QUALITY CHANGES IN LAMB/SHEEP AND BEEF MEAT RELATED TO OXIDATIVE PROCESSES IN MUSCLE SYSTEMS

KVALITETSENDRINGER I LAM/SAU OG STORFEKJØTT RELATERT TIL OKSIDATIVE PROSESSER I MUSKELSYSTEMER

Philosophiae Doctor (PhD) Thesis

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A doctoral thesis was submitted to the Department of Chemistry, Biotechnology and Food Sciences at the Norwegian University of Life Sciences (NMBU, Ås, Norway). The thesis has been carried during the time period of 2012-2015 and consists of a theoretical introduction with five scientific papers.

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Dedication

To my family

Preface

The thesis consists of a theoretical introduction and five scientific papers discussing the importance of oxidative processes as involved in tenderization mechanisms and flavour development in *post mortem* muscle. **Paper I** and **Paper II** emphasize mitochondria as antioxidants using beef as meat matrix. **Paper I** defines the relation between mitochondrial activity early *post mortem* and mechanical properties of beef meat. In **Paper II**, mixtures of Krebs cycle substrates that stabilize colour of minced meat were tested for their ability to provoke or retard (lipid) oxidation.

In the next three papers (**Paper III**, **IV** and **V**) sheep and lamb meat quality was studied, from grading to production of dry cured hams. The quality characteristics of fresh lamb and sheep meat from different production systems were first investigated (**Paper III**). Flavour compounds and sensory attributes of adipose tissue and lean meat were then studied subsequently (**Paper IV**). **Paper IV** also returns to the importance of Krebs cycle compounds. Flavour development and sensory properties of sheep hams produced from the raw material characterized in **Paper III** and **IV** using two different production processes from Western Balkan countries, were defined in the **Paper V**.

Acknowledgements

Creating PhD thesis presents a teamwork and implies many people who I would like to acknowledge. First of all, I would like to thank my main PhD supervisor, Professor Bjørg Egelandsdal for being enthusiastic, energetic and supportive during these years. I feel fortunate to have collaborated with Professor Egelandsdal who has been patient and encouraging during difficult moments. Professor, your high scientific and professional standards were inspiring and helped me to grow as a researcher. In addition, I would like to thank to my PhD co-supervisors, Professors Gunnar Klemetsdal and Božo Važić for their help and suggestions. Also, I would like to express my gratitude the leader of the HERD Project, Milena Bjelanović, for a fruitful cooperation and support in all segments of the project. I owe a big thank to Professor Erik Slinde for inspiring research discussions, support and assistance in completing my doctoral program.

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I would like to thank the members of HERD project: Professor Aleksandra Martinović, Professor Božidarka Marković, Dr. Biljana Rogić, MSc Sandra Stojković, MSc Goran Vučić, master students Sanja Kostić and Slobodan Momić for cooperation and assistance in realization of lamb/sheep studies. I owe a great thank to Per Berg from Nortura SA for support and encouragement. The staff at Nortura Gol, "BB" Kotor Varoš and "Franca" Bijelo Polje are thanked for their great assistance in sample collection.

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Summary

The oxidative processes in meat *post mortem* maintain the formation of free radicals. Behavior of reactive oxygen species is closely related to the energy metabolism of the muscle cell, mitochondrial activity and efficiency of antioxidant systems. The role of mitochondria as a natural antioxidant in a cell has been investigated. Biochemical changes in meat activated by oxidation processes provoke different pathways in tenderization and flavour formation.

In **Paper I**, a predictive model for meat tenderness was created based on the oxygen consumption rate measurements early *post mortem* using partial least square regression (that explained minimum 15-20% variation in beef tenderness). A major challenge was related to mitochondria isolation approximately 2.5 hours *post mortem* at the slaughter-line. The protein expression of isolated mitochondria was studied for tender and tough beef samples. Mitochondrial proteins were separated on 2-dimensional SDS-PAGE gels and image analysis was performed using Delta2D. Protein spots of interest were analyzed by mass spectrometry techniques (MALDI-TOF or LC-MS). Higher mitochondrial respiration and antioxidant activity, abundant Krebs cycle and electron transport system proteins as well as energy production enzymes were correlated with tender beef.

In **Paper II,** the effect of Krebs cycle substrates (KCS) used as colour stabilizers of oxidation processes in minced meat during chill-storage was investigated. Mixtures of succinate, pyruvate, glutamate, malate and citrate in various combinations were added to minced beef meat with either pork or beef fat, and packaged in modified atmosphere or high oxygen atmosphere for 13 and 8 days, respectively. Development of lipid derivated volatiles was to a large extent affected by the type of fat tissue. Lipid oxidation increased when the KCS were metabolized to eliminate oxygen and keep colour stable. The increase in lipid oxidation was too small to deteriorate flavour and nutritive characteristics of meat, and was offset by the positive effect of KCS additives on meat colour.

In **Paper III** and **IV**, significant differences in quality characteristics of lamb and sheep meat representative of three production systems were identified. The selected animals of different breed, age and from specific grazing regions reflected the consumption and cultural habits typical for Bosnia and Herzegovina, Montenegro and Norway. Animals belonging to autochthonous Pramenka breed (types Vlašićka and Pivska) were in general smaller, with higher fat content, better tenderness (only for Bosnia and Herzegovina muscles), and better colour stability and antioxidant capacity compared to the presently dominating Norwegian White breed, that had a better nutritional value. Additional differentiation of observed animal groups was done by sensory and flavour analysis. The volatile and metabolite profile of each animal group were described by specific compounds that were isolated from adipose tissue and lean meat, respectively. Large sensory and flavour differences among the two types of Pramenka breed were found. This is probably due to the local production systems and suggested that sheep/lamb sensory differences cannot be related to the distance between the grazing areas.

In **Paper V**, dry-cured sheep hams produced in Bosnia and Herzegovina (B&H) and Montenegro (MN) using old traditional recipes in the facilities commonly used in these two countries were investigated. The results for sensory, volatile and organic acid analysis were studied using multivariate analysis. Adding garlic and pepper during the salting phase of B&H ham production had a significant effect on sensory and flavour profile. Intense smoking of MN sheep ham gave a heavy, smoked flavour. The selected processing technology in B&H produced low-salt sheep hams, apparently by using a fermentation step, while MN hams were produced with higher salt levels.

Sammendrag

Oksidative prosesser i kjøtt opprettholder *post mortem* dannelsen av frie radikaler. Virkemåten til reaktive oksygen forbindelser er nært knyttet til energimetabolismen i muskelceller, mitokondrie aktivitet og effektiviteten av antioksidant systemer. Mitokondrienes rolle som naturlige antioksidanter i celler har blitt undersøkt. Biokjemiske endringer i kjøtt aktivert ved oksidasjons- prosesser leder til ulike metabolske veier som påvirker mørhet og smaksdannelse.

I **artikkel I** ble det laget en prediktiv modell ved bruk av partiell minste kvadraters metode for mørhet i kjøtt. Modellen forklarte minimum 15-20% variasjon i kjøttets mørhet. Denne modellen baserte seg på hvor høyt oksygen forbruket i muskelen er tidlig *post mortem*. En stor praktisk utfordring var knyttet til mitokondrie isolasjonen som ble utført ca. 2.5 timer *post mortem* på slaktelinjen. Protein uttrykket i de isolerte mitokondriene ble studert for møre og seige kjøttprøver. Mitokondriets proteiner ble skilt ved 2-dimensjonal SDS- PAGE og bildeanalysen ble utført ved hjelp av Delta2D. Protein flekker av interesse ble analysert med masse-spektrometri teknikker (MALDI-TOF eller LC-MS). Høyere mitokondrie respirasjon og antioksidantaktivitet, rikelig med Krebs syklus og elektron transport system proteiner samt energi produserende enzymer ble korrelert med kjøttets grad av mørhet.

I **artikkel II**, ble effekten på oksidative prosesser av å benytte Krebs syklus forbindelser (KCS) som farge stabilisatorer i kjølelagret kjøttdeig undersøkt. Blandinger av ravsyre, pyruvat, glutamat, eplesyre og sitronsyre i ulike kombinasjoner ble satt til kjøttdeig laget fra magert storfekjøtt tilsatt svine- eller storfefett. Kjøttdeigen ble pakket i modifisert atmosfære eller høy oksygen atmosfære i henholdsvis 13 og 8 dager. Utviklingen av lipid avledede flyktige forbindelser var i stor grad påvirket av type fettvev. Lipid oksideringen øker når KCS forbindelsene metaboliseres for å eliminere oksygen og for å holde fargen stabil. Økningen i lipid nedbrytningsprodukter var for liten til å gi harsk smak og redusere ernæringsverdi. Den positive effekten av KCS tilsetning på kjøttets fargen ansees som viktigere.

I **artikkel III** og **IV**, ble det identifisert betydelige forskjeller i kvalitet for lamme- og sauekjøtt fra tre produksjonssystemer. De utvalgte dyrene av ulike raser, alder og fra ulike beite regioner reflekterte konsum og kulturelle vaner typisk for Bosnia og Hercegovina (B&H), Montenegro (MN) og Norge. Dyr tilhørende autochtonous Pramenka rase (typene Vlašićka og Pivska) var generelt mindre, med høyere fettinnhold, bedre mørhet (dog bare for kjøtt fra Bosnia og Hercegovina), bedre farge stabilitet og antioksidant kapasitet sammenlignet med den dominerende norske hvite rasen, som til gjengjeld utviste en bedre ernæringsmessig verdi. Ytterligere differensiering av kjøttet fra observerte dyregrupper ble gjort ved hjelp av sensorikk og instrumentelle metabolitt analyser. Profilene av flyktige og mindre flyktige komponenter (metabolitter) fra hvert dyr ble beskrevet av bestemte forbindelser, som var isolert fra henholdsvis fettvev og fra magert kjøtt. Store sensoriske og metabolitt forskjeller, særlig mellom de to typer Pramenka typene, ble funnet. Dette tolkes som at stedet som sauen beiter på har mye å bety og at disse forskjellene ikke nødvendigvis kan måles i antall km mellom beite-stedene.

I **artikkel V** ble kvaliteten til en type spekede saueskinker produsert i Bosnia og Hercegovina (B&H) og Montenegro (MN) etter gamle tradisjonelle oppskrifter i anlegg brukt i disse to landene undersøkt. Resultatene for sensorisk, flyktige komponenter og organisk syre analyse ble studert ved bruk av multivariabel analyse. Hvitløk og pepper som benyttes i saltefasen i B&H sin spekeskinke produksjon hadde betydelig effekt på sensoriske profil samt på kjemiske metabolitter. Intens røyking som benyttes i MN for å produsere deres tradisjonelle sauespekeskinke, var kjennetegnet av intens røykt smak. Den undersøkte speketeknologien fra B&H produserer lav-salt spekeskinker fra sau, tilsynelatende med et fermenteringstrinn, mens MN sine spekeskinker fra sau produseres med høyere salt nivåer.

Садржај

Оксидациони процеси који се одвијају у месу *пост мортем* утичу на стварање слободних радикала. Дјеловање реактивних кисеоничних врста је уско повезано са енергетским метаболизмом мишићне ћелије, активности митохондрија и ефикасности антиоксидационог система. Улога митохондрија као природног антиоксиданта у ћелији је испитивана. Биохемијске промјене у месу активиране оксидационим процесима изазивају различите процесе омекшавања и формирања ароме.

У **Раду I**, предпостављени модел за њежност меса формиран је на основу мјерења потрошње кисеоника рано *пост мортем* примјеном парцијалне регресије (објашњава минимално 15-20% варијације у њежности говеђег меса у клаоничним условима). Највећи изазов представља изолација митохондрија приближно 2.5 часа *пост мортем* на линији клања. Експресије протеина из изолованих митохондрија у њежном и тврдим месу је истраживана. Протеини митохондрија су раздвајани примјеном 2-димензионалне СДС-ПАГЕ електрофорезе, док је обрада гелова извршена у програму „Делта2Д“. Протеини од значаја су анализирани масеном спектрометријском техником ("МАЛДИ-ТОФ" или ЛЦ-МС). Већа респирација митохондрија и антиоксидативна активност, већа концентрација протеина Кребсовог циклуса и система за транспорт електрона, као и ензима неопходних за производњу енегрије су у корелацији са њежнијим месом.

У **Раду II** се испитује утицај супстрата Кребсовог циклуса, који су коришћени као стабилизатори боје, на оксидационе процесе у охлађеном мљевеном месу током чувања. Мјешавине сукцината, пирувата, глутамата, малата и цитрата у различитим комбинацијама додате су мљевеном говеђем месу са свињском или говеђом масти, пакованом у модификованој атмосфери или у атмосфери високе концентрације кисеоника и чуваном у периоду од 13, односно 8 дана, респективно. На формирање испарљивих деривата из масти у великој мјери утиче врста масног ткива. Оксидација масти се повећава када су супстрати Кребсовог циклуса метаболизовани за уклањање кисеоника и стабилизацију боје. Међутим, повећање је релативно, сувише мало да би

погоршало арому и нутритивна својства меса и компезовано је позитивним утицајем субстрата Кребсовог циклуса на боју меса.

У **Раду III** и **IV** су идентификоване значајне разлике у својствима квалитета јагњећег и овчијег меса животиња узгојених у три различита производна система. Одабране животиње различитих раса, старости и узгајане у специфичним условима испаше одражавају културолошке и навике у исхрани типичне за Босну и Херцеговину, Црну Гору и Норвешку. Грла аутохтоне расе праменке (влашићки и пивски сој) су у основи мања, са већим садржајем масти, њежнијим месом (само животиње и Босне и Херцеговине), бољом стабилношћу боје и антиоксидационим капацитетом у односу на тренутно доминантну норвешку бијелу овцу чије месо има бољу нутритивну вриједност. Додатна диференцијација испитиваних група животиња је урађена на бази сензорских и инструменталних анализа. Профили испарљивих компоненти и метаболита свих испитаних група су описани специфичним компонентама изолованим из масног ткива, односно меса, респективно. Велике разлике у сензорским својствима и у ароми између два соја расе параменке су утврђене што указује на специфичност локалних продукционих система.

