells for energy production (Wittenberg, Wittenberg, & Caldwell, 1975). The mitochondrial activity is highest around pH 7 and a temperature of 37ºC (Cheah & Cheah, 1971). Cytochrome c oxidase is the mitochondrial enzyme that consumes oxygen *pm*, reducing the amount of oxygen available to bind to Mb, and consequently leads to DMb instead of OMb formation (Tang, Faustman, Mancini, Seyfert, & Hunt, 2005b). DMb is more susceptible to oxidation compared to OMb (Gill, 1996).

Muscles with lower colour stability have been linked with an elevated mitochondrial content (Tang, Faustman, Hoagland, Mancini, Seyfert, & Hunt, 2005a; Renerre & Labas, 1987). In *pm*

meat, the pH and temperature decline with time, due to glycolysis and decreased metabolism; lower final pH and faster rates of pH decline may inhibit the respiratory activity of mitochondria. Respiratory enzymes utilize more oxygen at higher temperatures and pH, and thus limit oxygen penetration and diffusion into muscle (Lanari & Cassens, 1991). Muscles with a very thin layer of OMb on the surface will appear darker due to subsurface DMb (Kropf, 1993).

The maturity of animals alters muscle oxidative capacity, which in turn has the potential to impact on meat during display time at wavelength 630 nm and 580 nm (R630/R580 known as a marker of the OMb/MMb ratio) (Calnan, Jacob, Pethick, & Gardner, 2014).
Priolo, Micol & Agabriel (2001) emphasised that meat from ruminants raised and finished on pasture is generally darker compared to animals fed concentrates.

3.5.3 Metmyoglobin Reducing Activity (MRA)

Mancini & Hunt (2005) emphasised that MRA is a property of meat where a series of reactions help reduce MMb to DMb. Once MMb is formed, it is reduced to a ferro-derivative form by an enzymatic system active both in anaerobic (Stewart, Zipser, & Watts, 1965a) and aerobic conditions (Ledward D. A., 1972).

Factors, such as the reduced form of nicotinamide adenine dinucleotide (NADH), muscle's oxygen scavenging enzymes, and reducing enzyme systems, help muscles to turn MMb to DMb (Mancini & Hunt, 2005). Enzyme (NADH-cytochrome b5 MMb reductase), the intermediate (cytochrome b5), and the cofactor NADH are assumed to be the major components required for the enzymatic reduction of MMb (Bekhit & Faustman, 2005). However, exact details are not well elucidated for comminuted, *pm* systems. Mohan et al. (2010) reported the reduction of MMb via oxidation of malate to oxaloacetate and the regeneration of reduced NADH via malate dehydrogenase (MDH). As a possible mechanism for MMb reduction, a malate MDH-NADH system was evaluated in two experiments. The results from the first experiment were that the kinetics of MDH and MMb reduction were evaluated, showing that increasing concentrations of oxidized nicotinamide adenine dinucleotide (NAD+) and L-malate also increased ($p < 0.05$) MMb reduction *in vitro*. In the second experiment, reducing activity of beef muscle extracts with added different concentrations of malate and NAD^+ was determined. The results showed that reduction of MMb in the muscle extracts via MDH was dependent on NAD^+, malate, and extract concentration ($p < 0.05$). In conclusion, the malate can replenish NADH via MDH activity in *pm*

muscle resulting in a greater colour stability. This was considered a new mechanism for nonspecific and specific enzymatic reduction of MMb.

3.5.4 The role of mitochondria in colour stabilization

Domination theory for colour stability relates to a strong contribution from the electron transport chain complexes. The electron transport chain consists of 4 complexes: Complexes I, II, III, and IV. Complex I is the largest complex (Chaban, Boekema, & Dudkina, 2014) and it is known from the literature that it can easily become dysfunctional. Phung, et al. (2013) reported that complex I is easily inactivated in meat during chill storage. Complexes II and III are regarded as important for MMb reduction. This is based on the fact that the electron receiver from Complex III cytochrome C is regarded as the protein that donates the electron to MMb. Complex II has a binding site for FAD and the transformation of succinate to fumarate takes place here and is a part of the Krebs cycle. The interplay between Complexes II, III, and IV in reducing MMb is, however, still poorly understood.

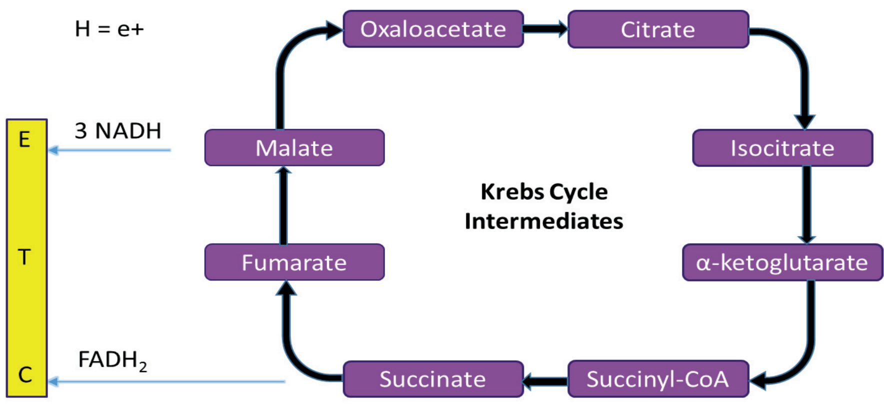

Figure 3: Oxaloacetic acid, which in water becomes oxaloacetate, is part of the Krebs Cycle which is involved in energy production within the mitochondria. Oxaloacetic acid is a critical metabolic intermediate in every mitochondria since malate and acetyl-CoA reacts and form oxaloacetate.

The Krebs cycle (KC) encompasses oxaloacetate, citrate, isocitrate, α- ketoglutarate, succinyl-CoA, succinate, fumarate, and malate (Figure 3). The cycle produces 3 NADH and 1 $FADH_2$. Since NADH and $FADH_2$ are used by the electron transport chain, it is often hypothesized that

the KC is important for fuelling the electron transport chain. We have no evidence that the KC is "running" *pm* as *in vivo* and then for how long.

3.6 Lipids and meat quality

Lipids in meat are composed of mainly triglycerides (TAG), diglycerides, monoglycerides and cholesterol (nonpolar compounds), free fatty acids, and several polar compounds such as phospholipids (PL) and sphingolipids (Ruiz, Antequera, Andres, Petron, & Muriel, 2004). The major roles of lipids are to store/provide energy and make cellular membranes stable. Energy is stored as TAG in adipose tissue (Lunn & Theobald, 2006), that makes up marbling, or IMF surrounding muscle fibres (Miller, 2004). IMF affects tenderness, juiciness, and flavour (FAO, 2010). Fat distribution in different carcass cuts is uneven, resulting in different amounts of fatty acids found there (dos Santos-Cruz, et al., 2012). Age and weight at slaughter are crucial factors influencing the total lipids in meat (Furusho-Garcia, Pérez, & Bonagurio, 2006; Jardim, Osório, & Osório, 2007).

De Smet et al. (2004) emphasised that scientists focused on FA composition of IMF because it cannot be removed from the meat before consumption. Animal fat is presumed to have a great impact on consumer's health. A reduction of daily fat intake to 30% of the total energy intake has been recommended since the 1970s although intake between 30-40% of total energy intake is presently acceptable.

There are still polemics about those recommendations, but according to the American Heart Association (2014), saturated fats (SFA) should be limited to 7% of this caloric intake. The recommended intake of total daily calories as fats from fish, nuts, and vegetable oils is 25-35%, while trans fats should not exceed 1 energy percentage. Fatty acid composition with a favourable *n*-6/*n*-3 ratio is presumed important for the regulation of inflammation in the human body. A sufficient intake of *n-3* (PUFA) can also neutralize the dietary effect of SFA (Dias, Garg, Wood, & Garg, 2014). Besides this desirable dietary effect, PUFAs impact odour, flavour, and meat colour because of its unstable chemical structure, which is prone to oxidation.

3.6.1 Lipid oxidation

Lipid oxidation (Figure 4) is the main cause of quality deterioration in muscle foods and is often the decisive factor in determining food product storage life (Frankel, 2005). It occurs when unsaturated fatty acids react with molecular oxygen via a free radical mechanism (Asghar, Gray, Buckley, Pearson, & Booren, 1988). The major primary products of this reaction, hydroperoxides (HP), are relatively unstable and essentially odourless. HPs decompose into a wide range of secondary compounds, as alkanes, alkenes, aldehydes, ketones, alcohols, esters, acids, and hydrocarbons. The aldehydes are considered to be the more important breakdown products regarding low threshold values and are the major contributors to the development of off-flavours and odours (Ross & Smith, 2006). Undesirable changes also include the destruction of valuable nutrients, generation of toxic elements, and deterioration of colour and texture (Kanner, 1994). Diets based on food containing peroxidized lipids have been related to far-reaching effects such as carcinogenesis, premature aging, and other diseases (Velasco & Williams, 2011; Kahl & Kappus, 1993). In general, lipid oxidation includes three phases: initiation, propagation, and termination. By mechanism, lipid oxidation can be divided into auto-oxidation, photo-and enzymatic oxidation (Tejero, Gonza´lez-Lafont, Lluch, & Eriksson, 2004).

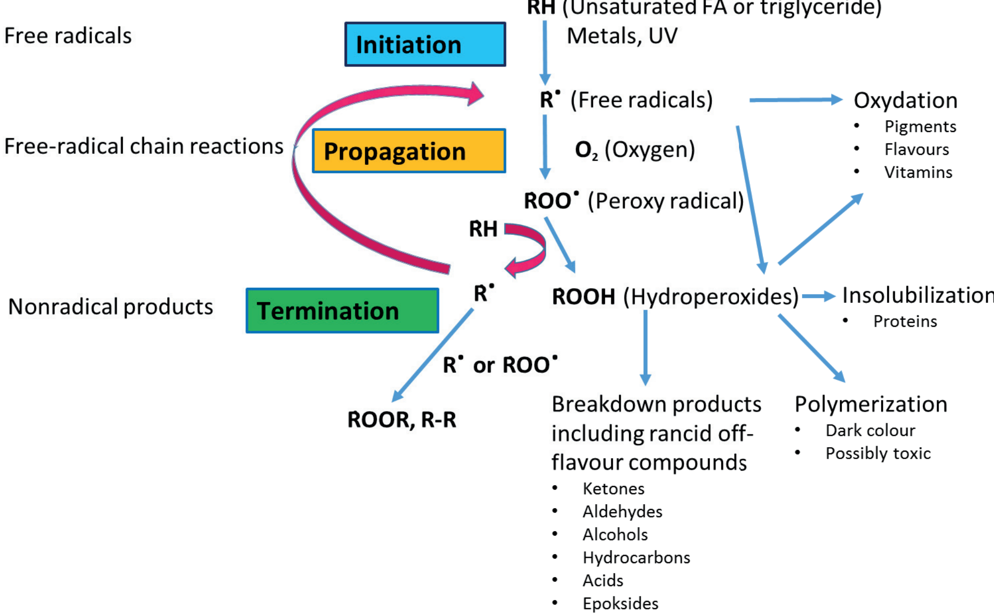

Figure 4: Lipid oxidation mechanism from initiation through propagation with termination through the formation of products with limited reactivity. The figure is adapted from (Cheng, 2016).

The major factors affecting lipid oxidation in meat include the composition of phospholipids, the content of PUFA, and the presence of free metal ions. The others are oxygen, haem pigments, mechanical processes, cooking, and the addition of salt during processing (Andreo, Doval, Romero, & Judis, 2003). The concentration of ferrous iron and its ability in lipid oxidation reaction is a key factor causing differences among species and cuts of meat. Mb that is prevalent in red meats has a very reactive iron. The iron from Mb catalyzes the lipid oxidation in the first phase (initiation). The oxidation of ferrous-OMb (Fe^{2+}) to ferric-MMb (Fe^{3+}) causes discolouration of meat during storage (Chaijan, 2008).

The lipid peroxidation process starts immediately after slaughtering and during the conversion of muscle to meat (*pm* ageing); causing the destruction of the balance between pro-oxidant and antioxidant factors. The rate and extent of lipid peroxidation in muscle tissues appears to depend on the degree of muscle tissue damage during pre-slaughtering events, such as stress and

physical damage and post-slaughtering events such as early *pm* pH fall, carcass temperature, shortening, and tenderising techniques such as electrical stimulation (Morrissey, Sheehy, Galvin, Kerry, & Buckley, 1998). The composition of raw meat, aging time, cooking or heating, size reduction processes (grinding, flaking, and emulsification), deboning, mechanical deboning, additives such as salt, nitrite, spices, and antioxidants, temperature, abuse during handling and distribution, oxygen availability, and prolonged storage are all factors that can influence the rate of lipid peroxidation in meat and meat products (Kanner, 1994; Rhee, 1988).

Differences in total lipid amount and fatty acid composition in meat depend on animal species, muscle type, and anatomical location of muscle (Wilson, Pearson, & Shorland, 1976). According to Pikul et al. (1984), the phospholipid fraction contributed to about 90% of the malondialdehyde (MDA) measured in total fat from chicken meat. The development of rancidity was positively related to the PUFA content of phospolipids (Igene, Pearson, Dugan, & Price, 1980). Level of lipid peroxidation is more influenced by the oxidative stability of membrane components than that of cytostolic components (Yin & Faustman, 1994).

3.6.2 Lipid oxidation and meat colour

Discolouration and off flavour development in meat are linked to the Mb and lipid oxidation, respectively. Generally, these processes influence each other and the oxidation of one of these leads to the formation of chemical species that can further accelerate oxidation of the other (Faustman, Sun, Mancini, & Suman, 2010). Mb and intramuscular lipid concentration are higher in the dark muscle type (Chaijan, Benjakul, Visessanguan, & Faustman, 2004).

Formation of free radicals and reactive oxygen species initiates lipid oxidation. The redox state of haem iron has a significant role in the generation of free radicals in meat (Li & Liu, 2011). Lipid oxidation is an oxygen-consuming process and OMb oxidation is dependent on oxygen level, whereby low oxygen tensions favour MMb formation. Lipid oxidation occurs after OMb oxidation, and further pigment oxidation can be enhanced by lipid oxidation primary products (O'Grady, Monahan, & Brunton, 2001). MMb (Fe^{3+}) with lower haemin affinity promoted lipid oxidation more efficient than OMb (Fe^{2+}) with higher haemin affinity, consequently OMb was less effective as pro-oxidant (Grunwald & Richards, 2006). Suman et al. (2007) reported that the Mb redox state affects meat colour and is destabilized by secondary lipid oxidation products, such as 4-hydroxy-2-nonenal (HNE). The experiment where porcine OMb was incubated with

HNE and analysed for MMb formation, resulted in greater MMb formation in the presence of HNE than in control samples. Meats with a high content of polyunsaturated fatty acids (PUFA) are more prone to oxidation (Pacheco-Aguilar, Lugo-Sanchez, & Robles-Burgueno, 2000).

Stability of stored meat can be extended with convenient packaging systems, by the addition of the exogenous antioxidants (Yin & Cheng, 2003), as well as adopting feeding systems able to improve the antioxidant status of muscle (Luciano, et al., 2014). Li & Liu (2011) mainly focused on on-farm practices via dietary supplementation of antioxidants to improve lipid stability, including lipid oxidation in an animal's body, and, consequently, meat colour stability, especially for beef and lamb production because colour change of these red meats during display is more critical compared with pork and poultry meats.

3.6.3 Lipid oxidation and flavour of meat

There are several sensory attributes used to describe lipid oxidation in the meat, but not all oxidation processes are negative. The characteristic flavour of cooked meat is generated from thermally induced reactions occurring during heating, particularly from the Maillard reaction and the degradation of lipids. Both reactions involve complex reaction pathways resulting in a wide range of products, which account for the large number of volatile compounds in cooked meat (Mottram D. S., 1998).

Warmed over flavour seen in cooked meats, known as off flavour, is typically present within 48h at refrigerated temperatures (Forrest, Aberle, Gerrard, & Mills, 2012). In some dry-cured domestic hams and fermented sausages, the desired end flavour is achieved when a specific level of hydrolysis and oxidation of fat occurs (Pearson, Love, & Shorland, 1977). According to Enser, Hallett, Hewitt, Fursey, & Wood, (1998), lipid oxidation immediately prior to cooking may be a source of intermediates that react with other components to contribute to desirable cooked flavour.

The products of Maillard reaction may interact with the products of lipid oxidation and cause a reduction in some compounds and the production of new ones (Resconi, Campo, Montossi, Ferreira, Sañudo, & & Escudero, 2010). Sheep meat flavour may be influenced by cooking/heating treatments in which flavour of cooked meat is a product of the reaction between carbohydrates and proteins and, between breakdown products of these compounds. Significant

flavour precursors are inosine, phosphate, and ribose, while heterocyclic, phenolic, and sulphur containing compounds are important flavour and end-products of this reaction. Lipids have a direct impact on Maillard-like reaction through their degradation products such as aldehydes, ketones, alcohols etc. If the final pH of lamb increased from 5.6-6.1, the production of sulphur containing compounds during cooking might increase in stressed animals (Wood, et al., 1999). Many compounds such as hydrocarbons, aldehydes, ketones, alcohols, furans, triphenes, pyrrols, pyridines, pyrazines, oxazols, thiazols, sulphurous compounds, and many others contribute to flavour and aroma (MacLeod, 1994; Ho, Oh, & Bae-Lee, 1994).

Hydrogen sulphide (H_2S), as the dominant sulphur compound in cooked meats volatiles, has a characteristic odour and might act as a precursor for other odour compounds (Young O. R., 1993). Forage fed-ruminants have a higher concentration of volatile sulphur compounds than concentrate fed-ruminants. However, some compounds, such as pyrazines are not influenced by the feeding system. Pyrazines and thiazoles as products of Maillard reaction might arise because of differences in the concentrations of free amino acids containing sulphur or reducing sugars, when sulphur-containing amino acids react with sugars to give rise to thiazoles and thiophenes and other aromas associated with meat products. Prolonged ageing time (20 days) can result in more amino acids and reducing sugars in muscle (Resconi, Campo, Montossi, Ferreira, Sañudo, & & Escudero, 2010; Resconi, Escudero, & Campo, 2013).

The volatile compounds from lipid oxidation contribute to off-flavour and foreign flavours observed with certain nutritional regime, but do not appear to be the defining factor in characterizing the lamb flavour (Duckett & Kuber, 2001). Ha & Lindsay (1991) reported that a higher concentration of alkylphenols might contribute to lamb flavour and proposed that these compounds relate to pasture-finished ruminants. Fat content dominates the lamb meat flavour that is associated with BCFA and with the presence of 3-methylindole (skatole- faecal smelly compound). The flavour of sheep meat can also be affected by lipid composition, fat content or by the formation of volatile compounds with special fat flavour such as 2.4- decadienal that originated from degradation of α-linoleic acid (Mottram D. S., 1998; Elmore, Mottram, Enser, & Wood, 2000). Young et al. (1993) reported that 4-heptenal is an undesirable degradation product from α-linoleic acid, the amount of which depends on the diet of sheep.

Despite considerable research, the answer about which volatiles that are essential for lamb aroma and how they differ from other red meats such as beef, is not conclusive (Watkins, Frank, Singh, Young, & Warner, 2013).

3.7 Inhibition of meat quality deterioration

During heating and long-term storage, fats, oils, and lipid-based foods are exposed to several degradation processes. Oxidation and decomposition of oxidation products are the main deterioration processes that decrease the nutritional value and the sensory attributes of the products. The oxidation process may be inhibited/prevented or reduced by different methods such as:

- Prevention of oxygen access, lower temperature, inactivation of enzyme catalysed oxidation, reduction of oxygen pressure and suitable packaging.
- Use of specific additives which inhibit oxidation, such as antioxidants (Pokorny, Yanishlieva, & Gordon, 2003).

3.7.1 Vacuum and modified atmosphere packaging of meat

Meat colour can exist in many shades, from desirable bright red to undesirable colour shades of brown, green, or yellow. In order to maintain an attractive fresh colour of meat, prolonged storage time and bacteriological safe products, the producers use different packaging methods such as vacuum packaging (VP), MAP, and air-permeable packaging (Lee, 2010).

VP is the most effective packaging strategy used to prolong the shelf life and palatability of beef during displaying time that became marketed successfully (Cornforth & Hunt, 2008; Seideman & Durland, 1983). Some authors emphasized that oxygen that permeates from the outside into the package through the packaging material cannot be removed by this method (Byun, Darby, Cooksey, Dawson, & Whiteside, 2011). From the consumer's point of view, VP does not necessarily contribute to the most desirable colour, and can also lead to a cheesy taste and odour, associated with the growth of anaerobic spoilage microorganisms (AMSA, 2001); (Seideman & Durland, 1983). However, microbial shelflife is longer in VP than in high oxygen packaging. A packaging atmosphere with less than 0.1 % oxygen generally results in the purplish red colour of

packaged meat (Sørheim, Westad, Larsen, & Alvseike, 2008), while high oxygen atmosphere packaging yields the bright red meat colour, and can promote lipid oxidation and sensory changes (Resconi, et al., 2012).

Modified atmosphere packaging (MAP) was designed to overcome some of the problems associated with VP (Church, 1994), such as improving the safety of meat products and other foodstuffs important in human nutrition. MAP is a method used to ensure the microbiological shelf life and the attractive red colour of the product (Sørheim, Aune, & Nesbakken, 1997) as well as decreased oxidation of the product, especially fat and aromatic substances as foodstuff constituents (Park, Kim, Lee, Yoo, Shim, & Chi, 2008). Meat packaged with CO_2, N_2, or a combination of both gases has the potential to satisfy good colour stability and extend shelf life (Tewari, Jayas, & Holley, 1999).

According to Gutiérrez, Tejeda, Parra, & Andrés, (2013) the gas mixture consisting of 69.4% argon, 30% carbon dioxide, and 0.4% carbon monoxide is recommended to MAP mixtures, based on a higher oxidative stability of meat during conservation in refrigeration.

Ground meat has a larger surface area, and incorporation of oxygen leads to lower colour stability compared to intact meat. Destruction of cellular integrity can liberate a variety of pro-oxidants that can accelerate the discolouration process, and thereby accelerate the formation of MMb from OMb and DMb (Faustman & Phillips, 2001). An MMb reducing environment involves reducing residual oxygen quickly and completely in order to avoid accumulation of MMb (Slinde, Phung, & Egelandsdal, 2011).

3.7.2 Antioxidants

Antioxidants are important in food processing and reduce oxidative rancidity-development in fat-based foods like meat, dairy products and fried foods. Recently, research has correlated antioxidants with the inhibition of cardiovascular disease and cancer (Pokorny, Yanishlieva, & Gordon, 2003). Protein oxidation is also prevented by antioxidants (Falowo, Fayemi, & Muchenje, 2014). Protein oxidation should be reduced because it leads to inactivation of proteases and thereby increased toughness.

Antioxidants added at low concentration to fresh and processed meat products, might delay or inhibit lipid oxidation (Bartusik, Aebisher, Bartosińska, Siluk, & Tomanek, 2014), retard

development of off flavours, and improve colour stability (Kumar, Yadav, Ahmad, & Narsaiah, 2015). The antioxidants can be endogenous or obtained exogenously through diet or as dietary supplements (Mates, Perez-Gomez, & De Castro, 1999). Due to their different solubility, antioxidants are divided into two groups: hydrophilic (water soluble) or hydrophobic (soluble in lipids). Hydrophilic antioxidants react with antioxidants in the cell cytosol and the blood plasma, while the hydrophobic antioxidants protect cell membranes from lipid peroxidation. A common feature in both antioxidant groups is cell protection from the damage caused by molecules known as reactive oxygen species (ROS) (Bajaj & Khan, 2012; Southorn & Powis, 1988).

3.7.3 Natural antioxidants

The most frequently used natural antioxidants are tochopherols, asorbic acid, carotenoids, flavonids, amino acids, phospholipids, and sterols. By scavenging free radicals, chelating prooxidative metals, quancing singlet oxygen and photosensitizers, and inactivating lipoxygenase, antioxidants inhibit the oxidation of food (Choe & Min, 2009).

The addition of antioxidants is necessary to increase storage stability, sensory quality, and the nutritional value of animal products. The food industry now chooses natural over synthetic antioxidants, due to suspected toxicological and carcinogenic effects of the synthetic ones (Kumar, Yadav, Ahmad, & Narsaiah, 2015).

Lipid and pigment oxidation in meat might be delayed by increased α-tocopherol concentrations in the membranes of muscles (Smith, Morgan, Sofos, & Tatum, 1996). However, vitamin E is an essential dietary vitamin for sheep and cattle and cannot be syntethized by the rumen or animal, and has to be supplied in the diet to create the maximum effect of incoorporation into muscle biomembranes (Sales & Koukolová, 2011). Green forrage and other leafy materials are very good sources of vitamin E. Dependent on their freshness, the concentrations of the vitamin in herbage may be 5-10 times higher compared to some cereals (McDowell, et al., 1966).

Lund et al. (2007) researched the incorporation of dietary antioxidants such as ascorbate/citrate and rosemary of meat packaged in MAP and evaluated their effects on optimal meat quality. They found that the antioxidants such as ascorbate/citrate were more efficient than the rosemary extract on lipid stability during storage time. It is also documented that vitamin C and α-tocopherol can act synergistically, where ascorbic acid is involved in the reduction of tocopherol.

However, high concentrations of vitamin C in the presence of O_2 may form H_2O_2 and has a detrimental effect on meat colour (Descalzo & Sancho, 2008).

The antioxidant nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide $FADH_2$ presumed formed through the metabolism of glutamate, malate, succinate, pyruvate, and citrate when added to ground bovine *M. semimembranosus*, altered the Mb redox forms in aerobic and anaerobic packaging systems (Bjelanovic, et al., 2016). OMb was preserved at the highest levels using glutamate-malate for meat packaged in aerobic systems, especially when combined with citrate. In anaerobic packaging, glutamate-malate and succinate induced DMb the quickest (Ibid). As glutamate was investigated in combination with malate, the sole effect of malate could not be extracted. However, it is suggested that it was of minor importance.

3.7.4 Mitochondria as antioxidants

In vivo metabolic processes in mitochondria (Figure 4) play an important role in maintaining a balance between free radical generation and antioxidant defence. Mitochondrial antioxidant capacity is related to antioxidant activity enzymes such as superoxide dismutase (SOD), catalase, thioredoxin reductase, peroxiredoxin and selenium-glutathione peroxidase (GSHPx) (Masella, Di Benedetto, Varì, Filesi, & Giovannini, 2005), in addition to the electron transport system itself that can reduce oxygen to water.

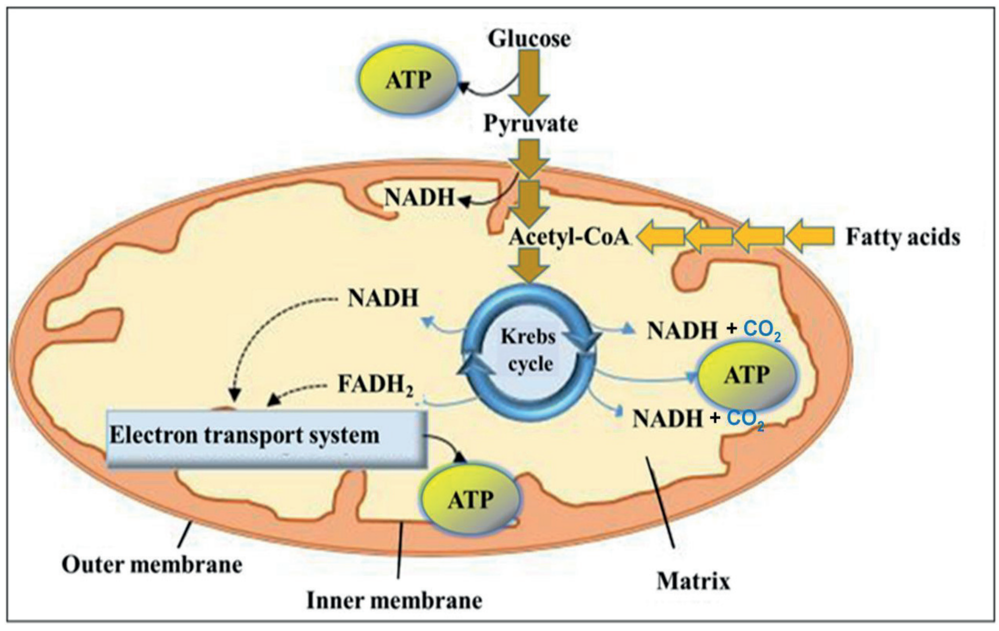

Figure 4: Metabolism in the matrix of mitochondria. Pyruvate and fatty acids are imported from the cytosol and converted to acetyl-CoA in the mitochondrial matrix. Acetyl-CoA is then oxidized to CO_2 via Krebs cycle, the central pathway of oxidative metabolism. The figure is adapted from (Cooper, 2000).

As long as mitochondria are in the functional state, the antioxidative defence system can be effectively regenerated after or during oxidative stress. The function of mitochondria depends mainly on the availability of suitable respiratory substrates which can provide hydrogen for the reduction of, among others, either the glutathione- or alpha-tocopherol-system, since glutathione (GSH) is likely regenerated by glutathione reductase with the substrate NADPH and the alpha-tocopheroxyl-radical by reduced coenzyme Q. Total inactivation of the mitochondrial electron transport system occurs when the antioxidative defence systems are exhausted, together with the degradation of mitochondria along with the oxidation and degradation of mitochondrial lipids and proteins. Extra mitochondrial antioxidants such as ascorbic acid can act both as pro-oxidant and as antioxidant and may assist the mitochondrial antioxidative defence systems in a complex way (Augustin, Wiswedel, Noack, Reinheckel, & Reichelt, 1997).

3.7.5 Selected additives in meat industry

Food additives are substances that directly or indirectly affect the characteristics of any food used in production, processing, treatment, packaging, transportation, or storage, in order to maintain or improve safety, freshness, nutritional value, taste, texture, and appearance (USDA - Food Safety and Inspection Service, 2015). The list of food additives in use is long, and some of them are mentioned below.

3.7.5.1 Glutamate (glutamic acid; E620)

Glutamate is a multifunctional amino acid, closely related to mitochondrial metabolism (Frigerio, Casimir, Carobbio, & Maechler, 2008), and is naturally present in our bodies and many foods. Glutamate is a compound that reacts to α-ketoglutarate that participates in the KC (Owen, Kalhan, & Hanson, 2002). Monosodium glutamate (MSG) is frequently added to different types of food as flavour enhancer (Freeman, 2006). According to Saleh & Watts (1968), addition of glutamate and malate separately to raw meat preserves colour and extends shelf life by increasing the reduction of MMb.

3.7.5.2 Malate (malic acid; E296)

The natural constituent of many fruits and vegetables preserved by fermentation is malic acid. Malate can be synthesized from fumarate by the enzyme fumarase and then oxidized to oxaloacetate by malate dehydrogenase with the accompanying reduction of NAD^+ (Edwards, Copes, Brito, Canfield, & Bradshaw, 2013). The addition of malate to raw meat can stabilize colour, and the degree of colour stabilization is muscle-dependent (Mohan, Muthukrishnan, Hunt, Barstow, & Houser, 2010).

3.7.5.3 Succinate (succinic acid; E363)

Succinate is an essential intermediate of cellular metabolism in microbiological fermentation, and also remains in final fermented food products (Cao, et al., 2013). The succinate is an intermediate of the Krebs cycle, and plays a crucial role in ATP generation in mitochondria (Mills & O'Neill, 2014) through the electron transport chain's complex II. MMb reduction is

therefore stimulated by succinate when mitochondria are present (Tang, Faustman, Mancini, Seyfert, & Hunt, 2005b).

3.7.5.4 Citrate (citric acid; E330)

Citrate is a natural metabolite in living cells, and is present in many natural products such as fruit, vegetables, meat, and milk. In the mitochondria, citrate is an intermediate in the Krebs cycle, which converts acetyl-CoA to two molecules of CO_2 with simultaneous generation of NADH and $FADH_2$ and ATP (reoxidation of NADH and $FADH_2$ via the electron transport chain) (Lawlis & Roche, 1980). Citrate also has regulatory roles in glycolysis, fatty acid synthesis, and oxidation (Denton & Randle, 1966). Holmer, Kutzler, McKeith, & Killefer (2009) reported that the use of sodium citrate in a brine solution resulted in visually darker steaks and less discolouration during the display period.

3.7.5.5 Pyruvate (pyruvic acid)

The oxidative breakdown of glucose to pyruvate via the Embden-Meyerhof pathway is known as the most common type of glycolysis, and is the backbone of ATP synthesis in eukaryotes (Müller, 2003). Pyruvate protected against lipid oxidation/degradation and its antioxidant activity has previously been reported (Lund, Hviid, & Skibsted, 2007). Pyruvate is not a common food additive, even if many studies support its health benefits (Owen & Sunram-Lea, 2011). Slinde et al. (2012) and Bjelanovic et al. (2016) reported that pyruvate was not a suitable compound to preserve the DMb state. In another experiment with ground beef patties packaged in polyvinyl chloride (PVC) and high oxygen, the addition of pyruvate increased colour stability and decreased lipid oxidation (Ramanathan, Mancini, Van Buiten, Suman, & Beach, 2012).

4 Materials and Methods

4.1 Muscles used in the study

To evaluate meat colour stability in beef muscle during the storage time, *M. semimembranosus (MSem)* was used in papers I and II. In addition, the same muscle was used in paper III to evaluate lipid oxidation using KCS.

M. longissimus thoracis et lumborum (LTL) from sheep/lamb was used to evaluate meat quality parameters in papers IV and V. These muscles are suitable and frequently used in similar studies. Mc Kenna et al. (2005) examined biochemical and physical factors that govern colour stability, and rate of discolouration of several individual bovine muscles. They identified *LTL* as a "high" colour stability muscle, while *MSem* was identified as a "moderate" colour stable muscle. The muscles with high colour stability had a high resistance to metmyoglobin formation (RIMF), nitric oxide reduction, and oxygen penetration, and possessed a low oxygen consumption rate (OCRs), Mb content, and oxidative rancidity.

4.2 Fat tissues used in the study

In the experiments with minced meat (Papers I-III), two different fat tissues from beef and pork were also used. Pork fat tissue was from Finnish pigs fed with rapeseed. The argument for choosing different fat tissues was related to the need of examining tissues with different oxidation rates (Bjelanovic, et al., 2013).

4.3 Statistical analysis

4.3.1 Factorial and Mixture designs

Factorial designs (Figure 5) are very common in research. In such designs, 2 or more factors at selected levels are defined and the experiment is composed of samples having all combinations of possible factors at the different levels. Such designs allow for identifying the effect of each

design variable without confounding. In this thesis, factorial designs were used in papers II and III.

Fractional factorial design is often used when there is a need for studying many variables. Often, some variables dominate, but it is not easy to “guess” which compounds are important. Fractionation subsets are chosen according to the principle that when one or more variables are not relevant, the design collapses to a full factorial design.

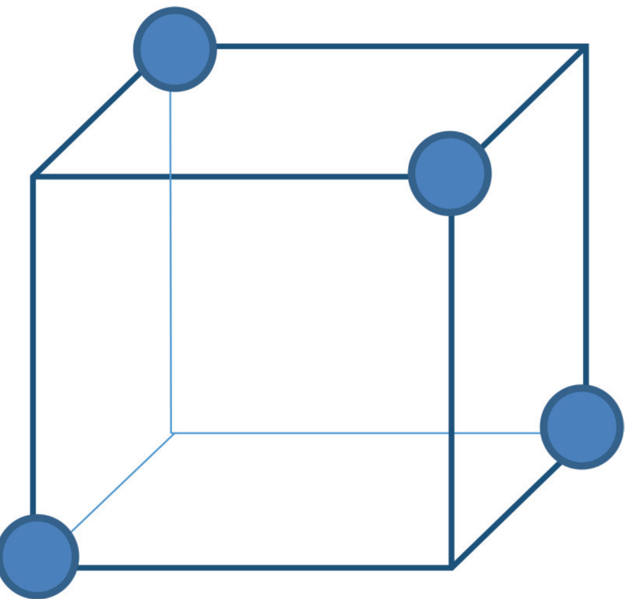

Figure 5: A 2^3 design fractionated to a 2^{3-1}. Fractionation was used in paper II and III.

A mixture design is formed by using two or more components (variables) together. When one variable decreases, one or more components must increase, making the sum of components the same, for example at a fixed molarity. The component are therefore portions or fractions, where the mixture components depend on each other, and the sum of the all fractions must be 1.0.

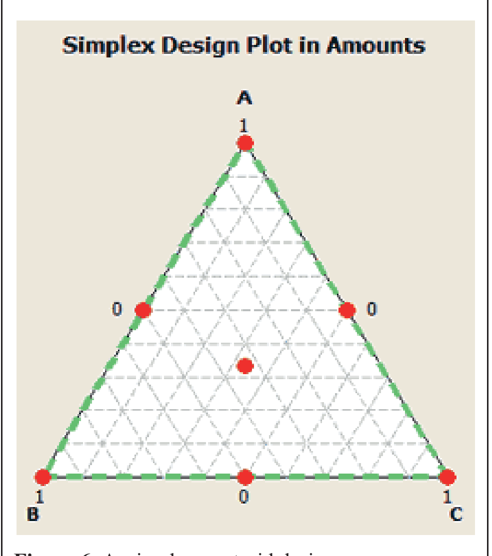

Figure 6. A simplex-centroid design

In the regression equation, the components (A, B, C) are used as factors, and the dependent response is presented as a line response or as the surface of a triangle (using 3 factors). Simple–lattice and simple-centroid designs (Fig 6., to the right, from Mintab) are common approaches to the mixture design. The three corners (pure "corners") of the resulting triangle represent the maximum allowed (1.0) for the specific component from a mixture. The Simplex Centroid Design has points at the corners, at the centre, and the midpoints of the sides. A simplex lattice design of degree m consists of m+1 points (0.5 and 1.0). In total, the design is 6 combinations with three "corners"; for the centroid, one more combination is added.

Paper III reveals a mixture design that is embedded in a factorial design and also fractionated. This is a highly unusual and creative design propelled forward by the need to reduce the number of systems to be investigated.

4.3.2 Analysis of variance (ANOVA)

One-way ANOVA is a method that compares variance among two or more independent groups of data, and decomposes the response variance into several parts that can be compared against each other for significance testing. When the response variance is larger than residual variance, the effect leans towards significance ($p<0.05$ is significant).

4.3.3 Principal component analysis (PCA)

Principal Component Analysis (PCA) is a simple yet popular and useful linear transformation technique used to transform a set of response variables (Y-matrix) into a smaller number of uncorrelated variables called principal components. Principal component analysis was used to

explore and diagnose the relationship between the three different chemically obtained myoglobin states (OMb, DMb and MMb). The first principal component has the highest explained variance.

4.3.4 Partial last square regression (PLS)

Partial last square regression was used to find the fundamental relations between two matrices (X and Y) – spectra and myoglobin states, but is also used in manuscript V. PLS regression is particularly suited when the matrix of predictors (here absorbance values at many different values) has more variables than observations, and when there is multicollinearity among X values.

4.3.5 Root mean square error of cross validation (RMSECV)

The RMSECV is a pre-processing technique used to determine a prediction confidence interval or validation of the prediction error. The quality of the model that provides higher R and lower RMSECV value is better, and the resulting model should have high precision in discriminating the Mb state's variability.

4.3.6 Variable selection

Variable selection is the process of selecting a subset of a relevant variable for use in model construction. This was needed both to make it easier to interpret models, and to reduce overfitting by removing variables that do not contribute to the model or, actually, in some cases, improve the model as well as making it easier to interpret. Typically, this is done when the variables exceed the number of samples. In manuscript V, variables were selected based on a significance test (Figure 7) of the regression coefficients obtained from using the optimal number of components in a PLS regression model.