У **Раду V** је испитивано суво саламурено овчије месо (бут) произведено у Босни и Херцеговини и Црној Гори примјеном традиционалне рецептуре и у условима производње типичним за ове двије земље. Резултати сензорских анализа, испарљивих компоненти и органских киселина су изучавани примјеном мултиваријативних анализа. Коришћење бијелог лука и бибера у фази усољавања у производњи суво саламуреног овчијег меса (бут) у Босни и Херцеговини у значајној мјери је утицало на сензорска својства и арому готовог производа. Интензивно димљење суво саламуреног овчијег меса (бут) у Црној Гори је довело до интензивне ароме дима. Специфичан начин производње суво саламуреног овчијег меса (бут) са ниским садржајем соли у Босни и Херцеговини је очигледно праћен процесом ферментације, док суво саламурено овчије месо (бут) из Црне Горе има већи садржај соли.

List of papers

I. Grabež, V., Kathri, M., Phung, V., Moe, K. M., Slinde, E., Skaugen, M., Saarem, K., & Egelandsdal, B. (2015) Protein expression and oxygen consumption rate of early postmortem mitochondria relate to meat tenderness. *Journal of Animal Science, 93*(4), 1967-1979.

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

III. Bjelanović, M., Grabež, V., Vučić, G., Martinović, A., Lima, L. R., Marković, B., & Egelandsdal, B. (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.

IV. Grabež, V., Bjelanović, M., Rohloff, J., Martinović, A., Berg, P., Tomović, V., Rogić, B., & Egelandsdal, B. (2015) Characterization of sensory profile and metabolites of lean lamb/sheep meat with flavour volatiles in adipose tissues: different breed, age and production system. Ready for submission to *Food Chemistry.*

V. Stojković, S., Grabež, V., Bjelanović, M., Mandić, S., Vučić, G., Martinović, A., Håseth, T. T., Velemir, A., & Egelandsdal, B. (2015) Production process and quality of two different dry-cured sheep hams from Western Balkan countries. *LWT – Food Science and Technology*, *64*(2), 1217-1224.

Abbreviations

ADP	Adenosine di-phosphate
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl / total antioxidant capacity
ES	Electrical stimulation
ETS	Electron transport system
$FADH_2$	Reduced flavin adenine dinucleotide
FAME	Fatty acid methyl esters
FCCP	p-trifluoromethoxy carbonyl cyanide phenyl-hydrazone
GC/MS	Gas chromatography mass spectrometry
HS-GC/MS	Headspace gas chromatography mass spectrometry
HPLC	High pressure liquid chromatography
LC/MS	Liquid chromatography mass spectrometry
MALDI	Matrix assisted laser desorption/ionisation
$NADH_2$	Reduced nicotinamide adenine dinucleotide
OCR	Oxygen consumption rate
PCA	Principal component analysis
PLSR	Part least square regression analysis
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TOF	Time of flight

Contents

Papers (*individual numbering*)

QUALITY CHANGES IN LAMB/SHEEP AND BEEF MEAT RELATED TO OXIDATIVE PROCESSES IN MUSCLE SYSTEMS

KVALITETSENDRINGER I LAM/SAU OG STORFEKJØTT RELATERT TIL OKSIDATIVE PROSESSER I MUSKELSYSTEMER

LIST OF ERRATA

	Written	**Should be**
Page xiii, Line 8	*postmortem*	*post mortem*
Page xiii, Line 10	*postmortem*	*post mortem*
Page 6, Line 7	post mortem	*post mortem*
Page 11, Line 14	(Huang & Lametsch)	(ETS)
Page 14, Line 9	(Huang & Lametsch)	(ETS)
Page 22, Line 10	(Andrés, Cava, Mayoral, Tejeda, Morcuende, & Ruiz, 2001)	(Cu)
Page 30, Line 20	can accurately way	can accurately
Page 35, Line 4	from microbial metabolites	from microbial metabolism
Page 38, Line 11	strategies how for	strategies for
Page 40, Line 6	the metallic flavour	the bitter flavour
Paper I, page 1969 Line 3	O2	O_2
Paper I, page 1971 Line 39	OCR3-4 h	$OCR_{3\text{-}4\ h}$
Paper III, page 204 Line 5	The purcase motives	The purchase motives
Paper III, page 205 Line 3-4	of C18:3 (n-3) fatty acids and intensity of acid taste	of C18:1 (t-11) fatty acid
Paper III, page 206 Line 1	*Longisimus thoracis at lumborum*	*Longissimus thoracis et lumborum*

Paper III, page 210 Line 20	samples lower protein content	samples had lower protein content
Paper III, page 210 Line 30	dependent	depend
Paper III, page 213 Line 17	isinteresting	is interesting
Paper III, page 214 footnotes	differencesat	differences at
Paper III, page 214 Line 4	NWS	NO
Paper III, page 215 Line 26	VP lamb	B&H lamb
Paper III, page 215 Line 37	PS meat	B&H meat
Paper IV, page 14 Line 310	($P \leq 0.05$)	($P < 0.05$)
Paper IV, page 29 Line 657	BH ord	BH lamb

1. Introduction

Meat consumption and the inclusion of different species of meat-producing animals in the diet are strongly related to the ethnic, cultural and religious backgrounds. Growing demands from consumers for specific quality characteristics of meat drive the industry towards new knowledge about underlying mechanisms which affect the final quality.

From consumers' perspective, meat quality is defined by "eating quality characteristics". Tenderness and flavour appear to be the most important sensory attributes in consumers' perception of meat quality (Joo, Kim, Hwang, & Ryu, 2013), in particular for meat from ruminants. Variations in meat quality are related to pre-slaughter factors, including genetics, diet, management practices, environmental conditions, and in addition post-slaughter handling and processing (Mullen, Stapleton, Corcoran, Hamill, & White, 2006). In individual consumer assessment, the most valuable beef sensory attribute is tenderness. A set of complex biochemical reactions during conversion of muscle to meat is strongly related to the final meat quality (Paredi, Raboni, Bendixen, de Almeida, & Mozzarelli, 2012). Controlled meat texture is the key priority for the red meat industry (Mullen et al., 2006). Some studies show that consumers are willing to pay more for tender meat (Boleman, Boleman, Miller, Taylor, Cross, Wheeler, et al., 1997; Huffman, Miller, Hoover, Wu, Brittin, & Ramsey, 1996).

Consumers' perception and acceptance of ruminant meat are related to the flavour characteristics (Watkins, Frank, Singh, Young, & Warner, 2013), although quantity of intramuscular fat and slaughter weight of animal could affect eating quality (Guerrero, Valero, Campo, & Sañudo, 2013). Sheep meat characterized by its unique flavour presents an obstacle for some consumers (Watkins et al., 2013). Animal feeding systems affect fatty acid composition of ruminant adipose and muscle tissue (Prache, 2009). Lipid oxidation products, lipid- and water-soluble components (Melton, 1990), may affect characteristic meat flavour. Adipose tissue is the main source of volatile compounds responsible for the species-specific flavour. In addition, formation of off-flavour and off-odours may be associated with microbial growth and meat spoilage. The accumulation of microbial metabolites, such as alcohols,

aldehydes, acids, amines, ketones, esters, and sulphur compounds may make the meat unacceptable for consumers (Ercolini, Ferrocino, Nasi, Ndagijimana, Vernocchi, La Storia, et al., 2011).

This thesis partly focuses on tenderization mechanisms governed by mitochondria and the activity of complex antioxidant system at early *post mortem* conditions. Prolonged mitochondrial activity preserves proteases' activities, modulates cell death (apoptosis/necrosis switch) and thereby increases meat tenderness. Further studies were focused on identification of biomarkers significant for flavour development in a different muscle system during the *post mortem* period.

In general, of practical importance for a stabile meat production system is the establishment of a suitable combination of breeds adapted to specific environmental conditions, with optimal growth performance and increased uniformity contributing to acceptability of meat and meat products. In addition, the combined effect of breed and environmental conditions of meat quality are of specific interest to enhance market opportunities.

Cattle production in Norway combines different systems such as milk and meat production and bull production, raised under semi-extensive or extensive systems. Feeding is 50% silage/ roughage and concentrate. However, beef production in Norway is based predominantly on "dual-purpose" Norwegian Red Cattle (Norsk Rødt fe; Fig.1).

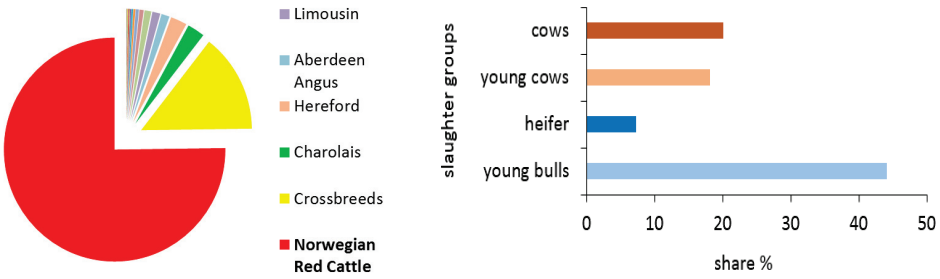

Figure 1. Norwegian production of beef meat (Source: Haugen, 2014). Minor breeds are not coded.

The Norwegian Red Cattle has been bred for important health and reproductive performances, feed efficiency, combining superior milk together with meat production (Kirkland, Patterson, Keady, Moss, & Steen, 2007). Furthermore, like most meat breeds, Charolais is produced predominantly for its meat, or more precisely, its large muscles. On the other hand, dominance of Norwegian Red Cattle in beef meat production is balanced with milk production and the quality characteristics (*e.g.* colour and tenderness).

Figure 2. Dominant cattle breeds in: 1.) Norway (Norwegian Red Cattle); 2.) Montenegro (Brown Swiss); and 3.) Bosnia and Herzegovina (Simmental)
(Source: http://www.genoglobal.com/Start/Norwegian-Red/about-norwegian-red/Norwegian-red-breeding-program/; http://brownswiss.org.nz/brown swisshistory.htm; http://cattleinternationalseries.weebly.com/simmental.html).

Ruminant production in Western Balkan countries is greatly influenced by environmental conditions, *i.e.* natural resources. Cattle production systems in Montenegro and Bosnia and Herzegovina are related to the farm production and combined farm/pasture feeding systems (4-

6 months grazing period). Dominant breeds are Brown Swiss, Holstein, Simmental, Tyrol Gray, Buša, and crossbreeds (Alibegovic-Grbic, 2009; Rajović & Bulatović, 2014).

Buša is an autochthonous breed from the Balkan Peninsula, with a relatively low live weight, high fertility, and high disease resistance (Jovanovic, Savic, & Trailovic, 2003). Domestic breed Gatačko cattle as a crossbreed of Buša and Tyrol Gray represents a small cattle population in Bosnia and Herzegovina (Alibegovic-Grbic, 2009). Similar to Norway, beef meat production in Western Balkan countries is connected to dairy production and fattening of calves for slaughtering, if not needed for reproduction and milk production. These breeds are medium sized, adopted to pasture feeding regime with efficient conversion of food into proteins (Rajović & Bulatović, 2014).

Norwegian lamb/sheep production is dominated by Norwegian White, a crossbreed composed of Dala, Rygja, Steigar, and Texel breeds, for meat and wool production (Eikje, Ådnøy, & Klemetsdal, 2008).

Figure 3. Dominant sheep breeds/types in: 1.) Norway (Norwegian White sheep); 2.) Montenegro (Pivska Pramenka), and 3.) Bosnia and Herzegovina (Vlašićka Pramenka) (Source: Christie, 2007; http://see.efncp.org/download/sofia2013/Montenegro.pdf; Alibegovic-Grbic, 2009).

It was accepted as a new breed in 2000/2001. Sheep farming meets consumers' demands through traceability, respecting ethical production values, and aims to provide unique meat quality. The economic advantage of Norwegian White is related to higher muscularity compared to Dala breed, high fertility and growth rate (Eikje et al., 2008). Highly appreciated on the Norwegian market is the Gourmet lamb meat, which originates from animals raised in Hallingskarvet mountain region in southern Norway. Although the meat carries an origin label that signals a special meat quality, the real quality properties are not well documented.

Sheep and lamb production in Western Balkan countries is predominantly a nomadic production, especially in mountain areas with dominant autochthonous triple-purpose Pramenka breed (Zackel sheep), reared for meat, milk, and wool (Gavojdian, Cziszter, Sossidou, & Pacala, 2013). Dominant types of Pramenka breed in Montenegro are Pivska and Sjenička, while Vlašićka (Dubska), Privorska, Kupreška, Stolačka and Podveleška are dominant in Bosnia and Herzegovina. As a primitive breed, Pramenka is adapted to almost exclusive pasture feeding in rough environmental conditions (Alibegovic-Grbic, 2009; Dubljević, 2009) together with high resistance to parasite infections (Savić, Baltić, Becskei, Dimitrijević, Dimitrijević, Savić, et al., 2014).

2. Objectives

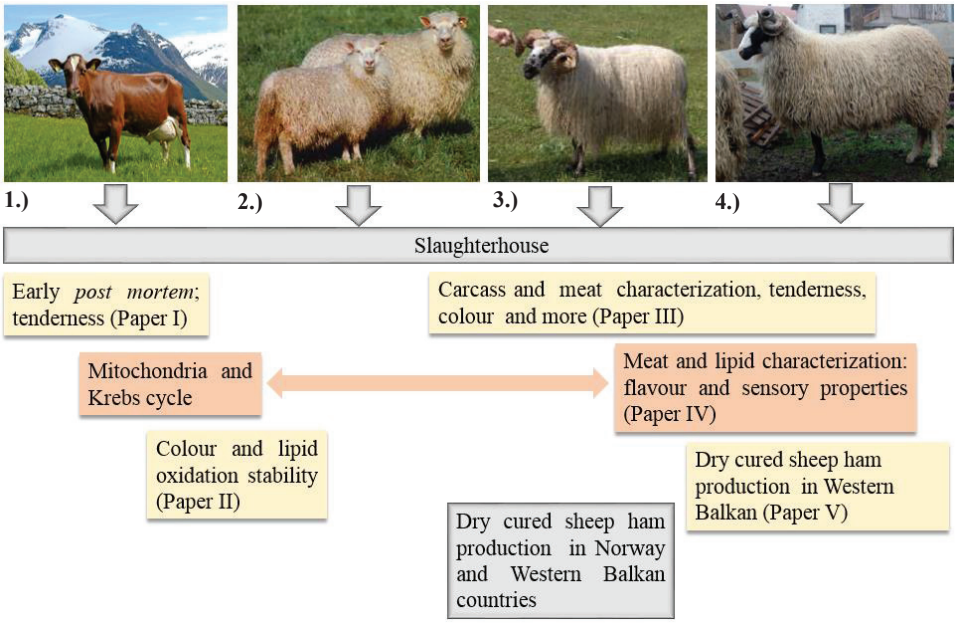

Figure 4. The picture links ruminants used from Norway and Western Balkans, the meat quality and the meat processing studies (the grey square is a Short Paper VI that is not included in the thesis). In addition, Fig. 4. shows how the papers are linked to each other. Ruminants presented in Fig. 4. are: 1.) Norwegian Red Cattle; 2.) Norwegian White Sheep; 3.) Vlašićka Pramenka; and 4.) Pivska Pramenka, respectively.

The five papers have the following specific objectives:

I. The proteomic study of mitochondria isolated ~ 2.5 h post mortem from the muscle system was set up to identify their protein modifications and relation to beef meat tenderization processes based on mitochondrial oxygen consumption early *post mortem*.

II. Krebs cycle substrates, as stabilizers of meat colour, were tested regarding whether they accelerate or suppress lipid oxidation in beef meat packed in low (60% CO_2 and 40% N_2) and high (75% O_2 and 25% CO_2) oxygen atmosphere at different storage conditions.

III. The meat and carcass quality characteristics and oxidative stability of lamb/sheep meat from different production systems, breed, and age were examined.

IV. Oxidation progression and metabolic status and its relation to sensory properties of meat.

V. The characteristics of sheep ham produced using different process technologies were studied. This was achieved by analyzing flavour compounds and metabolites developed with respect to production process.

According to our knowledge, the mitochondrial activity and protein modification early *post mortem* (isolated ~ 2.5 h *post mortem*) had not been examined previously with regard to its relation to tenderness. Of special interest are several enzyme complexes of the electron transport chain for studies of early *post mortem* events.

Similarly, the *in vitro* consumption of oxygen by the electron transport chain *post mortem* had not been examined before regarding oxidation progress and production of reactive oxygen species.

An effort was made to understand the quality of the meat from Pramenka breed, from the farm to a ready product, such as dry cured sheep. The focus was on lipid oxidation, energy metabolites, Krebs cycle compounds and amino acids. The sensory properties of two types of Pramenka breed were investigated. Knowledge about the quality of meat from the Pramenka breed is close to non-existing in the scientific literature.

3. Theoretical background

3.1. From muscle to meat

The conversion of muscle to meat starts with animal bleeding. The blood circulation is interrupted: loss of oxygen and nutrients transfer to the muscle cells leads to anaerobic metabolism. Levels of ATP production still remain low early *post mortem*. As a result of anaerobic glycolysis lactic acid is formed and the muscle pH declines from 7.44 to approximately 5.3−5.8. Cooling the carcasses too quickly may trigger a powerful contraction of muscle causing a toughening effect known as cold shortening when it is thawed. A rapid pH decline at body temperature results in greatly reduced water holding capacity and loss of colour stability, as commonly observed for pork meat and is termed PSE-meat (Pale Soft and Exudative). Thicker fat layers may prevent cold shortening during chilling, fast enzyme inactivation and improve tenderization process in the muscle system (Savell, Mueller, & Baird, 2005).

3.1.1. Muscle structure

Characteristics of skeletal muscles are influenced by various genetic and environmental factors. Skeletal muscles are composed (75−90% of the muscle volume) of different types of fibers (Lee, Joo, & Ryu, 2010). The morphological, contractile, and metabolic properties of muscle fibers have a direct effect on muscle biochemical pathways in *ante*- and *post mortem* period and characterize the differences between skeletal muscles or/and carcasses (Klont, Brocks, & Eikelenboom, 1998). There are four different fiber types: type I (slow-twitch, oxidative), type IIA (fast-twitch, oxidative and glycolytic), types IIB and IIX (fast-twitch, glycolytic) (Joo et al., 2013). Muscle fiber types' composition correlates with type of mechanism involved in the conversion of muscle to meat and the final meat quality. Muscle fiber type IA is rich in myoglobin and mitochondria with high oxidative mitochondrial enzyme activity, while type IIB has a high content of glycogen and glucose and a high glycogen enzyme activity (Choe, Choi, Lee, Shin, Ryu, Hong, et al., 2008).

3.1.2. Experimental units / muscles

Variation in meat quality may be large within animals, breed and production system and is affected by different metabolic factors (Klont et al., 1998). Thus the composition of muscle fiber types may provide large variation in *post mortal* biochemical processes and quality characteristics (Klont et al., 1998; Waritthitham, Lambertz, Langholz, Wicke, & Gauly, 2010). Different muscle types showed large variations in tenderness (Von Seggern, Calkins, Johnson, Brickler, & Gwartney, 2005). The round muscles were described as less tender compared to loin and chuck muscles, but from the perspective of the consumer, often appreciated as having a better quality (Hildrum, Rødbotten, Høy, Berg, Narum, & Wold, 2009; Voges, Mason, Brooks, Delmore, Griffin, Hale, et al., 2007). Improved tenderness of round muscles such as *M. semimembranosus* would have a great impact on consumers' acceptability (Miller, Carr, Ramsey, Crockett, & Hoover, 2001; Voges et al., 2007).

M. semimembranosus is a large round muscle primarily used during locomotion, and shows relatively large variation of sensory traits (Reuter, Wulf, & Maddock, 2002). According to Hwang, Kim, Jeong, Hur, & Joo (2010) a relatively high degree of type IIB fibers is present in bovine *semimembranosus* muscle.

The *M. longissimus thoracis et lumborum* (loin, LTL) is a back muscle, generally accepted as a tender muscle, and often studied and used as a reference muscle for quality assessment of other muscles and classification of carcasses in tenderness groups (Polkinghorne, Thompson, Watson, Gee, & Porter, 2008). Peinado, Latorre, Váquez-Autón, Poto, Ramírez, López-Albors, et al. (2004) reported high percentage of type IIB fibers with intense oxidative activity in the loin sheep muscle (53%) and of 90 days old lamb (59%).

3.2. Early *post mortem* biochemical pathways in the muscle system

Energy is produced in the *in vivo* cell through the conversion of glucose into pyruvate and by the Krebs cycle and oxidative phosphorylation. In *post mortem* phase, the cell is mainly fuelled with energy obtained through glycogen degradation by glycolysis to lactate (D'Alessandro & Zolla, 2013). Metabolic processes in the early *post mortem* period are presumed to be determinants of meat quality (Herrera-Mendez, Becila, Boudjellal, & Ouali, 2006; Klont et al., 1998; Lee, 1986; Luciano, Anton, & Rosa, 2007). Complex biochemical reactions during tenderization are mediated by a particular group of enzymes (Koohmaraie, 1994, 1996; Luciano et al., 2007; Takahashi, 1996). The enzymes cathepsins, calpains, multicatalytic proteinase complex (MCP) and caspases have been designated roles at different time points in the tenderization process (Herrera-Mendez et al., 2006; Kemp, Sensky, Bardsley, Buttery, & Parr, 2010; Ouali, Herrera-Mendez, Coulis, Becila, Boudjellal, Aubry, et al., 2006).

A possible route of conversion of muscle into meat was observed from the perspective of programmed cell death – apoptosis. This concept recognizes a central role of mitochondria in the cells' response to oxidative stress early *post mortem*. Apoptosis presents a normal mechanism and its function is to maintain cells during ageing in the living organism (Ouali, Herrera-Mendez, Coulis, Becila, Boudjellal, Harhoura, et al., 2007). The key role of apoptosis is in the elimination process of cells damaged by reactive oxygen species (Elmore, 2007; Ouali et al., 2007). A critical point in apoptosis involves caspase activation that depends on controllers (*i.e.* B-cell lymphoma 2, Bcl-2) (Nicotera & Melino, 2004). Intracellular energy (ATP) level maintained from the respiratory chain and the glycolytic process will affect the mode of the cell death (Leist, Single, Castoldi, Kühnle, & Nicotera, 1997). Overexpression of glycolytic and Krebs cycle enzymes early *post mortem* indicates great aerobic and anaerobic energy metabolism (Jia, Hildrum, Westad, Kummen, Aass, & Hollung, 2006). In addition, necrosis is considered as another mechanism of cell death, but is morphologically and biochemically different from apoptosis. Extreme conditions (*i.e.* hypoxia) will lead to rapid exhaustion of energy, extensive tissue damaging, cell lysis and necrosis (Nicotera, Leist, & Ferrando-May, 1998).

3.2.1. Mitochondrial energy production

Mitochondria are organelles of eukaryotic cells responsible for the energy production (ATP) through oxidative phosphorylation. Cell respiration occurs through three main metabolic pathways: glycolysis, Krebs cycle, and mitochondrial electron transport system. The key importance of these pathways is in energy production with wide range of other cellular functions (Fernie, Carrari, & Sweetlove, 2004).

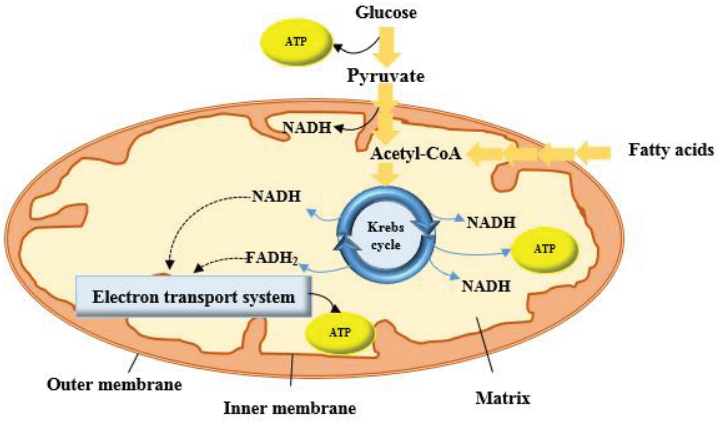

Figure 5. Mitochondrial energy production is located in the matrix.

The Krebs cycle (Fig. 6), located in the mitochondrial matrix is a chain of chemical reactions that contributes to ATP production by producing NADH. The first stage in aerobic energy production is degradation of proteins into amino acids, fats to fatty acids and carbohydrates to hexoses. Oxidized substrates are transferred through inner mitochondrial membrane (IMM) to the matrix as pyruvate, further converted into acetyl CoA (Nazaret, Heiske, Thurley, & Mazat, 2009). Energy rich molecules, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) donate electrons to the electron transport system (Huang & Lametsch), composed of respiratory complexes I-V (Fig. 7). The NADH, dominantly produced in the Krebs cycle, donate reducing equivalence to complex I (NADH dehydrogenase complex). The second molecule, $FADH_2$ generated from succinate, donates electrons to complex II

(succinate dehydrogenase). Electron transfer generates an electrochemical gradient generation across the IMM. Phosphorylation of ADP into ATP is a function of complex V (ATP-ase; Bratic & Trifunovic, 2010).

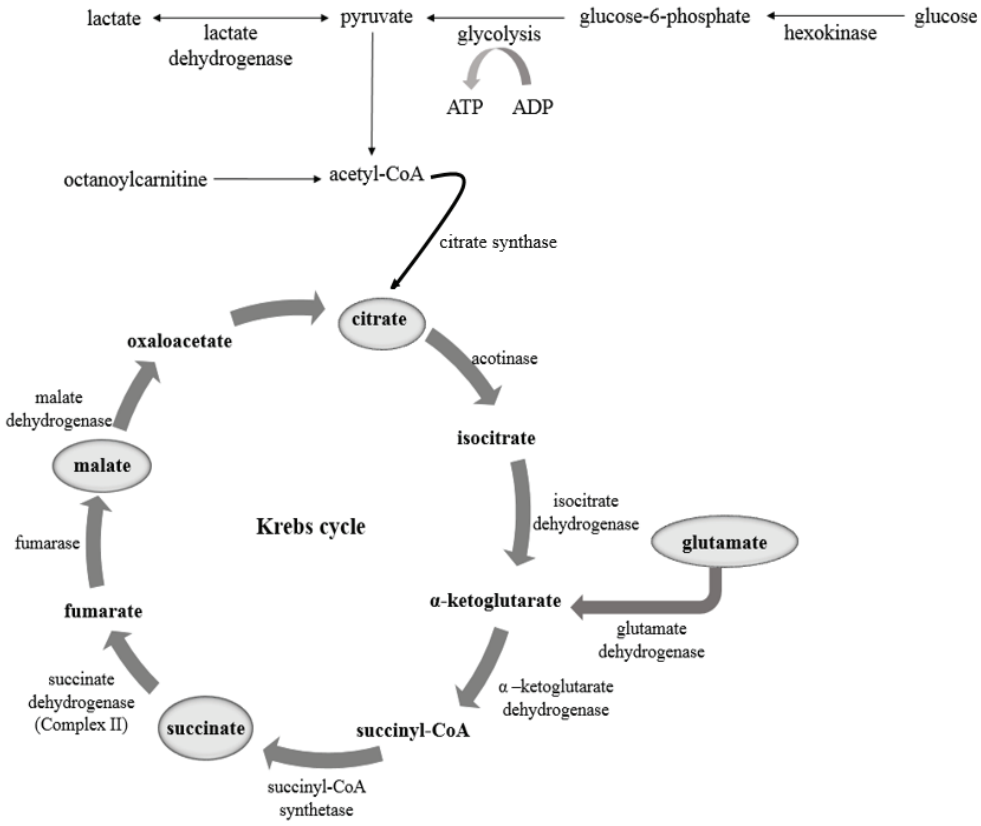

Figure 6. The sugars, fats and proteins are completely oxidized in various metabolic pathways within mitochondria. Glycolysis converts glucose into pyruvate with the production of ATP. A series of 8 biochemical reactions (Krebs cycle) convert pyruvate to high-energy molecules (NADH and $FADH_2$) that are fuelled into the electron transport system.