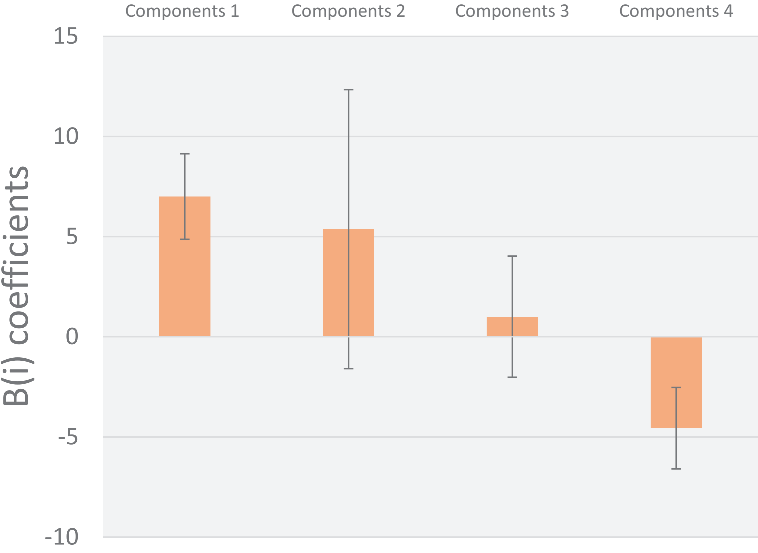

Figure 7. Regressions coefficients versus components (or variables). Compounds 1 and 4 would be selected as important variables as the 2 x standard deviation markers are not crossing the zero line.

4.4 Assessment of L*, a* and b*colour parameters

For the determination of the colour in practice, the most commonly used measuring instruments are colorimeters (Minolta versions) and spectrophotometers (Foss NIRSystems versions). Colour measurements are based on measuring the intensity of reflected light or the measurement of absorbed/ transmitted light. The values obtained by measuring the colour on any instrument, can be converted without problems from one colour system to another by using an appropriate mathematical formula. Bearing in mind, that colour measurements should be carried out under similar physical conditions.

CIE L * a * b * (Figure 8) are very common for assessing the discolouration of food. However, instrumental metamerism is a common and serious problem when using colorimeters.

In principle, spectrophotometers are more complex instruments, that supply spectral analysis with different degrees of resolution that are necessary in order to quantify myoglobin states.

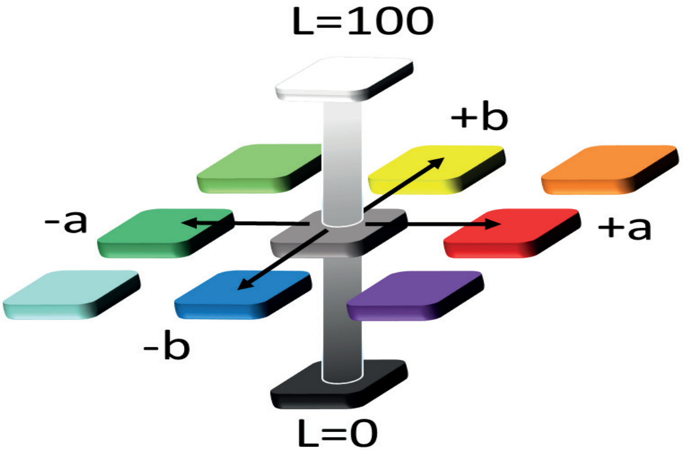

Figure 8: This three-dimensional model shows the L stands for the lightness of the colour, with 0 equal to black and 100 being a diffuse white. "a" is the redness versus greenness, while "b" is the yellowness versus blueness. The figure is adapted from (Saeed, Alsadi, Ahmad, & Rizvi, 2014).

Minolta is a frequently used colorimeter to measure tristimulus values (CIE L*a*b*). A Konica Minolta Spectrophotometer CM 700d (Konica Minolta Sensing Inc., Osaka, Japan) was used to measure surface meat colour. The colours were expressed as L*(lightness) = 0 to100 (black to white), a*(redness) = -128 to 128 (green to red) and b* (yellowness) = -128 to 128 (blue to yellow) (Pauli, 1976). Calibration of the instrument was done by a white ceramic calibration cap (CM-A177) built into the instrument.

4.5 Assessments of myoglobin states

The instrument used for the measurement of myoglobin redox forms in a sample was a Foss NIRSystems OptiProbe™ 6500 Analyzer (Foss NIRSystems Inc., Maryland, USA) equipped with an interactance fibre optic probe (NR-6770-A, Foss NIRSystems) and software from Vision (2001, NIRSystems). The probe head (attached to instrument) is mounted in the middle of a 40.0x40.0 mm^2 metal block consisting of seven rectangular parallel glass windows of about 1.0x20.0 mm^2, located about 2.0 mm apart (Isaksson, et al., 2012). By using both reflectance and absorbance spectra, different myoglobin states with different spectra can be measured and calculated. The first step for predicting Mb states (DMb, OMb and MMb) in stored ground beef is to make a calibration model, where pure states of Mb redox forms will be produced. The

calibration model created from samples of known composition, packaging material, and instrumentation can then be used in later experiments using the same type of samples. These pure state spectra were used to construct models for identifying the Mb states in an unknown sample (Egelandsdal, Bjelanovic, Khatri, & Slinde, 2013).

Isobestic points at 525 nm, where all three of the Mb species have the same reflectance/absorbance, might be used to calculate Mb states (AMSA, 1991; Tang, Faustman, Hoagland, Mancini, Seyfert, & Hunt, 2005a) and is often suggested to eliminate differences in Mb content between different meat samples. The first isobestic point for MMb and OMb is at 473nm, while 572nm is the isobestic point for DMb and OMb (Kryzywicki, 1979). Before quantifying Mb species with reflectance measurements on the meat surface, the physical characteristics of the meat samples should be known. The Mb layer can possibly vary between different redox states and it must be thick enough for minimising errors from another state existing below the prepared surface myoglobin state.

In order to prepare 100% pure Mb states, the preparation method was compared with guidelines (AMSA, 2012) and (Khatri, Phung, Isaksson, Sørheim, Slinde, & Egelandsdal, 2012) methods in which the different meat systems were used (Table 1).

Mb state	Preparation method	IA -Intact meat– (AMSA, 2012)*	IB- Intact meat (Khatri et al., 2012)	IIA- Comminuted meat (AMSA, 2012)*	II B- Comminuted meat (Bjelanovic et al., 2013)
MMb	Chemical	Immerse in 1.0% potassium ferricyanide for 1 min, drain, blot surface, package in oxygen-permeable film, store at 2 - 4°C in 1% oxygen for 48 hours. Use older meat.	Immersed in 1% potassium ferricyanide for 1 min, drained, blotted dry, packed in oxygen-permeable film, stored at 2 - 4°C for 12 hours. Four days old meat.	Not commented on.	Immersed in 1% potassium ferricyanide for 1 min, drained, blotted dry, packed in oxygen-permeable film, stored at 2 - 4°C for 16 hours. Examined in oxygen-impermeable film. Six to thirteen days old meat.
OMb	Packing	Expose samples at 0 - 2°C in a high-oxygen atmosphere (70 - 100%), store 24-48 hours at 0 - 2°C, (gas-to-meat volume ≥ 3 to 1).	Packed in 75 % O_2/ 25 % CO_2., 4°C, and measured after 24 hours, C, (gas: meat= 30 :1). Examined in oxygen-permeable film. Four days old meat.	For ground product, package in a thin layer.	Packed in 75 % O_2/25%CO_2 and measured after 45 min, at 4°C. Examined in oxygen-impermeable film. Three to thirteen days old vacuum-packed meat was used.
DMb	Packing	1) Make a fresh-cut surface on the sample's interior surface, scan immediately. 2)Vacuum package samples in oxygen-impermeable vacuum bag, store 24 -48 hours at 4°C. Holding the samples at 20°C for at least 50% of the time speeds up reduction time. Scan through the vacuum packaging film.	Samples were vacuum-packaged and kept at 2-4°C for 48 hours, (gas: meat= 30 :1). Repackaged in oxygen-permeable, scanned immediately. Four days old meat.	Not commented on.	Samples were vacuum-packaged and kept at 4°C for 0-13 days. Measured after 3 days. No re-packing.

Table 1. Comparison of preparation methods to prepare 100% pure myoglobin states.

4.6 Warner Bratzler measurements

The Warner Bratzler method for tenderness measurements is most widely used, and provides relatively fast, cheap, and objective measurements of meat tenderness (Holman, Alvarenga, van de Ven, & Hopkins, 2015). Heated meat (to 72 ºC) samples were measured. Samples cut had an area of 1cm x 1cm x 4-5cm; the longer direction was parallel to the fibre direction. Warner Bratzler shear cell (knife blade type HDP/BSK) with load cell 25 kg, was used in TA-HDi TextureAnalyser (Stable Micro Systems, Godalming, UK). According to (Davey, Gilbert, & Carse, 1976), meat with shear force scores above 50 N/cm^2 is considered tough, but this scale of relative tenderness of specific muscles has not always been in agreement with other scales (Calkins & Sullivan, 2007).

4.7 Gas Chromatography (GC) – Mass Spectrometry (MS)

4.7.1 Head space analysis HS/GC-MS

A specific HS-GC/MS technique was used to identify and or quantify volatile organic compounds (VOC) from meat samples.

A dynamic headspace technique known as “purge and trap” technique, does not change the nature of volatile compounds present in the sample and sample preparation is minimal (Narváez-Rivas, Gallardo, & León-Camacho, 2012). In addition, this method allows loading of many samples at the same time on an auto sampler. The method identifies mostly lipid degradation products as used in this thesis. The method was used targeted (compound of interest identified in advance, see below 4.7.3.) in paper III and untargeted in manuscript paper V.

4.7.2 Gas chromatography metabolite analysis following derivatization

Broad identification and quantification of small-molecule metabolites (<1500 Da); such as sugars, amino sugars, amino acids, vitamins, carboxylic acids, and nucleotides etc.) is often called metabolomics (Bowen & Northen, 2010). One of the suitable techniques for metabolomics analysis, is a gas chromatography coupled to mass spectrometry (GC–MS) due to the high

separation power, reproducible retention times, and sensitive selective mass detection. Derivatization of compounds was used before the GC/MS analysis to increase volatility. This improves detectability in GC, so more compounds would be detected (Erdman, MacDonald, & Zeisel, 2012) that otherwise would be more easily detected using Liquid Chromatography. Metabolomics research most often involves comprehensive nontarget analysis of at least as many as possible metabolites in cells, tissue or body fluids (Koek, et al., 2011). This method was only used untargeted, i.e. compounds of interest were identified post-analysis.

4.7.3 Target versus untargeted analysis

When targeted analysis is carried out, a decision is made pre-analysis regarding which components will be monitored. An example is the quantification of hexanal in paper III.
The components monitored, often only a few, are known, and quantification of these is easy using the components of interest that are often available from a commercial supplier.
Regarding targeted analysis, all components identified are in principle collected, pre-processing is carried out and then often the components are regressed to some type of response variable like a sensory attribute. Untargeted approaches were performed in paper V. Those methods imply an entire multidimensional sample profile and a comprehensive examination of qualitative and quantitative differences in the chemical composition characteristic between samples. The goal is to support classification of samples on the basis of degree of similarity/correlation (Cordero, et al., 2010) and deduce processes behind the relationships between variables and response.

To understand the specific molecular composition involved in specific lamb-sheep meat flavour, the non-targeted examination was performed on GC-MS data. For identification of the meat metabolites, the AMDIS software (version 2.71, National Institute of Standards and Technology, Boulder, CO, USA) in combination with NIST05 (National Institute of Standards and Technology/Gaithersburg, MD, USA) and GOLM metabolome data base (Max-Planck Institute for Molecular Plant Physiology, Golm, Germany) were used. For securing relationships, feature extraction techniques were used (Unscramler X10.4.1, Camo software AS, Oslo, Norway).

4.7.4 Flavour-omics

Flavour-omics is an expression used to investigate relationships between sensory properties and data obtained from gas chromatography or liquid chromatography. Flavour-omics is associated with use of statistical methods often covered under the name chemometrics (see above for multivariate regression methods). The concept was coined in 2008 by Reineccius (2008) and is still a young science. The chemometrical approach aids the identification of which components will be directly and indirectly related to sensory flavour. However, an extensive puzzle is thereafter needed to elucidate causal relationships. Using reference compounds with known thresholds and flavour descriptions (Sherman, Greenwood, Villas-Bôas, Heyman, Harbertson, & Fiehn, 2016) is one option to identify relevant sensory compounds.

4.8 Sensory analysis

For the sensory evaluation of the cooked sheep/lamb meat, the most important quality attributes include appearance and colour, flavour, juiciness, and tenderness (Mendiratta, Kondaiah, Anjaneyulu, & Sharma, 2008). Different production systems, age, ageing time, etc. affect sensory characteristics of sheep/lamb meat and are a cause of variability in the quality (Sañudo, et al., 2008). In a paper V sensory panel according to AMSA (1995) was used to define differences between lean meat samples.

4.9 Fatty acid analysis

Gas chromatography and mass spectrometry (GC-MS) (Figure 9) was used to analyse fatty acids present in meat. Fatty acid methyl esters (FAME) were derived by transesterification of fats with methanol from selected animal tissues and then analysed by using a method described by (Devle, Rukke, Naess-Andresen, & Ekeberg, 2009) with minor modifications.

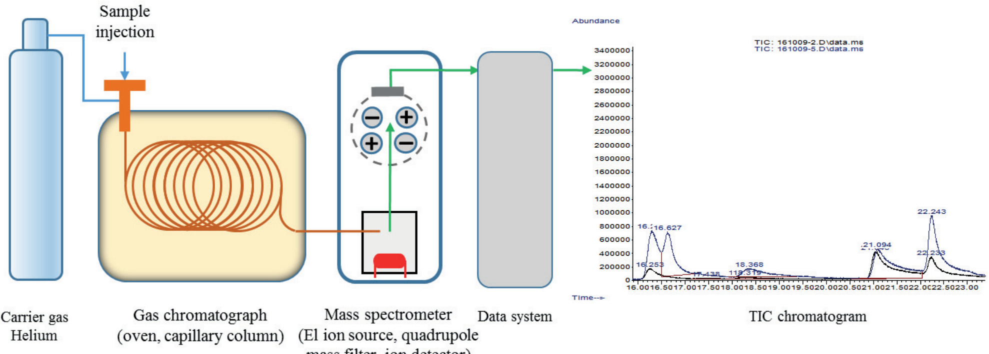

Figure 9: System of GC-MS

5 Main results and discussion

This thesis focuses on meat quality of ruminants (beef, sheep and lamb) with emphasis on their meat colour and flavour. Regarding flavour, the focus has been on compounds that were accessible using GC analysis.

The quality characteristics of meat and their variation are dependent on the complex conditions and mechanisms during the *pre mortem* and early *post mortem* periods. In addition to these conditions and mechanisms, the measuring methods, preparing of samples, and instruments also play a very important role in obtaining reliable results.

Below is a short reasoning of how this PhD project developed. It first had an in-depth focus on colour and then on flavour (Figure 10). The two-quality focus is linked through meat quality and lipid degradation and oxidation.

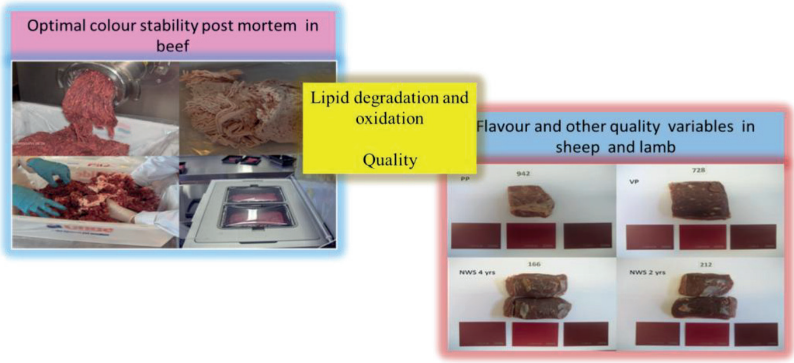

Figure 10: The first quality focus was on colour of meat while the second focus was on flavour and the other quality variable. Both meat quality parameters are dependent on lipid degradation and oxidation.

In the first article, the main goal was to make a calibration model for predicting Mb states in stored ground beef. The method was based on a similar method used by Khatri et al. (2012) on beef steaks where Mb states were determined according to the AMSA guidelines (1991). These guidelines are now updated.

Both experiments with steaks and ground beef gave significant results after validation of models. After correction and normalization, prediction errors of about 4 % and 5 % for Mb states on the

surface of steaks and ground beef were found. The AMSA method from 1991 resulted in prediction errors of Mb states of about 8 –18 % for whole steaks (Isaksson, Khatri, Bjelanovic, Sørheim, Slinde, & Egelandsdal, 2012). It is important to mention that there is a need to adapt the preparation methods in order to obtain pure Mb states.

The maintenance of raw meat colour *pm* is dependent on a number of factors such as the environment (MAP) in which the meat is stored, and also the inclusion of any colour-stabilising agents like the antioxidants that play an important role in colour and flavour stability. The combined effects of added KCS in article II and different MAP packaging in article I (Figure 3) during different storage times, affected the relative proportion of Mb redox states. Some chemical agents and/or substrates are known to improve colour stability in meat, but most previous studies were limited with respect to the number and concentration of used substrates. The substrates (specifically succinate, glutamate, citrate, malate, and, to a lesser extent, pyruvate) used in article (II), in combination with certain MAP, made it possible to improve the appearance (colour stabilization), shelf-life, and thus sale of raw meat.

After working with colour stability of ground beef in articles I and II, the subject was shifted towards lipid oxidation (article III) in meats packaged in MAP with KCS added since lipid oxidation (LO) is a logical and inseparable part of the transformation process of Mb redox states. LO directly affects this process and thus the colour change, meat taste, and otherwise the total meat quality. Some combinations of these compounds can also improve the storage-stability of meat products reducing the extent of rancidity. Compared to endogenous lipid oxidation in meat, the increase of lipid oxidation with addition of some KCS in high oxygen packaged meat was much less from the sensory point of view as described by Yi et al. (2015).

The last segment in this PhD thesis was dedicated to how the different animal groups, age, and sheep/lamb breed influenced meat quality (article IV) including colour, release of flavour compounds in stored meat (manuscript paper V), and sensory properties. The research was expanded to include factors other than colour and lipid oxidation, but they encompass "meat quality" and are equally important to the overall quality of meat. The three different animal groups were from: Bosnia and Herzegovina (B&H) Vlašićka Pramenka (VPr) sheep and lambs (indigenous); Montenegro (MN), Pivska Pramenka (PP) sheep (indigenous); and Norway (NO), Norwegian white sheep (NWS) sheep and lambs (crossbred). Using samples from these animal groups, general information about compounds affecting flavour of sheep/ lamb was approached.

5.1 Research interests

Bearing in mind that quality characteristics of post slaughter meat, specifically the intensity of colour and the flavour are main drivers of consumers acceptance of meat, efficient methods to produce/calculate and maintain desirable colour, flavour, and other quality attributes of the packaged meat were discussed and presented.

5.2 Target group

The varied population of food service groups, consumers, and researchers are the target groups of this scientific work. These packaging innovations are also important for industry research and development programs, and thus for the global and fast transport of food. Article I can be of particular interest for researchers because lower prediction errors for Mb states were obtained compared to AMSA results. Research article four and manuscript five are of particular interest for the Western Balkan states. Montenegro and Bosnia and Herzegovina participated and contributed in a bilateral project with Norway via the Norwegian Ministry of Foreign Affairs (No. 19028; HERD - Program for Higher Education, Research and Development, Agriculture) and provided samples. The Balkan region contains candidate countries for EU membership and the introduction of EU regulations, but there is a lack of information regarding their meat products. The results can also be of interest for the Balkan region overall since similar research has never been performed on Pramenka sheep/lamb.

5.3 Papers

5.3.1 Paper I

In the present study, a calibration model for DMb, OMb, and MMb states on the surface of a meat matrix composed of ground beef from *M. semimembranosus* and two types of fat tissues from either beef or pork of two 5 and two 1.5-year-old animals, respectively, was predicted.

The method comprised measuring the absorbance spectra of the minces packaged in MAP using spectroscopic equipment specifically designed for non-invasive measurements.

As opposed to the method study from Khatri at al. (2012) where intact meat was used to produce calibration samples, in the study of Bjelanovic et al. (2013) samples were composed of ground beef and fat. The samples were prepared to contain a predominantly DMb, OMb or MMb redox state on their surface. The data collection software delivered the spectroscopy data in absorbance (A) units equal to log (1 / R). Khatri et al. (2012) used K/S values (K and S are the absorption and scattering coefficients, respectively) at selected wavelengths to predict Mb states on unknown samples. Absorbance spectra were then corrected by using Extended Multiplicative Scatter Correction (EMSC), before PLS regression models were built. After calculations of states from PLS regression in ground beef, a post transformation was necessary in order to calculate the three Mb states and the lowest prediction errors (Egelandsdal, Bjelanovic, Khatri, & Slinde, 2013). Prediction of the Mb states of ground beef compared to measurements on whole steaks gave slightly poorer results (Table 2). The combination of EMSC/PLS was superior to the AMSA method in both studies with respect to providing good calibration models for predicting myoglobin states even under conditions where the state was not pure (as for their DMb state) (Bjelanovic, Sørheim, Slinde, Puolanne, Isaksson, & Egelandsdal, 2013; Khatri, Phung, Isaksson, Sørheim, Slinde, & Egelandsdal, 2012).

Table 2. The prediction error of the different Mb states on the surface of ground beef and beef steaks using multivariate regression methods.

Sample set	Mb state	RMSECV [fraction (# PLSR factors)]	R
Ground beef (Bjelanovic et al., 2013)	DMb	0.051 (2)	0.996
	OMb	0.055 (3)	0.995
	MMb	0.045 (5)	0.997
Whole steaks (Khatri et al., 2012)	DMb	0.042 (2)	0.997
	OMb	0.041 (3)	0.997
	MMb	0.039 (3)	0.997

Adapted from Egelandsdal, Bjelanovic, Khatri, & Slinde (2013).

5.3.2 Paper II

With the new calibration model for predicting myoglobin states in ground beef packaged in MAP, the ability of KCS (that stimulate formation of NADH and $FADH_2$) to stabilize red colour in stored ground beef was investigated. Moreover, under aerobic conditions, the metabolic

substrates could, on average, retain 66% OMb after 8 days of storage, while in anaerobic conditions, the substrates could, on average, retain 74% DMb after 13 days of storage. These values are high enough to give a favourable colour impression of the samples.

Response surface analysis showed that the DMb redox state dominated in anaerobic, while OMb predominated in aerobic packaging, as expected. In both anaerobic and aerobic packaging, the substrates glutamate, malate, succinate, pyruvate, and citrate were the main cause of variation in Mb redox forms. Two different concentrations of total mixture (0.1 and 0.05 mol/kg) were used, but their effects on Mb redox forms were quite similar.

In anaerobic conditions, the mixture composed of the additives succinate and glutamate-malate induced DMb most quickly. Using only the addition of pyruvate consequently resulted in prevalence of MMb, while addition of only succinate favoured DMb formation. The mixtures with an identical amount of all compounds lead to approximately 50% DMb in anaerobic packaging.

In aerobic packaging (75% O_2), the metabolic substrates glutamate-malate supported stabilization of OMb over the entire storage period. The addition of citrate was important for stabilization of OMb in aerobic packaging, especially when storage time was longer. Molar ratio of glutamate/malate to citrate of 3:1 induced the highest OMb level. Addition of the pyruvate and glutamate/malate induced MMb formation. Optimal additions of the KCS to ground beef were superior with regard to adding distilled water only.

5.3.3 Paper III

The lipid oxidative stability was studied in ground beef samples packaged in MAP where the KCS were added previously, in order to stabilize meat colour (paper II).
In anaerobic packaging, the combinations of succinate and glutamate (50:50 molar ratio) increased TBARS and the peroxide forming potential (PFP), while aerobic conditions glutamate-malate, in combination with citrate (56:25:19 molar ratio), was also followed by increased PFP and TBARS formation. Regardless of the fact that KCS apparently acted as pro-oxidants when added to ground beef packaged in aerobic and anaerobic conditions, the increase of PFP and TBARS was small and not believed to have any sensory importance.

The largest cause of variation (20-40%) in the lipid derived volatile compounds was explained by the type of added fat tissue. The level of PUFA was associated with lipid degradation, while KCS affected the concentration of volatile compounds (VOC). The mixture composed of pork fat tissue and beef meat, either from young or older cattle, gave the highest amounts of hexanal (marker of *n-6* FA degradation) and 2-octen-1-ol, known as degradation product from arachidonic acid (Volden, et al., 2011). Pyruvate was efficient in prevention of lipid oxidation in ground meat.

Hexanal production in MAP containing 75% O_2 was an indicator that addition of the KCS also increased reactive oxygen species formation (ROS).
In contrast to the succinate and glutamate, which metabolized in the MAP until oxygen was consumed, citrate was not metabolized, and this resulted in a decrease in lipid degradation.

The shortcoming of the addition of KCS such as glutamate and succinate is that they may be detected in ground meat because of the characteristic flavours if used at the level in paper II and III. The addition of citrate is only limited by its specific taste, since the citrate is on the *quantum satis* list, while glutamate is on the list where upper limits are defined.

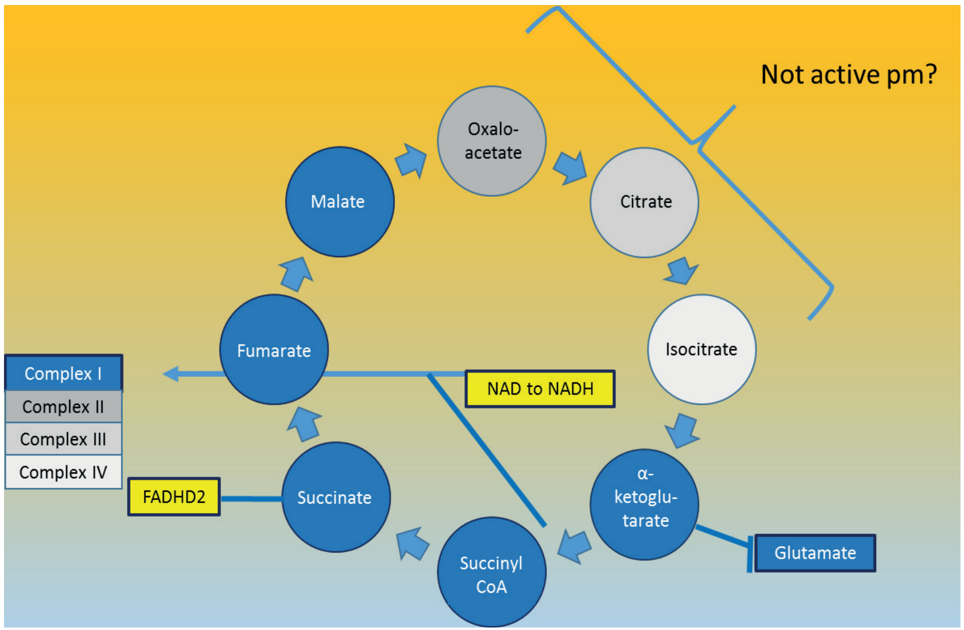

Figure 11: Flow of electron in low oxygen packaging.

The results from this paper suggest the following generation of reducing equivalents to the electron transport chain in low oxygen MAP (Figure 11). The buildup of malate leads to product inhibition.

5.3.4 Paper IV

The quality of ruminant carcasses and meat is of predominant importance in a competitive market where consumers tend to have certain ideas about the criteria defining meat quality characteristics.

The experimental work was initiated to determinate some of the quality characteristics of the indigenous Pramenka sheep breed (PS) from the Western Balkans (WB). For comparison, the Norwegian White Sheep breed (NWS), that had been subjected to the national selection program, (phenotypes for growth) was used.

Carcass performance like carcass weight, EU fatness and conformation, pH, tenderness and cooking loss were explored for sheep and lamb meat (*M. longissimus thoracis et lumborum* (*LTL*)) from animal groups in Bosnia and Herzegovina, Montenegro, and Norway. In Bosnia and Herzegovina, Pivska Pramenka (PP) sheep and lamb, and in Montenegro, Vlasicka Pramenka (VPr) sheep are raised more extensively, oriented towards utilization of grassland and pasture areas. Norwegian White sheep (NWS) and lamb rearing are produced more intensively, but lamb and sheep graze outdoors during the summer.

Carcass and meat quality characteristics such as meat colour, pH, tenderness, and fatty acid composition were significantly influenced by different animal groups. Higher haem and lower water contents were found in older animals, as expected. Pramenka sheep were smaller with more fat, lower protein content and antioxidant capacity, higher pH_{24}, IMF and n-6/n-3 ratio compared to NWS. Despite a higher fat α-tocopherol content, meat from Pramenka sheep became more easily rancid. Marketing advantages of Pramenka sheep rely on better colour stability and tenderness, while Norway's NWS lambs displayed a better nutritional profile.

Cathepsin B activity was similar for all animal groups. The results showed variation only within groups; the highest variation was for old NWS and PP sheep while the lowest variation was for NWS lamb and VPr lamb.

TBARS (thiobarbituric acid reactive substances) was used as a chemical measure of secondary oxidation products. After 7 days of aging at 4ºC, VPr sheep had the lowest TBARS value among sheep groups. TBARS accumulation was highest in PP sheep and VPr lamb, while in NWS sheep TBARS level was almost the same. TBARS value in NWS lamb was the lowest.

The marketing benefits of NWS relate to high protein/fat ratio, low *n- 6*/*n-3* ratio, and good antioxidant capacity. VPr sheep were muscular, with more fat, lower water content, cooking losses, L*a* b*values, and with higher *n-6*/*n-3* ratio. Meat from VPr became more rancid than meat from MN sheep. VPr lambs were smaller compared to NWS lambs, with a higher content of vitamin E and lower antioxidant capacity. NWS lamb meat had lower TBARS and higher EPA and lower n-6/n-3 ratio. The marketing potential of VPr lamb seemed only related to its higher vitamin E content. However, EC regulation demands that vitamin E content must be at least 15 % of the reference value that equals 12 mg/ 100g (in α-tokoferolekvivalenter = 1 mg D-α-tocoferol) (The Government of Norway - Ministry of Health and Care Services, 2009). This means that one must have 1.8 mg/100g for labelling the meat as a source of vitamin E and the highest value obtained was 0.6 mg/100g in a sheep from Montenegro. However, higher vitamin E level is beneficial for meat qualities like colour stability. The good oxidative stability with a favourable n-6/n-3 ratio showed the marketing potential of Norwegian lambs.

5.3.5 Manuscript Paper V

Volatiles of adipose tissue, metabolite, and sensory attributes of lean meat and fat tissue from different sheep and lamb groups were studied, in order to understand flavour variation associated with sheep and lamb.

The sensory attribute gamy flavour was most easily picked up by the sensory panel using the 92 animals for modelling the flavour with a PLS model. For this flavour, the metabolites and volatiles explained about 51-53 % of the variation when the 15 VOC were included in the model. This is a substantial explanation as most sensory analyses contain a significant error component.

In addition, the sensory scale was used well, meaning that the panellists found both samples with high and low gaminess. The flavour seemed to originate from different metabolite groups. One group was free amino acids present above taste thresholds. These may relate to the status of the animal at slaughter regarding feed, stress, fatigue etc. Another compound group seemed related to oxidative muscle types/ mitochondrial content as Krebs cycle components such as citric acid related to gaminess. Also, a volatile group contributed to gaminess but it was unclear if these were indirect markers, as in being above threshold, or simply correlated with the Krebs cycle component group.
Grassy flavour was less well explained (up to 50 %) by 19 VOC but had individual markers that did not correlate to gamy flavour. As an example, the diet marker caryophyllene, which originates from herbs correlated to caryophyllene.
The sensory panel found it more demanding to identify bitter and metallic flavours and thus also to identify compounds that related to this flavour was more demanding. Hypotaurine was among the more convincing markers for bitterness and metallic flavour.

Some focus was also put on compounds specific for animal groups. The volatile profile of BH lamb did not clustered together with NW animals. A clear differentation in volatile profile were found between BH and MN sheep. The third PCA analysis showed a clear differentiation for all animal groups, and the animals from the same production system clustered together. The antioxidant compounds that suppressed BH lamb off-flavor properties can probably contribute to their unique metabolite pattern.

5.4 Limitations

In papers IV and V the balance regarding the number of animals was not able to be respected. The lamb group from MN was lacking because there was not a sufficient number of 5-6 month old female lambs from the same herd (small production area).

It may also seem unusual from a strict scientific perspective that an indigenous breed in the Western Balkans is compared to more well-bred animals (Norwegian White) in Norway. However, for a region that has not initiated any breeding for sheep / lamb animals, it may be of relevance to have comparisons to a sheep where breeding has focused on rapid growth and lean meat fraction as well as reproduction. Norway has “Gammelnorsk sau” that could be compared

to the present Norwegian White. Such an extensive comparison as done here has not been carried out previously. The Western Balkans obviously cannot make such comparisons since crossbreeding is not present to a significant extent.

5.5 Main scientific achievements

This thesis contains the first prediction model of myoglobin states on minced meat surfaces that does not depend on the use of isobestic points that principally do not exist in meat and minces due to light scattering. Although Paper I resembles the paper of Kathri et al. (2012) using meat filets, the method used in **Paper I** is innovative as the prediction error is low bearing in mind the problems that are often experienced when minces have to fall down on the packaging film before measurements. When the mince falls down on the packaging film, the film tends to be smeared by fat. In addition, prediction errors are not described even in the new and revised AMSA guidelines (AMSA, 2012).

The optimal combination of KCS (**Paper II**) for maintaining or stabilising colour has never been arrived at by blending so many compounds. This may explain why the results differ from previous publications (*e.g.* for pyruvate and malate). **Paper III** is also the first paper to actually measure the metabolization of KCS. The results obtained are obviously an indication that the compounds are actually used according to the hypothesis that the electron transport chain system is involved in colour stabilization.

Many meat quality traits were examined in sheep/lamb meat (**Paper IV**) from different lamb/ sheep groups. Paper IV gives the broadest introduction ever to the quality of meat from animals of the Pramenka breed grazing in the Western Balkans. This is in fact also the case for Norwegian White Sheep.

Finally, this seems to be the most extensive flavour-omic approach using lamb and sheep as samples (**manuscript Paper V**). The literature has hitherto focused on volatiles with well-known off –flavour components that give an un-pleasant smell to sheep and make consumers dislike sheep meat. The frequency of this bad smell among sheep is unknown both in Norway and the Western Balkans.

Also, for some flavour compounds, their flavour description and threshold are attempted to be described for the first time. In addition, describing the components that dominated the gamy and grassy sensory flavour attributes in lamb/ sheep is novel.

The achievements referred to in this thesis can help with understanding the quality of lamb/ sheep meat. In addition to the scientific significance, these results will undoubtedly have practical relevance as well.

6 Conclusions

1) The MAP packaging maintains myoglobin redox stability forms of the ground beef mixed with fat tissues from either beef or pork.

2) Mitochondria and Mb play an important role in the stability and sustainability of the raw meat colour; a desirable dark appearance of meat packaged in MAP is the result of oxygen consumption and MetMb reduction, respectively.

3) The measurement of meat colour changes requires an accurate, non-invasive, reproducible, fast, and cheap measurement method. The most important region for predicting Mb states is between 400-700 nm according to results from paper I. NIR and Minolta are the adequate instruments for determining colour of ground meat as well as for meat slices. The surface of ground meat and meat cuts should be thicker than 2 cm, providing good light reflection from the surface of the samples.

4) Mitochondrial electron transport, generated by KCS, changed the Mb redox forms in ground beef packaged in MAP. Succinate and glutamate-malate played an important role in the sustainability of the DMb redox form in ground beef during storage time (13 days in MAP), while glutamate-malate, and citrate became increasingly important with storage time in high oxygen for maintaining the OMb redox form.

5) The KCS can temporarily increase lipid oxidation and degradation. However, the effects are small from a sensory, health, and, consequently, from an economic point of view when KCS is added for colour stabilization purposes.

6) Carcass and meat evaluations in the present study indicate that the marketing advantages of the Pramenka breed from BH rely on good colour stability and tenderness.

7) Gamy flavour was related to free amino acid status, components of the Krebs cycle, volatiles such as 3-methyl heptane, and the level of sweet tasting components.

8) Flavour attributes of sheep/lamb meat showed a clear separation for meat from BH, MN, and NW animals, presumably influenced by different production areas, age, and breed. However, to identify true markers of production systems, the results should be repeated with all breeds preferably grazing on the same locations for another year.

7 Future perspectives

The results in this thesis have shown the importance of the use of all wavelengths (400-1100nm) and PLS in combination with optimal preparation methods for quantification of surface myoglobin redox states on meat matrix.

Considering the increasing market of meat packaged in MAP, more understanding of the influence of MAP on meat colour is necessary since modern breeding has resulted in decreased pigment content.

Analytical methods for analysing a large number of flavour, in particular volatile, compounds are established and they are still developing, contributing to better understanding of meat flavour. For flavour/ taste components, taste thresholds of many components are still missing. Some metabolites were apparently present at concentrations lower than their detection thresholds, but their role in providing understanding of aroma in different meats and their possible effect on consumer acceptability may still be of importance. More work is needed on validating the importance of taste components in sheep, both with and without the badly smelling components such as skatole and 4-methylnonanoic acid for female animals. This would be of great practical interest for producers of sheep meat. Finally, and to the best of the researchers' knowledge, it has, for the first time, been observed that the *pm* colour stabilising compounds involved in the Krebs cycle are also affecting natural flavour/ taste in lamb/ sheep. It is therefore very interesting to use this type of meat to study the relationship between taste and colour stability.

Future research should focus on:

1) Building a colour calibration to be used on Minolta.
2) Investigation at low oxygen concentrations as this is often a problem in retail.
3) The changes in all Krebs cycle compounds (alpha ketoglutarate etc) and lactate.
4) Analysing the change in all Krebs cycle components *pm*.
5) Looking at quality of lamb from Montenegro.
6) Repeating the flavour measurements on meat from another slaughter period to validate markers.
7) Assessing if the flavour components that have been identified are relevant for bad smelling female sheep (skatole and 4-methylnonanoic acid tainted meat).
8) Elucidating the relationship between flavour and colour stability in sheep.

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Paper I

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Determination of the myoglobin states in ground beef using non-invasive reflectance spectrometry and multivariate regression analysis

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ABSTRACT

Seventy-two samples of ground beef from *M. semimembranosus* of two 5 and two 1.5 year old animals were prepared. Two types of fat tissues from either beef or pork were added to the ground beef. The samples were prepared to contain predominantly deoxymyoglobin (DMb), oxymyoglobin (OMb) and metmyoglobin (MMb) states on surfaces using selected methods based on chemical treatment (for MMb) and oxygen pressure packaging to induce the two other states. Reflectance spectra were measured on ground beef after three storage times. Partial least regression analysis was used to make calibration models of the desired myoglobin states. Validated models using leave-one-sample out cross validation gave, after correction and normalization, prediction errors of about 5%. Long term storage of ground beef was unsuitable for preparing pure MMb states due to gradual reduction of the pigment to DMb, presumably by bacteria.

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1. Introduction

Color is an important quality attribute influencing the consumer's decisions to purchase the meat because they use color as an indicator of freshness. Discoloration that induces quality deterioration of meat, leads to a $1 billion annual revenue loss in the American meat industry (Mancini & Hunt, 2005; Smith, Belk, Sofos, Tatum, & Williams, 2000).

Myoglobin and hemoglobin are the pigments responsible for the color of meat, of which myoglobin is the main component in well bled muscle (Fernández-López, Sayas-Barberá, Pérez-Alvarez, & Aranda-Catalá, 2004). Myoglobin in meat exists predominantly in the three redox forms; bright red oxymyoglobin (OMb), purple deoxymyoglobin (DMb) and brownish metmyoglobin (MMb). CIE L*, a*, and b* values have been used to monitor changes in meat surface color over time (De Marchi, Penasa, Battagin, Pulici, & Cassandro, 2011; Fernández-López et al., 2004; Sheridan et al., 2007; Tapp, Yancey, & Apple, 2011).

There are different methods for calculating myoglobin states. By adding the chemicals sodium dithionite ($Na_2S_2O_4$) or potassium ferricyanide $K_3[Fe(CN)_6]$ it is possible to produce DMb and MMb, respectively (Wilson, Ginger, Schweigert, & Aunan, 1959). These commonly used methods are recommended by AMSA (1991) and were called Chemically Induced Myoglobin States (CIMS) (Khatri et al., 2012). Myoglobin forms can also be produced by modified atmosphere packaging (MAP) by adjusting the Oxygen Partial Pressure (OPP method) to approximately zero to produce DMb, to low O_2 to produce MMb, and to high O_2 to produce OMb (Sørheim, Westad, Larsen, & Alvseike, 2009; Taylor, Down, & Shaw, 1990). Fresh ground beef packaged in vacuum-bags without oxygen access, will consume all the oxygen present in the system in a few days, and will result in DMb as the primary pigment (Mancini & Hunt, 2005). MMb is formed at low oxygen pressure due to the fact that fast transformation to MMb occurs at 0.1–2% oxygen (Mancini & Hunt, 2005; Sørheim et al., 2009). To produce OMb, samples should be placed at low temperature (from 0 to 2 °C) in a high oxygen atmosphere, and flushed with 100% oxygen for 10 min, packaged in oxygen permeable film and scanned immediately (AMSA, 1991).

The high precision and accuracy in production of these three myoglobin states in solution will give good and simple equations for calculating mixtures of myoglobin states in new samples. Such predictive equations are based on isobestic points (Tang, Faustman, & Hoagland, 2004). For meat samples, however, light scattering needs to be handled in addition to preparing pure myoglobin states. Transmission spectra are often replaced by reflectance spectra for

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meat systems. The Kubelka–Munk transformation (Kubelka, 1948) to K / S values (K = absorbance coefficient; S = scattering coefficient) is frequently used to improve on linearity, reduce the contribution from scattering and make the spectra look more like transmission spectra. Recently different methods for scatter correction have emerged so that chemical effects can be more easily modeled. One such method called Extended Multiplicative Scattering (EMSC) (Martens & Stark, 1991), has been successfully used for elimination of uncontrollable path length or scattering effects, such as those caused by *e.g.* light scattering in reflectance spectroscopy (Khatri et al., 2012; Thennadil, Martens, & Kohler, 2006).

A simple approach to handle scatter has been to define the absorbance at 730 nm as zero. This is often used as an alternative to Krzywicki (1982) based on the variable log (1 / R), where R is reflectance, to calculate the myoglobin concentration of DMb, OMb and MMb using the isobestic wavelengths 474 nm, 525 nm, 572 nm and 610 nm. The same wavelengths are recommended by AMSA (1991). The existence of true isobestic points for scatter matrices such as meat should be questioned as neither the path of light nor myoglobin concentrations are known for each sample where myoglobin states are calculated.

Another mathematical approach other than recommending specific wavelength for all systems should be considered. The successful use of reflectance spectra in near infrared for fat, water and protein determination has relied on multivariate regression techniques (Næs, Baardseth, Helgesen, & Isaksson, 1996). Martens and Næs (1989) suggested that the method of Partial Least Square regression (PLS) is efficient in finding fundamental relations between two matrices. PLS makes it possible to model the multidimensional direction in the X matrices (*spectra*) that covary with the Y matrices (*myoglobin states*) (Wold, Sjöström, & Eriksson, 2001). This regression method was used by Khatri et al. (2012) to predict myoglobin states with lower prediction errors than possible if a few selected wavelengths were used. They also suggested that the PLS regression method seemed to partly compensate for lack of accuracy in preparing pure myoglobin states.

The main objective of this study was to extend the method proposed by Khatri et al. (2012) for predicting myoglobin states of whole meat to ground beef systems that have different scattering properties. The sub-objectives were: 1) to make a calibration model for predicting myoglobin states in stored (0–13 days) ground beef; 2) to evaluate the stability of the preparation method of pure myoglobin states on different stored minced samples; and 3) to compare regression models for intact muscles with those from ground beef.

2. Materials and methods

2.1. Raw material

Four days *post mortem* beef muscle (*M. semimembranosus*) with adhering fat tissue; from two 1.5 and two 5 year old animals were supplied by a local slaughter house (Fatland A/S, Oslo, Norway). In order to get variation two different fat tissues (beef and pork) and water were included. The type of breed was not identified. The muscles were transported and stored at 0–4 °C. The pork fat was supplied by HK Scan (Ruokatalo, Finland) and transported vacuum packaged and frozen to the lab.

2.2. Preparation of materials

2.2.1. Preparation of ground beef and fat

The beef muscle and pork and beef fat tissues were weighed and ground separately with a Seydelmann ME-130 (Seydelmann, Stuttgart, Germany) grinder, through a plate with 3 mm openings. The ground beef was prepared separately from each of 4 animals and mixed with additional fat from pork or beef in ratios of 86% w/w and 14% w/w respectively, and was manually mixed for 3 min. The mixture was then re-ground. To the ground beef and fat tissue (360 g) was added 40 g distilled water that was then manually stirred for about 2 min. The minces were varied because the predictive model for myoglobin states was to be used in another experiment where different solutions and fats were added.

2.2.2. Preparation method of myoglobin states

2.2.2.1. OMb. The ground mixtures were placed in boxes of amorphous polyethylene terephthalate (APET) trays, which were sealed with top film of ethylene vinyl alcohol (EVOH). The dimensions of the boxes were 20.5 × 14.8 × 3.5 cm. The trays and films (Wipak Multipet and Wipak Biaxer, both Wipak, Nastola, Finland) had oxygen transmission rates of 7 and 5 $cm^3/m^2/24$ h at 23 °C/50% relative humidity, respectively, at 50% relative humidity. A tray sealing machine (Promens 511VG, Kristiansand, Norway) was used for packaging of the OMb samples. One third of the OMb samples (8) were placed in trays filled with an atmosphere composed of 75% oxygen and 25% CO_2 (a premixture supplied by AGA, Oslo, Norway) after preparing the minces, as described in Section 2.2.1. The spectroscopic measurements were taken through the top film (EVOH) about 45 min after packaging in a high oxygen atmosphere and this time point was denoted as zero time. The rest of the samples (16) to be prepared later for the OMb state, were stored in EVOH bags (Biaxer) at 4 °C. One third of these samples (8) were removed on day 6, opened in order to be placed in trays with a high oxygen atmosphere, sealed and measured after 45 min. This procedure was repeated on the last 8 samples of ground beef on day 13.

2.2.2.2. MMb. The ground mixtures were oxidized with a 1% potassium ferricyanide $K_3[Fe(CN)_6]$ solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The ground mixtures were thoroughly soaked for 1 min. The excess $K_3[Fe(CN)_6]$ solution was drained off, and thereafter left covered with oxygen-permeable polyvinyl chloride film (PVC) at 4 °C. After 16 h the PVC film was removed and replaced with top film (EVOH). At this point the spectroscopic measurement was done through the top film and the time was denoted as time 0. This procedure was repeated on the stored ground beef at days 6 and 13.

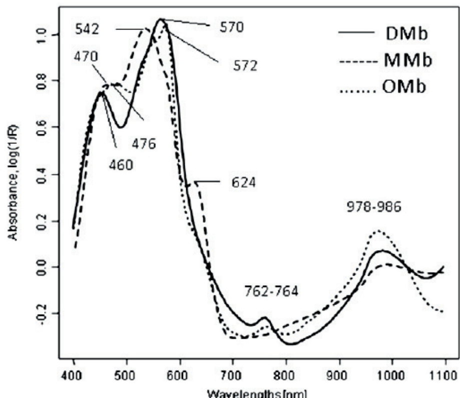

Fig. 1. Average EMSC (Extended Multiplicative signal Correction) spectra (from I = 64) *versus* wavelengths. "….." denotes OMb, "----" denotes MMb and "___" denotes DMb.

2.2.2.3. DMb. The ground mixtures were vacuum packaged in EVOH bags (Biaxer). The samples were stored at 4 °C from 0 to 13 days. Spectroscopic measurements of DMb were taken directly on the vacuum packaged sample; the first time after 3 days because this is the time needed for meat to form DMb transformed from OMb or MMb. This time was used as zero time for the DMb state. Thereafter, the spectroscopic measurements were taken directly on unopened stored ground beef samples at days 6 and 13 to eliminate oxidation of the DMb state through repacking. This procedure reflected an assumed optimal production method for DMb based on Khatri et al. (2012).

2.3. Measurements of reflectance spectra

During the trial 72 samples were analyzed. The samples were taken from the cold (4 °C) and the measurements were done for 1 min at an ambient temperature of 22 °C. The samples were scanned at three different random positions on the package's surface in order to increase the scanned area and reduce measurement error. Samples were scanned with a Foss NIRSystems OptiProbe™ 6500 Analyzer (Foss NIRSystems Inc., Maryland, USA) equipped with an interactance fiber optic probe (NR-6770-A, Foss NIRSystems) and software from Vision (2001, NIRSystems). The probe head consisted of seven rectangular parallel glass windows (1.0×20.0 mm^2), about 2.0 mm apart, mounted in the middle of a 40×40 mm^2 metal block. The optic probe emits monochromatic light every second (0, 2, 4, 6 and 8) into the sample and collects light every other second (*i.e.* every 1, 3, 5 and 7 s) by the detector. The design of the probe forces the emitted light to penetrate several mm into the meat sample. The instrument has an average spectral bandwidth of about 8.5 nm from the monochromator. The instrument was tested successfully (performance test and wavelength linearization) according to recommendations of the manufacturer. A reference spectrum of the apparent reflectance (R) was obtained on white ceramic tile ($L^* = 101.01$, $a^* = 1.74$ and $b^* = 5.3$) (Khatri et al., 2012) and re-calibrated every day before measurements. For absorbance ($A = \log(1 / R)$ where R is reflectance) measurements, thirty-two scans were made, at 2 nm intervals from 400 to 1098 nm, to give 350 spectral variables. The software further processed the data and stored them in absorbance units (A) equal to log (1 / R).

2.4. Design of the experiment, processing of spectral data and statistical analysis

The experiment was designed using a full factorial experimental design. Four factors were used; beef samples (4 animals), fat tissue (beef or pork), myoglobin states (OMb, DMb, MMb) and beef storage times (0, 6, and 13 days) for the different states were prepared. The levels for the three first factors were categorical and the last factor was a continuous variable. This gave $4 \cdot 2 \cdot 3 \cdot 3 = 72$ samples to be measured by spectroscopy. To ensure biological, physical and chemical variation, beef from two young (about 1.5 years) and two older (about 5 years) animals, were chosen. It is expected that older animals will have higher amounts of myoglobin compared to younger animals. The arguments for choosing two different fat tissues relate to increased tendency towards oxidation with pork fat tissue. To also include biological, physical and chemical variation during storage of ground mixture, myoglobin states were prepared after different storage times of the initially prepared ground beef samples.

2.4.1. Principal component analysis (PCA)
The method uses an orthogonal transformation to transmute the spectra into a set of values of uncorrelated variables called principal components (Martens & Næs, 1989). PCA was used to explore and diagnose the relationship between the three different chemically obtained myoglobin states.

2.4.2. Multivariate regression
Partial least square regression (PLS) is probably the most used method for multivariate calibration in near infrared applications (Næs et al., 1996). PLS is well suited when the matrix of predictors has more variables than observations, and when there is multicolinearity among X variables. The multivariate regression is usually carried out in four steps. First step: Spectra (with K variables) and a reference method, in the present case, samples with three specific myoglobin states (OMb, DMb and MMb) measured for a number of samples (I) represented as two matrices, $\mathbf{X}_{I \times K}$ (spectra) and $\mathbf{y}_{I \times 1}$(states), respectively. This may be referred to as a calibration set of samples. The myoglobin reference values were set to 1 for the myoglobin state prepared and to 0 for the two other states. Second step: A calibration model was determined. For PLS this can be done by

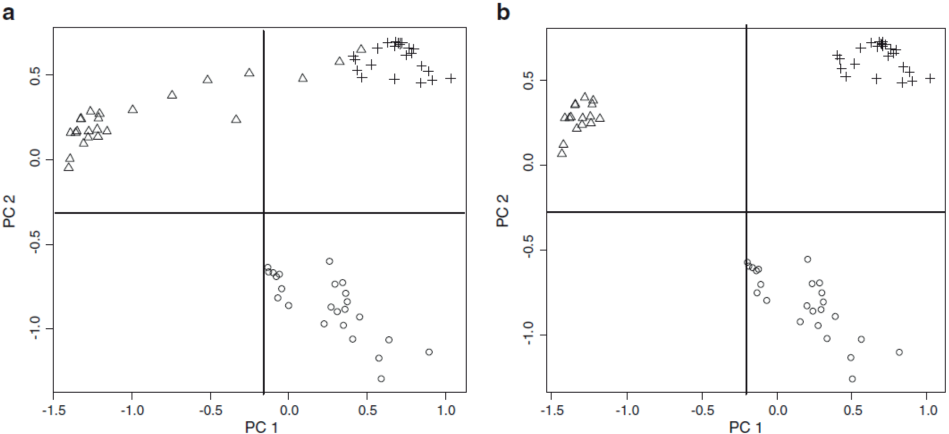

Fig. 2. Principal component analysis (PCA) score plots. PC1 *versus* PC2 for (a) all EMSC calibration spectra (I = 72) and (b) after the 8 outliers were taken out (I = 64), see text for further details. "o" denotes OMb, "Δ" denotes MMb and "+" denotes DMb.

Table 1
The prediction performances of the cross validated corrected and normalized predictions of DMb, OMb and MMb states using Extended Multiplicative Scatter Correction Absorbance EMSC(A) and PLSR.

Myoglobin states	*RMSECV*	Number of PLS factors	R
DMb	0.051	2	0.996
OMb	0.055	3	0.995
MMb	0.045	5	0.997

decomposing the centered **X** matrix into a score matrix ($\mathbf{T}_{I \times A}$) (where A is the number of factors), a loading weight matrix ($\mathbf{W}_{K \times A}$) and a residual matrix ($\mathbf{E}_{I \times K}$), formulated as $\mathbf{X}_c = \mathbf{TW}^t + \mathbf{E}$. Followed by estimation of the regression coefficient vector $\mathbf{q}_{A \times 1}$ for **T** as $\mathbf{q} = (\mathbf{T}^t\mathbf{T})^{-1}\mathbf{T}^t\mathbf{y}$ and the regression coefficient vector $\mathbf{b}_{K \times 1}$ for **X** as $\mathbf{b} = \mathbf{W}(\mathbf{P}^t\mathbf{W})^{-1}\mathbf{q}$, for regression model $\mathbf{y} = \mathbf{Tq} + \mathbf{e}$ and $\mathbf{y} = \mathbf{Xb} + \mathbf{e}$, where $\mathbf{e}_{I \times 1}$ is the residual vector, respectively. Each myoglobin state is modeled separately, and thus three models are obtained, one for each myoglobin state. Third step: The models are validated by leave-one-beef-animal-out cross validation, which give the quality of the models and the accuracy of the models, here expressed as root mean square error of cross validation, or validation prediction error, which can be expressed as $RMSECV = \left(I^{-1}\sum_{i=1}^{I}(\hat{y}_i - y_i)^2\right)^{1/2}$ where $\hat{y}_i$ is the predicted myoglobin states and y_i is the reference values for each myoglobin state, that is the pure states (set to 1 or 0) that were prepared. I is the number of samples. The quality of the model is better if the *RMSECV* value is lower. The *RMSECV* is used to determine a prediction confidence interval. If one predicts a sample to $\hat{y}_n$, there is about 95% confidence that y_n will be within the interval $[\hat{y}_n \pm 2RMSECV]$, presuming a normal distribution and no bias. The reference values for each myoglobin state and the linear correlation coefficient (R), between the predicted myoglobin states and actual myoglobin states were also calculated. The meaning of the validation step is also to identify the optimal number of PLS factors (A) to be used. Fourth step: When the best model is chosen and validated, any number of new samples ($\mathbf{x}_{i(1 \times K)}$) can be predicted as $\hat{y}_i = \mathbf{xb}$, for each myoglobin state. The predictions were corrected in step 3 ($\hat{y}_i > 1.00$ were set to 1.00 and $\hat{y}_i < 0.00$ were set to 0.00); in step 4:

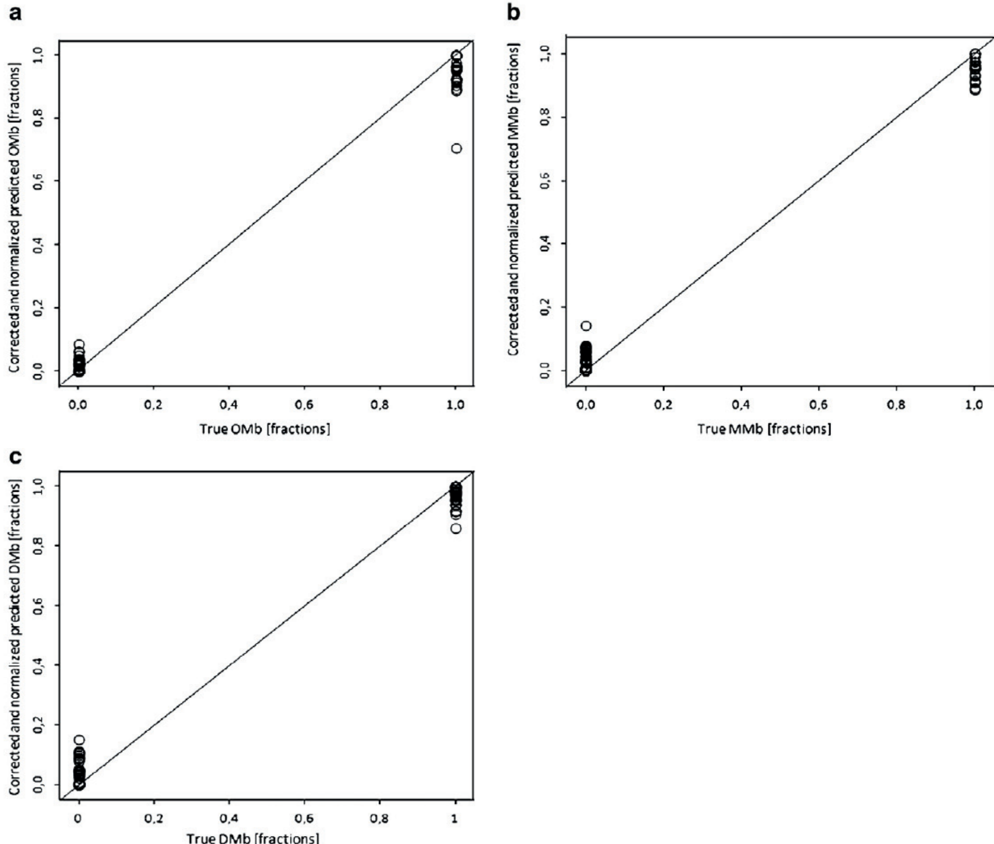

Fig. 3. Predicted, corrected and normalized myoglobin states *versus* true myoglobin states, for (a) OMb, (b) MMb and (c) DMb. The data were predicted (leave-one-sample-out cross validation) from EMSC log (1 / R) spectra (I = 64) using PLS with 3, 5 and 2 PLS factors for OMb, MMb and DMb, respectively. The diagonal line is the target line (y = x).

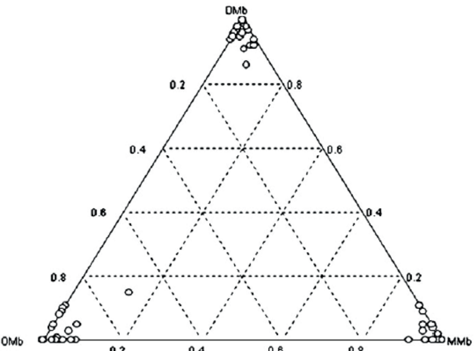

Fig. 4. The cross validated corrected and normalized predictions of DMb, OMb and MMb states using EMSC(A) and PLSR for the ground beef sample set (I = 64).

$\hat{y}_{i,OMb} + \hat{y}_{i,DMb} + \hat{y}_{i,MMb} = 100$, *i.e.* normalization. The regressions and predictions were performed in Unscrambler (Ver. 9.2, Camo ASA, Oslo, Norway), while the corrections were performed in Excel (Microsoft Office 2007, Seattle, WA, USA).

3. Results and discussion

3.1. Absorbance spectra of myoglobin forms prepared in ground beef

Fig. 1 shows average spectra for OMb, MMb and DMb. In general the three average spectra show largely overlapping absorbance spectra for these three myoglobin states. However, the average spectra also showed clear characteristic features for all the three myoglobin states, compared to the spectra obtained by Liu et al. (2003). The peaks at 978–986 nm in the near infrared wavelength region are mainly attributed to the water vibrations (Liu et al., 2003; Osborne, Fearn, & Hindle, 1993). The small peaks at 762–764 nm can probably be attributed to the vibration of fat and protein.

The characteristic peaks in the visual wavelength range were at 476 and 572 nm for OMb, responsible for cherry-red color of meat. While MMb, responsible for brownish color, had peaks at 470 and 542 nm with a shoulder at 624 nm. DMb, responsible for purple color, had peaks at 460 and 570 nm.

For myoglobin in solutions, UV–Vis spectra maximum peaks at about 582 nm for OMb are expected, at 503 nm for MMb and at 557 nm for DMb, with an isobestic point at 525 nm for all three myoglobin states (Tang et al., 2004). The present data gave wavelength shifts compared to the literature of about −10 nm for OMb, +39 nm for MMb and +23 nm for DMb. The reason for this difference is probably that the present data are from diffuse reflectance spectroscopy of ground beef. A deviation from Tang et al. (2004) was previously reported (Motoyama, Kobayashi, Sasaki, Nomura, & Mitsumoto, 2010; Thiansilakul, Benjakul, & Richards, 2011). No obvious isobestic point for three myoglobin forms were observed in the present data as was also expected since a reflectance mode was used.

3.2. Principal component analysis (PCA)

Fig. 2a shows the score plots of the three different myoglobin states using EMSC spectra of all 72 ground beef samples. The MMb spectra were not clearly separated from the DMb spectra. Some of the MMb spectra were similar to DMb. This result may be due to errors performed when adding ferricyanide, or that not enough ferricyanide was added. This result confirms the prior conclusion that optimizing the preparation methods to obtain pure Mb states is important for high precision and accuracy in determination of the different myoglobin states. It might have been better to reduce the storage time after application of ferricyanide (Faustman & Phillips, 2001). Some muscles may also have better reducing capacities than others (Phung et al., 2013) and this may affect the success of the preparation method. But it is also possible that the MMb layer was not thick enough for the detecting probe used.

In this experiment the preparation of the MMb spectra was made in agreement with AMSA guidelines for intact meat as part of the aim was to compare ground meat with intact meat (see Materials and methods). Another reason for the larger deviation of MMb spectra could be related to the fact that the 8 MMb samples that appeared to contain the most DMb were from day 13 and were microbially the most spoiled, as indicated by an unpleasant odor. Consumption of O_2 by the meat itself and bacteria over time can lead to a gradual shift from MMb to DMb in samples (Sørheim et al., 2009). Khatri et al. (2012) used 4-day old meat for calibration of intact steaks and micro-organisms might then be less influential for the stability of the myoglobin states. The changes in color were not visible to the naked eye; however, the reflectance spectra revealed it. These 8 samples with relatively more DMb in attempted pure MMb were removed as outliers, leaving 64 spectra for modeling. The models are discussed below without these 8 samples.

Fig. 2b shows the myoglobin forms after the 8 outliers were removed. The score plot for MMb spectra were now clearly separated from DMb spectra. The absorption spectra of OMb, DMb and MMb made three clusters that differed clearly from each other and allowed for a more accurate calibration equation.

A general increase in *RMSECV* when measurements are compared between steaks and ground meat may be technical. The fact that the ground meat is pressed against the sensor for contact may open cracks in the surface and these cracks may reveal underlying ground beef with a slightly different myoglobin composition.

3.3. Prediction of myoglobin states in the ground beef

The prediction performances of PLS models after cross validations using EMSC spectra on ground beef are shown in Table 1. The correlation coefficients between predicted and reference values were high. Compared to results from Khatri et al. (2012) where color changes were measured on whole steaks, the obtained prediction errors were slightly higher (approx 0.01 units), but the prediction performance was still good. The nominally lower prediction error may be because ground meat samples generally discolor much faster than whole steaks. Destruction of cellular integrity can liberate a variety of pro-oxidants that can accelerate the discoloration process. Such factors can accelerate the formation of MMb from OMb and DMb (Faustman & Phillips, 2001; Madhavi & Carpenter, 1993). Compared to the results from Khatri et al. (2012), the *RMSECV* prediction of MMb increased the most (0.016) in the present study, while the *RMSECV* of DMb increased the least (0.009). In addition, the use of meat with probably more bacteria and the options for cracks could provide less accurate predictions.

3.4. Accuracy of predicted OMb, MMb and DMb

Due to all three states being predicted independently of each other, negative values and values larger than 1 (100%) can be obtained. The sum of the predicted OMb, DMb and MMb forms was thus set as 1 (Fig. 3). When either DMb or MMb was calculated, the amount of OMb was defined as 0. The two other states were calculated similarly. When myoglobin forms were predicted, any value can in principle be obtained before normalization but only values between 0 and 1 after normalization. Fig. 3 gives the relationship between predicted, corrected and normalized myoglobin forms (leave-one-sample-out cross validation) from EMSC log (1 / R) spectra

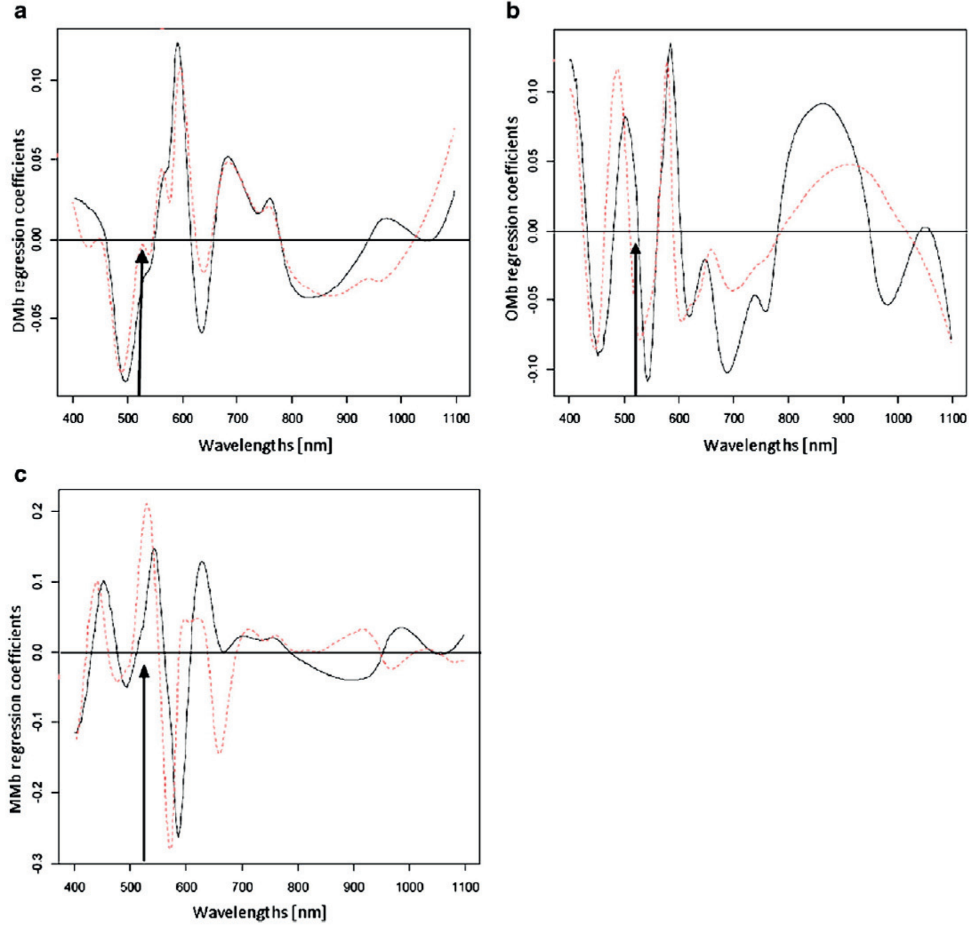

Fig. 5. Regression coefficients for PLS models of ground beef mixtures (------) and whole beef (____), for DMb, OMb and MMb. The models are from regressions of EMSC(A) spectra. The number of PLS factors for ground beef are 2, 3 and 5 for DMb, OMB and MMb respectively, and 2, 3 and 3 for whole steaks for DMb, OMb and MMb, respectively. The arrow points at wavelength of 525 nm.

(I = 64) using PLS with 3, 5 and 2 PLS factors for OMb, MMb and DMb, respectively, *versus* true myoglobin form. The results indicate a higher deviation for OMb and MMb compared to DMb when the predicted myoglobin form was 1. DMb is better predicted in this experiment due to the fact that no repacking of the state was done (Khatri et al., 2012).

When prepared OMb, MMb and DMb were 0, the results show that predicted DMb had the highest error. This can also be due to MMb and/or OMb being less well prepared on systems with bacteria since MMb and/or OMb are 1 when DMb is 0. The correlation coefficients (Rc) for OMb, MMb and DMb were 0.996; 0.997 and 0.996, respectively.

The prediction performances for ground beef are illustrated in Fig. 4. On the assumption that all predictions had no errors, *i.e.* the prepared myoglobin forms were 100% pure, the instrument and the modeling were perfect, it would be expected that all samples were perfectly placed in the corners of the triangle. Fig. 4 shows that the predicted samples were not perfect, but they are clustered close to the corners. All the three Mb states are clearly separated from each other.

3.5. Regression coefficients

To illustrate the similarities and differences between the calibration models from ground beef and whole steak (Khatri et al., 2012) the regression coefficients for both these data sets are illustrated in Fig. 5. In principle, for well resolved absorbance spectra, high positive regression coefficients mean that these regions of the spectra are emphasized and weighted high in the regressions. Due to strong overlapping of the absorbance peaks for the three states of myoglobin, it is not possible or meaningful to assign the spectroscopic peaks to the regression coefficients. The PLS regression

coefficients from the two models are clearly different for the three myoglobin states, DMb, OMb and MMb. The pattern for each myoglobin state is similar for ground beef samples and whole steaks. Thus there are identical or similar spectroscopic phenomena and interactions for both data sets. However, there are also differences between the regression coefficients for these two sets of samples. This indicates that there are differences in the spectra for the two data sets. These differences can be described as significant wavelength shifts of peaks, which indicate different degrees of overlapping of the spectra for the myoglobin states and/or effects of different physical light scatter of the samples.

It should also be mentioned that both the spectra (Fig. 1) and the regression coefficients (Fig. 5) clearly indicated that there is no wavelength region where any of these three myoglobin states do not overlap. This means that it is not possible to pinpoint any single wavelengths or wavelength regions where simple univariate regression against each of the three myoglobin states can be done. Consequently, multivariate regression is necessary.

At an isobestic point such as 525 nm for all three states, it should be expected that all regression coefficients were zero or close to zero in that region. This is not observed in the present regression coefficients. No distinct isobestic point at 525 nm for three states was found at any wavelengths below 750 nm for this light scattering system.

In the paper by Khatri et al. (2012) it was concluded that the combination of EMSC/PLS was superior to the AMSA method with respect to providing good calibration models for predicting myoglobin states even under conditions where the state was not pure (as for their DMb state). However, it is obvious that being able to produce pure states of sufficient thickness for the calibration model will always be an advantage. Thus it is possible that prediction error can be better than 0.04–0.05 if more is known about how thick a layer is measured by a specific instrument, and if this layer is homogenous in terms of myoglobin state. This issue was discussed by Khatri et al. (2012) for DMb and here it is quite relevant for the MMb state. This phenomenon may also influence the regression coefficient and should be further examined to reduce errors to below 0.04–0.05.

Previous experience with NIR spectroscopy reveals that multivariate regression with spectra where the species peaks strongly overlap, results in local models. Thereby different sample sets, with different constituents and/or different physical presentations result in different regression coefficients. Consequently, the models estimated from whole steaks cannot be used to predict other products such as ground beef mixtures. A full multivariate calibration must be done for each product type or fused to a common regression model. In the present study the fat level of 14% (w/w) was constant. The other fat levels were not investigated, but it can be supposed that OMb, DMb and MMb levels in the ground beef samples with variable fat levels could also be accurately predicted. Possibly not from the model generated here, but by a model produced according to the principles outlined here at the desired fat levels.

3.6. Use of this calibration model

The results above indicate that there is need for a local calibration model for myoglobin states, *i.e.* the calibration model determined by Khatri et al. (2012) should not be used on meat minces. The calibration model produced here must ultimately be validated by applying it to new, but similar minces.

4. Conclusion

Myoglobin states prepared from ground meat with added fat gave a prediction error of 5%. Metmyoglobin produced on ground meat stored in vacuum for 13 days reversed partly to deoxymyoglobin when the previously determined optimal procedure for metmyoglobin preparation in fresh steaks was used. Regression results do not support the idea of using selected wavelengths for predicting myoglobin states. The results also showed that calibrations for myoglobin state predictions cannot necessarily be transferred from one meat system to another.

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Paper II

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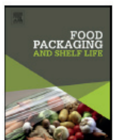

Effects of metabolic substrates on myoglobin redox forms in packaged ground beef

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ABSTRACT

Various direct (citrate, malate and succinate) and indirect (pyruvate and glutamate) Krebs cycle substrates were added to ground beef in order to investigate their effect on the inter-conversion of Mb redox forms in aerobic and anaerobic packaging. Glutamate, malate, succinate, pyruvate, and citrate added (up to totally 0.1 mol/kg) to ground bovine *M. semimembranosus* mixed with either ground porcine or bovine fat, altered the myoglobin redox forms in aerobic and anaerobic packaging systems. In anaerobic packaging, a mixture of succinate and glutamate formed deoxymyoglobin rapidly and it remained in this state for 13 days. In aerobic packaging (75% O_2), the highest oxymyoglobin level occurred with a molar ratio of (glutamate–malate) to citrate of 3:1. In this case, oxymyoglobin was more prevalent after 6–8 days of storage in aerobic condition than without addition of these compounds. Pyruvate induced metmyoglobin formation, acting as a pro-oxidant.

Succinate presumed leading to $FADH_2$; was most effective at converting metmyoglobin to deoxymyoglobin in anaerobic packaging. In aerobic packaging, NADH presumed formed by the oxidation of glutamate may maintain oxymyoglobin levels, but adding citrate as well is recommended. Overall, a combination of substrates relevant to mitochondrial oxygen consumption, improved meat color stability.
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1. Introduction

Consumers prefer meat with red color, which is associated with freshness. However, an attractive red color in meat is difficult to maintain in post mortem packaging. The shelf life of meat on display is, therefore, limited by color defects caused by the oxidation of surface myoglobin (Fraqueza et al., 2008).

Muscle is normally purple under anaerobic conditions due to deoxymyoglobin (DMb). In atmospheric oxygen, myoglobin (Mb) in meat turns the muscle transiently red as oxymyoglobin (OMb) forms. In low-oxygen packaging, muscle usually starts with a high percentage of OMb, which then forms metmyoglobin (MMb), transiently changing the color of meat to brownish, depending on whether the mitochondrial or cytosolic MMb reducing systems are intact (Ledward, 1985; Madhavi & Carpenter, 1993; Slinde, Phung, & Egelandsdal, 2011).

Prolonged storage demands anaerobic conditions to avoid oxidation (Resconi et al., 2012). Ground beef, however, has lower color stability than steaks, because of tissue disruption and the incorporation of oxygen. Oxygen removal involves packaging with either CO_2, N_2, or a combination of both, providing good color stability and extended shelf life (Tewari, Jayas, & Holley, 1999). Packaging in low-oxygen environments results in abundant surface MMb caused by residual O_2 (Sørheim, Westad, Larsen, & Alvseike, 2008). To get DMb on the meat surface, the packaging atmosphere needs to contain less than 0.1% O_2 (Sørheim et al., 2008), so residual oxygen must be removed quickly and completely to avoid accumulation of MMb and create an environment conducive to reducing MMb. The absence of oxygen allows the DMb to form (Mancini & Hunt, 2005). Thus, the mitochondria function as oxygen scavengers and as an MMb reducing system (Tang et al., 2005; Slinde et al., 2012).

Substrates that produce reducing equivalents like nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide ($FADH_2$) increase the rate of MMb reduction (Mancini, Ramanathan, Suman, Dady, & Joseph, 2011; Mohan, Hunt, Barstow, Houser,

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& Muthukrishnan, 2010; Ramanathan, Mancini, Van Buiten, Suman, & Beach, 2012). The reducing equivalents are oxidized by the electron transport system (ETS) at complex I (for NADH) and complex II (for $FADH_2$) in the inner mitochondrial membrane. The ETS must be sufficiently active to keep MMb low. The ETS remains partly active even after 3 weeks of storage at low post mortem pH (Phung, Saelid, Egelandsdal, Volden, & Slinde, 2011; Phung et al., 2013). However, the level of activity decreases with time, and some muscles do not have sufficient activity to complete these color conversions. Some intermediate metabolites from glycolysis and the Krebs cycle could help fuel the metabolic pathways for oxygen consumption and ferric heme reduction. Succinate improves color stability if added at levels higher than 0.02 mol/kg (Zhu, Liu, Li, & Dai, 2009), and Mancini et al. (2011) found it was a better substrate for color stabilization than pyruvate. Malate also stabilized color, but Mohan et al. (2010) suggested that the degree of color stabilization was muscle-dependent. With the exception of Mohan et al. (2010), who used malate, lactate, and pyruvate, most previous studies have used only one substrate, added at a fixed level. Information is limited, however, on glutamate, as well as the combined effects of added substrates on Mb stabilization, partly because we have lacked a suitable methodology to monitor redox states when meat remains in packages throughout the measurement period.

The aim of this study was to investigate the effect of substrates and their combinations at various concentrations on desirable myoglobin redox states of ground beef in both aerobic and anaerobic packaging conditions.

2. Materials and methods

Because formation of myoglobin redox forms is impacted by many factors, this investigation utilized a relatively new experimental design with accompanying multivariate analysis to help identify the major Krebs cycle substrates affecting myoglobin color stability.

2.1. Chemicals

The following chemicals were purchased from Alfa Aesar GmbH & Co., KG (Karlsruhe, Germany): Sodium salts of succinate hexahydrate, succinic acid, and pyruvic acid. From Sigma–Aldrich Chemie GmbH (Steinheim, Germany), we purchased sodium salts of pyruvate, glutamate, L-malic acid, and DL-malic acid disodium salts. Sodium salts of glutamate monohydrate were purchased from VWR International BVBA (Leuven, Belgium). Sodium salts of citric acid monohydrate and trisodium citrate were purchased from Merck KGaA (Darmstadt, Germany). All chemicals were analytical grade.

2.2. Animal tissues in the ground beef

Semimembranosus muscles and subcutaneous fat were removed from four 16–19 month-old bulls and from four 46–81 month-old cows on day 4 post mortem at a commercial plant (Fatland A/S, Oslo, Norway). Fresh, vacuum-packed subcutaneous fat from pigs fed rapeseed and vitamin E to enhance the content of

Table 1
The experimental design ($2^2 \times 2^3 \times 2^4$) design giving 512 samples which was reduced to 128 samples (2^{9-2}) according to fractional factorial methodology (see text).