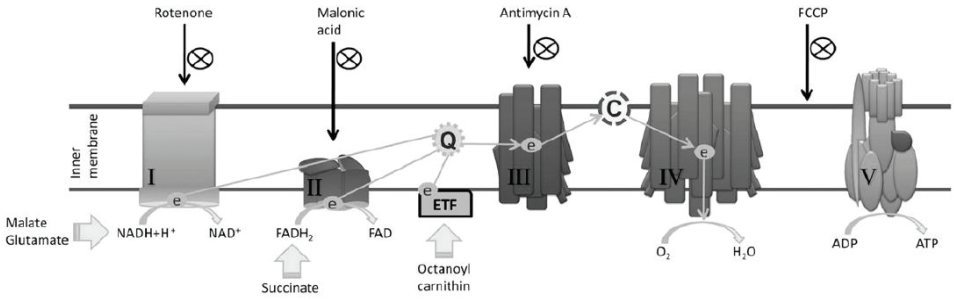

Figure 7. Four protein structures of electron transport system (ETS) involved in generation of an electrochemical gradient and the fifth protein for ATP production are located in the inner mitochondrial membrane. Mitochondrial complexes I, II, III, IV and V are marked with roman letters. Cross-circle represents some common ETS inhibitors and an uncouple FCCP, tick arrows show reducing equivalents, medium arrows show redox reactions and thin arrows show the flow of electrons. ETF – electron transferring flavoproteins; C – cytochrome C; Q − ubiquinone (Source: Phung, Khatri, Liland, Slinde, Sørheim, Almøy, et al., 2013)

3.2.2. Mitochondrial control over *post mortem* process

Mitochondria are involved in a number of cellular processes: cell cycle regulation, oxidative stress, and cell death (Sierra & Oliván, 2013). It is well known that mitochondria, during a normal respiration or reduced oxygen, produce less beneficial reactive oxygen species (ROS). The main sources of electron leak and ROS production are complex I and III (Bratic & Trifunovic, 2010). Although ROS generation is related to many sources, mitochondria produce approximately 90% of all reactive oxygen species (Balaban, Nemoto, & Finkel, 2005). Activation of enzymatic and non-enzymatic antioxidants leads to ROS detoxification that prevents mitochondrial damage and oxidative stress (Turrens, 2003). If normal mitochondrial respiration is disrupted, ROS accumulation leads to oxidative stress and oxidation of cellular proteins and lipids. Therefore, mitochondria have a pivotal role in metabolic pathway when cell death catabolism (apoptosis/necrosis) is triggered by oxidative stress (Chandra, Samali, & Orrenius, 2000; Proskuryakov, Konoplyannikov, & Gabai, 2003; Sierra & Oliván, 2013) influencing quality changes, *i.e.* flavour and tenderness. Immediately after slaughtering, the

programmed cell death (apoptosis) undergoes further morphological changes in muscle cells (Elmore, 2007).

3.2.3. Oxidative processes

Oxidative mechanisms and proteolysis are responsible both for tenderness and for flavour development (Ouali et al., 2006). The autocatalytic process in the muscle system starts immediately after death; free radicals are generated by the mitochondrial enzymatic system affecting apoptosis and complex oxidative processes. As mentioned above, mitochondrial respiration is the main source of ROS. The molecular oxygen is still consumed the first few hours *post mortem* by the mitochondrial electron transport system (Huang & Lametsch) which also forms various radicals (Ott, Gogvadze, Orrenius, & Zhivotovsky, 2007). The level of ROS will promote oxidation processes that affect flavour development and proteolysis (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). The increased accumulation of ROS provided by reduced ability of ETS to oxidize molecular oxygen immediately after slaughtering and during *post mortem* period leads to disruption of lipid membranes and damaging of mitochondrial metabolism (Ouali et al., 2006).

3.2.4. Lipid oxidation

The interaction between pre-slaughter phase conditions (stress level) and post-slaughtering factors (pH decline, chilling conditions, and electrical stimulation) will affect oxidative processes (Morrissey, Sheehy, Galvin, Kerry, & Buckley, 1998). Lipid oxidation appears to be affected by breed, muscle type, diet, age, sex, and the fatty acid composition of the meat (Kanner, 1994; Rhee, Anderson, & Sams, 1996). The polyunsaturated fatty acids (PUFAs) are the first targets of ROS and which leads to lipid oxidation.

The oxidative processes are implicated in several changes in meat quality due to lipid and protein oxidation, having both desirable and undesirable effects (Bekhit et al., 2013). Thermo-oxidation of the lipid fraction promotes formation of a range of volatile products, some desirable for meat flavour (Kanner, 1994). The undesirable effect of lipid oxidation is related to formation

of off-flavour. The number of volatile compounds generated by lipid oxidation includes hydrocarbons (alkane and alkene), carbonyls (aldehydes and ketones), alcohols, carboxylic acids and esters (Ba, Hwang, Jeong, & Touseef, 2012; Min & Ahn, 2005). Certain products may contribute to rancid flavour and odour. The interaction of lipid oxidation products with proteins compromises its stability and function (Lynch, Faustman, Silbart, Rood, & Furr, 2001).

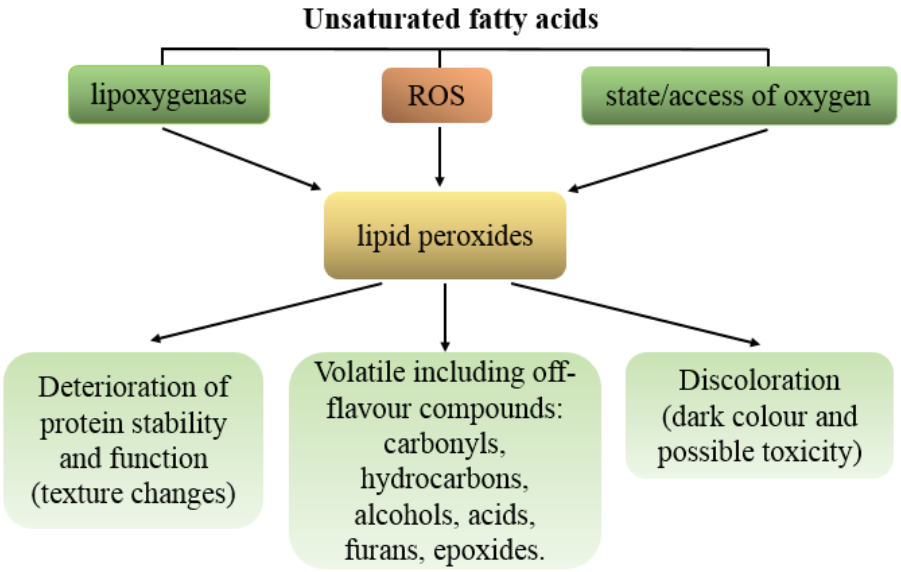

Figure 8. Schematic overview of lipid oxidation associated with undesirable changes in meat.

3.2.5. Protein oxidation

Protein oxidation in *post mortem* muscle is recognized as a significant factor for meat quality. The protein oxidation will lead to formation of carbonyl derivates, disulfide cross-links, and reduced activity of proteolytic enzymes (Bekhit et al., 2013; Huff Lonergan, Zhang, & Lonergan, 2010). In addition, these processes affect tenderization and modify metabolic pathway *post mortem*. According to Rowe, Maddock, Lonergan, & Huff-Lonergan (2004) tenderization of beef meat can be significantly reduced by increased protein oxidation during

the first 24 hours *post mortem*. The primary and secondary products of lipid oxidation promote protein oxidation and degradation. Peptides and amino acids formed by proteolysis clearly affect meat flavour, directly or by acting as precursors of flavour development (Gorbatov & Lyaskovskaya, 1980; Sentandreu, Stoeva, Aristoy, Laib, Voelter, & Toldrá, 2003). During the ageing period, the antioxidative defense of the muscle system decreases, while ROS and other oxidative species accumulate.

3.3. Factors affecting oxidative processes (tenderness/flavour) in meat

Meat flavour and tenderness biochemistry are largely influenced by *pre mortem* and *post mortem* factors. In agreement with approach "from farm to fork" each step in the production chain presents potential critical control point. Differences in oxidizing processes in a *post mortem* phase may increase due to differences in feeding regime, genetic, stress resistance, transportation, starvation, carcass manipulation, muscle type, etc.

3.3.1. Pre-mortem factors

Numerous factors, such as on-farm (nutrition, management practices, environmental conditions) and *pre mortem* factors (genetic, age, live weight), influence the development of ruminant meat traits. Production systems, observed as a multiple-factor effect, can induce differences in growth rate, fat content, fatty acid composition, and muscle energy status affecting final meat quality (Webb & Erasmus, 2013). An increased activity of animals relates to the abundance of different metabolites in a muscle system (Terlouw & Rybarczyk, 2008). Thus biochemical and oxidative processes in a muscle system are influenced by nutrient and caloric characteristics of feed (Bekhit et al., 2013; Gerhart-Hines, Rodgers, Bare, Lerin, Kim, Mostoslavsky, et al., 2007). Different environmental conditions of ruminant production may or may not improve meat quality traits. Extensive growth conditions, diet composition and feed availability, may significantly affect changes in energy metabolism, *i.e.* higher glycogen level.

Feeding systems, either based on grazing or on feeding on concentrate, are common for ruminants (Bouwman, Van der Hoek, Eickhout, & Soenario, 2005). High or low energy diet and pre-slaughtering fasting period determine the utilization of glycogen in the muscle system. McVeigh & Tarrant (1982) suggested that high-energy diet protects glycogen from fast depletion and provides higher concentration of residual glycogen in a muscle system when animals/heifers have been exposed to stress conditions. Animals fed on a high-energy diet are supposed to have higher glycogen levels which provide lower ultimate pH and more tender meat during pre-slaughter stress conditions (Devine, Graafhuis, Muir, & Chrystall, 1993). On the other hand, meat flavour can also vary with respect to the animal feeding system. Meat flavour of ruminants from pasture feeding system is often described as "pastoral" or "wild" and considered both as positive and negative attribute depending of the consumers' preferences (Sañudo, Nute, Campo, María, Baker, Sierra, et al., 1998). Lamb fed with concentrate has showed superior sensory qualities (lower intensity of undesirable flavour, stronger lamb flavour and better tenderness) than pasture-fed animals (Resconi, Campo, Furnols, Montossi, & Sañudo, 2009). In a similar study Resconi, Campo, Font i Furnols, Montossi, & Sañudo (2010) reported negative effects of high-energy diet (concentrate-fed animals) on beef flavour and meat tenderness. Differences in sensory attributes are related to various factors such as pre-slaughter handling of animals and/or post-slaughter manipulation of the carcass.

Genetics/breed is a complex source of carcass variation related to morphology and tissue characteristics. The total phenotype variation, genetic effect on beef quality traits, is approximately 5 − 30% (Warner, Greenwood, & Ferguson, 2011). In general, breed has a minor influence on sheep flavour compared to the feeding regime (Duckett & Kuber, 2001). New breeds, sometimes called synthetic breeds, may be less susceptible to pre-slaughter stress, *i.e.* during and after transportation, because they can adapt to environmental conditions (Mpakama, Chulayo, & Muchenje, 2014) if built into the breeding goals.

Live weight and age are often observed together, and greater weight implies older animals, except when the animals are exposed to feed restrictions (Guerrero et al., 2013). Sañudo, Alfonso, Sanchez, Berge, Dransfield, Zygoyiannis, et al. (2003) could not find differences in tenderness between lamb types by age alone, because neither chronological age nor

physiological maturity presents accurate predictor of lamb meat tenderness. Tough meat is often related to older and heavier lambs (Abdullah & Qudsieh, 2009) although muscle type and pH have significant effects. On the other hand, heavier carcasses showed more tender meat because of higher fat content (Landim, Castanheira, Fioravanti, Pacheco, Cardoso, Louvandini, et al., 2011; Martínez-Cerezo, Sañudo, Panea, Medel, Delfa, Sierra, et al., 2005).

Generally, greater amount of fat (or high energy diets) is associated with more tender ruminant meat. Light lamb carcasses with low subcutaneous fat thickness have much less marbling and may suffer a negative effect of chilling rate, while fatter carcasses are noticeably protected (Savell et al., 2005). Fat tissue possibly has more of an indirect effect on meat tenderness than a causative one. The effect of intramuscular fat on tenderness is generally small, but due to its link to sensory juiciness, it is very important to have some intramuscular fat (Sañudo, Enser, Campo, Nute, María, Sierra, et al., 2000).

Pre-slaughter manipulation (loading of animals, transportation, unloading in the slaughterhouse, slaughtering procedure and fasting period) of animals involves a number of stressful events. The stress activates hormonal mechanism, *i.e.* adrenaline, breakdown of glycogen and increased rate of glucose utilization in a muscle system is observed. In addition, stress hormones will induce protein catabolism, increasing the level of free amino acids (Hogan, Petherick, & Phillips, 2007). The type of diet and the level of stress animals are exposed to *pre mortem* will have strong impact on *post mortem* metabolism in muscle tissue. Daly, Young, Graafhuis, Moorhead, & Easton (1999) reported that grain fed cattle had 20% higher pre-slaughter glycogen level compared to pasture fed animals, although ultimate pH was low for both groups. In general, during the *post mortem* period, low glycogen reserves will lead to lower lactic acid content and high ultimate pH (Terlouw et al., 2005). Limited acidification (higher ultimate pH) has a profound effect on the tenderization mechanism (Hogan et al., 2007). Furthermore, Li, Wang, Mao, Zhang, Niu, Liang, et al. (2014) reported that each of the pre-slaughter factors, *i.e.* diet, pre-slaughter stress, and lairage time, can explain only 50% of variation in the ultimate pH.