Part	A	B	C
Design	2^2	Mixture or an apparent 2^3	2^4
Design point	Animal age Fat tissue	Glutamate and Malate Succinate Pyruvate	Total mixture Oxygen Glutamate and Malate ratio Citrate Total mixture Oxygen Glutamate and Malate ratio No Citrate
Variables	Type of fat tissue: Beef or pork Age of animal: 16-19 or 41-81 months	Succinate: 0-0.1 Molar Pyruvate: 0-0.1 Molar Glutamate Malate: 0-0.1 Molar	Total mixture: 0.05 or 0.1 Molar* Glutamate and Malate ratio: 1:3 or 3:1 Oxygen: 0 or 75 volume % Citrate: 0 or 25 Molar % of total mixture

*When one of the mixture compounds is zero, one or more of the other compounds defining the mixture add up to a total amount of either 0.05 mol or 0.1 mol/kg of mixture compounds.

polyunsaturated fatty acids was obtained from HKScan (Ruokatalo, Finland). Ground samples of each fat source were used for fatty acid analysis as methyl esters (Triumf, et al., 2012). The two groups of semimembranosus muscles and two groups of fat were ground twice through a plate with 3 mm holes using a Seydelmann ME-130 grinder (Seydelmann, Stuttgart, Germany). The ground beef of *M. semimembranosus* was prepared as described by Bjelanovic et al. (2013) and mixed with additional beef or pork fat in ratios 86% and 14% respectively. The combination of fat tissue from beef and pork lead to different oxidative stability of the samples. The assumption is that an elevated level of PUFA in animal diet will lead to increased lipid and protein oxidation in the tissues (Faustman & Cassens, 1990; Brewer, 2004; Yi et al., 2015). The ground blends were manually mixed (day 4 post mortem) with appropriate treatment solutions of substrates (see below). These blends had a pH of 5.8; the day the treatment solutions were added, was defined as day zero.

2.3. Preparation of substrate solutions

The solutions were prepared using succinate, pyruvate, glutamate, malate, and citrate in different combinations. To maintain pH at 5.8, the solutions were prepared by mixing the acid form with the corresponding sodium salt of these chemicals. Forty grams of either 1 M or 0.5 M solutions were added to the ground beef-fat mixture (360 g). The solution with substrates were manually stirred for about 2 min into the ground beef-fat mixture to ensure a uniform distribution of the solution, and the blend was then packaged. Reference samples with 40 ml of distilled water added to 360 g of ground beef were also prepared. Table 1 shows the total number of references and samples produced and measured.

2.4. Modified atmosphere packaging (MAP) and storage

The ground beef mixtures (360 g) was packaged in trays of amorphous polyethylene terephthalate (APET) with the dimension; $20.5 \times 14.8 \times 3.5$ cm, and sealed with a top film of ethylene vinyl alcohol (EVOH). The trays and films were of Wipak Biaxer (both Wipak, Nastola, Finland). The oxygen transmission rates for trays and films were 7 and 5 $cm^3/m^2/24$ h at 23 °C and 50% relative humidity, respectively. A tray sealing machine was used for packaging (Promens 511VG, RPC Group Plc., Kristiansand, Norway). Before flushing a vacuum cycle was applied for approximately 10 secs to 99% vacuum. Food grade gas mixtures were supplied by AGA (Oslo, Norway). The gas to meat ratio was approximately 2:1. The high oxygen (75% oxygen and 25% CO_2) samples were stored in darkness at 4 °C for 8 days, whereas the anaerobic (60% CO_2 and 40% N_2) samples were stored in the dark for 13 days.

2.5. Reflectance spectra

Without opening the package, each sample was scanned in the spectral range 400–1000 nm, with a Foss NIRSystems OptiProbe™ 6500 Analyzer (Foss NIRSystems Inc., Maryland, USA) with a remote reflectance fiber optic probe (NR-6770-A, Foss NIRSystems Inc., Maryland, USA). The optic probe has been described previously by Bjelanovic et al. (2013) and it consists of seven rectangular parallel glass windows (1.0×20.0 mm^2) about 2.0 mm apart, mounted in the middle of a 40×40 mm^2 metal block, which allows the emitted light to penetrate several mm into a meat sample. The optic probe emits monochromatic light every second (0, 2, 4 and 6 s) into the sample and collects light every other second (i.e., every 1, 3 and 5 s) to the detector. Packages were turned upside-down before scanning so that the meat would fall on the packaging film. Both references and each sample were scanned thirty-two times at three random places on the surface, and the results were averaged for regression analyses. All measurements were performed at room temperature (approximately 20 °C; Bjelanovic et al., 2013). The reflectance spectra were used to predict OMb, MMb, and DMb levels as reported in Khatri et al. (2012), but using the predictive model developed by Bjelanovic et al. (2013) for ground meat samples. The reflectance spectra of the samples packaged in aerobic packaging was measured on days 0, 1, 3, 6, and 8; anaerobic packaged samples were measured on days 0, 3, 6, 8, 10, and 13.

2.6. Experimental design and statistical analysis

One of the pitfalls of experiments designed with only 2–3 variables is that they do not adequately reflect the multiple variables interacting in meat raw materials and packaging systems (Esbensen, Guyot, Westad, & Houmoller, 2002). This investigation was possible because of a relatively new, advanced multivariate method for determining the redox states of myoglobin where the hitherto most likely causes of low prediction accuracy of myoglobin redox states are built into the predictive model (Bjelanovic et al., 2013).

2.6.1. Creation of the design

Table 1 shows the full experimental design (512 experiments) which were constructed by crossing three designs. A (2^2), B (8- point mixture) and C (2^4) the mixture design B is a simplex lattice mixture (0, 50 and 100%) with 2 additional centre experiments. This full design was reduced to 128 experiments (32 samples each day) by utilizing the theory of two-level fractional factorial designs (Mee, 2009). To achieve this, the 8 mixture design points were each allocated to the corners of a cube. This mixture design could then be considered as a 2^3 design using three fictive variables. Thus the full design was regarded as 2^9 design ($2^2 \times 2^3 \times 2^4$). The actual reduced design was chosen from standard 2^{9-2} designs taking into account such considerations as (i) an optimal confounding pattern for the fictive mixture variables, (ii) good individual designs at each single day, (iii) alternative models based on the original variables should behave well according to classical optimal design criteria (Berger & Wong, 2009).

The 128 samples represented a balanced design. However, seven extra samples (making it 32 + 7 samples each day) were added, while still keeping the system balanced, to enhance modeling capability. These 7 samples were replicates. At each working day two reference samples with only water were produced, and packaged in aerobic and anaerobic conditions in order to verify the difference between adding water and an average combination of substrates. Thus, the experiment started with 41 (32 + 7 + 2) samples on 4 different days.

2.6.2. The statistical analysis

A relatively complicated model was chosen to ensure that all terms needed to model the response variables as a function of the design variables were included. The effect of the 3-component mixture design (see Table 1, column B) for the variables succinate, pyruvate and glutamate–malate were grouped as one model element. More specifically the model element (two degrees of freedom) representing a linear response surface (over the mixture) was referred to as "Mixture" and the additional parameters needed to model a quadratic response surface was "Mixture^2" (second order) these, together with the other main terms (mixture components, total mixture concentration, ratio malate/glutamate, citrate, fat tissue, and animal age) were included in the model together with interactions, resulting in a model with terms up to third order. To allow a possible maximum/minimum value inside the mixture region a quadric response surface was needed

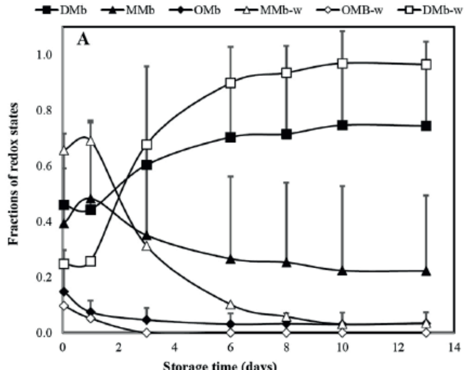
(similarly parabola has max/min, straight). With as many as 40 degrees of freedom for error, and because the analysis was based on a hierarchical approach, no further model reduction was needed. The observed effect of oxygen level was large, so the results were later modeled at each oxygen level (2 regression models for each myoglobin redox state).

We used the statistical analysis approach described by Langsrud, Jorgensen, Ofstad, and Naes (2007), implemented in the 50–50 MANOVA software (http://www.langsrud.com/stat/ffmanova.htm, downloaded 20.03.2013). This program handles mixture designs as generalized categorical variables (generalized ANOVA), which can also be viewed as a specialized variant of linear modeling. Egelandsdal, Dingstad, Togersen, Lundby, and Langsrud (2005) gave an example of a food science application of this method. Important outputs from the analysis are significance (P-values), explained variance (based on sums of squares), and adjusted mean values (or mean predictions). Adjusted mean values can be calculated over the whole mixture region and the results can be presented as surfaces. Surfaces are also the most appropriate way of illustrating the effect of mixture in addition to using its explained variance from the ANOVA model. The surfaces were drawn in Minitab Statistical Software version 16 (State College, 2011).

The analysis used here is a specialized variant of linear modeling, but the significance tests can be interpreted as regression and ANOVA modeling (with F-statistics, P-values, and adjusted means) (Esbensen et al., 2002).

3. Results and discussion

Mitochondrial oxygen consumption has previously been linked to redox states; it causes the Mb redox form to change from MMb and OMb to DMb (Slinde et al., 2011). The mean changes in surface DMb, OMb and MMb of ground beef during dark storage at 4 °C in anaerobic and aerobic packaging are shown in Fig. 1A and B, respectively. The figures show large differences in Mb redox forms present in the aerobic and anaerobic packages. DMb dominance in anaerobic and OMb dominance in aerobic conditions were expected (Taylor, 1972; Seydim, Acton, Hall, & Dawson, 2006; Sørheim et al., 2008). The myoglobin fractions, however, show large variation, in particular for anaerobic packaging of ground beef containing Krebs cycle substrates. This means that unless the



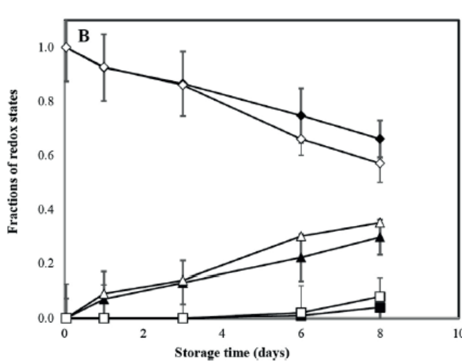


Fig. 1. Average fractions of DMb, OMb, and MMb determined from reflectance measurements of ground beef packaged in (A) anaerobic and (B) aerobic packaging. White and black symbols are fractions for samples with water and substrates added respectively. The error bars are standard deviations. The upper portion of the error bars are indicated for clarity.

correct combination of substrates is selected, the ground beef could end up with more MMb than meat without Krebs cycle substrates.

In anaerobic packaging (Fig. 1A), both OMb and MMb decreased and DMb increased over storage time. OMb was nearly depleted by day 6 in anaerobic packaging. With little O_2, MMb also began to decline, and electrons from the ETS reduced MMb to DMb. We saw little change after day six, suggesting that the oxygen in the packages was depleted and the system became stable.

In aerobic packaging (Fig. 1B) at day 0, OMb, averaged over all Krebs cycle substrate experiments, was essentially 100%, whereas DMb and MMb stood at 0. The mean OMb prevalence remained high but decreased throughout storage with a concomitant increase in MMb, indicating that OMb and DMb reacted to form MMb and that the transition through DMb was not rate limiting. The metabolic substrates could, on average, retain 66% OMb after 8 days of storage in aerobic and an average 74% DMb after 13 days in anaerobic system. The Krebs cycle substrates influenced standard deviation less in aerobic than in anaerobic system.

3.1. Comparison of Mb redox forms in ground beef samples added substrates with samples added only water

Fig. 1A and B also shows changes in myoglobin redox states with time when only water was added. In anaerobic packaging, the mean DMb fraction of the ground beef samples treated with substrates was slightly higher at an earlier stage than samples with just added water although after three days, the ground beef samples with additives had, on average, slightly lower DMb levels than samples where only water was added (Fig. 1A). The DMb fractions in the samples with added water were 0.25 on day 1 and 0.65 on day 3. MMb in samples with added water was initially higher (0.61) than samples with added Krebs cycle substrates, but after three days, the average value for MMb with added Krebs cycle substrates was similar to samples with only water added (Fig. 1A).

In aerobic conditions, by day 8, the average system with added Krebs cycle substrates had a higher fraction of OMb than the system with only water (Fig. 1). The lowest OMb fraction identified for systems with added Krebs cycle substrates was 0.59 on day 8 while the highest was 0.70 (Fig. 1B), so some combinations of Krebs cycle substrates may actually give undesirable redox state changes while other combinations may promote/preserve desirable Mb forms but only during the first 3 days.

3.2. The effect of the ratio of the substrates on Mb redox forms

Glutamate/malate, succinate, and pyruvate substrates were added using a mixture design (Table 1, column B), so the effects of these substrates on Mb redox forms can be predicted and illustrated in triangles (Figs. 2 and 3). We present the effects from day 6 because that was the day the mixtures of substrates explained most of the variation in the redox states (Table 2)

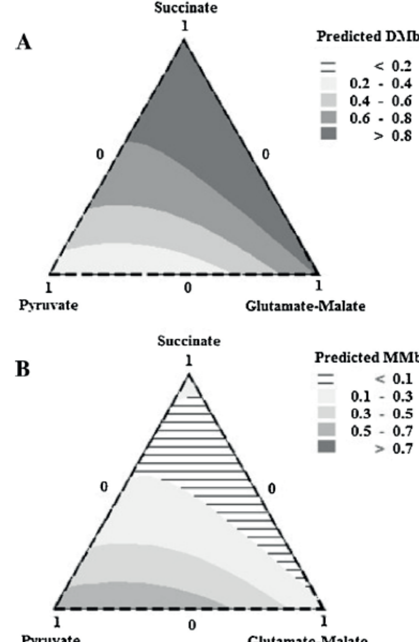


Fig. 2. Predicted amounts of DMb (A) and MMb (B) in ground beef on storage day 6 as a response to pyruvate, succinate, and glutamate–malate in anaerobic packaging. Each corner of the contour plot represents the maximum amount of succinate, pyruvate, and glutamate/malate, (i.e., 0.075 mol/kg) as the concentration for every position in the triangle. The slope of the response surface was significant ($P < 0.05$).

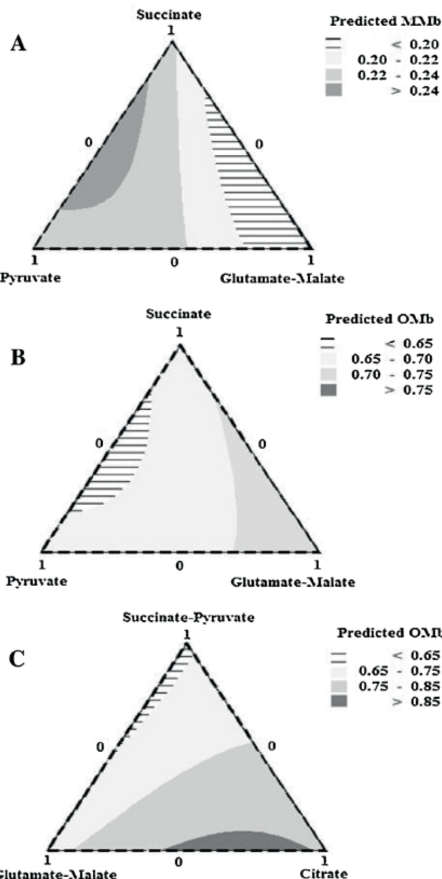


Fig. 3. Predicted amounts of myoglobin redox forms in ground beef on storage day 6 in aerobic packaging. Each corner of the contour plot represents the maximum amount (0.075 mol/kg) of succinate, pyruvate, glutamate–malate, citrate, or succinate, or succinate-pyruvate, (A and B) give OMb as response; (B) has succinate-pyruvate as a combined variable, (C) gives MMb as response. The slope of the response surface was significant ($P<0.05$).

although the citrate addition was most important on day 8 in aerobic system (Table 2). Data in Table 2 also support results shown in Fig. 1, where the substrates were the main cause of variation (largest explained variance) in myoglobin redox forms in anaerobic packaging.

Fig. 2 shows contour plots of DMb and MMb fractions in anaerobic packaging. When only pyruvate was added (lower left corner, Fig. 2A and B), DMb was low and MMb high. When only succinate was added (upper corners of Fig. 2A and B), the results showed a high fraction of DMb and a low fraction of MMb. In the study of Tang et al. (2005) the addition of succinate to bovine cardiac mitochondria stimulated tissue oxygen consumption and DMb formation. When glutamate–malate replaces succinate (sliding down along the right side of the triangle), the fraction of MMb eventually increases when only glutamate–malate replaced succinate (right corner, Fig. 2B). Replacing succinate with pyruvate (sliding down the left side of the triangles in Fig. 2A and B) shows a gradual increase in MMb content, which rises to rather high values (Fig. 2B). Mixtures with equal parts of all compounds, in the middle of the triangle, lead to approximately 0.5 DMb (Fig. 2A).

In anaerobic conditions, adding these substrates significantly ($P=0.002$) affected myoglobin states from day one through day thirteen. The added substrates were the most important design variables (out of the 9 listed in Table 1) affecting the Mb states. In anaerobic, the mixture explained as much as 65.8% (Table 2) of the variance in the DMb model and 65.9% (Table 2) of the variance in the MMb model (both values at day 6).

The MMb and DMb states were more affected by Krebs cycle substrates than the OMb state (Fig. 2). OMb was therefore not included in Fig. 2.

The patterns of the response surfaces for DMb and MMb (Fig. 2) did not change throughout the 13 days of the experiment in anaerobic system (data not shown). This means that the optimal

Table 2
Significantly explained variance (%)[a] in myoglobin states induced by added succinate, glutamate, malate, and pyruvate mixtures and citrate in high (75%) and oxygen excluded packaging.

Packaging	State	Day						
		0	1	3	6	8	10	13
Anaerobic MAP	DMb	34.5	56.6	59.3	65.8	61.9	60.7	41.8
	MMb	39.9	58.8	59.3	65.9	63.5	60.2	37.6
Aerobic MAP	OMb	26.5	31.3	29.8 *(17.2)*[b]	31.1 *(31.3)*	22.3 *(45.5)*		
	MMb	17.9	13.4	18.0	20.6 *(11.6)*	18.0 *(18.1)*		

[a] (P>0.05); each model had a myoglobin state as response. All effects used in the model in addition to mixture and citrate are described in Section 2. Model error was 15–20%.
[b] Figures in parenthesis indicate explained variance > 10% due to citrate additions (citrate contributing 25% of total mols added). Calculations followed Bjelanovic et al. (2013).

combinations of substrates remained the same from the beginning of the experiment to the end.

Two concentrations of additives were used, but their effects on Mb states were similar. This suggests that even when using lower concentrations of substrates, either the observed effects may be achieved, and/or the mitochondrial system was saturated with substrates when the oxygen concentration was low. After three days of storage, added citrate affected Mb states in anaerobic packaging, but inconsistently, explaining less than 5% of the variance in Mb states.

The anaerobic meat packaging keeps DMb levels at a maximum, when oxygen is not available to form OMb. The response surface gradient map (Fig. 2) shows DMb formation stabilized using a mixture of glutamate–malate and succinate in anaerobic packaging (Fig. 2). In a parallel example, in human skeletal muscles in vitro, a combination of succinate and glutamate–malate consumes the most oxygen (Rasmussen & Rasmussen, 2000).

In aerobic packaging, the best option is to keep OMb at a maximum because it is the most stable Mb state in high-oxygen environments, as well as being attractive, as a redox state, to consumers. The effect of adding substrates to the OMb and MMb states is shown in Fig. 3A–C. Adding these substrates significantly (P = 0.003) affected myoglobin states from day one through day eight, with maximum effect after 6 days for the 3 mixture design components and after day eight for citrate (Table 2). Under high-oxygen conditions, glutamate–malate favored OMb formation, while pyruvate–succinate favored MMb (Fig. 3, day 6).

DMb was not significantly affected by the mixture composition and was therefore not included in Fig. 3.

On day 6, the mixture explained 31.1% of the variation in OMb and 20.6% of the variation in MMb (Table 2). Citrate explained 31.3% of the variation in OMb on day 6 but was the most important Krebs cycle substrate supporting OMb on day 8 (Table 2). Adding glutamate/malate most strongly supported the formation of OMb over the entire storage period. However, by day 8, the optimal blend tended to drift toward including succinate (data not shown). Variation in MMb was not well explained by added Krebs cycle substrates in aerobic packaging.

When citrate was added, the amount of glutamate/malate, pyruvate, and succinate was reduced to keep the total amount of the additions constant. Thus, we can illustrate the effect of adding citrate using a triangle (Fig. 3C). Adding citrate had an effect that was only evident in aerobic environments, and readings on day 6 suggested increased stability of OMb when citrate was added (Fig. 3C). Optimally, then, to preserve OMb requires including both citrate and glutamate/malate (Fig. 3C); this blend appears to work better than using pure citrate or pure glutamate/malate in maintaining OMb. The relative importance of citrate in maintaining OMb increased and became significant (P < 0.001, 17.2% explained variance) on day 3; on day 8, citrate additions explained 45.5% of the variation in OMb (Table 2). These results show that citrate may be important to stabilizing meat color and that the optimum conditions for preserving OMb may change over time in aerobic packaging. Moreover, citrate is more important when storage times run longer.

Citrate works synergistically with ascorbate (Jaswir, Man, & Kitts, 2000) and to promote color and protect meat from lipid oxidation (Lund, Hviid, & Skibsted, 2007). Citrate is a metabolic intermediate of the citric acid cycle, but so are succinate and malate. Our results suggested that citrate is multifunctional and that its ability to chelate metals is equally important. It appears that it is not consumed, that means that there are other reasons for

Table 3
Predicted combinations of substrates that yield the minimum ([a]) or maximum ([b]) myoglobin redox forms with standard deviations after 6 days of ground beef storage.

Variable	Substrate (%) for anaerobic packaging				Mb-fraction (%)[***]	Substrate (%) for aerobic packaging			
	Citrate[*]	Succinate	Pyruvate	Glu–Mal[**]		Succinate	Pyruvate	Glu–Mal	Mb-fraction (%)
DMb	No	35	0	65	100.0 ± 7.5[a,***]	65	0	35	0.8 ± 0.8[a]
	Yes	48	0	52	100.0 ± 8.7[a,***]	0	60	40	0.0 ± 0.8[a,***]
	No	0	75	25	16.1 ± 4.8[b]	0	0	100	0.0 ± 0.6[b,***]
	Yes	0	85	15	13.7 ± 6.7[b]	0	33	67	0.0 ± 0.8[b,***]
Control	No	0	0	0	88.0 ± 5.9	0	0	0	0 ± 5
OMb	No	0	82	18	7.1 ± 0.7[a]	0	0	100	79.2 ± 1.4[a]
	Yes	0	78	22	7.2 ± 1.1[a]	0	0	100	86.6 ± 2.1[a]
	No	0	0	100	0.0 ± 1.4[b,***]	47	53	0	60.6 ± 2.1[b]
	Yes	59	0	41	0.0 ± 1.2[b,***]	48	52	0	69.4 ± 2.1[b]
Control	No	0	0	0	0 ± 3[*]	0	0	0	69 ± 6
MMb	No	0	78	22	67.9 ± 4.5[a]	47	53	0	25.9 ± 1.6[a]
	Yes	0	97	3	73.2 ± 7.9[a]	44	56	0	22.4 ± 1.5[a]
	No	42	0	58	0.0 ± 7.6[b,***]	0	0	100	14.9 ± 1.1[b]
	Yes	44	0	56	0.0 ± 7.6[b,***]	0	0	100	14.0 ± 1.7[b]
Control	No	0	0	0	11 ± 2.9	0	0	0	30 ± 3.9

[*] Results are shown as % of total amounts (0.05 mol/kg or 0.1 mol/kg). When citrate is present, it constitutes 25% of the mixture, so the combined amounts of succinate, pyruvate, and glutamate–malate are actually 75%.
[**] Glu–Mal = glutamate–malate. n = 156 samples. 8 reference samples with only water are not included in the table.
[***] These data were normalized to 100%, and therefore, predicted values below 0 appear as 0% and more than 100% appear as 100%, as described by Khatri et al. (2012).

its affect, since citrate is a known chelator (Keowmaneechai & Mcclements, 2002).

3.3. Mixtures giving maximum and minimum redox states

The substrate combinations with the maximum and minimum fractions for various Mb states using the two packaging methods are listed in Table 3. These are predicted values from the statistical models. Input values were from day six, and samples with or without added citrate provided the data for predictions. Data in Table 2 support the results in Figs. 2 and 3 by giving predicted (from the model) combinations where Krebs cycle substrates give the maximum and minimum results for redox states of myoglobin. For example, in anaerobic packaging, 0.35×0.05 mol/L succinate and 0.65×0.05 mol/L glutamate–malate resulted in 100% DMb on day 6 (first left line, no citrate added, Table 3). As mentioned above, the combinations of substrates that resulted in the highest relative amount of DMb in anaerobic packaging were largely independent of storage day. The optimal combination to preserve DMb in anaerobic packaging would be around 50% succinate and 50% glutamate–malate (Table 3, left side). This would allow DMb to develop rapidly in anaerobic packaging packaging and maintain the DMb state until the final storage day (13 days). The optimal total amount of substrates in mol/kg has not been established. Conditions that encouraged OMb and MMb to develop contained pyruvate (75–100% of total mol/L used) and glutamate–malate (Table 3).

Table 1 shows that the experimental ratio between glutamate and malate was either 3:1 or 1:3. These ratios were used in the analysis of variance but were not even close to being significant in the anaerobic packaging system, possibly because the added amount of both glutamate and/or malate was higher than what was needed to remove oxygen (Yi et al., 2015).

In the aerobic system, DMb formation was influenced by succinate and glutamate–malate, while pyruvate and glutamate–malate influenced MMb formation (Table 3, first line, right). Glutamate–malate (alone at 0.05 or 0.1 mol/kg) was the most important component in regulating OMb; 84.9% OMb was observed on day 6 when citrate was added (Table 3, left). Pyruvate–succinate (approximately 50:50) combinations did not promote OMb formation in aerobic packaging (Table 3), and this contradicts Mohan et al. (2010). The difference in results could be related to different observation times and temperatures. Ramanathan, Mancini, Dady, and Van Buiten (2013) reported that addition of the sole succinate in amount 2.5% increased redness in raw and cooked beef when packaged in high oxygen conditions. Glutamate-malate produces reducing equivalents as well as reactive oxygen species from complex I (Adam-Vizi & Chinopoulos, 2006). Saleh and Watts (1968) used malate and glutamate separately with different concentrations and also reported increased MMb reduction when added to meat. To preserve OMb in aerobic packaging environments, 75% glutamate/malate and 25% citrate was effective. The statistical models analyzed for both the total amount of substrates and the ratio of glutamate and malate among the various fractions of Mb states in aerobic packaging. The total amount of substrates in aerobic packaging tended to become significant as storage times grew longer; P was 0.058 for the effect of level of substrates on day 8. The ratio of glutamate/malate was also significant on day 8 ($P = 0.040$). From the actual model predications of OMb, a glutamate:malate ratio of 3:1 compared to a ratio of 1:3 favored slightly higher OMb percentages (calculated as 62% versus 57%) on day 8. This suggested that the substrates metabolized continuously to maintain OMb levels in aerobic packages. Thus, the results showed at least a tendency for the level of some Krebs cycle substrates to be more important in aerobic than anaerobic environments.

3.4. Metabolization of substrates

Succinate, glutamate, malate, and citrate are food additives that are specified by their E-numbers. The use of sodium and potassium citrate in meat formulations are limited to 1.3% and 2.5% respectively in USA (Agriculture, United States Departement of Food Safety and Inspection Service, 2015). In this experiment we used max 0.6% citrate. Pyruvate is not a common food additive, apparently not because of any undesirable side effects; studies support its health benefits (Owen & Sunram-Lea, 2011). On the other hand, monosodium glutamate as an additive has been debated although it is relatively well studied because free L-glutamate is an important and widely used flavor enhancer (Bellisle, 1999; Rangan & Barceloux, 2009; Rhodes, Titherley, Norman, Wood, & Lord, 1991; Williams and Woessner, 2009). The maximum amount of glutamate tested here (0.1 mol/kg ground beef) would not be acceptable as an additive because the daily intake of MSG would presumably double for people eating 100 g of ground beef or glutamate-enhanced meat every day (Rhodes et al., 1991). However, the results obtained here suggest that 20% of 0.1 mol/kg glutamate in ground beef would be sufficient to ensure the rapid reduction of MMb to DMb in anaerobic packaging systems.

Metabolization of glutamate post mortem has been documented by Yi et al. (2015). Glutamate probably metabolizes to 2-oxoglutarate to have its desirable effect on redox states, and 2-oxoglutarate, as a food additive, has a different health image from glutamate (Tocaj et al., 2003).

The exact concentration to be used in anaerobic packaging needs to be further studied. In addition, future studies should focus on elucidating if increasing the ratio of citrate would be useful for preserving OMb in high-oxygen packaging.

3.5. Influence of fatty acid composition

The fats used in formulating the ground beef samples meant that the samples had two different levels of polyunsaturation (Yi et al., 2015). The level of MMb was significantly affected from day one, but this effect was much smaller (data not shown) than the positive effects of Krebs cycle substrates on redox states. Mancini et al. (2011) suggested that 2.5% succinate can minimize lipid oxidation and premature browning in row patties and in cooked ground beef patties packaged in high oxygen and PVC. Nevertheless, the ground beef samples containing higher levels of unsaturated fats contained the most MMb.

3.6. Practical implications

Our method is in principle useful when there is a depletion in endogenous substances relevant for color stabilization. This happens when there is a substantial time before sample is packaged in the modified atmosphere, or temperature abuse as when meat is stored at too high temperature or when using poor primary chilling. More than anything else; it is a useful method in conventional meat packaging, if frozen and thawed minced meat is displayed and useful endogenous substrates have already been consumed. Succinate is a remarkable example of an endogenous compound that might be lost during storage, and this make reduction of metmyoglobin a challenge. The addition of such compounds may then be the best way to maximize meat color stability.

4. Conclusions

Substrates involved with the Krebs cycle added to ground beef affected the relative proportion of Mb redox forms in various

packaging systems. For meat packaged in aerobic systems, OMb was preserved at the highest levels using glutamate-malate, especially when combined with citrate. In anaerobic packaging, glutamate–malate and succinate induced DMb most quickly. Adding pyruvate increased MMb. Increasing the level of Krebs cycle substrates above 0.05 mol/kg had no added benefit on maintaining desired redox states in anaerobic packaging systems.

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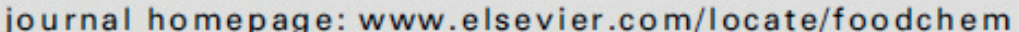

Lipid oxidation in minced beef meat with added Krebs cycle substrates to stabilise colour

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ABSTRACT

Krebs cycle substrates (KCS) can stabilise the colour of packaged meat by oxygen reduction. This study tested whether this reduction releases reactive oxygen species that may lead to lipid oxidation in minced meat under two different storage conditions.

KCS combinations of succinate and glutamate increased peroxide forming potential (PFP, 1.18–1.32 mmol peroxides/kg mince) and thiobarbituric acid reactive substances (TBARS, 0.30–0.38 mg malondialdehyde (MDA) equivalents/kg mince) under low oxygen storage conditions. Both succinate and glutamate were metabolised. Moreover, under high oxygen (75%) storage conditions, KCS combinations of glutamate, citrate and malate increased PFP (from 1.22 to 1.29 mmol peroxides/kg) and TBARS (from 0.37 to 0.40 mg MDA equivalents/kg mince). Only glutamate was metabolised.

The KCS combinations that were added to stabilise colour were metabolised during storage, and acted as pro-oxidants that promoted lipid oxidation in both high and low oxygen conditions.

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1. Introduction

Beef meat has been demonstrated to contain the strongest peroxide forming potential (PFP) among the most commonly consumed fresh meats in western population. The dominant reason for its high PFP is likely due to the meat's high myoglobin or haem levels (Yi, Haug, Nyquist, & Egelandsdal, 2013). All forms of myoglobin are reactive, and the highest iron oxidation levels are always the most reactive. Cycling of haem in myoglobin between different oxidation states is undesirable as it keeps oxidation in progress. This can lead to protein crosslinking (reduced tenderness) and degradation of lipids, resulting in an unwanted rancid flavour (Campo et al., 2006; Lepetit, 2008).

The Krebs cycle is an important metabolic pathway that oxidises Krebs cycle substrates (KCS) like succinate for the production of nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide ($FADH_2$) that feed into the electron transport system (ETS). In *vivo* the ETS produces adenosine triphosphate (ATP) and removes oxygen. The latter reaction is crucial for colour stability in meat. Specific combinations of Krebs cycle substrates (KCS) will stabilise and maintain myoglobin in the deoxymyoglobin (DMb) state in modified atmosphere (low oxygen) or in the desired full oxymyoglobin (OMb) state (high oxygen packaging) (Slinde et al., 2012). However, a certain amount of metmyoglobin (MMb) will always be present, in both low and high oxygen packaging, due to oxidation. It is the oxidation of KCS such as succinate and glutamate, that produces reducing equivalents that are transported from the mitochondrial membrane to reduce MMb (Phung et al., 2012, 2013; Tang et al., 2005). However, the mitochondrial membrane is a major source of reactive oxygen species (ROS) and this is especially true in meat due to the deterioration of the electron transport chain (ETC) (Barksdale, Perez-Costas, Melendez-Ferro, Roberts, & Bijur, 2010; Lenaz, 2001; Werner, Natter, & Wicke, 2010). This may suggest that some combinations of KCS could also act as pro-oxidants and promote lipid oxidation by feeding into the disintegrating ETC.

So far only KCS have been identified that have an effect on stabilisation of myoglobin states. Little is known about their effect on PFP or lipid degradation (Liu, Fiskum, & Schubert, 2002). However, the mitochondrial ETC as one of the major cellular generators of ROS, produces superoxide, hydrogen peroxide and hydroxyl free radicals which can lead to lipid degradation (Boveris, Oshino, & Chance, 1972; Loschen, Flohé, & Chance, 1971).

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Substrates of the ETC that can lead to ROS production are glutamate/malate through NADH to the flavine mononucleotide group (FMN) of complex I (Liu et al., 2002). Pyruvate/malate may act similarly (Chen, Vazquez, Moghaddas, & Hoppel, 2003). Some recent studies suggest that there are substantial changes in complexes I and III-V of the ETC in beef 4 h *post mortem*. Although oxygen consumption remains, sometimes for several weeks, it is reduced in intensity (Barksdale et al., 2010; Phung et al., 2012, 2013; Werner et al., 2010). It seems therefore relevant to identify if there are any indications of enhanced lipid oxidation when KCS are added *post mortem* to secure oxygen removal and myoglobin stability.

The aim of the present study was to explore how different combinations of KCS used to stabilise colour in minced meat may affect (lipid) oxidation using three different measurements: (1) volatile lipid degradation compounds; (2) peroxide; (3) thiobarbituric acid reactive substances (TBARS). We also aimed to document whether KCS were metabolised during storage, which would support involvement of ETC-ROS formation.

2. Materials and methods

2.1. Chemicals

All chemicals used in the study were of analytical grade. Butanedioic acid disodium salt (succinate hexahydrate disodium salt), butanedioic acid (succinic acid), and 2-oxopropanoic acid (pyruvic acid) were purchased from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany), whereas 2-oxopropanoic acid disodium salt (pyruvic acid sodium salt), 2-aminopentanedioic acid (glutamic acid), 2-hydroxybutanedioic acid (L-(−)-malic acid disodium salt) and DL-hydroxybutanedioic acid (DL-malic acid disodium salt) were from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Sodium 2-aminopentanedioate (sodium glutamate) was obtained from VWR International BVBA (Leuven, Belgium). 2-Hydroxypropane-1,2,3-tricarboxylic acid monohydrate (citric acid monohydrate) and 2-hydroxypropane-1,2,3-tricarboxylate (trisodium citrate) were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Animal tissues

Beef *M. semimembranosus* and beef fat tissue were collected four days *post mortem* (Fatland A/S, Oslo, Norway). The packaging day was defined as day zero, *i.e.*, four days *post mortem*. Pork fat tissues (from HKScan, Ruokatalo, Finland) were obtained from pigs fed rapeseed oil and vitamin E to obtain and preserve a high content of polyunsaturated fatty acids.

2.3. Preparation of minced meat

Minces (lean *M. semimembranosus*) were prepared as described by Slinde et al. (2012), and contained 14% w/w fat from beef or pork fat tissues. The ground meat was blended manually with solutions (all at pH 5.8) containing various KCS. The experiment used four combinations of raw materials: meat from young (1.5 years) cattle with pork or bovine fat; meat from old (4–5 years) cattle and pork or bovine fat.

2.4. Preparation of solutions

The solutions added to the minced meat were prepared using succinate, pyruvate, glutamate, malate and citrate in different combinations, either as pure sodium/acid based chemicals or as mixtures of 2, 3, 4 and 5 chemicals of varying concentrations. In order to maintain pH at 5.8 the solutions were prepared by mixing the acid form and the corresponding sodium salt of these chemicals. Forty grams of 0.5 M or 1 M solutions were added to ground 360 g minced meat. The solution was stirred into the mince and subsequently packed. The final concentration of KCS were 0.05 mol/kg succinate, 0.025 mol/kg citrate, 0.0125 mol/kg malate and 0.05 mol/kg glutamate. Eight control samples were included which comprised of 360 g mince with 40 mL of distilled water added.

2.5. Modified atmosphere packaging (MAP) and storage

The minced meat with solutions were packed as described previously by Bjelanovic et al. (2013). Briefly, packaging was carried out within 1 h after grinding. The samples were stored in darkness at 4 °C for 8 days in high-oxygen atmosphere (75% oxygen and 25% CO_2) and for 13 days in a low-oxygen atmosphere (60% CO_2 and 40% N_2). A shorter storage period in high-oxygen was chosen to reduce the influence of bacterial growth. Low oxygen is used as a concept because often approximately 1% oxygen prevails after MAP packaging. The food grade gas mixtures used for packaging were supplied by AGA (Oslo, Norway). The gas-to-meat ratio in a package was approximately 2:1. The thickness of the minced meat layer in the packages was approximately 3 cm.

2.6. Fatty acid composition analysis

Fatty acids of the selected fat tissues were transformed into methyl esters and then analysed by gas chromatography–mass spectrometry GC–MS (7890A GC, Agilent Technologies, Palo Alto, USA). Transesterification of lipids to fatty acid methyl esters (FAME) was performed by using method described previously with minor modifications (Devle, Rukke, Naess-Andresen, & Ekeberg, 2009). Briefly, 0.01 g fat was dissolved in 2.0 mL hexane and 1.5 mL of 3.33 mg/mL sodium methanolate solution was added. The mixture was then placed on a shaker for 30 min, left to settle for 10 min and 200 µL of the top layer was transferred into a new vial. Fatty acid analysis was performed by auto injection of 1 µL at a split ratio of 80/1; constant flow mode; velocity 20.4 cm/s; two replicates. To identify FAMEs, their retention times were compared to those of a known 37-component standard FAME mix, and the mass spectra were compared with spectra available from the NIST (National Institute of Standards and Technology) database.

2.7. Headspace analysis

The headspace analyses were performed according to a modified method by Volden et al. (2011). Each sample was randomly collected three times from inner and surface parts, blended and mixed. Two gram of this mixture was placed in a 20 mL tightly sealed headspace vial and used for volatile compound measurements. The headspace volatile compounds were analysed by a dynamic headspace analyser (Teledyne Tekmar HT3, Teledyne Tekmar, Ohio, USA) coupled to a gas chromatograph (Agilent 6890N, Agilent Technologies Santa Clara, CA, USA). The GC column was connected to the ion source (at 230 °C) of a quadruple mass spectrometer (Agilent 5975, Agilent Technologies, Santa, CA, USA, interface line 250 °C). The carrier gas was helium at a flow of 1.0 mL/min. The oven temperature programmed as 35 °C for 10 min, heating rate 1.5 °C/min up to 40 °C, 4.0 °C/min up to 70 °C, 7.5 °C/min up to 230 °C and 1 min at 230 °C. The retention times of the components of interest were compared with the retention times of the analytical standards and/or mass spectra of compounds in the NIST 05 Mass spectral Library (Agilent Technologies, Santa Clara, CA, USA). Minitab version 16 (mixture design procedure) was used for graphical representation of the

volatile mixtures presentations while the analysis of variance was performed according to Slinde et al. (2012).

Transformation to quantitative values of volatile marker compounds: In order to convert the measured area values of hexanal and 2-octen-1-ol to mg/kg mince, hexanal and 2-octen-1-ol were added to minced fresh meat samples that did not contain these components in detectable amounts. The samples were thereafter frozen at −80 °C. One sample high (2.5 mg/kg hexanal; 6 μg/kg 2-octen-1-ol) and one low (0.15 mg/kg hexanal; 0.25 μg/kg 2-octen-1-ol) in these two compounds were always measured together with unknown samples to keep track of the stability of the measuring system. A multipoint (5 different concentrations) calibration curve was run at the end of the measurements. The five-point calibration curve was used to calculate concentrations of hexanal and 2-octen-1-ol in unknown samples.

2.8. Hydroperoxide value (PV) measurements by using the ferric-xylenol orange method

Triplicates of minced meat (0.1 g) were incubated in 1 mL Ringer's solution for 2 h at 37 °C. The samples were then mixed with 1 mL chloroform and methanol (2:1, volume-ratio), vortexed and centrifuged at 25,186×*g* for 10 min at 4 °C. After centrifugation the mixture was separated in three phases: a polar phase, an interphase (the meat protein aggregate) and an unpolar phase (chloroform phase) containing soluble lipids. Each of the three phases was removed respectively for separate hydroperoxide measurements. Chemicals used were as described by Yi et al. (2013). Hydroperoxide values were calculated by first subtracting the absorbance of the negative control, then the absorbance was divided by the pigments' (ferric-xylenol orange) molar absorptivities of ε = 14,840 and 87,583 M^{-1} cm^{-1} for the upper phase/inter phase and the lower phase, respectively, before correcting for dilution. The peroxides distributed through the three phases represent the total amount of peroxides.

2.9. Thiobarbituric acid reactive substances (TBARS) measurements

TBARS were measured using the reaction between 2-thiobarbaturic acid (TBA) and compounds reacting with it in a meat extract. Two grams of comminuted meat were added to 10 mL of TBA-stock solution (0.375% TBA and 15% trichloroacetic acid in 0.25 N HCl). Following homogenisation, the suspension was heated in capped tubes at 98 °C for 10 min. The systems were then chilled on ice for another 30 min. Solutions (1.5 mL) under the upper fat layer were carefully removed and centrifuged for 25 min at 25,186*g* at 4 °C. After centrifugation, the absorption of the supernatant was measured by spectrophotometer at 532 nm using ε = 156,000 M^{-1} cm^{-1} (Buege & Aust, 1978).

2.10. High-performance liquid chromatography (HPLC) measurements for organic acids

Minced meat samples (2 g), encompassing the inner part of the mince, were transferred into glass tubes (Pyrex 22). Milli Q water (5 mL), 0.5 M H_2SO_4 (0.7 mL) and CH_3CN (20 mL) were added to each tube. All the tubes were hand-mixed for 2 min, then mixed in a Multifix turning machine (WEG, Balingen, Germany) for 30 min. Meat samples were then centrifuged at 14,243×*g* (Funke-Gerber, Germany) at room temperature for 15 min. The supernatant (1 mL) was filtered through a PTFE filter (pore 0.2 μm) into HPLC-vials before analysis. HPLC was used to analyse the organic acids, following a method modified from Marsili, Ostapenko, Simmons, and Green (1981). All the samples were analysed on an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) which was initially held at 30 °C, connected to the pump (Perkin Elmer Series 200), the autosampler and an LC oven 101 (Perkin Elmer, Waltham, MA). The Perkin Elmer Series 200 UV/VIS detector was used to detect organic acids. H_2SO_4 (5 mM) at a flow of 0.4 mL/min was used as the mobile phase. Standard solutions for external calibration were prepared similarly to the samples, and the compounds were identified according to their retention times compared with the standard solution.

2.11. Amino acid analysis for glutamate

Free AAs were analysed by adding 15 mL of internal standard solution (0.1 M HCl; 0.4 μmol/mL L-norvalin; Sigma, St. Louis, MO) to 1.5 g of the minced meat (inner part). The samples were homogenised on an Ultra-Turrax (IKA, USA) for 5 min at 20,000 rpm followed by sonication for 30 min. The sample were centrifuged (Thermo Scientific, Heraeus Multifuge X3R, Germany) at 3309×*g* for 40 min at 4 °C. One mL of 4% TCA (Sigma) was added to 1 mL of the supernatant, mixed in a mini shaker (Gene 2, New York, NY), and placed on ice for 30 min. After centrifugation at 15,700×*g* for 5 min at 4 °C, the samples were filtered (0.2 μm cellulose acetate filter, Advantec, Dublin, CA) and stored in a freezer (−20 °C) until analysis.

Prior to analysis, 350 μL of borate buffer (0.4 M, pH 10.2, Agilent Technologies) was added to 50 μL of sample. Separation of AAs was performed using an Agilent series 1200 pump and autosampler (Agilent Technologies, Singapore), a Perkin Elmer 200 column oven, and an Agilent 1200 series thermostat and fluorescence detector (Agilent Technologies). The system used software from EZChrom Elite (Agilent Technologies). An XTerra RP 18 column (150 × 4.6 mm; Waters, Milford, MA) was used for separation of AAs at 42 °C. Derivatisation with o-phthalaldehyde/3-mercatopro pionic (OPA/MPA) was done according to Bütikofer and Ardö (1999).

2.12. Reflectance spectra

The reflectance spectra were used to predict OMb, MMb and DMb according to a principle reported by Khatri et al. (2012). All the minced meat samples were scanned at 400–1100 nm, with a Foss NIRSystems OptiProbe™ 6500 Analyzer (Foss NIRSystems Inc., Maryland, USA) equipped with an interactance fibre optic probe (NR-6770-A, Foss NIRSystems) and software from Vision (2001, NIRSystems) without opening the meat package. The package was turned upside-down before measurements so that the meat would fall on the packaging film of ethylene vinyl alcohol (EVOH). The samples were scanned with three random placements of the probe on the package surface in order to increase the scanning area and reduce the measurement errors. Thirty-two scans of both the references and the samples were acquired and averaged for each measured area. All measurements were performed at room temperature (approx. 20 °C), otherwise in accordance to Khatri et al. (2012). Colour of the samples packed in O_2 was measured after 0, 1, 3, 6 and 8 days of storage from packaging day, while samples with trapped O_2 were measured after 13 days of storage using reflectance measurements.

2.13. Experimental design and statistical analysis

Animal and fat tissues were prepared as a 2 × 2 design (4 systems that contained a mixture design each). To each corner of this 2 × 2 design a 3-component (succinate, pyruvate and glutamate-malate additions; 0, 50 and 100%) simplex lattice mixture design with 2 additional centre experiments was added. Each of these eight experimental points had 4-factors (glutamate-malate ratio, total added mixture levels, citrate concentrations, oxygen concentrations) at 2-levels; *i.e.*, a 2^4 design.

A quarter fraction of the full design was prepared and the 128 samples were selected by using methodology for two-level fractional factorial designs (2^{9-2} design). Details are given by Slinde et al. (2012) regarding the calculation of effects from MANOVA for these data. Surfaces are the most appropriate way of illustrating the effect of mixtures. Response surfaces were drawn in Minitab Statistical Software version 16 (State College, PA 16801-3008, USA). It will be realised below that despite oxygen being a design variable when the experiment was planned, the nature of the data required the observations to be split into separate models for low and high oxygen packaging.

3. Results

3.1. Myoglobin state changes due to use of optimal combinations for preserving colour during storage

Two combinations were found optimal for colour stabilisation: a KCS combination containing succinate and glutamate/malate (molar ratio 50:50) in MAP and a KCS combination containing glutamate, citrate and malate (molar ratio 56:25:19) in high oxygen conditions during storage (Slinde et al., 2012). The effect of these two combinations on myoglobin states is shown in Fig. 1.

DMb quickly became the dominant state and remained in this state throughout the experimental period in Map (Fig. 1 A). Once formed, DMb remained stable at ~1.0 for the complete observations period of 13 days. Glutamate, citrate and malate addition kept the fraction of OMb higher and remained high for a longer time in high oxygen packaging; adding water gave OMb equal to 0.5 after 8 days of chill storage (Slinde et al., 2012).

3.2. The effect of type of fat tissue on volatile formation

Type of fat tissue explained the largest (20–40%; MANOVA analysis) cause of variation in the lipid derived volatile compounds on the final observation day (13 days chill storage in MAP; 8 days chill storage in high oxygen). Pork fat tissue combined with beef meat from either younger or older cattle gave the highest amounts of hexanal and 2-octen-1-ol (Table 1). Meat from young bulls (~18 months) and beef fat is most common in commercial minced meat in Norway.

As expected, a higher percentage of PUFA (16.2% in pork fat; 1.2% in beef fat) increased the prevalence of volatile lipid components (Table 1). Hexanal is a good marker of n-6 fatty acids degradation and in particular of linoleic acid degradation while

Table 1
Mean values of selected markers of lipid oxidation in minced meat (with 10% w/w of brine added) made from cattle of different ages and containing either pork or beef fat tissue.

Animal	Fat tissue	Low oxygen (8 days)		High oxygen (13 days)	
		Hexanal (mg/kg)	2-Octen-1-al (μg/kg)	Hexanal (mg/kg)	2-Octen-1-al (μg/kg)
Young	Beef	$0.00 \pm 0.14^{*,a}$	0.00 ± 0.34^{a}	$0.01 \pm 0.26^{*,a}$	0.00 ± 0.22^{a}
Young	Pork	0.65 ± 0.20^{b}	1.50 ± 0.37^{b}	0.81 ± 0.21^{b}	3.40 ± 0.43^{b}
Older	Beef	$0.00 \pm 0.06^{*,a}$	$0.00 + 0.40^{a}$	$0.01 + 0.21^{a}$	0.00 ± 0.33^{a}
Older	Pork	0.81 ± 0.16^{b}	1.50 ± 0.27^{b}	1.12 ± 0.21^{b}	5.20 ± 0.51^{b}

* Standard errors; negative values were predicted from the calibration equation for some samples with volatiles close to the detection threshold of marker compound. The letters in superscript, column by column, indicate significant differences ($P < 0.05$). The average CV was 0.34 for these measurements. The values are averaged over the combinations of KCS used. Minces in MAP and high oxygen were stored 8 and 13 days in low and high oxygen, respectively.

2-octen-1-ol is more typically a marker for degradation of arachidonic acid (Volden et al., 2011) although these markers may also be associated with other fatty acids (Elmore et al., 2004; Larick, Turner, Schoenherr, Coffey, & Pilkington, 1992).

The average amount (both pork and beef minces) of C18:2 (linoleic acid) was 11 g/kg mince and the average amount of C20:4 was 0.13 g/kg mince. Thus these suggest that, on average, the degradation of C18:2 and C20:4 was at a comparable rate provided that hexanal and 2-octen-1-ol were not also derived from other fatty acids. The fatty acid C20:2 has been reported to give 2-octen-1-ol, but this fatty acid is only present in small amounts compared to C20:4 (Raes et al., 2003). Hexanal and 2-octen-1-ol were, as expected, the highest in high oxygen packaging (Ullrich & Grosch, 1987).

3.3. Lipid degradation products from stored minced meat systems with added Krebs cycle substrates

The effect of KCS mixture on volatile formation was small (max 5% explained variance, ANOVA). This means that the effect of mixture is expected to be small if conditions for lipid oxidation prevails, *i.e.*, the presence of relatively larger amount of PUFA and oxygen. The effect of mixture depended on the measuring system ($P = 0.04$ for interaction effect). Thus the mixture had an effect on the volatile profile but the magnitude of the effect depended on the specific fat/beef muscle batch used. The nominally largest effect of KCS was observed for meat from older cattle with added

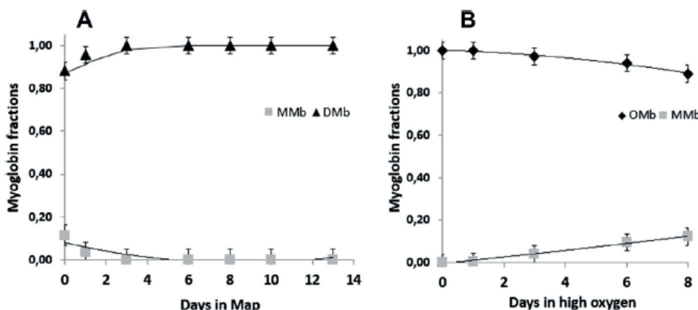

Fig. 1. Myoglobin fractions on surfaces of minces in two different packaging. (A) Myoglobin states with added succinate, glutamate and malate (molar ratio 50:37.5:12.5) in MAP; (B) myoglobin states with added glutamate, citrate and malate (molar ratio 56:25:19) in high oxygen. Total concentration of KCS was 0.075 mol/kg. Bars indicate standard errors. OMb was not included in panel A as its fraction was very low; the same was the situation for DMb in panel B.

beef fat tissue, *i.e.*, where the endogenous oxidation of the meat and fat tissue was low (Table 1).

Fig. 2 shows the effect of KCS on hexanal and 2-octen-1-ol levels, averaged over animal age and fat tissue. All 4 systems (Table 1) ranking KCS similarly regarding hexanal and 2-octen-1-ol production. After 13 days of chill storage, hexanal and 2-octen-1-ol formation was highest for glutamate/malate additions. To preserve DMb in MAP, a 50:50 mixture (molar ratio) of succinate: glutamate/-malate may be the most efficient (Slinde et al., 2012). Fig. 2 suggests that such a combination would not provide the highest hexanal values, but values just below 1 mg/kg. However, it seemed that pyruvate addition mixed with glutamate/malate worked best as a protector of lipid degradation. Regarding 2-octen-1-ol, it also seems that adding a 50:50 mixture of succinate: glutamate/malate would be acceptable with respect to lipid oxidation. For the water sample (control), hexanal was not detected after 13 days and 2-octen-1-ol was only detected in one of the three replicates (then at 0.8 μg/kg). Thus, the average water sample was less oxidised than many samples containing KCS.

Fig. 3 suggests that pure succinate and pure glutamate/malate should not be used if lipid degradation should be avoided. Pure glutamate/malate or glutamate/malate with added citrate have

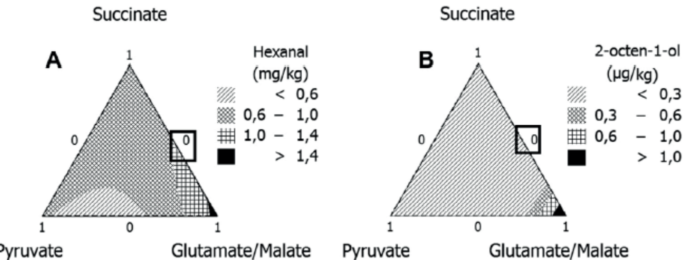

Fig. 2. Hexanal (A) and 2 octen-1-ol (B) concentration as a function of the composition of succinate, pyruvate and glutamate/malate (KCS, mean conc. = 0.075 mol/kg) for minces chill stored for 13 days in MAP. The slopes of the response surfaces were significant ($P < 0.05$). Square indicates the area with the most suitable (combination to preserve DMb). The control sample (added only water) had no detectable hexanal and maximum 0.8 μg 2-octen-1-ol/kg mince.

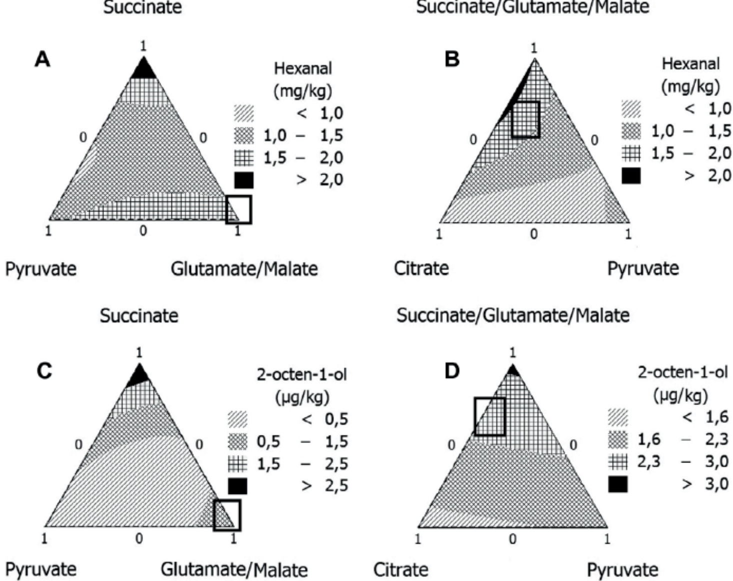

Fig. 3. Hexanal concentration (A) and 2 octen-1-ol (B) as a function of the composition of succinate, pyruvate and glutamate/malate (Krebs cycle substrates, mean conc. = 0.075 mol/kg) for minces chill stored for 8 days in high oxygen. The slopes of the response surfaces were significant ($P < 0.05$). Squares indicate area most suitable to preserve OMb. Hexanal (C) concentration and 2-octen-1-ol (D) as a function of the composition of succinate/glutamate/malate, pyruvate and citrate (KCS, mean conc. = 0.075 mol/kg) for minces chill stored for 8 days in high oxygen. The control sample (added only water) had no detectable hexanal and 2.5 μg 2-octen-1-ol/kg.

been suggested as the most suitable additives to preserve OMb (Slinde et al., 2012). In high oxygen the effect of citrate addition was significant on day 8. Most samples containing KCS had nominally lower values for the volatile 2-octen-1-ol than the minces containing only water (*i.e.*, control). This was not the case for the hexanal where many samples had higher amounts of hexanal than the control samples. There was a significant O_2 reduction in headspace on the third day of chill storage in response to KCS addition ($P = 0.026$, ANOVA).

3.4. PFP and TBARS in all the beef system

For the most common commercial minced meat system the time series was analysed. The total peroxides (Fig. 4A and B) were highest in the system with added KCS, at least at the end of the experiment. The total peroxides increased with time for all systems. Typically, PFP increased quickly in MAP when KCS were added. The major part of PFP was endogenous, however, and presumed dominantly due to the combination of haem and fatty acids (Yi et al., 2013). All types of peroxides (unpolar, polar and protein-bound peroxides) nominally increased with storage time although the increase in protein-bound peroxides in high oxygen was not significant ($P > 0.05$, not shown). Unpolar peroxides were always significantly higher, if KCS were added on the final day of chill-storage. However, the increase in PFP was small (max 12% increase in MAP).

TBARS were higher after 13 days when KCS were added compared to adding only water in MAP (Fig. 4C). This was also the case under high oxygen. In both systems, the nominal value a few hours after addition of KCS favoured lower TBARS values (significant in high oxygen). Again, the changes inducible by KCS were relatively small compared to the volatiles produced endogenously. The TBARS values were below the thresholds assumed detectable by sensory analysis, even after 8 days high oxygen storage (Resconi et al., 2012). The TBARS data suggest that the added KCS initially acted like antioxidants and then gradually become pro-oxidants. The increase in TBARS due to KCS addition was small (from 0.30 to 0.38 mg/kg after 13 days chill storage).

After 3 days succinate and glutamate were not reduced further in MAP. The nominal amount of succinate removed during 3 days was ~0.025 mol/kg mince. This value is in agreement with values suggested by Zhu, Liu, Li, and Dai (2009). The nominal reduction in glutamate was 0.019 mol/kg mince. It looks like malate is consumed in MAP, but the metabolism is unaffected (same slope) by addition of glutamate and succinate. Endogenous glutamate was low (~0.5 mmol/kg) in minces stored in MAP. Endogenous succinate was substantially higher (~5 mmol/kg) than the endogenous glutamate level. Apparently, the amount of succinate only declined initially when it needed to remove oxygen, and thereafter this substrate increased somewhat (Fig. 5A).

Apparently, the initial amount of succinate only declined when the oxygen present was removed, thereafter this substrate increased somewhat (Fig. 5A). In high oxygen, the added citrate metabolised only to a small extent, whereas glutamate consistently metabolised (Fig. 5B). However, the addition of citrate and glutamate may build-up malate in high oxygen packaging.

4. Discussion

Although fatty acid composition and other endogenous components were more important for the degradation of lipids than the effect of adding KCS, specific lipid degradation products (in this

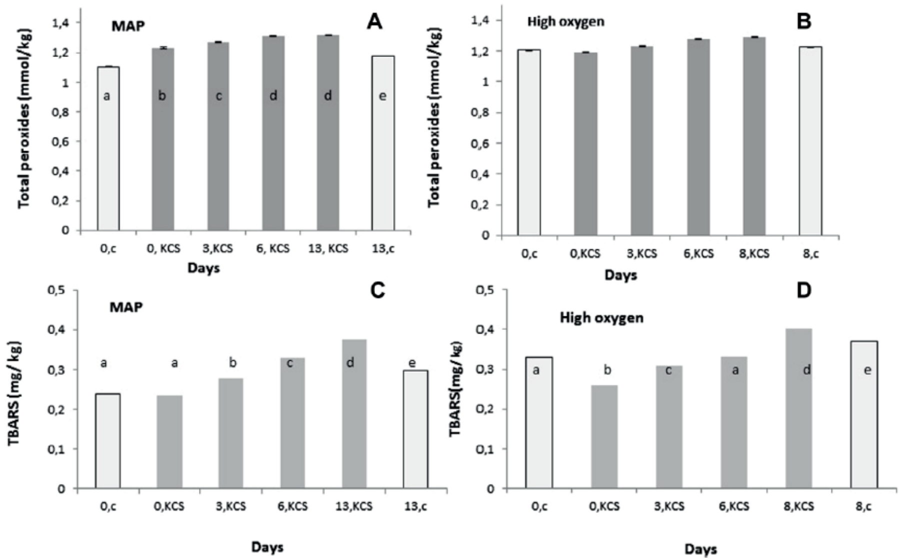

Fig. 4. (A) Total PFP over time for a succinate and glutamate mixture in MAP; (B) total PFP over time for a glutamate, citrate and malate mixture in high oxygen; (C) TBARS formed over time for a succinate and glutamate mixture in MAP; (D) TBARS formed with time for glutamate, citrate and malate mixture in high oxygen (succinate:glutamate was in molar ratio 50:50, totally 0.1 mol KCS/kg; glutamate, citrate and malate was in molar ratio 56:25:19, totally 0.1 mol KCS/kg; c was control with water).

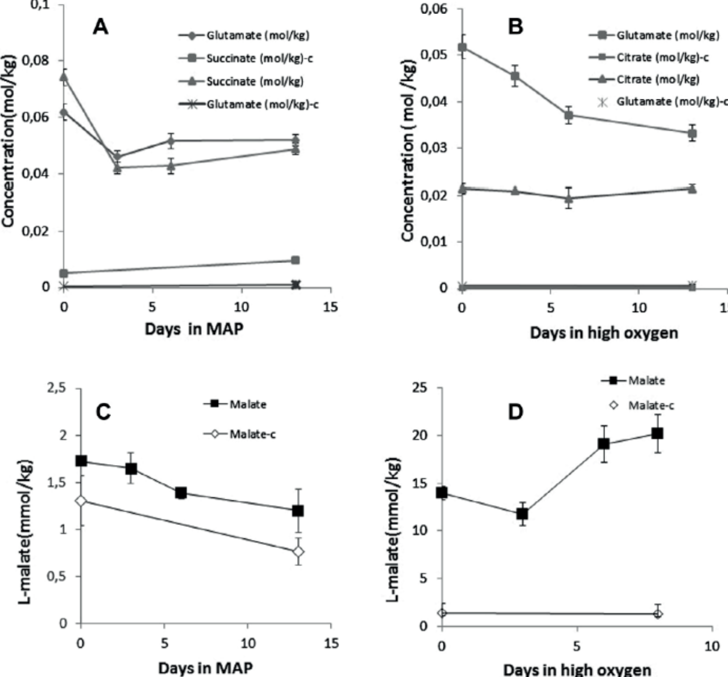

Fig. 5. (A) Succinate and glutamate concentrations (molar ratio 50:50, totally 0.1 mol KCS/kg) in minces stored in MAP; (B) glutamate, citrate and malate in high oxygen (molar ratio 56:25:19, totally 0.1 mol KCS/kg); (C and D) malate concentration in the same minces stored in MAP and in high oxygen, respectively (c = control with water).

study exemplified by the degradation products of hexanal and 2-octen-1-ol) still depended on the addition of KCS substrates.

4.1. Lipid degradation when the colour is optimally stabilised

The motivation for adding KCS to minced meat would be to induce and keep the DMb state in MAP. The substrates (succinate and glutamate) that rapidly resulted in a dominance of the DMb state also result in the lower production of 2-octen-1-ol though this was not evident for the oxidation marker hexanal. Peroxide and TBARS measurements also supported that a small increase in lipid oxidation may be inducible by the addition of succinate-glutamate. This would be in line with the ability of succinate and glutamate to generate H_2O_2 in mitochondria (Skulachev, 1996). No measurement of hemin degradation due to peroxide formation was carried out, but in principle, hemin could also be affected by ROS production.

Reducing the KCS addition to the minimum level needed may provide an insignificant increase in TBARS. Pyruvate protected against lipid oxidation/degradation and has previously been reported as an antioxidant (Lund, Hviid, & Skibsted, 2007) but the compound is not suitable to preserve DMb (Slinde et al., 2012).

Correspondingly, the best combination to maintain the OMb state in high oxygen would be a 1:3 M ratio of citrate-glutamate (Slinde et al., 2012). However, maintaining OMb seemed generally in conflict with maintaining low lipid oxidation at least after 8 days of chill storage. This interpretation was supported by TBARS measurements.

Pyruvate seems more useful to prevent lipid oxidation while the citrate, glutamate and malate combination (molar ratio 1:3) was actually providing maximum hexanal formation and was not optimal regarding the marker 2-octen-1-ol either. These results mean that at least in high oxygen packaging, it may not necessarily be possible to use only the four compounds tested here to stabilise colour and lipid oxidation. However, before concluding this, it is worth examining the conditions in which the citrate ratio is increased beyond 1:3 to glutamate. Maintenance of oxymyoglobin cannot take place without some production of ROS and thereby lipid degradation after some time. Despite the fact that the KCS addition increased total peroxide formation, the TBARS increment seemed small and may not have any contribution to the products' sensory profile.

4.2. Metabolism of substrates

As expected, succinate and glutamate were metabolised in MAP until all the oxygen was removed. This happens within a few days; presumably within a few hours for some parts like the surface of the meat (Slinde et al., 2012). The later increase in succinate may partly be from other metabolites, including glutamate. Since succinate and glutamate were only consumed to some extent, it is expected that it is possible to reduce these additives to 0.03 and 0.02 mol/kg mince, respectively, if added as a brine to the whole

mince. Lower amounts of KCS may be used if it is only added to improve surface colour. It also seemed that the endogenous succinate in mince may provide reducing equivalent to the electron transport system (ETS) in fresh meat (here 4 days), but that would not be the case for glutamate since its endogenous concentration is quite low. Malate seemed to be used in MAP but we have no indication of it affecting colour stability or lipid degradation.

In high oxygen, a very important additive is citrate. Citrate did not become metabolised and it minimised lipid degradation. It seems therefore that its presence is not important regarding providing reduction equivalents to ETS, but that a different mechanism prevails as a chelator for iron (Ke, Huang, Decker, & Hultin, 2009). However, glutamate is clearly metabolised and thus a very important additive in high oxygen, if the OMb state is desired. It cannot be concluded how much glutamate is needed as OMb was measured on the surface, whereas glutamate was measured in the complete sample. Thus, it is possible that glutamate is exhausted and therefore it is the limiting substrate for preserving OMb in the meat surface, but that glutamate still remains in the inner layer of the mince. The need for glutamate may be localised; high need (>0.1 mol/kg mince) in the surface but not needed in inner layers. This hypothesis needs to be further explored, but some support has already been given by Slinde et al. (2012). Succinate was not a very relevant additive in high oxygen packaging. Addition of succinate seemed to boost lipid oxidation and degradation. Ramanathan, Mancini, and Dady (2011) reported that OMb formation is not supported by succinate.

Malate seemed to be an end point substrate when citrate and glutamate were added. It is not metabolised, but produced. It is possible that oxidation of (endogenous) succinate with the glutamate available creates malate. When pyruvate is unavailable, the Krebs cycle will stop at malate as large amounts of oxaloacetate give negative feedback on malate dehydrogenase.

4.3. ROS formation

It is not desirable to increase the PFP of foods and in particular not in meat as it is speculated to already be high due to the presence of hemin (Yi et al., 2013). MAP packaged meat seemed to retain increased PFP when KCS was added. This could be because the remaining succinate and glutamate was oxidised and produced peroxides when assayed. With time there may also be some exhaustion of antioxidants, also in MAP packaging and thus this could explain the increased PFP observed in the control (added only water). It is questionable whether the small increase in PFP had any relevance for health as this increase, upon addition of KCS, relies largely on enzymatically formed ROS and therefore this source of increased PFP may not be present in heated foods and in heated, digested foods. However, KCS should not be added beyond what is needed to stabilise colour. Since hexanal was observed in higher concentration when KCS was added, it appears that O_2 reduction generates ROS, and that glutamate/malate is involved as suggested by Liu et al. (2002).

In high oxygen packaging, additions of KCS also increased ROS formation as assessed from hexanal production, but the difference between the control and the sample with the additives was small after 8 days. This may actually suggest that some of the ROS formation is actually exhausted during the storage in high oxygen packaging. This is in agreement with the formation of volatiles; lipid breakdown products were more abundant in high oxygen packaging. These compounds emerge after peroxide formation.

4.4. Flavour aspects and legislation

We are left with the suggestion that up to 0.03 mol/kg succinate and 0.02 mol/kg glutamate should be added to minces in MAP. Succinate's taste (seashell) threshold of 0.03% in pure water is by far exceeded then. Even the endogenous concentration of succinate exceeds this taste threshold. The taste threshold in a meat matrix is not established. There are reasons to believe that the recommended addition of succinate (up to 0.03 mol/kg) and glutamate (up to 0.02 mol/kg) could be identified by sensory assessors (Baroň & Jaromír, 2012; Byrne, Brediea, Mottram, & Martensa, 2002). Succinate is transformed to fumarate (accepted as a food additive as well, and has an acid taste) but even this flavour's threshold is not defined in a meat matrix. Oxoglutarate (produced from glutamate) flavour is not well described in the literature.

The flavour threshold of L-glutamate (only L has the umami flavour) is 1–8% (g/kg) depending on sensor physiology (Luscombe-Marsh, Smeets, & Westerterp-Plantenga, 2008). This means that at least some people will detect the minimum addition required. Succinate has been suggested as an umami (glutamate) taste enhancer (Gangyang Flavors & L., 2014). In principle, the combination of glutamate and succinate may be appreciated, even if detected, while the glutamate/fumarate taste needs to be examined.

The amount of citrate that can be added appears limited only by taste. Thus, an upper limit of addition needs to be defined. Glutamate addition is challenged by concerned consumers and it should be verified if simply adding high glutamate concentrations to the surface could be a better alternative. What is said about oxoglutarate above is also valid here.

L-malate is not really needed as an additive, but it should be addressed if it would accumulate in meat with KCS addition. L-malate is an accepted food additive. However, we do not think adding this component is needed for colour stability Slinde et al. (2012).

5. Conclusion

The optimal KCS combinations succinate and glutamate in MAP and glutamate, citrate and malate in high oxygen for colour stabilisation increased lipid degradation when oxygen was removed in the package. The increase was too small to be sensory relevant compared to endogenous lipid oxidation. Succinate and glutamate in MAP was consumed when oxygen was removed, but the citrate needed in high oxygen packaging was not.

Acknowledgements

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EFFECTS OF DIFFERENT PRODUCTION SYSTEMS ON CARCASS AND MEAT QUALITY OF SHEEP AND LAMB FROM WESTERN BALKAN AND NORWAY

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Abstract: The identification of meat quality characteristics from selected breeds grazing in specific regions is particularly relevant to achieve a marketing advantage. *Longisimus thoracis at lumborum* (*LTL*) from the indigenous Western Balkan (WB) sheep - VlašićkaPramenka (VP) sheep and lambs, and Pivska Pramenka (PP) sheep grazing in Bosnia & Herzegovina (B&H) and Montenegro (MN), respectively, was compared regarding carcass and meat qualities to the crossbred Norwegian white sheep (NWS) - sheep and lambs, grazing in wide Hardangervidda and Jotunheimen regions where the lamb meat is marketed as gourmet meat. The WB sheep had lower average carcass weights and antioxidant capacity, higher ultimate pH, intramuscular fat and *n*-6/*n*-3 ratio, but better tenderness and color stability compared to NWS. The WB lambs were lighter, had higher *n-6/n-3* ratio, lower antioxidant capacity and became more easily rancid despite a higher fat α-tocopherol content. The marketing advantage of WB meat is its tenderness properties while NO's NWS lambs displayed a better nutritional profile.

Key words: production system, sheep meat quality, physical and chemical traits, meat color, fatty acid composition.

Introduction

The consumers' have an increasing interest in more healthy meat products and lower production costs. EU's Common Agricultural Policy stimulates

at the same time pasture-based production systems resulting in meat with higher content of omega 3 polyunsaturated fatty acids (PUFA) (*Enser et al., 1998; Carrasco et al., 2009*). The consumers in Western Balkan (WB) are becoming more aware of claimed organic meat advantages, but prefer domestic meat from non-conventional production systems. The purcase motives for such meat are safety, "natural" content, health, good meat quality and a distinctive taste (*Vukasovič, 2013*). The Norwegian consumers also prefer domestic meat from mountain pastures with perceived elements of naturalness, healthiness and environmental friendly production combined with good meat quality (*Hersleth et al., 2012*).

Meat quality differ among animal species (*Guerrero et al., 2013*), and can be used to promote sheep and lamb sale, such as done for the Texel sheep (*Cockett et al., 2004*) and lamb from Aragosa (*Martinez-Royo et al., 2008*). The producers in EU were encouraged to continue producing lamb meat according to the traditional methods (*Texiera, 2005*) in agreement with consumers' requirements and acceptance. In Europe the Spanish scientists have carried out a substantial amount of research on their autochthonous breed Aragonese in order to obtain the PGI (Protected Geographical Indication) label (*Martínez-Cerezo et al., 2005*).

The predominant sheep breed in the WB is the Pramenka sheep (PS). It makes up 80 to 90% of the sheep population and belongs to indigenous primitive sheep type (*Robic, Liker, and Rupic, 1992*). In the 20th century, most PS types were crossed with different exotic breeds, mostly Merino, but the last indigenous PS types remain in the high mountain regions of the Balkan Peninsula, where the environmental conditions and quality of pastures are less favorable for conventional sheep grazing (*Cinkulov et al., 2008*).

In B&H, the dominant sheep is Vlašićka Pramenka (VP) (synonym Dubska) with female adults weighing 60-70 kg (*Porcu and Markovic, 2006*), while PP (synonym Jezeropivska) is the predominant sheep in MN, with female adults weighing 51-54 kg (*Markovic, Markovic, and Adzic, 2007*). Farming in WB is done semi extensively, oriented towards utilization of grassland and pasture areas.

A predominant sheep breed in Norway is the Norwegian White Sheep (NWS). It constitutes 76.2% of all sheep flocks in Norway (*Domke et al., 2011*). NWS is a crossbreed composed of Dala, Rygja, Steigal and Texel breeds selected for fast growing lambs, good reproduction and high meat yield (*Boman, et al., 2010*). NWS rearing is intensive, but lamb and sheep graze outdoors during the summer. An adult sheep can reach up to 100 kg live weight. Norwegian lambs grazing in specific regions are marketed by origin (*e.g.* Gourmet lamb from the mountains in Central Norway; Lofot-lamb from the mountainous islands of North Norway).

The research on NWS meat quality began in 1990, but is still not extensive. Meat quality characteristics such as typical EU grade scores, fat content, fatty acid composition (only adipose tissue), color, flavor and sensory traits have

been reported to depend on grazing regions (*Ådnøy et al., 2005; Lind et al., 2009*). The fattening of lambs on nutrition rich pastures lowered n-6/n-3 FA ratio, while fattening on a concentrate-based diet lowered the content of C18:3 (n-3) fatty acids and intensity of acid taste (*Lind et al., 2009*).

The aim of this study was to: 1) describe the meat quality characteristics of Western Balkan PP and VP breeds grazing in typical regions; 2) compare sheep and lamb meat quality from WB regions with a crossbreed NWS from Norwegian mountains developed for intensive meat production; 3) describe the meat quality variations within each meat production group.

Materials and Methods

Grazing regions

All three grazing regions are characterized by a complex, but different floristic composition.

WB: PP animals were collected in 2012 from the grazing region Ljubišnja, at an altitude of 900-1300m. The MN pastures are unique areas of fragmented mountain grasslands with trees and bushes. *Poetum violaceae, Festucetum ovinae, Festucetum rubra-falax, Festucetumvalesiaca, Nardetum strictae, Brometum erectistrictae* predominate the floristic composition of the grasslands up to 1200 m (*Dubljevic, 2009*). VP animals were collected in 2012 from the Vlašić grazing region, at an altitude of about 1500 m. The grazing region of VP is characterized by fragmented mountain grasslands, separated by trees and bushes. *Poa pratensis, Bromus racemosus, Dactylis glomerata, Briza media, Lotus corniculatus, Trifolium pratense, Trifolium repens, Vicia sativa and Pteridium aquilinum* dominate floristic composition (*Alibegovic-Grbic, 2009*).

Norway: NWS animals were collected in 2012 from grazing regions in central and southeast Norway at an altitude 500-1700 m. The region is about 40 000 km^2, and covers the production of Gourmet lamb. At an elevation of 500-900 m, the grazing area is characterized by spruce and pine forests, while at an elevation of 900-1700 m by scarce birch forests with little grass. *Avenella flexuosa, Luzula pilosa, Festuca ovina, Anthoxanthum odoratum, Agrostisca pillaris, Deschampsia cespitosassp.cespitosa, Carex spp.* are floristically predominant (*Lunnan and Todnem, 2011*).

Only the 4 years old NWS were fed indoor their last 3 months after the outdoor grazing period on the concentrate and local grass silage.

Slaughtering

Totally 92 *Longisimus thoracis at lumborum* (*LTL*) sheep/lamb samples were collected from 3 countries.

B&H: *LTL* was collected at "BB" Kotor Varoš, a traditional slaughterhouse, from 15 female sheep (age 4-5 years) and 15 lambs (age 5-6 months). Traditional slaughtering without stunning was used. The handling of *post mortem* (*pm*) was set up to reduce the effect of cold shortening, i.e. by a controlled temperature drop. **MN:** *LTL* was collected from 15 female sheep (age 4-5 years) at the meat production company Franca, Bijelo Polje. We were not able to collect the lambs from MN, because there was not a sufficient number of female lambs ageing 5-6 months from the same herd in a small production area. In addition, lambs are not commonly raised to age 5-6 months to be slaughtered for meat consumption. **Norway:** *LTL* from 14 female sheep (age 4-5 years) and 15 female sheep (age 2 years) as well as from18 lambs in an early fattening phase (9 ecologically fed) were collected at the Nortura Gol slaughter plant. The only difference between ecological and conventional production was the lower level of the fatty acid C22:6 (*n*-3) in ecological lamb, and therefore these two groups were merged into a single group in all analysis.