3.3.2. Post-mortem factors

Electrical stimulation (ES) of a hot carcass immediately after slaughtering initiates increased pH decline and anaerobic glycolysis (Mombeni, Mombeini, Figueiredo, Siqueira, & Dias, 2013; Savell et al., 2005). The application of ES is linked to rapid *rigor mortis* and prevention of cold shortening when carcasses are cooled below 10 °C (Tornberg, 1996). Nevertheless, low voltage (and high) ES enhances tenderness due to ATP depletion and prevention of muscle contraction in cold storage (Adeyemi & Sazili, 2014). In addition, the magnitude of changes induced by ES may be affected by pre- and post-slaughtering manipulation steps and/or differences between individual carcasses and muscles (Adeyemi & Sazili, 2014).

The chilling phase is considered important in meat processing since it reduces *post mortem* glycolysis, pH decline, and microbial growth giving an extended shelf life. Phung et al. (2013) suggested a positive effect of rapid chilling on colour stability maintaining mitochondrial oxygen consumption after storage with possible negative effect on meat tenderness.

The quality of fresh meat, *i.e.* odour, texture and flavour, can be affected by storage temperature, oxygen concentration, endogenous enzymes (lipolysis and proteolysis), microbial growth, and oxidation processes (Zhou, Xu, & Liu, 2010). Packaging of fresh meat in high oxygen atmosphere (20% CO_2 and 80% O_2) accelerates lipid and protein oxidation processes involved in generation of typical off-flavours and odours (Mohamed, Jamilah, Abbas, & Rahman, 2008; Zakrys-Waliwander, O'Sullivan, Walsh, Allen, & Kerry, 2011) and increased level of protein-crosslinks with decreased tenderness (Lund, Heinonen, Baron, & Estévez, 2011; Lund, Hviid, & Skibsted, 2007), respectively. However, modified atmosphere (CO_2, N_2, CO_2/N_2) prevents microbial growth and extends the shelf life maintaining oxidation processes at lower level (Jongberg, Wen, Tørngren, & Lund, 2014; Zakrys, Hogan, O'Sullivan, Allen, & Kerry, 2008).

3.3.3. Antioxidants

Antioxidant defense system of the muscle tissue is a complex system equipped with various enzymes and antioxidants that protect cells from ROS attack. System of endogenous antioxidants prevents uncontrolled formation of free radicals and their reaction with biological structures (Chaudière & Ferrari-Iliou, 1999). Natural (exogenous) antioxidants incorporated in a muscle system, within cell membranes, through dietary delivery retard lipid and protein oxidation, preserve the colour and prevent rancid flavour development in fresh and stored meat (Descalzo & Sancho, 2008). Pasture feeding increases the level of natural antioxidants; the most powerful being tocopherols (Descalzo & Sancho, 2008). Additionally, supplementation of diet with vitamin E can improve oxidative stability, although benefits of vitamin supplementation may vary with animal species, diet and muscle type (Castillo, Pereira, Abuelo, & Hernández, 2013).

3.3.3.1. Endogenous antioxidants

In vivo mitochondria have a crucial role in metabolic processes regulating the balance between free radical generation and antioxidant defense. Naturally high antioxidant capacity of the organelle is related to several antioxidant enzymes, *i.e.* superoxide dismutase (SOD), catalase, thioredoxin reductase, peroxiredoxin, and selenium-glutathione peroxidase (GSHPx) (Masella, Di Benedetto, Varí, Filesi, & Giovannini, 2005). The superoxide dismutase enzymes in animal tissue may be present as mitochondrial matrix enzyme Mn-SOD and mitochondrial/cytosol Cu/Zn-SOD. A reactive molecule of superoxide can be converted to hydrogen peroxide by SOD and further to oxygen and water by catalase or glutathione peroxidase (GPx) (Milbury & Richer, 2008). In mammalia, the GSHPx family are involved in cellular, extracellular, phospholipid hydroperoxide, and gastrointestinal selenoproteins (Arthur, 2000). Daun & Åkesson (2004) reported significance of both soluble and total content of selenium for GSHPx activity.

Some studies have suggested a relation between endogenous antioxidant activity in a muscle system and genotype component. Hernández, Zomeño, Ariño, & Blasco (2004) reported the effect of genotype on SOD and catalase activity in pork meat, while GSHPx was not affected.

Differences of antioxidant enzymes activity appeared in different muscles depending on the post-slaughter conditions (Pastsart, De Boever, Claeys, & De Smet, 2013). The relation between antioxidative mechanism of the cell and diet has been investigated but is not fully understood. Pasture finishing diet showed positive effect on SOD activity, negative effect on GPx activity in beef meat, while catalase were not diet dependent (Mercier, Gatellier, & Renerre, 2004). Furthermore, Petron, Raes, Claeys, Lourenço, Fremaut, & De Smet (2007) reported the effect of different pastures on antioxidant enzyme activities of lamb meat, *i.e.* type of pasture affected GPx activity, but had no effect both on SOD and catalase activity. In stressed conditions cell mitochondrial antioxidant system has limiting efficiency when ROS production is enhanced (Masella et al., 2005).

3.3.3.2. Exogenous antioxidants

Diet rich in plants is a significant source of bio-active substances affecting activity of antioxidant enzymes in a muscle system (Park, Kang, Shin, & Shim, 2015). Pasture feeding is often associated with the efficient exogenous antioxidants such as vitamin E, ascorbic acid, carotenoids and flavonoids. Uptake of additional antioxidant agents as dietary supplements increase the cell protection scavenging ROS produced in stress conditions decreasing damage to mitochondria. Vitamin E (α-tocopherol) as a lipid soluble antioxidant which has s specific position in the membrane, interacts with unsaturated fatty acyl chains and molecules' mobility, providing protection of highly unsaturated fatty acids from ROS. It can directly react with molecular oxygen (O_2), as well as with oxide radicals ($O\cdot_2^-$, $\cdot OH$, $ROO\cdot$) (Machlin & Bendich, 1987). The effect of animal diet and type of plant species on vitamin E content and its prevention of damage caused by free radicals was recently reviewed by Howes, Bekhit, Burritt, & Campbell (2015). Pasture-fed animals showed higher β-carotene (pro-vitamin A) content compared to grain-fed (Descalzo & Sancho, 2008). Furthermore, Walshe, Sheehan, Delahunty, Morrissey, & Kerry (2006) found no significant difference in α-tocopherol (isomer of vitamin E), β-carotene and retinol (vitamin A) between organically and conventionally produced beef. Carotenoids and tocopherols have synergistic relationship in radical scavenging within lipid membranes. Addition of plant compounds, *i.e.* polyphenols, has been used to increase

antioxidant effect of vitamin E and suppress lipid oxidation (Gobert, Gruffat, Habeanu, Parafita, Bauchart, & Durand, 2010). Ascorbic acid and polyphenols are involved in regeneration of tocopherol molecules, maintaining antioxidant status of the cells. Ascorbic acid on the other hand can act as a pro-oxidant in the presence of Fe^{3+} and Cu^{2+}, promoting ROS generation (Descalzo & Sancho, 2008).

Antioxidant elements delivered through the diet in sufficient amounts are important for normal intracellular function of metalloenzymes. The protective role of selenium (Se) as a structural component of selenoenzymes is related to the metabolic regulation of oxidative processes (Hefnawy & Tórtora-Pérez, 2010). Antioxidant/free radical balance also involves several other trace elements, such as zinc (Zn), copper (Andrés, Cava, Mayoral, Tejeda, Morcuende, & Ruiz, 2001), iron (Fe) and magnesium (Mg). The role of Zn and Cu is essential for SOD enzymes, as previously mentioned. Many biochemical processes are related to iron, *i.e.* catalase which is involved in oxido-reduction processes, the electron transport chain and other metabolic processes (Andrieu, 2008).

3.4. Selected additives in meat industry

3.4.1. Pyruvate

The presumed antioxidant effect of pyruvate is related to its capacity to scavenge ·OH species preventing peroxidation of mitochondria, activity of metabolic enzymes, and the effect is concentration-dependent (Dobsak, Courderot-Masuyer, Zeller, Vergely, Laubriet, Assem, et al., 1999). Furthermore, pyruvate and its derivates have shown a protective role of mitochondrial function in oxidative stress conditions both *in vivo* and *in vitro* models, suppressing ROS generation and maintaining mitochondrial membrane potential (Esposito, Capasso, di Tomasso, Corona, Pellegrini, Uncini, et al., 2007; Wang, Perez, Liu, Yan, Mallet, & Yang, 2007). As a glycolytic end product and a metabolic intermediate, pyruvate can be decarboxylated by pyruvate dehydrogenase to acetyl CoA and then transported into mitochondrial matrix (Crestanello, Kamelgard, & Whitman, 1995) with NADH production. In the Krebs cycle

condensation of acetyl CoA and oxaloacetate forms citrate. Jia et al. (2006) suggested that during the *post mortem* period when oxygen is depleted, lactate dehydrogenase converts pyruvate into lactate, regenerating NADH and decreasing pH in a muscle. Addition of lactate generates pyruvate and stabilizes colour due to formation of NADH.

In the industry, pyruvate is used as an enhancer of fatty acid metabolism and a dietary supplement, which stimulates weight loss (Stanko, Reynolds, Lonchar, & Arch, 1992). Some have suggested pyruvate as an efficient additive that stabilizes meat color during storage (Ramanathan, Mancini, & Dady, 2011; Ramanathan, Mancini, Van Buiten, Suman, & Beach, 2012), while others found small, no or even a negative effect on colour (Mohan, Hunt, Barstow, Houser, & Muthukrishnan, 2010; Phung, Bjelanovic, Langsrud, Slinde, Sørheim, Isaksson, et al., 2012).

3.4.2. Citrate (citric acid; E330)

Citrate is the first intermediate in the Krebs cycle and an organic acid that creates bioactive complexes with Fe^{2+} or Fe^{3+} ions (Puntel, Nogueira, & Rocha, 2005). These complexes can act both as antioxidants and pro-oxidants in a muscle system (Ke, Huang, Decker, & Hultin, 2009). Jerez, Calkins, & Velazco (2000) suggested a positive effect of a mixture sodium citrate and sodium fluoride on beef meat tenderness, by increasing pH in pre-rigor muscle. In addition, citrate in a combination with ascorbate inhibited lipid oxidation but promoted protein oxidation in minced beef patties (Lund et al., 2007). Phung et al. (2012) suggested a positive effect of citrate on the color of minced beef, in particular when oxygen was present in the headspace of the package with meat.

3.4.3. Glutamate (glutamic acid; E620)

Glutamate participates in mitochondrial respiration through an oxidative metabolism. Glutamate conversion by glutamate dehydrogenase yields α-ketoglutarate and NADH (Frigerio, Casimir, Carobbio, & Maechler, 2008). ROS production in ETS (complex I, II, and III)

provokes glutamine catabolism (Weinberg, Hamanaka, Wheaton, Weinberg, Joseph, Lopez, et al., 2010).

Glutamate is a dietary amino acid with multiple functions: it is involved in the mitochondrial metabolism, provides umami taste, and acts as a flavour enhancer often used for dietetic purposes (Jinap & Hajeb, 2010). Naturally, glutamate presents an important taste component of vegetables, cheese, meat, and seafood (Kurihara, 2009). Glutamate also functions as neurotransmitter in the brain (Meldrum, 2000).

3.4.4. Succinate (succinic acid; E363)

Succinate is oxidized by succinate dehydrogenase (complex II) to fumarate giving FADH at the inner membrane of mitochondria and is involved in both the Krebs cycle and ETS. The stimulation of mitochondrial respiration and ATP synthesis, when complex I is inhibited, can be observed by succinate addition (Protti, Carré, Frost, Taylor, Stidwill, Rudiger, et al., 2007). Antioxidative effect of succinate is attributed to ubiquinol generation, prevention of lipid oxidation induced by Fe^{2+}, ADP, NADH, and NADPH (Puntel, Nogueira, & Rocha, 2005).

Succinate is a flavour enhancer used in ham and sausage production. As a taste active compound, succinate contributes to both sour and an umami-related taste (Chen & Zhang, 2007).

3.4.5. Malate (malic acid; E296)

Malate is a Krebs cycle intermediate that is synthesized from fumarate by the enzyme fumarase. The conversion of malate and oxaloacetate is catalyzed by malate dehydrogenase with utilization of NAD^+/NADH. Puntel et al. (2007) reported malate, oxaloacetate and citrate as effective antioxidants decreasing TBARS production.

3.4.6. Lactate (lactic acid; E270)

Lactate is formed from pyruvate by lactate dehydrogenase in the glycolytic pathway. Anaerobic conditions in *post mortem* muscle yield lactic acid from glycogen degradation, decreasing pH and promoting *rigor mortis* development. Starvation periods and the level of stress animals are exposed will affect the final concentration of lactic acid in meat (Peres, Bridi, da Silva, Andreo, Tarsitano, & Stivaletti, 2014; Pighin, Brown, Ferguson, Fisher, & Warner, 2014).

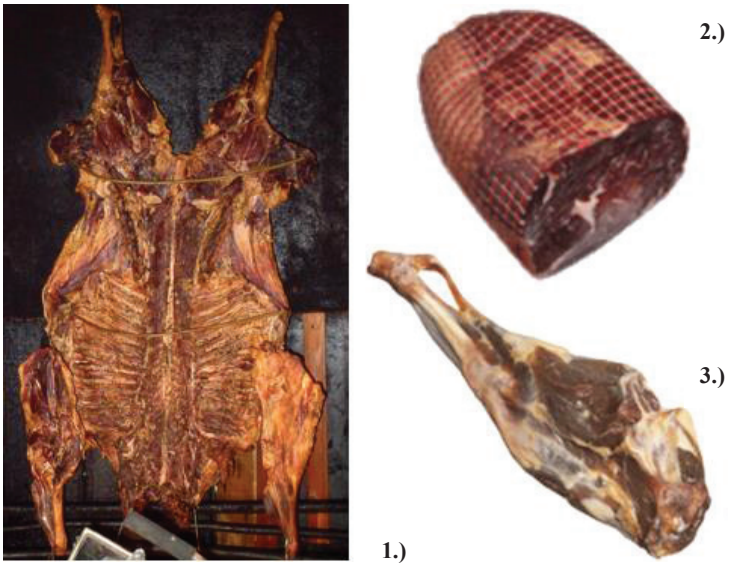

Figure 9. Traditional sheep and lamb products: 1.) smoked dry-cured sheep carcass in Bosnia and Herzegovina (typical Western Balkan sheep product); 2.) deboned, salted and dried sheep ham in Norway; and 3.) salted and dry-cured sheep ham in Norway called Fenalår (Source: Egelandsdal, Stojković, Grabež, Bjelanović, Vučić, Martinović, et al., 2014).

Lactic acid is a product of lactic acid bacteria and functions as a biopreservative of fermented meat products (Stiles, 1996). Spontaneous lactic acid bacteria growth in ham production is less pronounced due to minute amounts of carbohydrates on the surface of the green ham (Arnau, Serra, Comaposada, Gou, & Garriga, 2007). In ham production, sometimes, pure culture of

lactic acid bacteria is used to reduce pH and prevent *Clostridium botulinum* from growing on the surface (Nip, 2004). Spontaneous (natural) fermented meat products often have different sensory properties compared to fermented products under controlled conditions. The disadvantage of natural fermentation caused by "house-flora" is the lack of uniformity (Ojha, Kerry, Duffy, Beresford, & Tiwari, 2015) and it is more difficult to control safety.

3.5. Basic analytical methods in meat science and technology

3.5.1. Warner Bratzler measurements

Globally used Warner-Bratzler shear force (WBSF) value, expressed in Newtons (N), is an instrumental measure for meat tenderness. Compared to sensory analysis, WBSF is an objective, relatively fast, cheap, and gives comparable measurement, which provides insight into consumers' expectations of tenderness (Holman, Alvarenga, van de Ven, & Hopkins, 2015). A typical protocol for WBSF measurements requires: heating steaks to 71 °C (internal temperature), cooling to a constant (room) temperature, than meat is cut in pieces parallel to the fiber direction and sheared across the fiber direction on Warner-Bratzler shear head connected with Texture analyzer (our laboratory performs measurements with shear cell HDP/BSK Warner Bratzler, load cell 25 kg, TA-HDi Texture Analyser, Stable Micro Systems, Godalming, UK). Different protocols will give different results. Thus, the American ranking scale presented in Table 1 may not be relevant in other countries.

Table 1. Relationship between WBSF values and meat tenderness (Daveya, Gilberta, & Carsea, 1972)

WBSF range (N/cm^2)	**Descriptors for tenderness**
< 30	very tender to tender
30−40	marginally tender
40−50	tough
> 50	very tough

3.5.2. Meat colour

Instrumental evaluation of meat colour with a chroma meter (CR-400/410, Konica Minolta Sensing Inc., Osaka, Japan) is based on measuring CIE L*, a* and b* of the colour space. The CIE L*, a* and b* system allows colour specification in three co-ordinates (dimensions), the colour components present: L* − lightness, a* − red/green, and b* − yellow/blue, *i.e.* white/black (AMSA, 1991; CIE, 1976). Meat colour largely depends on the content and state of myoglobin; oxymyoglobin (OMb), deoxymyoglobin (DMb), and metmyoglobin (MMb).

3.5.3. Water binding/ cooking loss

An important functional characteristic of proteins is their ability to retain water in a muscle, described as drip loss. This characteristic is of huge importance for quality of fresh meat and meat products, *i.e.* juiciness and tenderness. Difference in weight between fresh and heated meat presents cooking loss as one of predictors of the sensory traits (Hopkins, Hegarty, Walker, & Pethick, 2006). Water binding is most often measured gravimetrical but low field Nuclear Magnetic Resonance (NMR) is often suggested as an option.

3.5. Statistical analysis

Analysis of variance (ANOVA)

One-way ANOVA compares the variance between groups with the variability within the group indicating whether significant differences in mean scores are present.

Principal component analysis (PCA)

Principal component analysis is a member of multidimensional factorial methods used to identify the group of variables which contribute to the specific phenomena (Pasikanti, Ho, & Chan, 2008). PCA allows simplified plot, clustering data set in different patterns. In order to

minimize dimensionality, complex data are compressed, maintaining the maximum information. The first principal component has the highest explained variance presenting the main piece of information, followed by the second principal component which represents smaller amount of information, and so forth with respect to decreasing order (Cordella, 2012).

Partial least square regression (PLS regression)

PLS regression, a technique similar to PCA, is used to correlate matrix **X** (predicted variables) to a matrix **Y** (response variables). The aim of PLS models is to predict the set of the relevant components in matrix **X** that define the maximum covariance of matrix **Y** (Abdi, 2010). PLSR approach allows quantitative modeling of **Y** (Wold, Sjöström, & Eriksson, 2001).

4. Methodology

The methods used in this thesis were employed to study tenderization mechanisms and flavour development in a muscle system. An overview of analytical methods is presented below and a detailed description is given in **Papers I−V**.

4.1. Direct volatile (headspace) analysis

The analysis of volatile compounds (VOC) in meat and meat products in normally accomplished by gas chromatography (GC) / mass spectrometry (MS). An often used analytical technique for separation and identification of VOC is the dynamic headspace/GC-MS (HS/GC-MS). This method does not require sample preparation that may affect the nature of the volatile compounds (Nam, Cordray, & Ahn, 2004). The advantage of this method is loading of many samples on an auto sampler. The four steps in volatile analysis are: (i) collection and removal of volatiles from the headspace, (ii) extracted volatiles are trapped on porous polymer, (iii) thermal desorption from adsorption trap to capillary column, (iv) separation of volatiles on GC (Kolb & Ettre, 2006), and (v) data processing.

The method was used in **Paper II** to identify volatile as lipid degradation products in minced meat with added Krebs cycle components that could lead to rancid flavour development. In **Paper V**, the applied method was aimed to detect volatile of different origin, *i.e.* lipid degradation products, smoke components, spices, etc. in dry-cured ham. In **Paper IV**, adipose tissue was subjected to HS/GC-MS, to define the influence of breed × age × diet on volatile profile. In **Paper IV** and **V** we used the untargeted method, meaning that we were not, in advance, looking for a specific compound in meat. However, in **Paper II** we used targeted approach where the concentration of few flavour compounds was investigated.

4.2. Gas chromatographic metabolite analysis following derivatization

Metabolomic research is focused on identification and quantification of small molecules (Mw 1500), *i.e.* peptides, amino acids, amines, nucleic acids, organic acids, carbohydrates, vitamins, etc. (Wishart, 2008). The complete set of molecules in a biological system is unique and influenced by the environmental conditions.

Another non-targeted study was performed on auto-sampler/GC-MS to understand the specific molecular composition which participates in characteristic lamb/sheep meat flavour. Compared to the volatile analysis above, we used derivatization to reduce polarity and provide volatility so more compounds would be detected. Derivatization reaction implies replacement of hydrogen atom in a polar group ($–NH_2$, –OH, –COOH, and –SH) by alkylation, acylation, or silylation. Typical silylation reagent N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) gives stabile derivates with higher volatility and forms more distinct MS spectra (Dettmer, Aronov, & Hammock, 2007).

In **Paper IV**, this improved metabolomic GC/MS method was used to identify intermediates and products of metabolism contributing to the sensory profile of lamb/sheep meat.

4.3. Sensory analysis

Sensory analysis of meat and meat products consider evaluation of quality characteristics such as colour, flavour and tenderness. Relationships between preferences and sensory attribute (hedonic profile) can be obtained from consumer sensory evaluation or trained sensory panel that can accurately way describe the sensory attributes. Quantitative descriptive analysis is accepted in sensory evaluation of various types of food, with line scales used to describe the intensity of specific attributes. The sensory method can be used for comparison of meat from different production systems, or to investigate effect of ingredients and processing variables on the final quality of a product. Trained panelists are calibrated for narrow differences between samples (Murray, Delahunty, & Baxter, 2001).

In **Paper IV**, sensory evaluation was used to define differences between lean meat samples of different animal groups, while in **Paper V** it was used as the instrument to define differences between traditional and industrial sheep ham.

4.4. Proteomic studies

Proteomics is an important tool in research related to animal production and meat quality. Developments in mass spectrometry and bioinformatics help proteomic studies in identification and characterization of a whole set of proteins (Bendixen, 2005) that are involved in complex molecular events, *i.e.* muscle growth, conversion of muscle into meat, meat quality variations, and effects of meat processing (Yu, Morton, Clerens, & Dyer, 2015). In the meat science area proteomic analysis provides information about protein composition, post-transitional modifications (PTMs), and expression patterns (Huang & Lametsch, 2012).

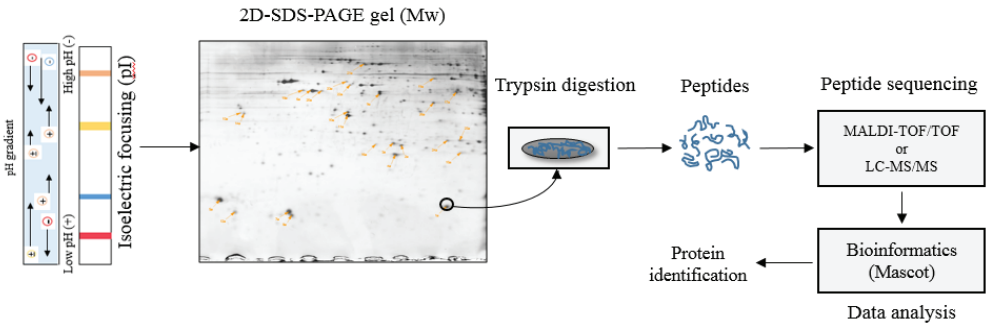

Figure 10. Scheme of two-dimensional gel-based proteomic analysis. The first two steps are: (i) first dimension isoelectric focusing (IEF) separation according to the isoelectric point; (ii) second dimension SDS-PAGE separation based on molecular size. After 2D-gel development image analysis is performed in order to determinate the spots of interest. Those spots are subjected to trypsin digestion. Eluted peptides are analyzed by mass spectrometry. Mass spectra of analyzed peptides are compared to database masses leading to protein identification.

Challenging point in proteomic studies is sample preparation, performed in order to avoid additional proteome transformation. Gel-based proteomic approach can be used for studying protein oxidation, phosphorylation, and degradation, presenting a combination of two-step protein separation (see Figure 10). 2-D gel electrophoresis has the advantage of being easier to control if a large protein appears as degraded or with its intact Mw before trypsin digestion.

In **Paper I**, a gel proteomic approach was applied to define mitochondrial protein modification and its possible role in protein structure, energy production, cell signaling and defense pathways in an early *post mortem* phase.

5. Main results and discussion

Tenderness and flavour have been identified as the most important characteristics of ruminant meat, determining consumers' acceptance of the product. The large variations in eating quality characteristics of meat are presumed to be related to complex underlying mechanisms ongoing in the *pre mortem* and early *post mortem* period.

The aim of **Paper I** was to identify the protein modification of mitochondria (mitochondria isolated ~ 2.5 h *post mortem*) and its relation with measured and predicted beef meat tenderness from oxygen consumption rate measurements 3−4 h and 3 weeks *post mortem*. Significant relations ($P < 0.05$) were found between oxygen consumption rate and Warner−Bratzler shear force measurements. High mitochondrial respiration and low residual oxygen-consuming side reactions early *post mortem* in bovine *M. semimembranosus* had a positive effect on tenderization mechanism, correlating with decreased Warner-Bratzler shear force.
Mitochondrial proteins isolated from tender (22.92 ± 2.2 N/cm^2) and tough (72.98 ± 7.2 N/cm^2) meat samples were separated on 2-dimensional gels showing significant expression difference ($P < 0.05$) in a total of 45 proteins and peptide fragments. The higher abundance of glycolytic, Krebs cycle, and electron transport system (ETS) enzymes in tender meat suggested the following hypothesis: energy supply of ETS and prolonged oxygen removal stimulated by ETS activity may reduce reactive oxygen species accumulation and prevent proteolytic inactivation in the muscle system. Furthermore, prevalent antioxidant and chaperon enzymes (*e.g.* thioredoxin; thioredoxin-dependent peroxide reductase; superoxide dismutase [Mn] and stress−70 protein) may protect proteases and ETS enzymes, and modulate cell death (apoptosis/necrosis switch) with beneficial effects on meat tenderness.

Combinations of Krebs cycle substrates used for colour stabilization were evaluated in **Paper II** whether the mixture stimulated generation of reactive oxygen species and lipid oxidation progression in minced beef meat stored in modified atmosphere packaging (MAP; 60% CO_2 and 40% N_2) and high (75% O_2 and 25% CO_2) oxygen atmosphere for the maximum storage period of 13 and 8 days, respectively. Stabilization of colour with the combination of

mitochondrial substrates was previously investigated (Phung, et al., 2012; Slinde, Bjelanovic, Langsrud, Sørheim, Phung, & Egelandsdal, 2012). MAP keeps deoxymyoglobin at a maximum level when a combination of succinate and glutamate/malate (50:50 molar ration) was used. On the other hand, in high oxygen atmosphere maximum oxymyoglobin was maintained by glutamate/malate and citrate. The level of lipid degradation was related to the level of PUFA, while the mixture of Krebs substrates affected the concentration of volatile compounds (hexanal and 2-octen-1-ol). Pyruvate showed a beneficial effect in prevention of lipid oxidation, while citrate, glutamate and malate mixture increased hexanal formation together with 2-octen-1-ol. This result is a clear indication that colour stability and lipid oxidation are not of the same kind. Although KCS increased the total of peroxides, the TBARS values were still regarded as too low to affect sensory properties of the product. Peroxide forming potential was not influenced by KCS added to MAP packaged meat, although higher amounts of KCS should be avoided due to higher hexanal/ROS formation. High oxygen atmosphere increased ROS production and volatile formation when KCS were added, but the small increase of lipid oxidation products may not be sensory relevant.

The aim of **Paper III** and **Paper IV** was to define quality characteristics of lamb/sheep meat from different production systems in order to maintain competitive advantage based on a set of unique attributes. These papers present 3 different production systems that are horizontally compared as opposed to following each production system from slaughter to dry-cured sheep products. The three different production systems were: 1.) Bosnia and Herzegovina (BH) – Vlašićka Pramenka, 2.) Montenegro (MN) – Pivska Pramenka, and 3.) Norway (NW) – Norwegian White. Ecologically and conventionally produced NW lamb meat was compared. The samples of *M. longissimus thoracis et lumborum* (LTL) and surface adipose tissue of the muscle were collected.