The carcasses in Norway and MN were exposed to low electrical stimulation, and then returned to the chiller (4°C). All *LTL* samples were cut along the carcass length and vacuum-packed in the cutting room ≤ 5 h at 10°C, before being returned to the chiller. The vacuum packaged samples were transported on ice to the laboratories 24 h *pm*.

One *LTL* from each animal was stored at 4°C for 7 days and then sliced, vacuum-packed and frozen. The second *LTL* was cut in pieces suitable for the intended measurements, vacuum-packaged and stored at – 80°C, for tenderness measurements at – 40°C.

Meat quality assessments

pH: In Norway and MN, the pH value was measured 24 h *pm* (pH_{24}) using the Knick Portamess Model 913 (Knick, Berlin Germany), while in B&H using the HANNA Model 99161 (Cluj-Napoca, Romania). Both instruments were calibrated with commercial standard solutions.

Color stability: Fresh meat samples (24 h *pm*) were sliced into 2 cm thick cuts, and placed on trays (Polystyrene Weigh Boats 85x85x24mm, VWR International, Darmstadt, Germany) over-wrapped with oxygen-permeable polyvinyl chloride film (PVC) and stored at 4°C. One hour after slicing was denoted as time zero. The meat color was determined in triplicates on slices after 4, 72 and 144 h chill storage. The meat surfaces were turned up, towards the cling wrap, during measurements at a temperature of 19°C. Norway: Konica Minolta Spectrophotometer CM 700d (Konica Minolta Sensing Inc., Osaka, Japan)

calibrated by a white ceramic calibration cap (CM-A177) was used. The light source was a pulsed xenon lamp. Illuminant D65 (Daylight, color temperature 6504 K) with a 10º observer (CIE Konica-Minolta 1964) was used. B&H: Konica Minolta Spectrophotometer CM 2600d (Konica Minolta Sensing Inc., Osaka, Japan) calibrated by a white ceramic calibration plate (CM-A145). The light source, standard illuminant and observer was the same as in Norway. MN: Color-Tec PCM+ (ColorTec, Clinton-New Jersey, USA) 20 mm reflectance colorimeter was used. The light source was a light emitting diode (LED) array.

To secure that the measurements were comparable in the 3 countries, seven paint codes (black, white and 5 shades of red) from "JOTUN" A/S (Sandefjord) were measured in Norway, B&H and MN and used to calculate and correct for instrumental differences.

***Warner Bratzler tenderness measurements*:** Slices (4 cm), thawed overnight and heated at 72°C in the core of the samples, were cooled on ice up to approximately 20°C. Sensors inserted in dummy samples recorded internal temperatures. Muscle samples (1×1×4 cm) were cut in parallel to the fiber direction, and sheared across the fiber direction. Norway: shear cell HDP/BSK Warner Bratzler, load cell 25 kg, TA-HDi Texture Analyser, Stable Micro Systems, Godalming, UK. MN/B&H: Shear cell HDP/BS Warner Bratzler, load cell 25 kg, TA.XT, PLUS, Texture Analyser, Stable Micro Systems, Godalming, UK. The number of replicates was 6-8. In order to transfer data between labs, a rubber was split in two and each half was measured in each country, and a factor was calculated to transfer data from one instrument to another one.

***Cooking loss (% weight loss)*:** Cooking loss (%) was calculated as a percent difference between the fresh and heated samples weights.

Chemical composition

***Protein Content*:** Nitrogen content was determined using the Kjeldahl method as described by ISO 937:1992 (ISO, 1992). Total Kjeldahl nitrogen was converted to protein by conversion factor 6.25.

***Water content*:** Water content in meat samples was determined, according to the AOAC Official Method (AOAC 950.46, 1950) in three replicates.

***Fat content and fatty acid composition*:** Fat content was determined according to the AOAC Official Method (AOAC 991.36, 1996), and fatty acid composition according to the O'Fallon method (2012).

***Vitamin E content*:** The measurements were carried out by applying the procedure of *Triumf et al. (2012)*, with modification of the centrifugation time.

2,2-diphenyl-1-picrylhydrazyl (*DPPH), total antioxidant capacity*: The antioxidant capacity was determined by using DPPH, according to the procedure

described by *Brand-Williams et al. (1995),* with some modifications. Meat pieces (0.5 g) were added to 4 ml of DPPH in ethanol (0.050mg/ml). The homogenates were incubated (50 min) in the dark at room temperature. Trolox solutions were used as a standard. The samples were shortly vortexed and centrifuged at 2534 x g for 5 min. The reduction of DPPH was measured by Synergy H4, Hybrid Multi-Mode Microplate Reader from BioTek Instruments Inc., P.O. Box 998 (Highland Park, Winooski, Vermont 05404-0998 USA) at 515 nm after 60 min incubation (until stable absorptions values were obtained). The percentage of DPPH-scavenging activity was calculated as (Ao-At)/(Ao)x100, where Ao was the absorbance of the control and At was the absorbance in the presence of the sample after 1 h of incubation.

Cathepsin B analysis: The assay was based on the procedure of *Barret and Kirschke (1981)*, with some modifications. The frozen meat was pulverized (IKA 11 basic Analytical mill, Germany). Meat (1 gram) was mixed with 10 ml extraction buffer (containing 0.25 M of sucrose and 1 mM EDTA in 0.2 M KCL; pH 6.0, adjusted with NaOH). After adjusting the pH of the extraction buffer 0.2 (w/v) Triton X100 was added. The meat homogenates were vigorously shaken and centrifuged (VWR by Hitachi Koki, CT 15E, Japan) at 1946 x g for 20 min at 4°C.The supernatant was mixed with 100 µl buffer, 50 µl Milli-Q water and 100 µl stock solution (15mM Z-Arg-Arg-AMC in 100% DMSO). The blank sample contained 150 µl Milli-Q water, 100 µl assay buffer (containing 0.2 sodium acetate, 4mM EDTA and 8 mM DTT, the final pH 6.0 was adjusted with NaOH) and 50 µl supernatant.

The stock solution of the standard contained Milli-Q water, 7-methyl-coumarin amide MCA (1mM MCA in 100% DMSO) and assay buffer. The assay buffer and the diluted extract were incubated in Synergy H4 Hybrid Multi - Mode Microplate Reader (BioTek Instruments. Inc. USA) at 40°C for 30 min. The excitation wavelength was 340 nm, and the emission was monitored at 460 nm.

Heme pigment /hemin analysis: The method was based on the procedure described by *Lombardi-Boccia et al. (2002),* adapted to Eppendorf tubes.

Total peroxide value using the ferric-xylenol orange method: The frozen and aged samples were prepared according to the procedure described by *Yi et al. (2013).*

TBARS: Lipid oxidation was assessed by the TBARS (thiobarbituric acid reactive substances) assay on the aged samples.Two g frozen meat was pulverized (IKA 11 basic Analytical mill, Germany) and mixed with 10 ml stock solution (0.375 % TBA and 15% TCA in 0.25 N HCl). All samples were treated in a water bath at 98 °C for 10 min and cooled on ice for the next 30 min. Solutions under the upper fat layer (1.5 ml) were carefully removed and centrifuged for 25 min at

25 186 x g and 4°C. The absorption (at 532 nm) of the supernatant was measured immediately after centrifugation using Shimadzu UV-1800 (Shimadzu corp. Kyoto, Japan).

Statistical analysis: All statistical analyses were performed using one way ANOVA or a general linear model (Minitab version 16 or 17, Minitab Ltd., Coventry) in combination with Tukey's test for individual comparisons. Significant differences were reported for $P \leq 0.05$.

Results and Discussion

Physical characteristics of sheep/lamb LTL

Carcass characteristics: Carcass weight, fat and conformation grading, tenderness, cooking loss and pH_{24} for the six different age and breed categories are shown in Table 1. NO carcasses had nominally higher slaughter weights when compared to carcasses from WB. The carcasses from NO and B&H lambs had similar slaughter weights. The B&H sheep were small, had more fat, but good conformation score (Table 1), while the B&H lamb had the lowest fat and conformation score. The conformation score was highest for NO lambs. Due to unusual WB weather conditions in 2012 with pasture in surplus, the WB sheep and lamb were slaughtered one month later than usual; consequently the animals were also fatter (*Bjelanovic et al., 2013*). A significant difference ($P < 0.001$) in fatness and conformation score was found between groups.

Table 1. Carcass and meat physical quality assessments (mean and standard error square).

	Norwegian white sheep			WB Pramenka sheep		
	NO old	NO young	NO lamb	MN sheep	B&H sheep	B&H lamb
Age (years)	4-5	2	0.5	4-5	4-5	0.5-0.6
Carcass w. (kg)	30.4(±5.2)^{ab}	33.1(±3.2)^{a}	17.1(±2.6)^{d}	27.3(±3.6)^{bc}	25.0(±3.1)^{c}	16.0(±1.7)^{d}
EU fatness s.*	8.0(±1.4)^{b}	7.4(±0.8)^{b}	5.6(±1.3)^{c}	7.7(±1.3)^{b}	9.8(±1.0)^{a}	5.1(±1.2)^{c}
EU conformation s.**	5.0(±0.0)^{b}	7.6(±0.6)^{a}	8.0(±0.0)^{a}	5.3(±1.5)^{b}	7.9(±1.6)^{a}	3.4(±0.9)^{c}
pH	5.55(±0.12)^{b}	5.61(±0.07)^{ab}	5.64(±0.07)^{ab}	5.75(±0.08)^{a}	5.75(±0.25)^{a}	5.75(±0.15)^{a}
>pH 5.8	0/14	0/15	0/18	4/15	2/15	0/15
SF (N/cm^2)***	52.4(±10.4)^{a}	54.6(±12.3)^{a}	40.1(11.06)^{bc}	47.4(±7.9)^{ab}	38.9(±6.1)^{bc}	31.8(±5.9)^{c}
Range	38-70	37-77	25-60	28-83	25-66	25-42
>50 (N/cm^2)	4/14	8/15	4/18	3/15	1/15	0/15
Cooking loss (%)	20.5(±5.1)^{ab}	19.3(±4.2)^{b}	21.8(±5.1)^{ab}	25.4(±4.9)^{a}	18.1(±1.7)^{b}	21.5(±5.2)^{ab}

*Scale 1-15 points:1=P-; 2=P (poor);3=P+; 4=O-; 5=O(normal); 6=O+; 7=R-; 8=R (good), 9=R+; 10=U-; 11=U(very good); 12=U+, 13=E-; 14=E (excellent), and 15=E+
**Scale 1-15 points:1=1-; 2=1(very scarce); 3=1+; 4=2-;5=2 (scarce); 6=2+; 7=3-; 8= 3 (medium); 9=3+; 10=4-; 11=4 (important), 12=4+; 13=5-; 14=5 (excellent), and 15=5+
***8 days p.m.
^{abcd}Row means within factors with different letters indicate statistically significant differences at ($P < 0.001$).

Sheep and lamb meat quality related characteristics:

Mean pH_{24} ranged from 5.55 to 5.75 (Table 1). A significant difference between groups in pH_{24} ($P < 0.001$) was found. pH was higher in WB than in NO samples. This may indicate less stress in NO animals when slaughtered (*Martínez-Cerezo et al., 2005*), or less type I fibers (*Park et al., 1987*). PS is an indigenous breed, and may uphold its natural instincts (i.e. fear) and sensitivity to stress. Stress results in excretion of adrenaline causing a series of biochemical changes that indirectly catalyze the breakdown of glycogen ante mortem (*am)*, leading to an elevated muscle pH_{24} (*Voisinet et al., 1997*). *Priolo et al. (2002),* also connected higher ultimate pH to physical activity of animals and extensive production system.

Generally, the samples from WB sheep and lamb were significantly tenderer when compared to NO sheep and lamb, and this may depend both on breed and production system in agreement with *Guerrero et al., (2013).* Meat samples from B&H sheep and lamb were tenderer compared to the other groups. The samples from young NO were the toughest, while the MN sheep varied the most (Table 1). Meat with shear force scores above 50 N/cm^2 is regarded as tough (*Davey, Gilbert, and Carse, 1976*) and will be discounted by consumers. The breeding aim for higher muscular mass is often at the expense of lower tenderness and lower *IMF* content (*Więcek et al., 2008*). Cooking losses were highest in the MN samples (Table 1). This may reflect these samples lower protein content (Table 2).

The average changes in surface meat color parameters (L*a*b*) during the aerobic storage were significantly different among groups (Figure 1 a,b). The first measurement (4 h) would reflect a bloomed sample with dominantly oxy-myoglobin (OMb) in the surface. A decline in L* and a* with time would be interpreted as conversion to meat-myoglobin (MMb). Surface L* may increase due to microbial growth after prolonged storage in air.

L* (lightness) was always higher in WB animals (Figure 1a) with B&H lamb having the highest initial L* value. L* increased/remained the same for 72 h, except for the young NO and B&H sheep. L* may dependent on production system. Some authors have reported darker meat from extensive production systems (*Mancini and Hunt, 2005;Priolo et al., 2002*), but *Lorenzo et al. (2014),* reported a higher L* value in meat from a free extensive production system. This phenomenon may be explained by a higher *IMF* level in meat from extensive production systems (*Priolo et al., 2002*).

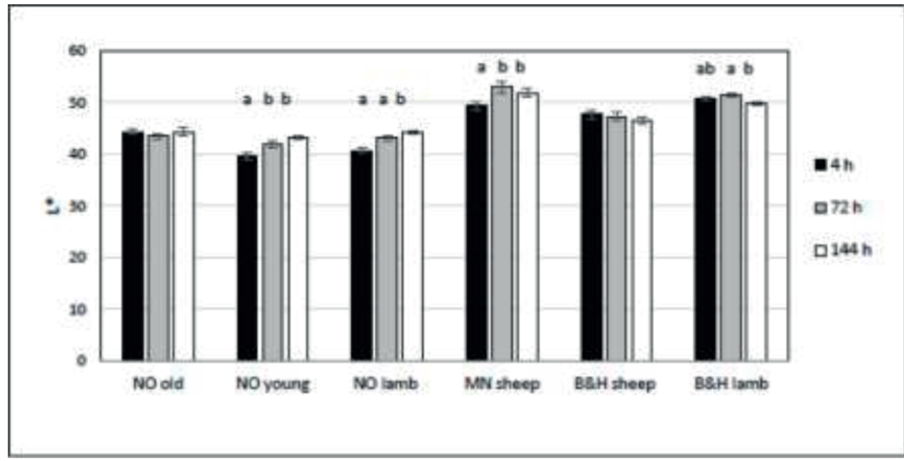

Figure 1a. The average changes in L* during aerobic incubation for different sheep/lamb groups and times. Different letters indicate significant (*P*<0.05) differences.

The variable a* was not dependent on production system. Four h post mortem, only the NO lamb and B&H sheep had low a* values. This could be due to low color stability for the NO lamb or the higher fat level in B&H sheep (Table 1). The variable a* of MN sheep declined after 72 h, but still retained a higher level than in the other groups. a* of the B&H sheep declined only moderately from 4 to 72 h. The color stability of NO sheep, using a* as an indicator, was lower than in MN sheep and B&H sheep (Figure 1b). For lamb, a* declined the least for the NO lamb.

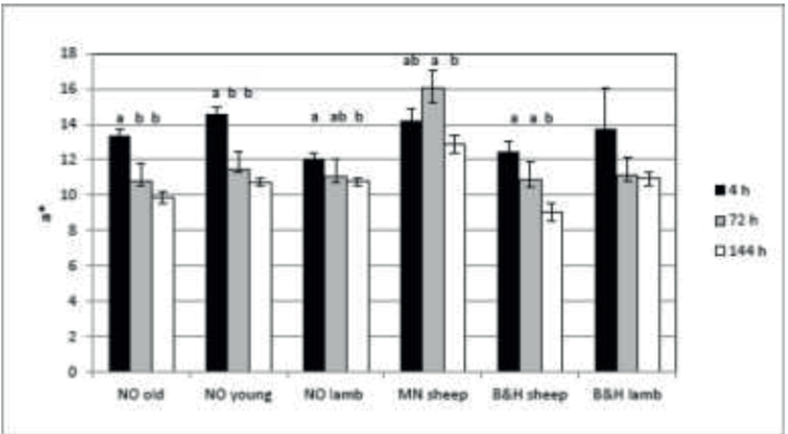

Figure 1b. The average changes in a* during aerobic incubation for different sheep/lamb groups and times. Different letters indicate significant (*P*<0.05) differences.

NO young sheep and NO lamb had the lowest b* and a much lower b* than NO old (not presented). Interestingly, b* was also high in B&H meat. Differences in muscle lightness and yellowness can be attributed to dietary effects on pre-slaughter glycogen and on marbling levels (*Mancini and Hunt, 2005*) while differences in a* depend largely on heme amount, myoglobin states plus marbling.

Composition of sheep/lamb LTL

The iron concentration in meat is highly dependent on breeding, age, sex and muscle type of the animal (*Lombardi-Boccia et al., 2002*). As expected, heme was highest in older sheep and lowest in lambs (Table 2). There was no difference in heme between NO and B&H lambs, but NO lambs had the nominally lowest heme concentration (0.15 mg/ml).

Water content depended on age and was higher in younger compared to older and more fatty animals. The low water content in B&H sheep meat was related to its higher fat content (supported by Table 1 and 2). Breed combined with production system had no significant impact on dry matter.

Table 2. Meat chemical quality assessments (mean and standard error square).

	Norwegian white sheep			WB Pramenka sheep		
	NO old	NO young	NO lamb	MN sheep	B&H sheep	B&H lamb
Heme (mg/ml)	0.23(±0.05)^{a}	0.21(±0.04)^{ab}	0.15(±0.03)^{c}	0.24(±0.04)^{a}	0.21(±0.05)^{ab}	0.18(±0.03)^{bc}
Water content*	73.13(±0.6)^{b}	73.42(±1.0)^{b}	75.30(±0.9)^{b}	73.15(±0.4)^{b}	70.93(±0.6)^{c}	75.83(±0.4)^{a}
Dry matter*	26.87(±0.6)^{b}	26.58(±0.9)^{b}	24.69(±0.6)^{b}	26.85(±0.4)^{b}	29.07(±0.6)^{a}	24.17(±0.4)^{c}
Protein content*	21.38(±0.9)^{a}	21.61(±0.9)^{a}	20.56(±0.6)^{b}	17.12(±0.4)^{c}	20.49(±0.6)^{b}	20.63(±0.4)^{b}
Fat content*	3.88(±0.5)^{b}	3.38(±0.2)^{b}	2.58(±0.6)^{c}	7.46(±0.8)^{a}	7.39(±0.4)^{a}	2.35(±0.1)^{c}
Vitamin E (mg/100g)	0.23(±0.04)^{ab}	0.12(±0.05)^{c}	0.09(±0.07)^{c}	0.29(±0.15)^{a}	0.22(±0.08)^{ab}	0.16(±0.06)^{bc}
Vitamin E/Fat (mg/100g)	0.07(±0.05)^{ab}	0.03(±0.03)^{b}	0.05(±0.04)^{b}	0.04 (±0.02)^{b}	0.04(±0.02)^{b}	0.11(±0.05)^{a}
DPPH (total antioxidant)*	66.2(±5.2)^{b}	66.5(±3.3)^{b}	66.3(±4.8)^{b}	70.9(±2.6)^{a}	68.7(±3.7)^{ab}	72.7(±3.7)^{a}
Cathepsin B**	0.33(±0.07)^{ns}	0.33(±0.04)^{ns}	0.30(±0.03)^{ns}	0.32(±0.05)^{ns}	0.31(±0.04)^{ns}	0.32(±0.03)^{ns}
TBARS***	0.33(±0.13)^{ab}	0.33(±0.21) ^{ab}	0.22(±0.05)^{b}	0.47(±0.25)^{a}	0.23(±0.23)^{b}	0.43(±0.03)^{a}

* expressed in %
** µM MCA/min/g meat
*** 8 days p.m. / mg malondialdehyde/kg
^{abcd}Row means within factors with different letters indicate statistically significant differences at(P< 0.001) except TBARS (P<0.005).

Protein content was significantly different among all animal groups (Table 2). Both old and young NO had higher protein content than B&H and MN sheep. MN sheep had the lowest protein content, but with no difference for lamb groups. *Hofman et al. (2003)* reported that the muscles with the highest protein content were characterized by lower fat content. NO sheep had a more favourable fat/protein ratio (Table 2) in agreement with general breeding goals. The results also indicated that old and young NO sheep, with the highest protein content, were

less tender (Table 1). This can again relate to types of muscular fibers. *Wood et al. (1999)* suggested that genetic selection for modern breeds with increased meat yield and lean content increases the proportion of white glycolytic fibers (type IIB), and consequently less tender meat (*Karlsson et al., 1993*).

Vitamin E (α-Tocoferol) is a fat-soluble vitamin. Its content was significantly different among all six animal groups (Table 2). Green pasture or supplementation in feeds increase vitamin E in meat (*Jose et al., 2008*). Vitamin E can delay OMb oxidation via inhibition of lipid oxidation (*Faustman et al., 1998*). Color and lipid stability of fresh beef *longissimus muscle* can be improved if α-tocopherol concentrations of tissues is between 3.0 to 3.3 μg α-tocopherol/g meat (*Faustman et al., 1989*). MN sheep had a high concentration of vitamin E (0.29 mg/100g), close to this threshold. This can be a possible explanation of the delayed OMb convertion to MMb in MN sheep. Older sheep groups had a higher vitamin E concentration than younger groups. Unexpectedly, vitamin E/fat (mg/100g fat) was nominally highest in B&H lamb, and significantly different from the other groups (Table 2).

α-Tocoferol level isinteresting from a nutritional perspective, assuming that its antioxidative power protects cells against the effects of free radicals which can contribute to the development of chronic diseases like cancer and cardiovascular diseases. This vitamin can enhance the immune function and block the formation of cancerogenous nitrosamines in the stomach from nitrates used as additive in food products. Vitamin E also prevents against cataracts (*Daley et al., 2010*).

Cathepsin B is a relevant enzyme for dry cured sheep production since its level is closely related to textural defects during the ripening phase of pig hams (*Priolo et al., 2002*). The activity of cathepsin B in *LTL* (Table 2) did not differenciate between groups, only within groups; the highest variation was for old NO and MN sheep. The variation was lowest for NO lamb and B&H lamb.

Table 3 shows average values and standard errors (SE) of intramuscular fatty acid composition (mg/100 g meat). The concentrations of total fatty acids were age dependent. Sheep had more total fatty acids than lambs, and WB sheep more than NO in agreement with their amount of total fat (Table 2). The concentration of the polyunsaturated fatty acids C18:2 (*n*-6) and C18:3 (*n*-3) showed the greatest variation, as indicated by their SE, while the concentration of C20:4 (*n*-6), C20:5 (*n*-3), C22:5 (*n*-3) and C22:6 (*n*-3) showed the lowest SE. The total amount of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and PUFA was also age dependent, and significantly higher in older animals. The percentage of PUFA dropped with age, but was also significantly dependent on production systems, as described by *Enser et al. (1998)*. The nominally highest % SFA was found in MN sheep.

Table 3. Fatty acid composition (mean and standard error square).

	Norwegian white sheep			WB Pramenka sheep		
	NO old	NO young	NO lamb	MN sheep	B&H sheep	B&H lamb
C18:2 n-6 Linoleic acid*	1.81(±1.58)[ab]	1.93(±0.72)[ab]	0.98(±0.27)[c]	2.34(0.84)[a]	2.21(±0.69)[a]	1.17(±0.24)[bc]
C18:3 n-3 α-Linolenic acid*	1.15(±1.33)[a]	1.49(±0.73)[a]	0.49(±0.17)[b]	1.42(±0.76)[a]	1.06(±0.45)[ab]	0.43(±0.10)[b]
C20:4 n-6 Arachidonic acid*	0.32(±0.03)[b]	0.39(±0.08)[ab]	0.33(±0.06)[b]	0.33(±0.04)[b]	0.38(±0.04)[ab]	0.43(±0.07)[a]
C20:5 n-3 Eicosapentaenoic acid*	0.20(±0.02)[b]	0.24(±0.05)[a]	0.20(±0.04)[b]	0.17(±0.03)[bc]	0.17(±0.03)[c]	0.16(±0.03)[c]
C22:5 n-3 Docosapentaenoic acid*	0.26(±0.11)[b]	0.36(±0.10)[a]	0.21(±0.04)[b]	0.27(±0.06)[b]	0.25(±0.05)[b]	0.22(±0.04)[b]
C22:6 n-3 Docosahexaenoic acid*	0.07(±0.02)[bc]	0.10(±0.04)[a]	0.06(±0.02)[c]	0.09(±0.03)[ab]	0.09(±0.01)[ab]	0.09(±0.02)[ab]
n-6/n-3*	1.37(±0.17)[b]	1.12(±0.15)[c]	1.44(±0.15)[b]	1.46(±0.15)[b]	1.73(±0.20)[a]	1.84(±0.14)[a]
SFA*	30.87(±42.48)[a]	27.66(±14.77)[ab]	8.67(±1.87)[b]	47.41(±23.20)[a]	29.51(±10.61)[a]	7.51(±2.69)[b]
MUFA*	29.12(±41.93)[a]	22.11(±12.69)[ab]	6.78(±1.68)[c]	36.77(±16.54)[a]	24.97(±10.41)[ab]	6.5(±2.57)[bc]
PUFA*	3.88(±3.09)[ab]	4.58(±1.65)[a]	2.33(±0.55)[c]	4.71(±1.70)[a]	4.23(±1.21)[a]	2.55(±0.46)[bc]

* mg/100g meat

[abcd]Row means within factors with different letters indicate statistically significant differencesat (P< 0.001).

Total amounts of C18:2 (*n*-6) was higher in sheep compared to lamb. Total amounts of α-Linolenic acid C18:3 (*n*-3) tended to follow this pattern. Sañudo et al. (2006) reported similar results for Spanish and British lambs. Old NWS had the greatest amount of *n-3* LC-PUFA.

The ratio *n*-6/*n*-3 was still favorable for lamb/sheep (*Russo, 2009*). Interestingly, this ratio showed no variation with age in both NO and B&H systems. But the *n-6/n-3* ratio was significantly higher for B&H sheep and lamb (Table 3) than other systems. The ratio *n*-6/*n*-3 was the lowest in young NO sheep. C18:3 (*n*-3) is regarded as the preferred fatty acids leading to C20:5 (*n*-3), docosapentaenoic acid C22:5 (*n*-3), and docosahexaenoic acid C22:6 (*n*-3) (*Brenna et al., 2009*). Additionally, it inhibits the conversion of C18:2 into the others *n*-6 LC-PUFA (*Smink et al., 2012*).

A favorable *n*-6/*n*-3 ratio is important for the regulation of SFA in human body. The dietary SFA can raise unfavorable blood lipids, but sufficient intake of *n-3* PUFA can neutralize this effect (*Dias et al., 2014*), and prevent coronary heart diseases, diabetes 2, obesity and cancer. The SFA intake is a major contributor to calcium, vitamin D, vitamin B12 and the other essential nutrients absorption; a reducing of SFA without substituting lower-fat versions may result in serious unintended nutritional consequences (*Huth et al., 2013*).

Oxidative stability measurements

The total antioxidant activity method detects the ability of a matrix to eliminate an unpaired valence electron in DPPH (*Dawidowicz, Wianowska, and Olszowy, 2011*). Low DPPH values are therefore favorable. The total antioxidant activity was highest in NO meat (Table 2). Antioxidant activity was not affected by the age of the animals.

TBARS values above 0.5 are considered as critical and indicate a lipid oxidation level which produces a rancid odor and taste that can be recognized by consumers (*Wood et al., 2008*). TBARS was significantly different among the groups (Table 2). After 7 days of aging at 4ºC, TBARS acumulation in NO old and young was equal. NO lamb had the lowest TBARS value, while MN sheep and B&H lamb had the highest. B&H sheep had the lowest TBARS among sheep groups. The TBARS value of 0.47 in MN sheep was near the threshold of 0.5 suggesting that the high fat content and poor ratio vitamin E/fat content may have some impact on its low oxidative stability (Table 2). All together factors such as concentration of the fat, heme pigment and antioxidant status in the muscle tissue can influence color stability and FA oxidation, and are tightly related to the diet (*Ponnampalam et al., 2012*). *Lourenço et al. (2007)* suggested that different grazing regions can induce changes in the rumen microbial population, and therefore differences in the biohydrogenation of PUFA. Dietary effects in form of different grass types might have an impact on the FA composition in ruminants. *Lee et al. (2003)* suggested that white clovers (*Trifolium repens*) can limit biohydrogenation of *n-3* PUFA. It seems that vitamin E had a positive impact on color stability in MN sheep, but not on FA oxidation stability.

Polar peroxides (0.12-0.39 mmol/kg meat) originating from lipids (*Volden et al., 2011*) were highest in VP lamb followed by NO old and young. Proteins bound peroxides (*Yi et al., 2013*) also varied significantly among groups from 0.09 in MN sheep to 0.191 mmol/kg in NO old. No significant difference was found for unpolar (chloroform soluble) peroxides. These data are partly in agreement with TBARS (Table 2).

Conclusion

The different production systems influenced meat color, pH, tenderness and fatty acid composition. Pramenka sheep, collected from their natural grazing areas, were smaller animals with more fatty carcasses relative to NWS from Hardangerevidda and Jotunheimen regions. WB meat (*LTL*) had higher pH_{24}, and a low protein to *IMF* ratio. Its total antioxidant capacity was lower, and the *n*-6/*n*-3 ratio tended to be higher. The marketing potential of PS meat seems to be related to its higher color stability and good tenderness. This quality can be used to

encourage the production of B&H sheep and lamb in future. The marketing advantages of NO carcasses seemed related to their high protein/fat ratio, low *n-6*/*n-3* ratio and good antioxidant capacity.

B&H sheep were muscular but with more fat, lower water content and lower cooking losses, lower L*a* b* with higher *n*-6/*n*-3 and became more rancid than MN sheep. The B&H lambs were smaller than NO lambs, with a higher level of vitamin E, but lower antioxidant capacity, more TBARS and less EPA and higher *n*-6:/*n*-3 ratio. Its marketing potential seemed only related to its high vitamin E content while the marketing potential of NO lamb seems related to its good oxidative stability with a favorable *n*-6/*n*-3 ratio.

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Uticaj različitih proizvodnih sistema na kvalitet mesa trupova ovaca i jagnjadi Zapadnog Balkana i Norveške

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Rezime

Definisanje kvaliteta mesa odabranih rasa ovaca i jagnjadi koje su bile na ispaši u posebnim regijama je od velike važnosti u postizanju tržišne konkurentnosti. U ovom eksperimentu korišten je mišić *Longisimus thoracis at lumborum* (*LTL*) autohtonih zapadno-balkanskih(WB) ovaca i jagnjadi vlašićke pramenke (VP) koje su bile na ispaši na planiniVlašić u Bosni i Hercegovini. Također je korišten *LTL* od ovaca pivske pramenke (PP) koje su bile na ispaši na planini Ljubišnja u Crnoj Gori. Kvalitet mesa trupova i *LTL*-a autohtonih balkanskih ovaca upoređivani su sa trupovima norveških belih ovaca i jagnjadi (NWS), koje su bile na ispaši u regionu hardangerske visoravni i Jotunheimen regiona. Jagnjeće meso iz ovih regiona smatra se gurmanskim proizvodom.

U poređenju sa NWS ovcama rase pramenka ovaca imale su nižu prosečnu težinu, manji oksidativni kapacitet, veću konačnu pH vrednost, intramuskularnu masnoću kao i viši odnos n-6/n-3, bolju mekoću mesa i stabilnost boje. Jagnjad zapadno-balkanske pramenke su imala nešto manju masu, viši odnos n-6/n-3, slabiji oksidativni kapacitet, njihovo meso je veoma brzo užeglo, bez obzira na viši sadržaj α- tocopherola. Tržišna prednost mesa zapadno-balkanskih rasa je u njihovoj mekoći, dok NWS jaganjci imaju bolji nutritivni profil.

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Paper V

1 **The relationship between volatile compounds, metabolites and**
2 **sensory attributes: a case study using lamb and sheep meat**
3

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46 **ABSTRACT**

47 The aim of this study was to use a flavoromics approach to identify
48 key compounds responsible for sensory flavor of lamb and sheep
49 meat. The investigation was confined to volatile compounds from
50 adipose tissue and metabolites in lean meat using headspace-gas
51 chromatography/mass spectrometry (HS-GC/MS) and solvent
52 extraction-GC/MS, respectively. Partial least square regression
53 analysis supported with variable selection were used to correlate
54 identified compounds to sensory attributes. Several metabolites
55 involved in energy production via Krebs cycle and Embden-
56 Meyerhof-Parnas pathway contributed to gamy and grassy flavor.
57 Gamy flavor was strongly and positively correlated with aspartic
58 acid, cyclo-leucine, gluconic, citric and pyruvic acid. Gluconic and
59 pyruvic acid together with formic acid, β-caryophyllene, 3-
60 methylphenol, 2-ethylfuran showed strong positive correlation
61 with grassy flavor. Sugars (glucose, mannose-6-phosphate and
62 myo-inositol) were negatively correlated with gamy and grassy
63 flavor, suggesting a role in suppression of off-flavors in lamb and
64 sheep meat. Bitter flavor was strongly correlated with hypotaurine
65 and (*E*)-2-pentenal. Metallic flavor and bitterness were influenced
66 by almost the same compounds. Acidic flavor was not explained
67 by any compound identified, while rancidity was not detected by
68 panelists. Finally, the flavor components describing grassy and

69 bitter flavor could be used to discriminate animals from different
70 production systems.
71
72 **KEYWORDS:** sheep; adipose tissue volatiles; lean meat
73 metabolites; sensory attributes
74
75 **INTRODUCTION**
76
77 Meat flavor is an important quality criterion with a key role in the
78 overall lamb/sheep meat acceptability (Wood et al., 1999).
79 Significant attention has been given to the characteristic mutton
80 and pastoral flavor that negatively affects consumers' acceptance
81 of lamb/sheep products (Sink and Caporaso, 1977; Young et al.,
82 2003). Mutton flavor is described by Wong (1975) as sweaty, sour,
83 urinary, fecal, barnyard, oily, sharp and acrid. This flavor note was
84 associated with branched chain fatty acids (BCFA; C_8 – C_{10}),
85 specifically 4-methyloctanoic, 4-ethyloctanoic and 4-
86 methylnonanoic acids that are more abundant in adipose tissue of
87 aged animals (Wong et al., 1975 a,b; Watkins et al., 2013).
88 However, it is noteworthy that discrimination of lamb from sheep
89 meat according to BCFA concentration has not nevertheless been
90 possible (Watkins et al., 2010).

91 Pastoral flavor described as sheepy, gamy, barnyard, animal, fecal,
92 is related with pasture-fed animals (Schreurs et al., 2008). This
93 flavor note has also been associated with higher concentrations of
94 3-methylindole (skatole) and 4-methylphenol in lamb adipose
95 tissue (Young et al., 2003).
96 The chemistry of flavor is very complex and depends of interaction
97 between volatile (aroma) and non-volatile (taste) compounds. A
98 number of studies have been carried out to identify and define key
99 volatile compounds associated with the characteristic flavor in
100 cooked sheep meat (Almela et al., 2010; Bueno et al., 2014;
101 Caporaso et al., 1977; Elmore et al., 2000; Hornstein and Crowe,
102 1963; Resconi et al., 2010; Young et al., 1997). Generally, limited
103 work has been done on non-volatile (metabolites) compounds and
104 their role in lamb/sheep flavor (Watkins et al., 2013). In addition,
105 the complex nature of meat flavor requires understanding of the
106 essential flavor-active compounds isolated both from adipose
107 tissue and lean meat and their joint contribution to perceived
108 flavor.
109 To understand flavor properties of lamb/sheep meat, in the present
110 study an untargeted approach called flavoromics was applied
111 (Ronningen, 2016). This novel approach in flavor research
112 combines three phases: characterization of volatile and non-
113 volatile (metabolites) compounds, model development and

114 validation of compounds. The analytical information, as an
115 outcome of three steps, is correlated with sensory properties in
116 order to define compounds responsible for specific attributes.
117 Using this approach, the aim was to: 1) Identify and quantify
118 volatiles and metabolites as constituents of lamb/sheep meat
119 flavor; 2) Evaluate sensory properties of lamb/sheep meat; 3)
120 Elucidate how volatiles and metabolites from different metabolic
121 pathways correlate with sensory attributes using a flavoromics
122 approach.

123

124 **MATERIALS AND METHODS**

125

126 **Experimental design**. Ninety-two female animals were used in the
127 study. In order to get high variability in flavor profiles the
128 following animals were chosen: lambs (5−6 months), young sheep
129 (~2 years) and old sheep (4−5 years) belonging to two different
130 breed representative for the production system of three country of
131 origin (Bosnia and Herzegovina – BH, Montenegro – MN, and
132 Norway – NW). Lamb (18 animals; NW lamb), young (15 animals;
133 NW 2y) and old sheep (14 animals, NW 4y) belonging to the
134 Norwegian White Sheep breed were selected. Furthermore, lamb
135 (BH lamb) and old sheep (BH 4y), 15 animals each, belonged to
136 Vlašićka Pramenka, being the most common phenotype of

137 Pramenka breed in BH. Thus, fifteen old sheep of Pivska
138 Pramenka from Montenegro (MN 4y), as a second Pramenka
139 phenotype, were included in this experiment. Six months old lambs
140 of Pivska Pramenka from the same herd could not be obtained.
141
142 **Tissue sampling.** All animals were slaughtered in the country of
143 origin (for more details see Bjelanović *et al*., 2015). The *M.*
144 *longissimus thoracis et lumborum* (LTL) from left side of carcass
145 was removed and adipose tissue available on the surface of the
146 muscle was excised within 24 h *post mortem*, wrapped in
147 aluminum foil, vacuum-packed and stored at -80°C. A slice of
148 LTL was vacuum packed and stored at -80ºC for intramuscular
149 fatty acid analysis. The rest of LTL was vacuum-packed,
150 refrigerated for 7 days (at 4°C), divided into slices of 2.5 cm
151 thickness, vacuum-packed and stored at -80°C for sensory and
152 GC/MS analysis. All samples were analyzed in the same
153 laboratory.
154
155 **Fatty acid composition.** Intramuscular fat was extracted according
156 to AOAC Official Method (AOAC 991.36, 1996). Fatty acid
157 methyl esters (FAME) synthesis was performed according to
158 modified method by Yi et al. (2013). The fatty acids were analyzed

159 by accredited laboratory (http://vitas.no/) according to the O'Fallon
160 method (2007).
161
162 **Headspace gas chromatography/mass spectrometry (HS-**
163 **GC/MS) analysis of volatile compounds.** Frozen adipose tissue
164 was homogenized with a crushing machine (IKA® A11 Basic
165 Analytical Mill, Staufen, Germany) to a fine powder. Four grams
166 of homogeneous powder were placed in a glass vial (50 mL) and
167 stored at -80°C until the next preparation step. In order to increase
168 the volatile compounds extraction and generate representative
169 volatile profiles, the homogenized sample was heated at 75°C in
170 water bath for 30 min on the day of analysis. The liquid fat phase
171 (1 g) was transferred to a clean glass vial and kept at 4°C for ~ 4 h
172 before measured. All samples were analyzed in two replicates.
173 A mixture of five compounds in Mygliol (AXO INDUSTRY,
174 Warve, Belgium) was used as a control sample throughout the
175 measurement period, at the beginning and end of sequences. These
176 compounds were: butanal (99%), *cis*-2-penten-1-ol (95%), 2-
177 undecanone (99%), and dimethyl sulfone (98%) (Sigma-Aldrich
178 Chemie GmbH, Schnelldorf, Germany) and acetic acid (100%,
179 VWR, Fontenay-saus-Bois, France).
180 HS-GC/MS analysis was performed according to a modified
181 method by Volden et al. (2011). Fat volatiles were extracted by

182 dynamic headspace analyzer Teledyne Tekmar HT3 (Teledyne
183 Tekmar, Ohio, USA) coupled to an Agilent gas chromatograph
184 6890N (Agilent Technologies, Santa Clara, CA, USA). The DB -
185 WAXetr fused silica capillary column (30 m × 0.25 mm i.d., 0.50
186 μm film thickness; J&W Scientific, USA) was connected to the ion
187 source (230°C) of a Agilent 5975 (Agilent Technologies,
188 SantaClara, USA) quadrupole mass spectrometer (interface line
189 250°C). The carrier gas was He with a flow rate of 1.0 mL/min.
190 Samples were heated at 150°C in the headspace trap. The
191 temperature program for GC was: 35°C for 10 min, ramped
192 1.5°C/min up to 40°C, ramped 4.0°C/min up to 70°C, ramped
193 7.5°C/min up to 230°C, and 1 min at 230°C. Analysis time was
194 54.62 min, and recorded mass range was *m/z* 33−300. Volatiles
195 were identified by: (i) computer-matching of generated mass
196 spectra with NIST05 database (National Institute of Standards and
197 Technology/Gaithersburg, MD, USA) and (ii) comparison of
198 retention indices (RIs) with published RI values. Identified
199 compounds (Table S-1) were used for statistics (see below). All
200 compounds referred to below except butyrolactone that we failed
201 to acquire, have been re-identified using pure compounds. The
202 standard solutions run during measurement period were run at four
203 different concentrations (R^2= 0.996–0.999 for regression line). The
204 concentration for all volatiles was standardized to the calibration

205 curve for most relevant chemical compound present in the standard
206 mix described above.
207
208 **Extraction, derivatization, and GC/MS analysis**
209 **(GC/MS$_{extraction}$) of meat metabolites.** One gram of lean meat was
210 transferred into a 15 mL tube, and 5 mL of a water: methanol:
211 chloroform (1: 2.5: 1) mixture with internal standard ribitol (66
212 μg/mL) was added. The sample was incubated at 60°C for 60 min
213 in sonication bath and centrifuged for 10 min at 3 000 rpm at 4°C.
214 An aliquot of 1 mL was transferred into a 1.5 mL Eppendorf tube,
215 dried in a SpeedVac (Thermo Scientific, Waltham, MA, USA)
216 overnight and stored at -80°C. The dried residues were
217 resuspended in 80 μL methoxyamine hydrochloride with pyridine
218 (20 mg/mL) at 30°C for 60 min and sonicated at 30°C for 30 min.
219 Finally, samples were treated with 80 μL of N-methyl-N-
220 (trimethylsilyl) trifluoroacetamide at 37°C for 30 min.
221 GC/MS analyses were performed according to Sissener et al.
222 (2011). Derivatized samples (1 μL) were analyzed on an Agilent
223 6890 GC connected with an Agilent 5975 MS detector. A HP-5MS
224 capillary column (i.d. 30 m × 0.25 mm, film thickness 0.25 μm)
225 was used. The carrier gas (He) flow rate through the column was 1
226 mL/min. The GC temperature program: 70°C for 5 min, ramped at

227 5°C/min until 310°C. Analysis time was 60 min. The MS was
228 operated at 230°C, and the recorded mass range was *m/z* 50−700.
229 MS files from Agilent ChemStation (Agilent Technologies,
230 Waldbronn, Germany) were exported in the netCDF format
231 (OPENChrom, Eclipse Public License 1.0) to MetAlign (version
232 041012, RIKILT Wageningen UR, Plant Research International)
233 for data pre-processing and alignment. Metabolites were identified
234 with the AMDIS software (version 2.71, National Institute of
235 Standards and Technology, Boulder, CO, USA) in combination
236 with NIST05 (National Institute of Standards and
237 Technology/Gaithersburg, MD, USA) and GOLM metabolome
238 database (Max-Planck Institute for Molecular Plant Physiology,
239 Golm, Germany). Normalization of the peak area was performed
240 on the internal standard ribitol and expressed as mg/kg of meat.
241 Samples were run randomized. Metabolites are presented in Table
242 S-2.
243
244 **Sensory analysis.** For sensory testing meat samples were
245 defrosted at 4°C overnight. The 2.5 cm slices of lean meat were
246 heated in water bath set to 80°C until internal temperature of 71°C
247 was achieved (AMSA, 1995) and served as 1×1×1 cm pieces to
248 each assessor. A panel consisting of 8 trained (ISO 8586–1:1993)
249 assessors (4 females and 4 males 30−59 years old) was selected for

250 the sensory analysis. The laboratory for sensory analysis at Faculty
251 of Technology in Novi Sad was designed according to ISO
252 8589:2007. During the evaluation, water and bread were served to
253 assessors to cleanse their palate between samples. Animal group
254 was randomly selected, and then the whole group was analyzed.
255 Three samples were served per session and two sessions were
256 performed. Sensory traits of lamb/sheep meat were evaluated by
257 the quantitative-descriptive analysis (Lawless and Heymann,
258 2010), using a scale from one (none) to nine (very intense)
259 according to ISO 4121:2003. Assessors were asked to evaluate the
260 following odor (gamy, grass, rancid) and taste (acidic, bitter,
261 metallic) attributes. Gamy was defined like leather/ horse saddle
262 and grassy like cut grass. These attributes were selected as they
263 have been observed to distinguish different Norwegian lamb
264 samples earlier (Lind et al., 2011). The other tastes were defined as
265 in basic taste tests (see below). All samples were analyzed in the
266 same sensory laboratory.
267
268 **Flavor threshold determination**. There were no literature data for
269 the flavor thresholds of some relevant compounds dissolved water.
270 Flavor thresholds for six compounds (2-heptadecanone, gluconic
271 acid, dimethyl sulfone, hypotaurine, mannose-6-phosphate and
272 uridine) that correlated with sensory attributes (gamy, grass, bitter)

273 were identified using 2-AFC method. In order to define min and
274 max concentration of each compounds for threshold study,
275 preliminary survey was performed based on Maximized Survey-
276 derived Daily Intakes value (MSDI-EU;
277 http://www.thegoodscentscompany.com.).
278 The four basic tastes sweet (sucrose), salty (sodium chloride), sour
279 (citric acid monohydrate) and bitter (caffeine) were prepared as
280 solutions in deionized water and stored at 4°C in screw glass
281 bottles. Six sensory experienced persons (31-43 yrs old) were
282 assembled at the Norwegian University of Life Sciences. Prior to
283 flavor threshold analysis panelists were re-trained by tasting easily
284 recognizable solutions of sucrose (90 mM), sodium chloride (340
285 mM), citric acid monohydrate (3260 μM) and caffeine (1.75 mM)
286 as suggested by Gomez et al. (2004) and Torrico et al. (2015). For
287 "blanks" deionized water was used.
288 On the day of analysis, participants were invited at 11.00 o'clock
289 and instructed to have a light breakfast and avoid smoking,
290 drinking coffee, tea, refreshments or chewing gum for at least 2 h
291 before the test (Gomez et al., 2004). Five solutions of each
292 compound, from 4.7 to 75 mg/kg for 2-heptadecanone, gluconic
293 acid and dimethyl sulfone, from 6.25 to 100 mg/kg for hypotaurine
294 and mannose-6-phosphate, and from 0.625 to 10 mg/kg for uridine
295 were prepared in 250 mL graduated closed flasks using deionized

296 water and stored at 4°C. The samples for flavor threshold analysis
297 (10 mL) were presented in 15 mL plastic tubes labeled with a 3-
298 digit random code.
299 For the series of trial, participants were presented with five
300 different concentrations of each chemical in order of increasing
301 concentration until they report difference between chemical
302 solution and distilled water. Upon comparing the samples, being
303 two distilled water samples and one chemical solution sample, the
304 subjects expressed freely their impressions about flavor profiles for
305 chemical solutions using their own expressions. Subjects were
306 informed about chemical safety information for all compounds and
307 the purpose of the test. Threshold concentration was defined as a
308 concentration of compound at which panelists could detect a
309 difference from deionized water 50% of the time. Some
310 compounds (see below) remained without flavor thresholds; one
311 because no pure compound was available and the rest were
312 excluded since they were described as hazardous or there were no
313 available data about their toxic effect.
314
315 **Statistical analysis.** Sensory scores and fatty acid composition
316 were analyzed using Microsoft Excel 2016, considering the animal
317 group as a single unit. The sensory data have only been used for
318 regression analysis where analysis of variance of sensory data were

319 not relevant. In order to explore the relationship between chemical
320 compounds and meat flavor, Partial least squares regression (PLS)
321 and Principal component analysis (PCA) were carried out using
322 Unscrambler, version X10.1 software (Camo, Trondheim,
323 Norway). The PLS routine was used and the calculations were
324 made in 3 manners. First, the data were kept as is, secondly only
325 the volatiles were multiplied by 100 so that the dimension was
326 changed to (μg/kg meat×10). The third data set was generated by
327 multiplying only the volatiles by 1000 so that data originally
328 calculated with dimension mg/kg now appeared with the
329 dimension μg/kg meat. This dimension weighting was done to
330 keep the compounds at comparable magnitudes and thereby
331 increase the possibility that a compound present in low quantities
332 could be included in the explanatory model. In addition, the
333 concentrations defining the 3 concentration weighted matrices
334 above were used for modelling both with and without weighting
335 according to standard deviation (SD) of a specific
336 variable/compound (mean/SD). The latter was an additional
337 principle to changing dimension (from mg to μg or μg×10) to
338 secure that both compounds in low and large quantities could enter
339 the explanatory models. No compound was selected as influencing
340 a sensory attributes unless the compound's regression coefficient β
341 (w) significantly ($P < 0.05$) differed from zero (equal to zero

342 defined no influence) for all 3 different weighting (×1, ×100,
343 ×1000) principles.
344 The PLS model was set up with random validation using segments
345 of 7 samples; that is approximately 50% of an animal group. As an
346 example, one group would be lamb from a specific region. It was,
347 however, not critical for the results whether the segment number
348 for validation was higher (*e.g.* 10) or lower (*e.g.* 4) than 7.
349 Principal component analysis (PCA) were performed to visualize
350 flavor (local/global) markers significant for different animal
351 groups in a reduced dimension plot. The PCA models included
352 volatile compounds isolated from adipose tissue and metabolites
353 from lean lamb and sheep meat that were significantly different (P
354 < 0.001) between animal groups. Sample names were coded as
355 described in the Experimental design section.
356
357
358 **RESULTS AND DISCUSSION**
359
360 **Fatty acid composition**
361 Fatty acid (FA) composition indirectly plays an important role in
362 characteristic meat flavor in various animal species (Kosowska et
363 al., 2017). Fatty acids are directly or indirectly involved in
364 generation of the volatile compounds and flavor constitution (Ba et

365 al., 2012). In our study, the total fatty acids of intramuscular fat of
366 *M. longissimus thoracis et lumborum* in 92 female animals was 50
367 mg/g of meat, with saturated fatty acids (SFA), monounsaturated
368 (MUFA) and polyunsaturated fatty acids (PUFA) presenting
369 50.5%, 42.1% and 7.4%, respectively (for more details see
370 Bjelanović et al., 2015). PUFAs, namely, α-linolenic acid (2.0%),
371 eicosapentaenoic acid (EPA; 0.4%), docosapentaenoic acid (DPA;
372 0.5%), docosahexaenoic acid (DHA; 0.2%) were considered.
373 These FA may cause flavor defects as a result of the oxidation
374 induced by cooking (Watkins et al., 2013). Higher levels of
375 oxidation products were previously found for grilled meat from
376 lambs fed supplement rich in EPA and DHA (Elmore et al., 2000;
377 Elmore et al., 2005). Campo et al. (2003) found that mixtures of α-
378 linoleic acid, cysteine, ribose and iron were associated with
379 'grassy' flavor and related to meat from grazing animals.
380

381 **Sensory attributes**

382 The sensory attributes of all animal groups are presented in Table
383 1. The assessors used the scale from 1−9 to evaluate odor and
384 taste. Gamy and grass odor were clearly identified, bitterness was
385 less well identified, while metallic and acidic taste had limited
386 variation for the examined samples. The assessors did not identify
387 rancidity for the samples and the attribute was therefore excluded.

388

389 Table 1. Sensory quality profile (evaluated on a 1−9 scale)

390 assessed by trained assessors on lean meat.

Sensory attributes	Mean[a]	SD[b]	Min value	Max value
gamy	4.4	1.0	2.6	6.5
grass	2.6	0.5	1.8	3.8
acidic	2.1	0.3	1.4	2.8
bitter	2.0	0.5	1.1	3.4
metallic	1.6	0.4	1.0	2.5

391 [a] Mean = average scores for each attribute for 92 animals

392 [b] SD = standard deviation

393

394 **Correlation among volatile compounds, metabolites and**

395 **sensory attributes**

396 In the present study an untargeted approach was used to identify

397 volatile compounds and metabolites in lamb and sheep adipose

398 tissue and lean meat, respectively. Adipose tissues of 92 animals

399 were subjected to HS-GC/MS analysis simulating oven-roasting

400 temperature effect (150°C). Seventy-five volatile compounds were

401 identified and classified according to their chemical nature

402 (Supplementary Material, Table S-1). They were alkanes (15),

403 alkenes (8), alcohols (11), aldehydes (19), ketones (7), acids (5),

404 lactone, terpene, sulphur compound, phenol, ester and others.

405 Among the selected compounds some were found in adipose tissue

406 of one animal group but not in others, i.e. 3-methylhexane, 3-

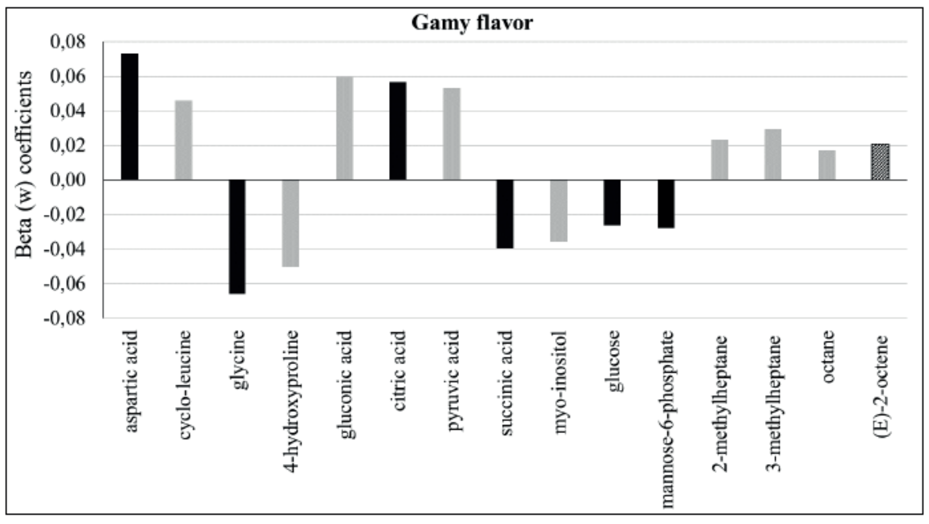
407 methylphenol, 3-methylphenol identified only in adipose tissue of

408 MN 4y sheep. Approximately 50% of all identified volatile
409 compounds were lipid oxidation products. High temperatures
410 (150−200°C) have been reported to increase formation of
411 aldehydes and ketones with reduction in hydrocarbons and furans
412 (Almela et al., 2010). In addition, 69 metabolites were separated
413 and identified in the lean meat using $GC/MS_{extraction}$ analysis (Table
414 S-2), although unlike volatiles these metabolites were identified in
415 all animals but at different level.

416 Interaction between odor (volatiles) and taste (metabolites)
417 compounds is important for meat flavor although taste is
418 dominating response. However, in further discussion for odor and
419 taste attributes we will use term flavor since taste were assessed
420 with open nostrils.

421 PLS models were calculated between sensory attributes and all
422 measured chemical compounds. Only 17 volatile compounds and
423 19 metabolites significantly ($P < 0.05$) correlated with sensory
424 attributes in PLS regression analysis (Fig.1). Chemical compounds
425 that did not pass the selection criteria are listed in Table S-3
426 (Supplementary Material). Compounds could be left out, *i.e.* not
427 selected, because: 1) they do not correlate significantly to sensory
428 attributes despite being present above flavor threshold; 2) their
429 measured value varied too much for significance despite having a
430 relevant flavor. Twenty-six of the metabolites and 31 volatile

431 compound were eliminated by first selection criteria. Metabolites
432 presumed above sensory threshold, but left out were: malic acid
433 (acid), sucrose (sweet), fructose (sweet), cysteine (sulpherous) and
434 4-aminobutyric acid (savory). Volatiles that were above flavor
435 thresholds but left out were pentanal and octanal (fruity note), (*Z*)-
436 4-heptanal (green), dimethyl sulphone (sulfurous/ metallic) and
437 toluene (complex note). To some extent (*Z*)-4-heptanal (green),
438 dimethyl sulphone (sulfurous/ metallic) may seem relevant for
439 grass or metallic flavor but nevertheless these compounds clearly
440 failed the selection criteria.



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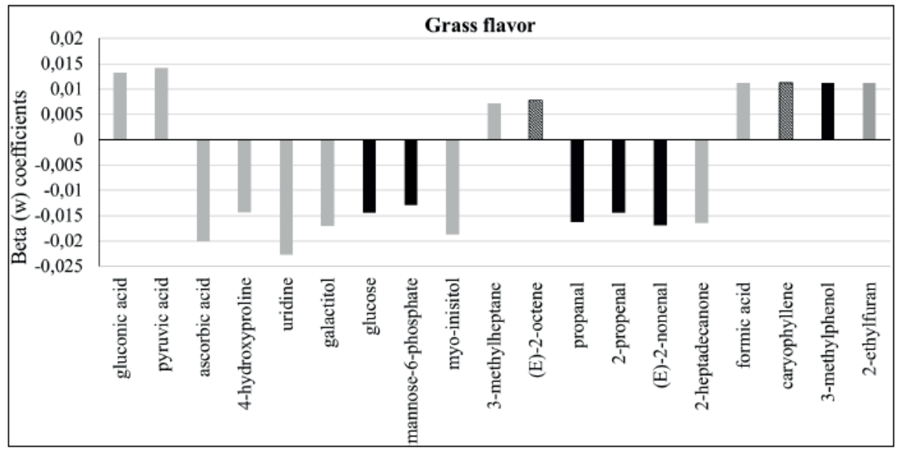

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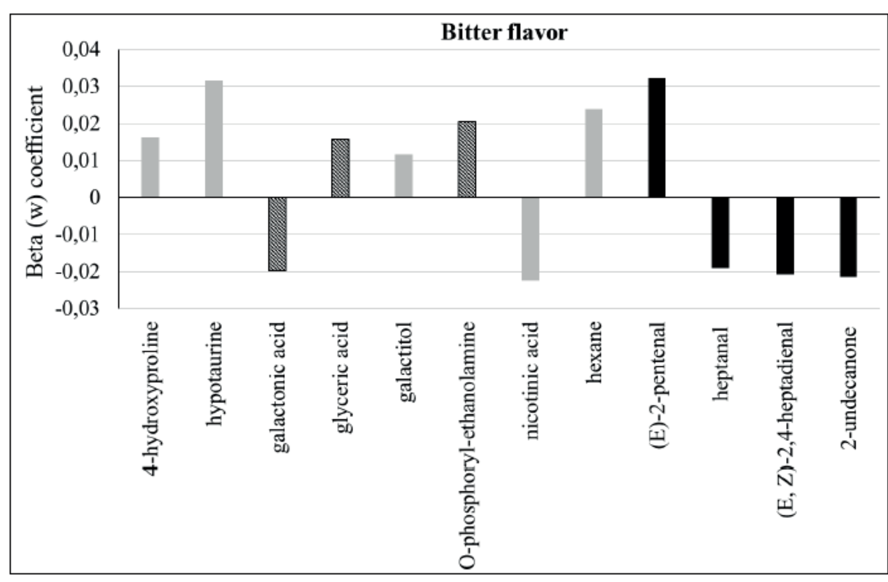


443

444 **Fig.1.** Estimated regression coefficients (β_W; dimension (1/(µg/kg)
445 for VOC and (1/(mg/kg)) for metabolites) obtained from Partial
446 least squares regression (PLS) analysis of chemical compounds (X)

447 and sensory attributes (Y). Beta (w) coefficients were used for
448 relation between sensory attributes and volatiles and between
449 sensory attributes and metabolites, respectively. Black bars in a
450 plot represent compounds with concentrations higher than flavor
451 threshold (odor, taste or both), grey bars are compounds with
452 concentrations lower than flavor threshold, and bars with pattern
453 are compounds with unknown flavor thresholds.

454

455 **Gamy flavor:** Gamy flavor was well explained (53-51%) and 15
456 compounds were included in the model (Fig. 1a). The explanation
457 was highest when components present in low quantities were
458 selected in all 3 models (×1, ×100, ×1000) by weighted variables
459 (1/SD).

460 In principle, there could be 6 independent compounds or cluster of
461 compounds that contributed to gamy flavor. This is because
462 maximum 6 uncorrelated principal components (PC) were
463 identified. Thus, it is possible that more components are associated
464 with gamy flavor since compounds metabolically can correlate
465 with other compounds. Krebs cycle components (*e. g.* citric acid,
466 succinic acid) can be related since these are involved in respiration.
467 In this way malic acid may indirectly contribute to flavor, albeit
468 not being selected, since succinic acid affected flavor. In addition,
469 it is also important to note that the compounds that apparently

470 suppress gamy flavor can also camouflage this sensory attribute
471 and therefore indirectly affect the flavor, i.e. glucose can
472 modify/reduce other flavors (Meinert et al., 2009).
473 Amino acids reflect several physiological situations in the animal
474 species: fatigue, stress, postprandial time etc., possibly reducing
475 flavor acceptability (Warner et al., 2007) and alter the experience
476 of a gamy flavor. Maruri and Larick (1992) reported a positive
477 correlation between diterpenoids and off-flavor of grass-fed beef
478 described by sensory panelists as gamy/stale. The actual gamy
479 flavor is so far not well explained in terms of chemical compounds.
480 Aspartic acid and cyclo-leucine showed positive, while glycine
481 negative correlation with gamy flavor. Aspartic acid and glycine
482 were identified as having concentrations above flavor thresholds,
483 where glycine with its slightly sweet note (Table S-2) may reduce
484 gamy flavor. Cyclo-leucine is a non proteinogenic amino acid that
485 may be a product of ruminal bacteria. Cyclo-leucine can be related
486 to the level of methionine that correlated positively to gamy flavor
487 but did not pass the selection criteria. In addition, cyclo-leucine
488 correlated significantly ($P < 0.001$, linear regression) to aspartic
489 acid, glycine, homocysteine, leucine, lysine, phenylalanine,
490 tryptophan and tyrosine. Therefore, cyclo-leucine together with
491 aspartic acid possibly presents a marker not only for gamy flavor
492 but also for the general catabolic/anabolic status in lamb/sheep that

493 may affect flavor. The concentration of 4-hydroxyproline was
494 below flavor threshold (Fig.1a) but with a positive correlation to
495 compounds like hypotaurine ($r = 0.60$, $P < 0.001$), gluconic acid (r
496 $= 0.68$, $P < 0.001$), ascorbic acid ($r=0.55$, $P<0.001$), urdine ($r =$
497 0.61, $P<0.001$) and hexanal ($r = 0.57$, $P<0.001$). In addition, 4-
498 hydroxyproline showed positive correlation to only one amino
499 acid, β-alanine ($r= 0.51$, $P<0.001$) but this amino acid is not
500 important in protein biosynthesis. This cluster of compounds is
501 discussed below.

502 Three organic acids (citric acid, pyruvic acid and succinic acid)
503 were correlated with gamy flavor, where citric and pyruvic acid
504 were positively correlated. Pyruvic acid was below flavor
505 threshold, but correlated to several compounds in the citric acid
506 cycle including a positive correlation to malic acid ($r = 0.30$, $P=$
507 0.004). Krebs cycle components are likely involved in the
508 development of gamy flavor as many wild animals have oxidative
509 muscles (Curry et al., 2012) that need to be furnished by the Krebs
510 cycle to produce ATP for extensive movements. In addition,
511 pyruvic acid correlated to sucrose ($r = 0.61$; related to pyruvate
512 through glycolysis, $P<0.001$) that was present in concentration
513 above flavor threshold. The sugar acids (gluconic, glyceric and
514 ribonic acid) also correlated to pyruvic acid suggesting that the
515 metabolic status of sugar polymer degradation may be involved in

516 defining gamy flavor. The fact that pyruvic acid is a key compound
517 in several metabolic pathways may explain why it is directly
518 related to gamy flavor.
519 Glucose and mannose-6-phosphate can be regarded as one variable
520 due to their correlation (r = 0.57, P<0.001). Glucose was present in
521 a far higher concentration than the other metabolites and above
522 flavor threshold, possibly it reduced the intensity of gamy flavor.
523 Mannose-6-phosphate strongly correlated to two metabolites from
524 Embden-Meyerhof-Parnas pathway, fructose-6-phosphate and
525 glucose-6-phosphate (r= 0.80 and r= 0.90, respectively, P<0.001),
526 but also to many other components like fructose. Strangely enough
527 sucrose tended to enhance gamy flavor (r = 0.39, P<0.001). Myo-
528 inositol is a carbohydrate/sugar alcohol with half the sweetness of
529 sucrose and it was negatively correlated with gamy flavor.
530 The volatiles 2-methylheptane, 3-methylheptane, octane and (*E*)-2-
531 octene can actually be looked upon as one variable due to the high
532 correlation between the 4 selected compounds (r = 0.89 – 0.94, P <
533 0.001, Fig.1a). Only (*E*)-2-octene may be above threshold since the
534 other volatiles were below. However, (*E*)-2-octene is classified as
535 dangerous (European Chemicals Agency - ECHA) organic
536 compound and its threshold was therefore not determined. In
537 addition, this volatile compound may not be a universal marker for
538 gamy flavor since the compound was not identified in all animals,

539 it was most typical for sheep from Montenegro. (*E*)-2-octene has
540 previously been detected in beef fat obtained from the renal
541 periphery of beef carcasses (Umano and Shibamoto, 1987) and
542 grilled lamb meat (Madruga et al., 2013). The selected volatiles
543 had a high correlation to citric and pyruvic acid ($r = 0.67$-0.78, $P <$
544 0.001) in addition to several other lipid degradation products like
545 octane ($r = 0.88$, $P < 0.001$).
546 Metabolites that correlated with gamy flavor were identified in all
547 animals at various levels (Table S-2) and can therefore be universal
548 markers of gamy flavor, while important volatiles were commonly
549 only found in a fraction of the samples.
550
551 **Grassy flavor:** As for gamy flavor, maximum 6 compounds or
552 clusters of correlated compounds were suggested for grassy flavor
553 by the PLS analysis. Nineteen compounds, volatiles and
554 metabolites (Fig.1b), explained grassy flavor up to 50% depending
555 on weighting. Grassy flavor significantly correlated ($r = 0.62$,
556 $P < 0.001$) with previously described gamy flavor. Therefore it is
557 expected that some compounds that contributed to gamy flavor
558 will also be relevant for grassy flavor. It should be noted here that
559 no amino acid was positively correlated to grassy flavor or above
560 its threshold. However, 4-hydroxyproline was included as relevant
561 based on its correlation to other compounds.

562 Only two acids, gluconic and pyruvic acid, were positively related
563 to grass flavor. Gluconic acid may originate from microbiological
564 oxidation of glucose in rumen of animal or it can also be a plant
565 metabolite not metabolized in the rumen. It has complex taste
566 (acid, bitter, metallic). Gluconic acid was negatively correlated to
567 pyruvic acid, 3-methylheptane and (*E*)-2-octene, with the two latter
568 compounds positively correlated to grass flavor. The acid was
569 found in all animals and can possibly be a universal marker of
570 grassy flavor if microflora of rumen or plant biodiversity is not
571 causal and region dependent.
572 Volatiles, formic acid, β-carophyllene, 3-methylphenol, 2-
573 ethylfuran, were highly correlated and positively correlated to
574 grass flavor. 3-methylphenol was above threshold in this group and
575 actually had a flavor that makes it likely to influence grassy flavor
576 (see Table S-1), but not as a universal marker. In addition, four
577 volatiles were identified as negatively affecting grass flavor.
578 Propanal ($r = -0.39$, $P<0.001$) and 2-propenal ($r = -0.33$, $P = 0.002$)
579 showed weak correlation with grassy flavor, although both
580 compounds were above threshold and described as pungent. (*E*)-2-
581 nonenal and 2-heptadecanone correlated more weakly ($r = -0.33$, P
582 $= 0.024$) with grassy flavor. (*E*)-2-nonenal was above threshold
583 and has a flavor that would normally be associated with lipid
584 oxidation (Kosowska et al., 2017).

585 Group of antioxidants composed of ascorbic acid, 4-
586 hydroxyproline, galactitol and uridine where negatively correlated
587 with grassy flavor. Ascorbic acid may function as an antioxidant
588 (Howes et al., 2015) with possible repressing effect on grassy
589 flavor despite the fact that it showed weak correlation ($r = -0.47$,
590 $P<0.001$) with this sensory attribute. Antioxidant compounds were
591 below thresholds and therefore have no direct influence on flavor.
592 Although the trained panelists could not identify rancidity as a
593 discriminator this cannot exclude lipid oxidation as undesirable
594 process that ultimately leads to development of off-flavors.
595 Antioxidant capacity of muscle is associated with vitamin E as a
596 fat soluble vitamin (Howes et al., 2015). Hopkins et al. (2013)
597 found that vitamin E can prevent lipid oxidation, when PUFA was
598 present at high levels, i.e. in at a concentration above 2.95 mg/kg
599 of muscle. The old sheep studied here had high content of vitamin
600 E (2.5 mg/kg; Bjelanović et al., 2015), i.e. close to reported
601 threshold. This can possibly explain the relatively low number of
602 lipid-oxidation products that correlated with grassy flavor and the
603 absence of rancidity.
604 Glucose was above threshold and its sweetness suppressed grassy
605 flavor. This sugar had strong negative correlation to grass flavor (r
606 $= -0.50$, $P<0.001$). In addition, glucose is metabolically related to
607 many compounds; most strongly to fructose and galactose (r =

608 0.83-0.84, P<0.001) and nominally less to mannose-6-phosphate (r
609 = 0.50, P<0.001). Sugars seems to play a significant role in
610 suppression of off-flavors in lamb/sheep and this is most obvious
611 for gamy and grassy flavor. Myo-inositol possibly has the same
612 role as suggested for the gamy flavor.
613
614 **Bitter flavor:** Bitter flavor was the 3rd best explained flavor
615 attribute (27%) that did not show high correlation to gamy or
616 grassy flavor. Maximum 6 clusters were suggested by PLS
617 analysis. If variables with no correlation to bitter flavor were
618 removed, *i.e.* the data set of volatiles was reduced it was possible
619 to explain 40% of the variation in bitter flavor. Fig. 1c shows
620 correlation of hypotaurine and (*E*)-2-pentanal (r = 0.52 and r=0.51,
621 respectively, P<0.001) with bitter flavor. Although, the threshold
622 concentration for hypotaurine was by the criteria used, higher than
623 identified concentration in lamb and sheep, participants recognized
624 bitter flavor at the lowest tested concentration (6.25 mg/kg). It can
625 be speculated that hypotaurine may take longer to leave taste cells
626 what caused carry-over effect and late response of participants
627 when distilled water was tested. Thus, since the lowest tested
628 concentration was close to highest identified concentration in some
629 animals (4.7 mg/kg) it is plausible that hypotaurine contributed to
630 bitterness in sheep.

631 The metabolites 4-hydroxyproline, hypotaurine, O-phosphoryl-
632 ethanolamine, were positively correlated with bitter flavor.
633 Galactonic acid was close to being in the same group due to its
634 significant correlation coefficient (r= -0.46, $P<0.001$) with
635 hypotaurine. Additionally, galactonic acid correlated with several
636 sugar phosphates (e.g. glucose-6-phosphate, r=0.53, $P<0.001$),
637 known as sweet compounds, and that may explain its negative
638 contribution to bitter flavor.
639 Glyceric acid and galactitol showed weak correlation (r= 0.31,
640 $P=0.003$) and had a small positive influence on bitter flavor.
641 Glyceric acid correlated with gluconic acid (r = 0.60, $P<0.001$) and
642 pyruvic acid (r= 0.54). Galactitol was below threshold and
643 correlated with glucuronic acid (r =0.73) together with cysteine (r=
644 0.54), arabitol (r= 0.53) and inosine (r= 0.55), all with $P<0.001$.
645 Cysteine was above threshold in all samples and showed weak
646 positive correlation to bitterness, without being picked out directly
647 as important for describing bitterness. Strong negative correlation
648 (r= -0.28, $P=0.01$) of nicotinic acid with bitter flavor was
649 surprising since it has a bitter flavor. Possible explanation for this
650 phenomenon is the low concentration of this metabolite that was
651 far below flavor threshold. The positive correlation of nicotinic
652 acid to glycine (r = 0.49, $P<0.001$) may actually be the source of it

653 reducing bitterness, bearing in mind that glycine was not directly
654 selected as involved in the flavor.
655 Other significant correlation was observed between two volatiles,
656 hexane and (*E*)-2-pentanal, and bitter flavor. The contribution of
657 hexane to bitter flavor was small since it was below threshold and
658 relatively weakly related to hypotaurine (r= 0.39). Despite strong
659 correlation of (*E*)-2-pentanal with bitter flavor, and its presence in
660 concentration above threshold, this volatile does not have a flavor
661 profile that appear associated with bitter flavor. In addition,
662 heptanal, (*E,Z*)-2,4-heptadienal and 2-undecanone with fatty
663 perception possibly modify the perception of a bitter attribute.
664
665 **Metallic flavor:** Metallic flavor was the 4th best explained flavor
666 attribute (19% explained, validated). There were max 3
667 independent factors. The issue with metallic flavor was that it
668 correlated significantly to bitterness (R-square = 0.63, $P<0.001$,
669 not validated). This was apparent from the compound listed below.
670 There were no really new compounds to explain metallic flavor but
671 arabitol (was selected) that was indirectly involved in bitter flavor.
672 By selecting a subset of compounds it was possible to explain 30%
673 of the variation in metallic flavor; this means that it was not a well
674 explained attribute. Among volatiles hexane may have a small

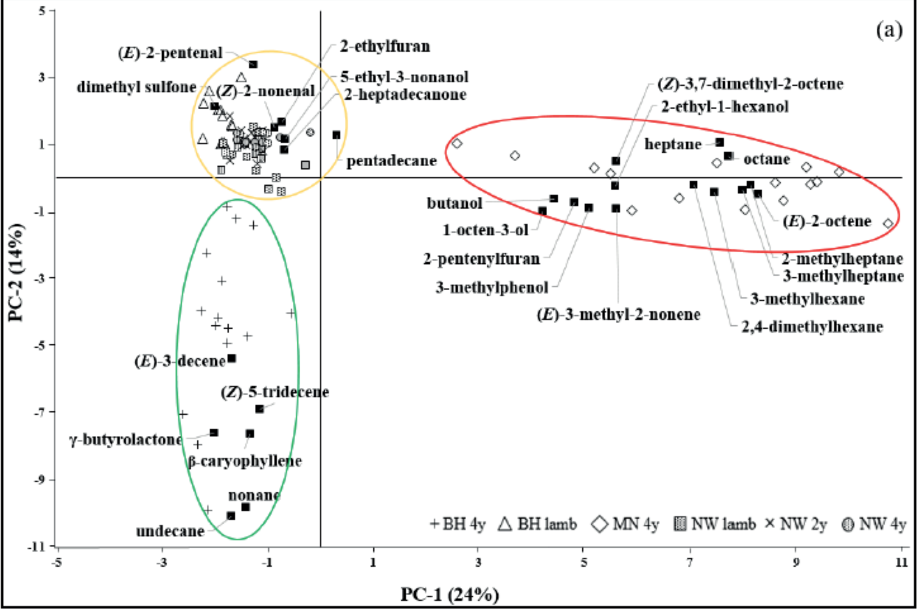
675 positive (r= 0.33, *P*=0.002) influence on metallic flavor. Most lipid
676 volatiles had negative effects on metallic flavor.
677 Acidic flavor was not explained when model validation was used.
678 The nominally lowest P value was to galactonic acid (r = 0.1,
679 *P*=0.37).
680
681 **Relationship between identified flavor compounds and meat**
682 **origin**
683 Principal component analysis (PCA) was used to differentiate the
684 animal population based on identified volatile compounds isolated
685 from adipose tissues and metabolites from lean meat of lamb and
686 sheep. Tracing the origin of products is important for
687 authentication of meat from different production systems.
688 PCA, the first two components, carried out on the volatile
689 compounds isolated from lamb, young and old sheep from BH,
690 MN, and NW, are shown in Fig. 2a. A clear differentiation
691 between volatile profiles of 4y old sheep that belonged to two
692 phenotypes of the Pramenka breed was observed. MN 4y sheep
693 volatile profile was determined by 14 compounds, including gamy
694 and grass-related (2-methylheptane, 3-methylheptane, (*E*)-2-octene
695 and 3-methyphenol) flavor compounds. Butanol and 3-
696 methylphenol were only identified in MN animals presenting
697 potential biomarkers of this production system. Furthermore, β-

698 caryophyllene, almost exclusively synthesized in plant tissue, was
699 identified only in BH 4y sheep. The role of sesquiterpenes in lamb
700 and sheep flavor profiles needs further investigation regarding
701 seasonal changes.

702 Four animal groups (BH lamb, NW lamb, NW 2y sheep, NW 4y
703 sheep) that showed poor separation in first PCA, was used to
704 develop a second PCA model. Fig. 2b shows that the volatile
705 profile of BH lamb did not clustered together with NW animals.
706 Two volatiles were associated with BH lamb profile, among them
707 (*E*)-2-pentenal which has been proposed as a potential, indirect
708 biomarker of bitter flavor in our data.

709 In order to obtain more information about relationship between
710 metabolites and animal population, a third PCA analysis were
711 performed. The PCA plot in Fig. 2c showed a clear differentiation
712 for all animal groups, and the animals from the same production
713 system clustered together. Although NW animals clustered, no
714 characteristic metabolite was identified. Gluconic and pyruvic acid
715 related to MN animals' meat. BH 4y meat pattern was defined by
716 high concentration of essential amino acids that clustered with
717 bitter flavor. Furthermore, antioxidant compounds that suppressed
718 off-flavor properties may contribute to the unique BH lamb
719 metabolite pattern.

720 Our results indicate that PCA plots offer an interesting approach
721 regarding discrimination of animals from different production
722 system using flavor markers. However, some of the markers might
723 change depending of pasture season.