Paper III and **Paper IV** are largely dedicated investigating how the raw material from sheep relates to the sheep ham production in Western Balkans (Table 2). In addition, flavour and sensory difference between lamb and sheep and between ecological and conventional lamb was included. In particular, the focus on oxidation and tenderness was maintained. The comparison

between Norwegian dry-cured sheep ham and Western Balkan dry-cured sheep ham showed that most of the discriminating volatiles were in higher amounts in Western Balkan meat products. The components were from smoke, lipid degradation and derived lipid degradation products, as well as from microbial metabolites. This indicated more intense flavours in the Western Balkan products.

Table 2. Sheep meat characterization from carcass to dry-cured sheep meat (**Paper III**, **IV** and **V**)

Production system	Bosnia and Herzegovina	Montenegro	Norway
Paper III	Carcass and meat quality characteristics		
Paper IV	Volatiles of adipose tissue, metabolite and sensory profiles of meat		
Paper V	Sensory and volatile analysis		Sensory and volatiles*

* Egelandsdal, B., Stojković, S., Grabež, V., Bjelanović, M., Vučić, G., Martinović, A., Pallin, E., Marković, B., & Berg, P. (2014). In Proceedings from 60th International Congress of Meat Science and Technology, 17-22nd August 2014, Punta del Este, Uruguay.

In **Paper III**, characteristics of the carcasses (carcass weight, EU fatness and conformation, pH, cooking loss, and Warner-Bratzler shear force values) and meat quality characteristics were examined. In general, smaller carcasses of MN and BH animals and higher pH_{24h} compared to NW animals can be explained as due to variations among autochthonous and new breed, *i.e.* Norwegian White. Lower Warner-Bratzler value and better colour stability was found for BH and MN sheep. Older animals had higher heme and lower water content compared with young animals. In addition, NW animals showed higher protein, lower fat content and less tender meat.

There were no significant variations in the activity of cathepsin B among production systems (only within animal groups). Cathepsin B is an important enzyme in ham production (Toldrá, Rico, & Flores, 1993). Fatty acid composition and the oxidative stability of LTL muscle were studied. Older and fattier animal groups, *i.e.* MN animals, had higher total fat and vitamin E content. Furthermore, NW animal groups showed the lowest values for 2,2-diphenyl-1-picrylhydrazyl/total antioxidant capacity (DPPH) and higher oxidative stability. Development of lipid oxidation was studied using thiobarbituric acid reactive substances (TBARS) and

hydroperoxide value (PV) measurements. High TBARS value for MN animal group together with low vitamin E/fat ratio and high fatty acid content led to low oxidative stability in a muscle system. TBARS and PV measurements of sheep showed similar pattern: MN sheep > NW sheep > BH sheep and NW sheep > MN sheep > BH sheep, respectively. In addition, TBARS and PV were BH lamb > NW lamb. Taking into consideration quality characteristics of NW lamb meat from ecological and conventional production, the significant difference was found in a lower level of C22:6 (n-3) fatty acid in ecological lamb meat.

In **Paper IV** the variation among flavour profiles of different lamb and sheep, volatiles and metabolites isolated from adipose tissue and lean meat were examined. In addition to the flavour precursors, sensory attributes of lamb/sheep meat were evaluated. Volatile and metabolite profiles of heated adipose tissue and lean meat of NW lamb produced under ecological and conventional conditions showed significant differences in twenty-three compounds. However, no differences were found in sensory attributes among NW lamb meat from different production systems and was further observed as one animal group.

Volatiles extracted and identified from heated adipose tissue varied between animal groups, reflecting the level of accumulated diet tracers and thermal oxidation products. Principal component analysis (PCA) revealed the importance of specific volatiles of different production systems. Abundance of lipid degradation products differed the MN profile from other animal groups. Although relatively minute differences were found among NW young and old sheep volatile profiles, NW lamb had an unique profile. Thus, large differences were found among BH animals (lamb vs. sheep). Metabolite profiles and evaluated sensory attributes of BH (lamb, old sheep), MN (old sheep), and NW (lamb, young sheep, and old sheep) animals showed broad variations attributed to differences in energy metabolism of the muscle system, breed, age, and production conditions (diet and environment). Despite substantial metabolite differences, these are not necessarily useful for picking out one biomarker and using it to claim a specific sensory quality of meat originating from a specific mountain region. All identified compounds must be validated over several grazing years and preferably related to positive traits.

Paper V was designed to describe the traditional processing of sheep ham in facilities commonly used in Bosnia and Herzegovina (B&H) and Montenegro (MN) using the raw material in **Paper III** and **Paper IV**. The flavour differences between two typical sheep ham products from the two Western Balkan regions were obtained through sensory and aroma-active compound (volatile and organic acids) analysis. For better understanding of sheep ham flavour development and the effect of production process, the differences in raw material were defined. The volatile compounds were extracted from two types of sheep ham and identified using the dynamic headspace-GC/MS method. In addition, sensory profiles on final hams were measured. Volatile and sensory profiles of B&H and MN hams were defined in bi-plot applying principal component analysis. The flavour differences between two typical sheep ham products from the two Western Balkan regions were obtained through aroma-active compounds (volatile and organic acids). For better understanding of sheep ham flavour development and the effect of production process results from **Paper III** and **Paper IV** were used. Salting phase has also a strong influence on flavour development in BH ham, while on the other side, MN ham flavour was strongly affected by smoking. Organic acid content and specific volatiles identified in B&H ham suggested that fermentation occurred in the salting phase.

6. Conclusions

The mechanism of biochemical processes in a muscle system is partly modulated by mitochondrial antioxidant activity. The implication of mitochondria in mechanical properties of meat was demonstrated by oxygen consumption profiles of tender and tough beef meat. Protein expression profile defined status of mitochondria in early *post mortem* phase, the complexes I, III, IV and V most probably modulated cell death (apoptosis/necrosis switch). Furthermore, added mitochondrial substrates, Krebs cycle substrates, can stabilize colour of minced meat during storage (low and high oxygen atmosphere). The substrates support lipid oxidation to a limited extent, but below the amounts that would be detected by sensory analysis.

Lamb/sheep meat quality was defined for animals traditionally consumed in the three geographical regions. In order to define future strategies how for achieving consumers' acceptance of lamb/sheep fresh meat and meat products, sensory and flavour characteristics were studied. Quality differences between fresh sheep meat produced in two Western Balkan regions and differences between Norwegian lamb and sheep are of the specific interest. Specific processing technology applied in Bosnia and Herzegovina sheep-ham production and flavour characteristics of product have the potential to position such products in the market.

7. Future perspectives

- Studies of mitochondrial respiration and protein expression have shown the significance of oxygen removal through electron transport system (ETS) in early *post mortem* conditions to be beneficial for meat tenderness. Our study showed that complexes I, III, IV, and V are of special interest for apoptosis/necrosis switch, and the pathway of electrons formed in ETS *post mortem*. The investigation carried out here could be towards better understanding of what proteolytic enzyme systems are effected by the activity of respiratory enzymes and how different endogenous antioxidants affect this.

- The area of application (whole sample or surface) and minimum amount of Krebs cycle substrates used for colour stabilization in minced meat should be further tested in order to reduce lipid degradation and protect antioxidants. Taste characteristics of minced meat generated with minimum amount of additives should be further tested.

- The increased consumers' interest in the authenticity of the meat leads towards better characterization of lamb and sheep meat from Western Balkan and Norway. It is of the special interest to measure characteristic values for lamb and sheep meat which can be presented to the consumers based on meat attributes: geographical location, sensory properties, and nutritional value. The acceptable level of tenderness as an important sensory trait that should be explored as an asset for sale.

- From an analytical point of view, the authentication of flavour properties of lamb/sheep meat is challenging. Further studies should be focused on seasonal effect on meat quality and more precise relation between identified flavour compounds (volatiles and metabolites) and sensory attributes. Biomarkers should be validating for robustness regarding ecological and conventional meat by carrying out these types of measurements for several consecutive years. The same can be said for using biomarkers as measures of region or origin.

- The fermentation seemed to be involved in a salting phase of the B&H ham production and may be of critical importance for the safety of low-salt dry cured ham. The beneficial microbial flora in B&H ham production should be isolated and characterized, and a starter culture should be produced and marketed. It would be relevant to do consumer studies to identify how important the fermentation process is for acceptance, and also to find out to what extent the metallic flavour originating from the raw material is disliked in the dry-cured hams.

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Paper I

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Protein expression and oxygen consumption rate of early postmortem mitochondria relate to meat tenderness[1]

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ABSTRACT: Oxygen consumption rate (OCR) of muscle fibers from bovine semimembranosus muscle of 41 animals was investigated 3 to 4 h and 3 wk postmortem. Significant relations ($P < 0.05$) were found between OCR measurements and Warner–Bratzler shear force measurement. Muscles with high mitochondrial OCR after 3 to 4 h and low nonmitochondrial oxygen consumption gave more tender meat. Tender (22.92 ± 2.2 N/cm^2) and tough (72.98 ± 7.2 N/cm^2) meat samples (4 samples each), separated based on their OCR measurements, were selected for proteomic studies using mitochondria isolated approximately 2.5 h postmortem. Twenty-six differently expressed proteins ($P < 0.05$) were identified in tender meat and 19 in tough meat. In tender meat, the more prevalent antioxidant and chaperon enzymes may reduce reactive oxygen species and prolong oxygen removal by the electron transport system (ETS). Glycolytic, Krebs cycle, and ETS enzymes were also more abundant in tender meat.

Key words: apoptosis/necrosis, bovine semimembranosus muscle, mitochondria, oxygen consumption rate, proteomics, tenderness

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INTRODUCTION

Tenderness is an important aspect of meat quality and an important criterion in acceptance of the product (Huffman et al., 1996; Moloney et al., 2001). Despite extensive research, tenderness variation in beef meat is still too large and presumed to be related to metabolic processes in the early postmortem period (Lee, 1986; Klont et al., 1998; Herrera-Mendez et al., 2006; Luciano et al., 2007) but still not understood.

The conversion from muscle to meat and the subsequent tenderization process are complex phenomena related to enzymatic (proteolytic) degradation of myofibrils and weakening of structural proteins (Koohmaraie, 1994, 1996; Takahashi, 1996). Recent approaches to better understanding of meat tenderization are connected to apoptosis (controlled cell death; Ouali et al., 2007). Apoptosis is of specific interest as it involves the release of proteases (caspases) to achieve cell destruction (Fuentes-Prior and Salvesen, 2004). In addition, mitochondrial proteins are implicated in the apoptosis and caspases activation; for example, mitochondrial Bcl-2 proteins control protease release (Adams and Cory, 1998). Phung et al. (2013) found that the condition of mitochondria through early postmortem slaughter procedures (e.g., chilling rate) clearly affects meat color. Thus, mitochondria may determine changes in the tenderization pathway in postmortem muscle. However, it is not known how large this effect may be and if it can be identified in the rather variable conditions of commercial slaughterhouse.

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The aim of this study was to 1) measure the condition of the electron transport system (**ETS**) early postmortem, 2) isolate mitochondria at the earliest possible time point at a commercial slaughter plant and subject their proteins to a proteomic analysis, 3) measure tenderness and identify if tenderness could be related to the early postmortem condition of the ETS, and 4) identify if such a relationship could be explained by the presence of mitochondrial proteins.

MATERIALS AND METHODS

Sampling

Forty-one bovine semimembranosus muscles (**SM**) were collected approximately 2 h postmortem directly from the hot boning slaughter line in a commercial abattoir (Nortura SA, Rudshøgda, Norway). Each carcass was low voltage stimulated 15 to 20 min after death at 85 V, 35 s duration, 5 ms pulse duration, and 65 ms pulse pause (Carometec A/S, Herlev, Denmark). The samples were collected during 4 wk (2 animals per day) in September and October. Twenty samples were removed from the hot boning line during the first and second week (called group 1), and 21 samples were obtained in wk 3 and 4 (called group 2). The availability of a specific cut carcass was timed to suit the progress of the laboratory activities (see below). The slaughter order of a specific animal was adjusted to the sample collection. This was done in order to get a more balanced data set (regarding weight, age, and sex). Otherwise, the collected samples represented the diversity of animals that arrived in this slaughterhouse plus their slaughter routines. Selected animals were 34 ± 23 mo (mean ± SD) old, carcass weight was 285 ± 74 kg, and 19 female and 22 male animals were used. Norwegian meat production is dominated by dual-purpose Norwegian Red cattle (75.2%) and crossbreeds with Norwegian Red cattle (14.3%). The rest are several common breeds selected for meat or milk production. This is reflected in our animal choice. To the best of our knowledge, the animal selection is representative of Norway's meat production.

Muscle pH was measured 4 h postmortem ($\mathbf{pH_{4h}}$) and 24 h postmortem using a portable Knick Portamess 913 pH meter (Knick, Berlin, Germany). The temperature on the surface of the proximal end of the SM at 3 to 4 h postmortem was recorded using Ebro TLC 1598 (Ebro Electronic GmbH & Co., Ingolstadt, Germany). Slices from the hot-boned SM were individually vacuum packed in polyamide bags (type PA/PE, 30–40 cm^3/m oxygen; LogiCon Nordic A/S, Kolding, Denmark) and stored overnight at 10°C as an extra precaution to prevent cold shortening and subsequently at 4°C for 3 wk before Warner–Bratzler shear force (**WBSF**) measurements. For mitochondria isolation, tissue permeabilization, and oxygraph measurements, a neighboring slice to the slice for WBSF was used. The samples were taken from the middle of the proximal side of the SM.

Warner–Bratzler Shear Force Measurements

After chill storage, the vacuum-packed samples were cooked in a water bath until they reached an internal temperature of 70°C. Temperature measurements were performed by injecting a temperature logger (EB 1-2t-313; Ebro Electronic GmbH & Co.) in a dummy sample. Cooked samples were kept in an ice bath until the temperature reached approximately 20°C and stored vacuum packed at –40°C. For WBSF measurements, samples were thawed at 4°C overnight and subsequently allowed to reach room temperature. The WBSF was measured in 10 pieces. Warner–Bratzler shear force was performed on 1 by 1 by 4 cm piece of each sample cut parallel to the muscle fibers using a texture analyzer (HDP/BSK knife blade, 25 kg load cell, and 4 cm/min cross head speed; TA-HDi Texture Analyser; Stabile Micro Systems, Godalming, UK).