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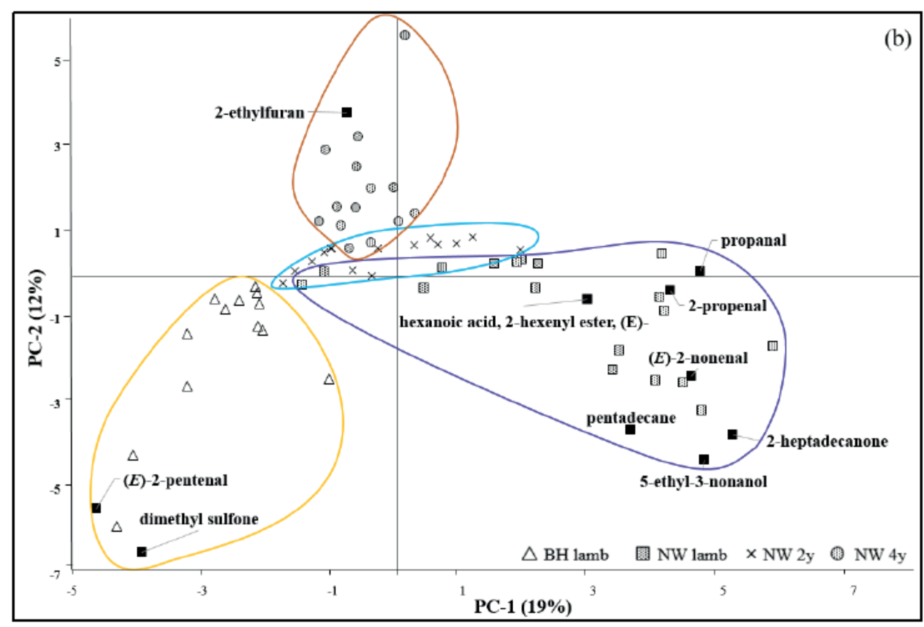

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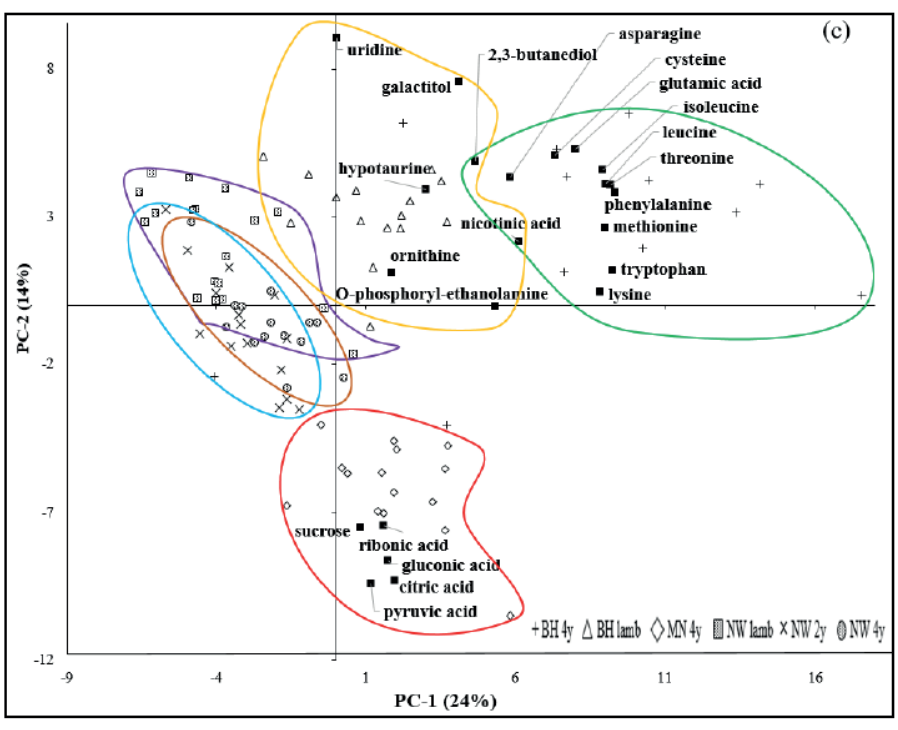

727 **Fig.2.** Differentiation of animal groups ($P < 0.001$) based on: (a)
728 volatile compounds isolated from heated adipose tissue of all
729 animal groups (BH lamb, BH 4y, MN 4y, NW lamb, NW 2y, and
730 NW 4y); (b) volatile compounds isolated from heated adipose
731 tissue of four animal groups (BH lamb, NW lamb, NW 2y and NW
732 4y) that showed poor separation when all animal groups were
733 included in PCA; (c) metabolite compounds isolated from lean
734 meat of all animal groups.
735
736
737
738 **Notes**
739 Per Berg is employed in the Research Unit of the cooperative meat
740 production in Norway. He has provided the Norwegian animals,
741 but not influenced the data treatment and conclusions. All authors
742 declare no competing financial interest.
743
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920 **Supplementary material**

921 Table S-1. The volatile compounds identified in aliquot of 1g lamb/sheep fat by HS-GC/MS with corresponding literature values for

922 odor/flavor threshold and odor/taste attributes

RI	RT	Volatiles	Concentration (mg kg^{-1})						Odor thresholds (air) by D = dilution (mg/m^3)	Flavor threshold in water (mg L^{-1}) / fat-oil (mg kg^{-1})	Odor / Taste
			BH lamb	BH 4y	MN 4y	NW lamb	NW 2y	NW 4y			
Alkanes											
2.09	600	hexane	0.173	n.d.[a]	n.d.	0.024	0.152	0.020	5.3[1]	0.5[2] / > 500[3]	gasoline[4], petroleum-like[5]
2.27	660	3-methylhexane	n.d.	n.d.	0.039	n.d.	n.d.	n.d.	3.4[1]	n.a./ n.a.	n.a.
2.39	700	heptane	0.011	n.d.	0.097	0.017	0.019	0.007	2.7[1]	n.a./ 47[6]	gasoline-like odor[5]
2.52	715	2,4-dimethylhexane	n.d.	n.d.	0.090	n.d.	n.d.	n.d.	n.a.	n.a/ n.a.	n.a.
2.83	751	2-methylheptane	n.d.	n.d.	0.193	n.d.	n.d.	0.005	0.52[1]	n.a. / n.a.	strong, petrol, petroleum[7]
2.93	763	3-methylheptane	n.d.	n.d.	0.375	n.d.	n.d.	n.d.	7.1[1]	n.a./n.a.	strong, petrol, petroleum[7]
2.98	769	methylcyclohexane	n.d.	n.d.	n.d.	0.019	n.d.	n.d.	0.60[1]	n.a./ n.a.	petroleum[4]
3.25	800	octane	0.004	0.006	0.235	0.047	0.023	0.054	8.0[1]	n.a./ 0.94[8]	gasoline, oil[9]
5.25	900	nonane	n.d.	0.005	n.d.	n.d.	n.d.	n.d.	12[1]	n.a./n.a.	gasoline, oil[9]
8.44	954	2-methylnonane	n.d.	n.d.	n.d.	n.d.	n.d.	0.036	n.a.	n.a./n.a.	faint odor[9]
17.34	1100	undecane	0.035	0.134	0.030	n.d.	0.021	n.d.	5.6[1]	n.a./n.a.	faint odor[9]
25.83	1204	2-propyl-1,1,3-trimethyl-cyclohexane	n.d.	n.d.	0.004	n.d.	n.d.	n.d.	n.a.	n.a./ n.a.	n.a.
32.62	1400	tetradecane	n.d.	n.d.	n.d.	0.001	n.d.	n.d.	5[10]	n.a. / 300 – 400[11]	n.a.
34.96	1500	pentadecane	n.d.	n.d.	0.001	0.004	n.d.	n.d.	n.a.	n.a. / > 13.000[12]	odorless[13]
37.06	1600	hexadecane	n.d.	n.d.	n.d.	n.d.	n.d.	0.001	0.5[10]	n.a./ 300 – 400[11]	odorless[13]
Alkenes											
2.20	637	(E)-2-hexene	n.d.	n.d.	n.d.	n.d.	n.d.	0.004	n.a.	n.a./ n.a.	n.a.
4.60	868	(*E*)-2-octene	0.034	n.d.	1.870	n.d.	n.d.	n.d.	n.a.	n.a./ n.a.	gassy[14]
11.23	1000	(*E*)-3-decene	n.d.	0.035	n.d.	n.d.	n.d.	n.d.	n.a.	n.a./ n.a.	n.a.
12.73	1025	(*Z*)-5-tridecene	n.d.	0.008	n.d.	n.d.	n.d.	n.d.	n.a.	n.a./ n.a.	n.a.
14.87	1059	(*E*)-3-methyl-2-nonene	n.d.	n.d.	0.003	n.d.	n.d.	n.d.	n.a.	n.a./ n.a.	n.a.
14.77	1060	(*Z*)-3,7-dimethyl-2-octene	n.d.	n.d.	0.006	n.d.	n.d.	n.d.	n.a.	n.a./ n.a.	n.a.

26.76	1220	(*E*)-3-dodecene	n.d.	n.d.	0.007	n.d.	n.d.	n.d.	n.a.	n.a./ n.a.	n.a.
40.63	1736	[R-[R*,R*-(*E*)]]-3,7,11,15-tetramethyl-2-hexadecene	n.d.	0.011	n.d.	0.003	n.d.	n.d.	n.a.	n.a./ n.a.	n.a.
Alcohols											
7.36	936	ethyl alcohol	n.d.	14.864	15.319	15.266	n.d.	n.d.	0.17[15]	10[16] / 900[6]	vinous, alcohol[4]
16.16	1082	5-ethyl-3-nonanol	n.d.	n.d.	n.d.	0.080	n.d.	n.d.	n.a.	n.a./ n.a.	n.a.
22.57	1162	butanol	n.d.	n.d.	0.045	n.d.	n.d.	n.d.	0.015 - 0.214[17]	0.5[18]/ 20-200[19]	harsh fusel odor with banana[20], similar to amyl alcohol[21], rancid, sweet[22], strong characteristic, mildly alcoholic odor[23]/ banana, fusel taste[20], dry, burning taste[21]
23.59	1172	1-penten-3-ol	n.d.	0.545	0.228	0.453	2.478	0.336	0.01-0.1[24]	3[25]/ 1-10[25]	powerful, mild grassy-green odor[21]/ green vegetable and fruity[21]
25.83	1204	2-heptyn-1-ol	n.d.	n.d.	n.d.	n.d.	n.d.	0.063	n.a.	n.a./ n.a.	n.a.
28.36	1256	1-pentanol	n.d.	0.325	0.280	0.655	0.471	0.604	0.02[26]	4.5[27]/ 0.47[8]	fusel-like sweet and pleasant odor and burning taste[21] / intense fusel, fermented, bready and cereal with a fruity undertone[21]
31.74	1362	hexanol	n.d.	0.142	n.d.	0.159	n.d.	0.080	0.01[28]	0.2[29] / 0.4[30]	sweet alcohol, pleasant[22] / fatty, fruity[20]
34.25	1470	1-octen-3-ol	n.d.	0.165	0.189	0.108	n.d.	0.064	0.001-0.1[24]	0.001[31]/ 2.3[32]	powerful, sweet, earthy odor with a strong, herbaceous note reminiscent of lavender–lavandin, rose and hay[21]/ mushroom,

										earthy, fungal, green, oily, vegetative, umami sensation and savory-brothy[21]
35.01	1502	2-ethyl-1-hexanol	n.d.	n.d.	0.059	n.d.	n.d.	n.d.	0.40[33]	n.a./ n.a.	mild, oily, sweet, slightly floral odor reminiscent of rose[21], intense and unpleasant[34] / sweet, fatty-floral taste with fruity note[21], intense and unpleasant[34]
36.57	1577	1-octanol	n.d.	n.d.	0.033	0.031	n.d.	0.024	0.005[35]	0.054[36] / n.a.	fresh, orange-rose odor, quite sweet[21] / oily, sweet, slightly herbaceous taste, waxy, green, citrus, orange and aldehydic with a fruity nuance[21]
42.69	1867	benzyl alcohol	n.d.	n.d.	0.023	n.d.	n.d.	n.d.	n.a.	5.5[37] / n.a.	characteristic pleasant, fruity odor[21] / slightly pungent, sweet taste[21]
Aldehydes										
2.58	722	acetaldehyde	0.566	1.608	n.d.	1.149	0.342	0.858	0.0027[1]	0.01[38]/ 0.0071[39]	fresh[4], characteristic pungent, penetrating, ethereal[21]
3.37	806	propanal	n.d.	0.178	n.d.	0.265	0.144	n.d.	0.0024[1]	0.007[40]/ 0.05[41]	characteristic sharp, pungent odor similar to acetaldehyde[21]
3.41	808	butanedial	n.d.	0.143	n.d.	0.161	0.002	0.109	n.a.	n.a/ n.a.	n.a.
4.20	848	2-methylpropanal	n.d.	n.d.	n.d.	n.d.	0.129	n.d.	0.001[1]	0.0004-0.0006[42]/ 0.0034[43]	extremely sharp[20], pungent[44] / fruity[20]
4.38	857	2-propenal	n.d.	0.193	n.d.	0.339	n.d.	n.d.	0.0083[1]	n.a./ n.a.	extremely sharp,

										extremely acrid, pungent, burnt sweet, hot fat[45], disagreeable choking odor[46]
5.01	888	butanal	n.d.	0.098	n.d.	n.d.	n.d.	n.d.	0.00084[47]	0.00526[36]/ 0.005[41]	pungent[21] / musty, fusel, fermented, bready and yeasty with a malty nuance[21]
6.33	919	3-methylbutanal	0.512	0.199	n.d.	0.194	0.394	n.d.	0.00035[1]	0.00025[40]/ 0.0108[48]	choking, powerful, acrid, pungent, apple-like odor; also reported to have a fruity, fatty, animal, almond odor[21]
6.88	928	pentanal	0.118	0.089	n.d.	0.147	0.108	n.d.	0.0014[1]	0.008[16]/ 0.05[41]	powerful, acrid, pungent odor[21]/ warm, slightly fruity and nut-like[21]
14.20	1049	2-butenal	n.d.	n.d.	0.011	n.d.	n.d.	n.d.	0.067[1]	0.5[16]/ 5[42]	pungent[4]
17.37	1101	hexanal	1.090	n.d.	0.345	1.264	0.832	0.633	0.00082[49]	0.0036[36]/ 0.02[41]	fatty, green, grassy, powerful, penetrating characteristic fruity odor[21]/ green, woody, vegetative, apple, grassy, citrus and orange with a fresh, lingering aftertaste[21]
21.23	1148	(*E*)-2-pentenal	0.967	n.d.	0.039	n.d.	n.d.	n.d.	1.4[50]	0.1-0.15[51]/ 0.6[18]	pungent green fruity apple orange tomato[52], grass, tomato[53] / green, waxy and fruity[52]
24.89	1186	heptanal	n.d.	0.868	0.876	1.337	1.310	0.731	0.00085[1]	0.005[54]/ 0.02[41]	strong, fatty, harsh, pungent odor[21] /

										unpleasant, fatty taste[21]
26.77	1209	(*E*)-2-hexenal	n.d.	0.102	0.042	0.070	n.d.	0.073	0.0031[55]	0.0493[36]/ 0.257[39]	sharp, penetrating fresh leafy green, clean, fruity with herbal and spicy herbal nuances[56] /Fresh green, leafy, fruity with rich vegetative nuances[56]
27.90	1243	(*Z*)-4-heptenal	n.d.	0.048	n.d.	0.010	n.d.	n.d.	0.0034[50]	0.00006[57]/ 0.0005[58]	oily fatty green dairy milky creamy[59], biscuit, cream[53] / sharp green milky creamy tea[59]
29.61	1288	octanal	0.083	n.d.	0.034	0.142	0.215	0.201	0.000052[1]	0.0001[60]/0.040[58]	fatty, citrus, honey odor on dilution[21] / aldehyde, green with a paly, citrus, orange note[21]
32.79	1407	nonanal	0.269	0.164	0.142	0.477	0.518	0.329	0.0003[60]	0.0035[61]/ 0.20[58]	strong, fatty odor developing an orange and rose note on dilution[21] / aldehydic citrus orange body with waxy and oily melon-like nuances[21]
33.82	1451	(*E*)-2-octenal	n.d.	0.053	0.021	0.068	0.061	0.035	0.0027[61]	0.0002-0.0003[62]/ 0.061[63]	green, nut, fat[53], fresh cucumber fatty green herbal banana waxy green leaf[59] / sweet green citrus peel fatty[59]
34.76	1491	(*E*,*Z*)-2,4-heptadienal	n.d.	0.206	0.140	0.213	0.171	0.232	n.a.	0.0035[64]/ 0.05[18]	fried, tallowy[65]
36.24	1561	(*E*)-2-nonenal	n.d.	0.026	n.d.	0.038	n.d.	n.d.	0.00009[66]	0.000065[67]/ 0.045[68]	fatty, green cucumber aldehydic citrus, green, soapy,

										cucumber, melon-like with an aldehydic / fatty nuance[52]
Ketones										
4.16	838	acetone	5.761	2.429	n.d.	2.563	2.575	0.251	0.94[69]	50-100[42]/ 50[41]	ethereal[4], fruity odor[70]/ pungent, sweetish[70]
29.61	1288	3-hydroxy-2-butanone	n.d.	24.042	n.d.	n.d.	n.d.	7.358	0.014[71]	0.75[72]/ n.a.	bland, woody, yogurt odor[21]/fatty creamy "tub" butter taste[21]
30.88	1329	2,3-octanedione	7.702	46.664	44.601	49.606	77.788	27.982	0.00000009[73]	0.11[74]/ 2.0[73]	warmed-over" flavor[21]
37.44	1608	2-undecanone	n.d.	0.359	0.447	0.622	0.374	n.d.	0.03-0.05[75]	0.03[72]/ 2.4[48]	characteristic rue odor with a sweet flavor reminiscent of peach[21] /waxy and fruity with creamy cheese notes[21]
38.83	1655	acetophenone	n.d.	0.248	0.144	n.d.	n.d.	n.d.	0.0012[76]	3.0[77]/ n.a.	sweet, pungent and strong medicinal odor[21] / bitter, aromatic cherry branch taste, pungent[21]
44.78	2010	2-pentadecanone	n.d.	1.473	1.441	1.208	n.d.	n.d.	n.a.	n.a./ n.a.	fresh, jasmin, celery[59]/ fatty, spicy, floral[59]
48.01	2245	2-heptadecanone	n.d.	n.d.	n.d.	0.286	0.393	n.d.	n.a.	37.5*/ n.a.	acidic* / bitter, metallic, acidic*
Acids											
35.72	1536	formic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.218	0.98[78]	4.6[79]/ 500[41]	pungent, penetrating odor[21]/ sour[20]
36.84	1590	2-methylpropanoic acid	n.d.	0.082	0.054	0.049	n.d.	n.d.	0.005[41]	1[80]/ n.a.	strong penetrating odor of rancid butter[21]/acidic, sour dairy, creamy, cheese,

											cultured dairy nuance[21]
38.01	1619	butanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.089	0.00035-0.00086[81]	1[80]/ 0.5[41]	persistent, penetrating, rancid, butter-like odor[21] / burning, acid taste[21]
45.38	2049	octanoic acid	n.d.	0.028	n.d.	0.096	n.d.	0.071	0.000065[78]	0.5[16]/ 100[41]	mildly unpleasant odor[21]/ creamy, waxy, dirty, sweaty, dairy cheese like[21]
48.50	2361	decanoic acid	0.219	0.290	0.306	0.350	n.d.	0.066	0.05[82]	0.5[80]/ 200[83]	fatty, unpleasant, rancid odor[21] / soapy waxy fruity[59]
Lactone											
38.49	1642	γ-butyrolactone	n.d.	0.977	n.d.	0.381	0.065	0.250	n.a.	n.a./ n.a.	faint, sweet, aromatic, slightly buttery odor[21] / milky, creamy with fruity peach-like afternotes[21]
Terpene											
37.32	1604	β-caryophyllene	n.d.	0.214	n.d.	n.d.	n.d.	n.d.	64[84]	n.a./ 60-90[85]	woody-spicy, dry, clove-like aroma[21] / spicy, pepper-like, woody, camphoraceous with a citrus background[21]
Sulphur compound											
43.33	1901	dimethyl sulfone	0.377	0.217	0.145	0.131	n.d.	0.094	n.a.	37.5*/ n.a.	sulfurous, burnt[87] / sweet, acidic, metallic*
Phenol											
45.91	2106	3-methylphenol	n.d.	n.d.	0.245	n.d.	n.d.	n.d.	0.00022-0.035[87]	0.002[88]/ 0.01[89]	dry, tarry, medicinal–leathery odor[21] /phenolic, smoky, balsamic, medicinal and spicy eugenol-

											like[21]
Esters											
38.32	1636	hexanoic acid, 2-hexenyl ester, (E)-	n.d.	0.018	n.d.	0.028	n.d.	n.d.	n.a.	n.a./ n.a.	green[90]
Others											
7.89	945	2-ethylfuran	n.d.	n.d.	n.d.	n.d.	n.d.	0.216	n.a.	n.a./ 8[91]	smoky burnt, sweet[21] / sweet, chemical with clean, fresh, fruity lift[21]
13.51	1040	toluene	n.d.	n.d.	0.065	n.d.	n.d.	n.d.	0.08-0.19[87]	0.14[92]/ 100 – 200[93]	sour, burnt[4], sweet, pungent, benzene-like odor[5]
28.76	1264	1-methyl-4-(1-methylethyl)-benzene	0.334	n.d.	0.105	n.d.	n.d.	n.d.	n.a.	n.a./ n.a.	citrusy aroma reminiscent of lemon/ rancid with slightly woody, oxidized citrus notes, green pepper and oregano[21]
30.14	1304	2-pentylfuran	n.d.	n.d.	0.123	n.d.	n.d.	n.d.	n.a.	n.a./ 8[91]	fruity, green bean, metallic, vegetable odor[21] /green, waxy with a cooked caramel nuance[21]
37.93	1623	benzonitrile	0.324	0.429	0.453	0.518	0.004	0.098	n.a.	n.a./ n.a.	almond[70] / sharp taste[94]

923 [a] n.d. = Not detected in the sample
924 *Measured in our study
925 [1]Nagata, Y. (2003); [2]Palamand et al. (1969); [3]Kaneda et al. (1974); [4]Murnane et al. (2013); [5]NIOSH (2010); [6]Halek et al. (1994);
926 [7]Čížková et al. (2009); [8]Morales et al. (2000); [9]ECHA (2015); [10]Patte et al. (1979); [11]Motohiro et al. (1976); [12]Champagne et al. (1969);
927 [13]Hajaratul et al. (2012); [14]Umano et al. (1987); [15]Cain et al. (2005); [16]Rothe et al. (1972); [17]Hermans et al. (1989); [18]Belitz et al.
928 (1992); [19]Granzer et al. (1986); [20]Furia (1980); [21]Burdock (2005); [22]Verschueren (2001); [23]NIOSH (1997); [24]Schieberle (2001); [25]Stark
929 et al. (1967); [26]Ziemer et al. (2000); [27]Siek et al. (1971); [28]Dravnieks et al. (1972); [29]Guth (1996); [30]Aparicio et al. (1998); [31]Stark et al.
930 (1964); [32]van den Ouweland et al. (1978); [33]Hellman (1973); [34]Vitali et al. (1993); [35]Rocén (1920); [36]Ahmed et al. (1978); [37]Keith et al.
931 (1968); [38]Milo et al. (1997); [39]Reiners et al. (1998); [40]Milo et al. (1996); [41]Hvolby (1961); [42]Rothe (1963); [43]Wagner et al. (1998);
932 [44]Lewis (1997); [45]Verschueren (1996); [46]Larranaga et al. (2016); [47]Japan Environment Agency (1980); [48]Kubickova et al. (1998);

[49]Ueno et al. (2009); [50]von Ranson et al. (1992);[51]Moshonas (1973); [52]Mosciano et al. (1991); [53]Zhu et al. (2015); [54]Täufel et al. (1960); [55]Young et al. (2008); [56]Moscino (2000); [57]Grosch et al. (1994); [58]Badings (1970); [59]http://www.thegoodscentscompany.com/data/rw1027821.html; [60]Andersson et al. (2005); [61]Buettner et al. (2001); [62]Meilgaard (1972); [63]Widder et al. (1997); [64]McGill et al. (1977); [65]Belitz et al. (2009); [66]Yang et al. (2008); [67]Chatonnet et al. (1998); [68]Widder et al. (1994); [69]Makeicheva et al. (1978); [70]O'Neil (2013); [71]Boonbumrung et al. (2001); [72]Rothe et al. (1967); [73]Pampizzi (1999); [74]Sigrists (2000); [75]Backman (1917); [76]Randebrock (1986); [77]Meilgaard (1975); [78]Cometto-Muñiz et al. (2010); [79]Becker et al. (1907); [80]Guth (1996); [81]Wise et al. (2007); [82]Passy (1892); [83]Feron et al. (1961); [84]Pino et al. (2006); [85]Burdock (2001); [86]Arn et al. (1998); [87]Nader (1958); [88]Dietz et al. (1978); [89]Urbach et al. (1970); [90]FEMA (https://www.femaflavor.org/flavor/library/e-2-hexenyl-hexanoate); [91]Evans et al. (1971); [92]Alexander et al. (1982); [93]Motohiro et al. (1976); [94]Lewis (2007)

Table S-2. List of metabolites detected in lamb/sheep *M. longissimus thoracis et lumborum* and identified by $GC/MS_{extraction}$ with corresponding literature values for odor/flavor threshold and odor/taste attributes

RI	Metabolites	Concentration (mg kg^{-1})						Odor thresholds (air) by D = dilution (mg/m^3)	Flavor threshold in water (mg L^{-1}) / fat-oil (mg kg^{-1})	Odor / Taste
		BH lamb	BH 4y	MN 4y	NW lamb	NW 2y	NW 4y			
	Amino acids									
1090.70	alanine	174.5	196.9	146.7	150.0	108.0	122.1	n.a.	600[1] / 135[2]	sweet[2]
1162.23	2-aminobutyric acid	0.9	1.2	0.6	1.0	0.7	0.7	n.a.	1340[3] / n.a.	sweet[3]
1526.53	4-aminobutyric acid	8.4	10.5	5.1	5.0	3.2	3.6	n.a.	2.1[4] / n.a.	savory, meat like flavor[5]
1814.43	arginine	0.6	0.3	0.5	0.7	0.4	0.4	n.a.	100[6] / n.a.	bitter[7]
1666.44	asparagine	1.6	2.7	1.2	1.4	0.9	1.1	n.a.	214- 1232[8]/ n.a.	umami[9]
1509.77	aspartic acid	6.0	11.0	15.4	2.6	2.5	5.3	n.a.	14.8[10]/ n.a.	slight acid taste[5], sour[11]
1424.73	β-alanine	29.5	13.9	11.3	31.3	11.4	12.4	n.a.	107[12]/ n.a.	slight sweet taste[5]
1140.39	butyro-1,4-lactam	0.2	0.9	0.6	0.2	0.2	0.3	n.a.	n.a./ n.a.	n.a.
1366.92	cyclo-leucine	5.3	6.6	6.3	3.7	3.9	5.0	n.a.	388 – 775[13]/ n.a.	sweet[13]
1549.83	cysteine	4.8	7.4	2.1	2.3	1.9	2.6	0.0045[14]	7.6-47[8] / n.a.	sulfureous aroma[5] /salty[15]
1615.40	glutamic acid	86.5	228.7	28.1	56.0	40.8	53.1	n.a.	9.2[16]/ n.a.	high strength, roasted type[11] /umami, sour[11]
1304.47	glycine	184.4	220.2	180.6	248.4	139.7	176.7	n.a.	3.9 – 35.67[17] / n.a.	odorless[2]/oily, bitter with a fatty, nutty aftertaste, slightly sweet[18]
1661.30	homocysteine	0.5	0.7	0.6	0.40	0.4	0.5	n.a.	n.a. / n.a.	n.a.
1518.00	4-hydroxyproline	8.6	2.4	2.7	6.1	2.4	2.3	n.a.	500[1] / n.a.	sweet[19]
1605.89	hypotaurine	2.7	0.9	0.9	0.5	0.4	0.4	n.a.	75* / n.a.	sweet, bitter, metallic*
1288.60	isoleucine	42.6	69.0	32.7	26.8	22.4	25.8	n.a.	900[1]/ n.a.	bitter[20]
1264.43	leucine	69.7	123.6	56.7	41.7	36.8	41.6	n.a.	846 – 1692[8]/ n.a.	green, citrus, lemon, floral, linalool, spice and chamomile-like[5]
1912.30	lysine	16.0	35.1	22.3	13.5	12.6	15.2	n.a.	104-327[8]/n.a.	odorless[5] / bitter[21]
1514.50	methionine	18.2	29.6	17.4	10.4	10.5	13.3	0.0018[14]	300[1] / n.a.	faint[22]/sweet[23], sulfurous[24]
1809.10	ornithine	5.9	12.6	4.9	2.4	3.2	3.2	n.a.	460[20] / n.a.	sweet[20]
1629.33	phenylalanine	45.4	83.9	41.9	28.5	24.9	30.7	n.a.	530[10]/ n.a.	slight bitter taste[5]
1519.43	pyroglutamic acid	64.3	90.1	44.3	52.1	49.8	54.8	n.a.	500[25]/ n.a.	sour[26]
1379.43	threonine	32.2	63.0	29.9	25.4	19.1	20.8	n.a.	1250[27]/ n.a.	sweet[19]
2214.74	tryptophan	6.9	12.9	7.5	3.4	3.7	5.3	n.a.	102[28]/ n.a.	bitter[28]
1932.60	tyrosine	24.3	64.1	44.2	25.0	24.2	30.1	n.a.	725[20]/ n.a.	odorless/bland taste[5]
1208.77	valine	52.1	98.4	42.2	33.8	28.5	30.5	n.a.	188[29]/ n.a.	sweet, bitter[3]
	Acids									

1937.87	ascorbic acid	0.9	0.8	0.2	0.8	0.4	0.4	n.a.	50-132[30]/ n.a.	sour[20]
1804.57	citric acid	4.8	2.0	23.1	2.2	1.9	2.7	n.a.	0.04-1.4[31] / 314[32]	pleasant, sharp, acidic[5]
2010.00	diethyl tartrate	1.1	0.9	0.6	0.7	0.4	0.4	n.a.	n.a. / n.a.	mild, fruity, wine aroma[5]
1346.23	fumaric acid	9.8	3.8	4.3	7.5	4.9	8.5	n.a.	400[33]/ n.a.	tart, acidic-sour flavor[5]
1980.49	galactonic acid	0.1	0.3	0.2	0.5	0.5	0.5	n.a.	n.a. / n.a.	n.a.
1989.07	gluconic acid	6.2	2.3	51.4	2.4	2.5	3.4	n.a.	75* / n.a.	refreshing sour[34], bitter, metallic*
1927.57	glucuronic acid	0.4	0.5	0.1	0.2	0.2	0.1	n.a.	15[35]/ n.a.	n.a.
1321.67	glyceric acid	32.3	6.9	42.7	23.9	26.4	23.0	n.a.	n.a. / n.a.	n.a.
1149.20	3-hydroxybutyric acid	8.4	12.1	5.0	3.0	2.5	2.9	n.a.	n.a. / n.a.	n.a.
1228.67	4-hydroxybutyric acid	3.0	2.6	1.1	2.0	1.1	1.4	n.a.	n.a. / n.a.	n.a.
1477.30	malic acid	17.3	11.6	22.5	18.8	7.1	10.1	n.a.	9.6-99[36]/ n.a.	nearly odorless, sometimes a faint, acrid[5]/tart, acidic, nonpungent[5]
1037.80	pyruvic acid	0.8	0.4	8.6	0.5	0.7	0.4	n.a.	176[37]/ n.a.	n.a.
1750.53	ribonic acid	1.2	1.1	7.0	0.9	0.8	1.1	n.a.	n.a. / n.a.	n.a.
1310.17	succinic acid	228.6	273.0	95.0	190.8	142.0	172.9	n.a.	15.6-22.2[30] / n.a.	umami[38]
1546.43	threonic acid	1.0	0.6	0.8	0.8	0.6	0.7	n.a	n.a / n.a.	n.a.
	Alcohols									
1707.80	arabitol	5.7	8.3	1.7	5.9	5.1	7.7	n.a.	6560[39]/ n.a.	sweet[39]
1031.00	2,3-butanediol	8.1	28.2	4.5	7.0	11.7	14.2	n.a.	1000[40]/ n.a.	odorless[41]/ sweet[41]
1927.45	galactitol	24.4	34.1	11.1	14.0	14.5	12.0	n.a.	8000[42]/ n.a.	sweet[42]
2083.87	*myo*-inositol	319.2	326.7	206.8	320.5	315.7	206.3	n.a.	3190[39]/ n.a.	sweet[39]
	Sugars									
1856.23	fructose	237.1	130.6	114.3	251.7	186.4	228.3	n.a.	160[43] / n.a.	sweet[38]
1897.67	galactose	607.0	502.7	330.7	617.9	570.3	634.1	n.a.	1800[44]/ n.a.	sweet[21]
1878.20	glucose	926.0	676.4	454.2	1047.6	789.8	867.9	n.a.	700 – 1000[45] / n.a.	sweet[46]
2629.63	sucrose	0.2	0.1	2.2	0.5	0.4	0.2	n.a.	0.00008[47] / 1580[48]	characteristic caramel[49], odorless[50]/sweet[49]
	Phosphates									
2292.77	fructose-6-phosphate	217.0	165.9	164.0	338.6	299.1	328.3	n.a.	570[51] / n.a.	sweet[51]
2329.63	glucose-6-phosphate	720.4	530.5	537.6	1153.7	909.1	1020.3	n.a.	360[51] / n.a.	sweet[19]
2236.42	glycero-3-phosphoethanolamine	7.1	5.6	36.4	6.7	4.1	5.1	n.a.	n.a. / n.a.	n.a.
1748.53	glycerol-3-phosphate	25.0	58.1	36.4	34.8	40.6	16.8	n.a.	n.a. / n.a.	n.a.
2307.10	mannose-6-phosphate	37.2	21.9	26.7	73.1	47.1	52.1	n.a.	25* / n.a.	sweet, metallic tomatoes
1779.12	O-phosphoryl-	4.3	2.7	2.9	2.0	1.7	1.6	n.a.	n.a. / n.a.	n.a.

	ethanolamine									
	Lipids									
2451.63	eicosanoic acid (C20:0)	3.6	2.2	2.7	7.1	4.5	5.0	n.a.	20[52] / n.a.	n.a.
2144.58	heptadecanoic acid (C17:0)	0.5	0.7	0.9	0.4	0.5	0.6	n.a.	20[52] / n.a.	n.a.
2382.00	1-monomyristin	0.8	0.1	0.3	0.4	0.2	0.3	n.a.	n.a.	n.a.
2208.23	octadecadienoic acid, 9,12-(*Z,Z*)- (18:2)	1.1	2.2	1.8	0.6	0.8	0.9	n.a.	n.a. / 4000[53]	bitter[54]
2214.83	octadecanoic acid, 9-(*Z*)- (C18:1)	4.5	7.6	14.9	2.8	3.9	5.6	44[55]	n.a. / > 10000[53]	bitter[56]
2217.41	octadecatrienoic acid, 9,12,15-(*Z,Z,Z*)- (C18:3)	0.1	0.3	0.4	0.1	0.1	0.1	n.a.	n.a. / 1000[53]	bitter[56]
	Other N-containing compounds									
1135.77	3-hydroxypyridine	1.2	0.9	1.1	1.4	1.1	1.3	n.a.	n.a. / n.a.	n.a.
2577.30	inosine	215.9	180.2	154.4	179.2	140.0	171.3	n.a.	60-250[57] / n.a.	bitter[23]
1274.00	nicotinic acid	1.7	3.1	2.2	2.1	1.9	2.0	n.a.	2460 – 3080[58] / n.a.	bitter[54]
1984.50	panthotenic acid	0.5	0.8	0.4	0.3	0.7	0.8	n.a.	n.a. / n.a.	n.a.
1396.91	thymine	2.0	2.2	1.4	1.1	1.1	1.4	n.a.	95-150[59] / n.a.	bitter[54]
1910.63	tyramine	46.8	58.7	3.7	0.9	0.6	0.9	n.a.	70[60]/ n.a.	medium strength odor, meaty type[5]/mild, sweet, vegetative phenolic[5]
1235.60	urea	70.6	75.3	114.1	114.4	115.7	68.2	n.a.	0.19 – 6610[61]/ n.a.	almost odorless[62]/ cooling, saline taste[49]
2463.67	uridine	6.8	4.3	2.9	4.9	3.5	3.3	n.a.	> 10* / n.a.	n.a.

955 *defined in our study

956 [1]Yoshida et al. (1966); [2]Hofmann et al. (2005); [3]Ishibashi et al. (1988); [4]Rotzoll et al. (2005); [5]Burdock (2005);[6]Takahashi (1990);
957 [7]Otagiri et al. (1985); [8]Schiffman et al. (1979); [9]Toelstede et al. (2008); [10]Tanimura et al. (1993); [11]Kirk-Othmer Encyclopedia of
958 Chem. Technology (1992); [12]Stark et al. (2006); [13]Wieser et al. (1977); [14]Laska (2010); [15]Rotzoll et al. (2006); [16]Graham et al. (1995);
959 [17]Hahn et al. (1948); [18]Drauz et al. (2007); [19]Sonntag et al. (2010); [20]Scharbert et al. (2005); [21]Stark et al. (2006); [22]Lewis (1993);
960 [23]Dunkel et al. (2009); [24]Gerhartz (1985); [25]Petró-Turza et al. (1989); [26]Dashdorj et al. (2015); [27]Faurion et al. (1992); [28]Schwarz et al.
961 (2007); [29]Haefeki et al. (1990); [30]Schiffman (1993); [31]Powers et al. (1971); [32]Thurgood et al. (2010); [33]Meilgaard (1975);
962 [34]Ramachandran et al. (2006); [35]Maga et al. (1973); [36]Stevens (1997); [37]Taylor et al. (1930); [38]Watkins et al. (2013); [39]Hufnagel et al.
963 (2008); [40]Rothe et al. (1972); [41]Steffen et al. (2005); [42]Dziedzic et al. (1983); [43]Pangborn (1963); [44]Becker et al. (1907); [45]Rothe et al.
964 (1963); [46]Nagai et al. (2006); [47]Land (1968); [48]Thurgood et al. (2010); [49]Budavari (1996); [50]NIOSH (1997); [51]Sonntag et al. (2010);

965 [52]Cherchinski (1961); [53]Ladahudec et al. (1991); [54]Grosch et al. (1984); [55]Nagy (1991); [56]Wieser et al. (1984); [57]Kuchiba-Manabe et al.
966 (1991); [58]Jugel (1979); [59]Fischer (1967); [60]Warmke et al. (1996); [61]Hahn et al. (1938); [62]Lewis (1997)
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981 Table S-3. Volatile compounds from adipose tissue and metabolite from lean meat that were of no relevance for flavor based on PLS
982 regression analysis

Not identified as important in any statistical model independent of weighting			
Volatiles		*Metabolites*	
methylcyclohexane	above threshold	asparagine	below threshold
undecane	below threshold	ornithine	below threshold
2-propyl-1,1,3-trimethyl-cyclohexane	n.a.	pyroglutamic acid	below threshold
pentadecane	below threshold	tyrosine	below threshold
(*E*)-3-decene	n.a.	valine	above threshold
(*Z*)-5-tridecene	n.a.	malic acid	above threshold
(*Z*)-3,7-dimethyl-2-octene	n.a.	galactose	below threshold
2-heptyn-1-ol	n.a.	sucrose	above threshold
benzyl alcohol	below threshold	glycerol-3-phosphate	n.a.
butanal	above thresholds	heptadecanoic acid	below threshold
pentanal	above thresholds	octadecatrienoic acid, 9,12,15-(Z,Z,Z)- (C18:3)	below threshold
2-butenal	above threshold for odor	panthotenic acid	n.a.
(*Z*)-4-heptenal	above thresholds		
octanal	above thresholds		
acetone	above threshold for odor		
acetophenone	above threshold for odor		
dimethyl sulfone	above threshold		
toluene	above thresholds		
Identified as significant once in 2 different models with special weighting but not in the 3rd model attempted			
Volatiles		*Metabolites*	
heptane	below thresholds	2-aminobutyric acid	below threshold
pentadecane	below threshold	cysteine	above threshold
(*E*)-3-dodecene	n.a.	threonine	below threshold

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2-hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	n.a.	fumaric acid	below threshold
2-ethyl-1-hexanol	below threshold	fructose glycerol-3-phosphate octadecanoic acid, 9-(Z)- (C18:1)	above threshold n.a. above threshold for odor
Identified as significant once in 2 different models with special weighting but not in the 3rd model attempted			
Volatiles		***Metabolites***	
3-methylhexane (*E*)-2-hexene 3-methyl-2-nonene 1-octanol 3-hydroxy-2-butanone	below threshold n.a. n.a. above threshold above thresholds	4-aminobutyric acid butyro-1,4-lactam leucine lysine phenylalanine	above threshold n.a. below threshold below threshold below threshold
decanoic acid	above threshold for odor and flavor in water	ribonic acid	n.a.
γ-butyrolactone	n.a.	tyramine	above threshold

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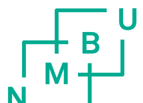

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