Preparation of Fibers for Oxygen Consumption Measurements

The muscle fibers were permeabilized 2.5 to 3.5 h postmortem prior to oxygen consumption rate (**OCR**) measurements. The samples of muscle were placed in a relaxing solution containing 15 m*M* phosphocreatine, 10 m*M* Ca–ethylene glycol tetraacetic acid (**EGTA**; 0.1 m*M* free calcium), 20 m*M* imidazole, 20 m*M* taurine, 6.6 m*M* $MgCl_2$, 50 m*M* K-2-(*N*-morpholino) ethanesulfonic acid, 0.5 m*M* dithiothreitol (**DTT**), and 5.8 m*M* ATP adjusted to pH 7.1. Small fiber bundles were separated to an approximate length of 5.0 mm with a diameter of approximately 1.0 mm, weighed, and permeabilized with 0.052 mg/mL of saponin for 30 min (Sperl et al., 1997). The fiber bundles were subsequently washed for 10 min at 4°C in the respiration medium (see OCR measurements below).

Oxygen Consumption Rate

Oxygen consumption rate measurements were carried out at both 3 to 4 h postmortem ($\mathbf{OCR_{3–4\ h}}$) and after 3 wk chilled storage ($\mathbf{OCR_{3\ wk}}$). All chemicals were of analytical grade and purchased from Sigma Chemicals Corp. (St. Louis, MO), with the exception of pyruvate (Applichem; VWR International AS, Oslo, Norway). High-resolution respirometry was carried out with Oroboros Oxygraph-2K instruments (Oroboros Instruments, Innsbruck, Austria) as

described by Gnaiger (2001). The OCR of permeabilized tissue was measured at 20°C and approximately 200 μ*M* O2. The closed chambers had a volume of 2.1 mL, and results were reported as (picomoles O_2/second)/milligram protein. Injection of substrates was done with a Hamilton syringe of 10 to 50 μL.

The OCR measurements were carried out on permeabilized muscle fibers by stimulating mitochondrial enzyme complexes in a sequence. Adding chemicals is possible as the responses are additive and incremental, so that no response is masked by the previous chemical added. A similar protocol was used by Scheibye-Knudsen and Quistorff (2009). Complex I respiration was initiated by malate and glutamate followed by testing β-oxidation using octanoylcarnitine. Beta-oxidation donates electrons to an electron-transferring flavoprotein on the mitochondrial inner membrane. Oxidative phosphorylation was stimulated by the addition of ADP. Complex II respiration was tested by succinate. Uncoupling was measured by carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (**FCCP**) addition. At this point, rotenone addition blocked complex I and revealed the maximum capacity of complex II. Inhibition of complex II was achieved by adding malonic acid. Finally, inhibition of complex III was achieved with antimycin A. Background oxygen consumption after complete inhibition of the ETS using antimycin A (Gnaiger, 2008) was recognized as a residual oxygen-consuming side reaction (**ROX**). Background adjustment was done for all OCR responses. The respiration medium consisted of 0.5 m*M* EGTA, 3 m*M* $MgCl_2$, 60 m*M* potassium methanesulfonate, 20 m*M* taurine, 10 m*M* KH_2PO_4 (Calbiochem, Darmstadt, Germany), 20 m*M* HEPES, 110 m*M* sucrose (Alfa Aesar, Karlsruhe, Germany), and 1.0 g/L BSA (Pesta and Gnaiger, 2012). The respiration medium had pH 7.1. For more details, see Phung et al. (2013).

Mitochondria Isolation

Mitochondria were isolated from 10 g fresh SM (muscle center at proximal end) after fat and connective tissue had been removed. The isolation was initiated approximately 2.5 h postmortem. Briefly, the muscle was washed twice with phosphate buffer containing 5 m*M* K_2HPO_4, 2 m*M* KH_2PO_4, and 0.25 *M* sucrose supplemented with 10 m*M* EDTA at pH 6.0. Meat was incubated in 10 m*M* PBS, 10 m*M* EDTA (pH 7.0), and 0.05% trypsin on ice for 30 min. After 10 mg/mL of albumin was added, the samples were mixed and incubated for 5 min. Minced meat was homogenized in Teflon-glass Potter Elvehjem homogenizer (at 400 rpm; 10 mL). The homogenized meat was filtrated twice through medical gauze with 10 mL of extraction buffer (100 m*M* 3-(*N*-morpholino)propansulfonic acid [pH 7.5], 550 m*M* KCl, and 5 m*M* EGTA). To isolate mitochondria, filtrate and supernatant were centrifuged (CT15RE, VWR International AS, Oslo, Norway by Hitachi Koki Co. Ltd.) at 600 × *g* for 5 min at 4°C and 11,000 × *g* for 10 min at 4°C. Finally, the pellets were frozen in liquid nitrogen and stored at –80°C for protein analysis. All chemicals used for mitochondria isolation were of analytical grade and purchased from Sigma Chemicals Corp.

The method above was initially compared with the Mitochondria Isolation Kit (MITOISO1 Sigma; Sigma-Aldrich, St. Louis, Mo.). Although the time efficiency of this kit was satisfactory, the yield was too low. The presented isolation method was selected based on the principles of the tested kit and other commercial isolation kits as well as on our in-house laboratory routine (Phung et al., 2011). The isolation procedure for mitochondria was finally selected based on yield, measured respiratory profile, and protein concentration analysis.

Two-Dimensional Electrophoresis (Isoelectric Focusing and Two-Dimensional SDS-PAGE)

Protein concentration was measured for the frozen and thawed mitochondria based on total protein yield by colorimetric RC DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) at 760 nm in a SHIMADZU UV-1800 Spectrophotometer with UVProbe software version 2.33 (Shimadzu Corp., Kyoto, Japan) using BSA as protein standard. No significant difference was found in the concentration of mitochondrial proteins between tender (**Te**) and tough (**To**) samples. Seventy-five micrograms (analytical gels) and 400 μg (preparative gels) of mitochondrial pellets were diluted in rehydration solution (8 *M* urea, 2% [wt/vol] 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 2 *M* thiourea, and 50 m*M* DTT) with immobilized pH gradient (**IPG**) buffer (GE Healthcare Bio-Sciences, Little Chalfont, UK) and 0.5% bromophenol blue (**BPB**). Samples (final volume of 450 μL) were applied onto the isoelectric focusing cell system (Bio-Rad Laboratories, Inc.) with IPG strips (pH 4–7; 24 cm; Bio-Rad, Sundbyberg, Sweden) for passive rehydration during 16 h under mineral oil. Protein focusing was performed at approximately 20°C under the following conditions: 30 min of linear ramp to 250 V, 1 h rapid ramp to 500 V, 1 h to 1,000 V, and from 10,000 V to 70,000 V for the last 6 h. Strips were stored at –40°C until the next step in analysis.

Reduction of IPG was performed for 15 min in equilibration buffer (6 *M* urea, 50 m*M* Tris-HCl [pH 8.8], 30% [vol/L] glycerol, 2% [wt/vol] SDS, and 1% [wt/vol] DTT) followed by 15 min alkylation in equilibration buffer with 2.5% iodoacetamide (**IAA**). Proteins

were separated in the second dimension on 12.5% SDS-PAGE gel at 10°C at 5 mA/gel for 3 h and then 15 mA/gel until BPB reached the bottom of gel using an Ettan DALTtwelve system (GE Healthcare, Uppsala, Sweden). Preparative gels were stained according to Shevchenko et al. (1996) and analytical according to Görg et al. (2007; modification of Blum et al., 1987).

Gel Image Analysis

Analytical gels were produced in triplicate and scanned using an Epson Perfection 4990 Photo scanner (16-bit grayscale and 400 dots per inch; Epson, Long Beach, CA). Image analysis of 24 gels (3 technical replicates × 8 biological samples) was carried out with Delta2D (DECODON, Greifswald, Germany). Gel images were automatically warped and manually refined to correctly align spots. The Te sample with the lowest WBSF value (19.9 N/cm^2) presented the reference gel. As a measure of relative protein abundance, spot volumes for each spot were normalized by dividing each spot's volume by the sum of volumes of all valid protein spots in a gel. Classical student *t*-test (available in Delta2D) was used to identify expression differences between 2 groups of samples at the 5% significance level; 4 Te samples were considered one group and 4 To samples were considered the second. The protein percentage differences, used to establish differences among proteins identified both in Te and To samples, were calculated for average mean protein value for Te vs. To.

Protein Preparation for Mass Spectrometry

Pieces of gels (approximately 1 mm) were individually washed for 15 min with 100 µL 50% acetonitrile (**ACN**) and 50 m*M* NH_4HCO_3 at room temperature with shaking. The solution was discarded and gel pieces dehydrated in 200 µL 100% ACN for 15 min. After removing the solution, the gel pieces were dehydrated in a fume hood for 15 min. In addition, the gel pieces were incubated in the reduction solution (0.1 m*M* NH_4HCO_3 and 10 m*M* DTT) for 30 min at 56°C. After removing all liquid, incubation with alkylation solution (0.1 m*M* NH_4HCO_3 and 55 m*M* IAA) was continued in the dark for 30 min at room temperature and then with 100% ACN for 15 min. The solution was removed, and the gel pieces were dried in a fume hood for 15 min and afterwards transferred to ice for trypsin digestion (10% ACN, 25 m*M* NH_4HCO_3, and 10 ng/mL modified porcine trypsin; ProMega Corp., Fitchburg, WI) for 30 min. Surplus liquid was removed and a solution of 10 m*M* NH_4HCO_3 and 10% ACN was added. Proteins were digested for 12 to 16 h at 37°C. Centrifuged (600 × g during 15 s at room temperature) samples (Sorvall RC 5-5C; Thermo Scientific, Asheville, NC) were mixed with 10 and 0.1% trifluoracetic acid (**TFA**) and sonicated for 10 min. Peptides were desalted on GELoader tips (20 µL; Eppendorf, Sigma-Aldrich, Schnelldorf, Germany) packed with C18 extraction material (3M Empore C18 extraction disk, 3M Company, St. Paul, MN; Varian, Neuss, Germany; Rappsilber et al., 2003), eluted in 70% ACN/0.03% TFA, and dried for liquid chromatography–tandem mass spectrometry (**LC-MS/MS**) or applied directly to a matrix-assisted laser desorption/ionization target plate (Bruker Daltonics, Bremen, Germany) with matrix solution (50% [**ACN**] and 0.1% TFA saturated with α-cyano-4-hydroxycinnamic acid mixed 1:1 with 100% ACN).

Protein Identification by Matrix-Assisted Laser Desorption/Ionization–Tandem Time-of-Flight and Liquid Chromatography–Tandem Mass Spectrometry

Mass spectra and tandem mass spectra were recorded using an Ultraflex MALDI-TOF/TOF (Bruker Daltonics) in reflection mode. External calibration was performed using a standard peptide mixture (Bruker Daltonics) prepared in the same matrix solution as the samples. Peak lists were generated using the FlexAnalysis software (version 2.4; Bruker Daltonics) and used in Mascot (www.matrixscience.com) searches against the NCBInr database (www.ncbi.nlm.nih.gov/Database/), taxonomy *Other Mammalia*. Other search parameters were 100 mg/L mass tolerance, 1 trypsin miscleavage, fixed carbamidomethylation of cysteine, and variable methionine oxidation. Protein identifications based on peptide mass fingerprinting were confirmed by MS/MS analysis on at least 2 most intense ion peaks in the MS spectra.

Proteins with low abundance were identified using LC-MS/MS. The dried peptides were dissolved in loading solution (0.05% TFA and 2% ACN in water), loaded onto a trap column (Acclaim PepMap100, C18, 5 µm, 100 nm, 300 µm i.d. by 5 mm; Thermo Fisher Scientific, SE-126 26 Hägersten, Sweden), and then flushed back onto a 50 cm × 75 µm analytical column (Acclaim PepMap RSLC C18, 2 µm, 10 nm, 75 µm i.d. × 50 cm, nanoViper; Thermo Fisher Scientific, SE-126 26 Hägersten, Sweden). The gradient profile used for peptide separation was from 4 to 40% solution B (80% CAN and 0.1% formic acid) for 45 min at a flow rate of 300 nL/min. The Q-Exactive mass spectrometer (Thermo Fisher Scientific, SE-126 26 Hägersten, Sweden) was set up as follows (Top5 method): a full scan (300−1600 m/z) at R = 70.000 was followed by (up to) 5 MS2 scans at R = 35000, using a normalized collision energy setting of 28. Singly charged precursors were excluded for MS/MS, as were precursors with $z > 5$. Dynamic exclusion was set to 30 seconds.

Raw files were converted to Mascot generic format (.mgf) using the msconvert module of ProteoWizard (http://proteowizard.sourceforge.net) and compared to the SwissProt database (taxonomy *Other Mammalia*; www.ebi.ac.uk/uniprot) on an in-house Mascot (version 2.4; www.matrixscience.com) server. The selected parameters were 1) 10 mg/L/(3.34 x 10-29 kg) tolerance for MS and MS/MS, respectively; 2) up to 2 missed cleavages; and 3) fixed cysteine carbamidomethylation and variable methionine oxidation. The interpretation of MS/MS results was based on the following criteria: 1) protein sequence coverage and 2) isoelectric point (**pI**) and molecular weight (**Mw**) of the identified protein vs. spot position in a gel.

Statistical Analysis

The study consisted of a total of 164 $OCR_{3–4\ h}$ and $OCR_{3\ wk}$ (2 times × 2 replicates × 41 animals). Minitab (version 16 from Minitab Inc., State Collage, PA) was used for *t*-test ($P < 0.05$), 1-way ANOVA, and univariate regression analysis.

The relation between OCR measurements and WBSF peak values was calculated using partial least squares (**PLS**) regression (Unscrambler X 10.1; CAMO, Trondheim, Norway) with full cross-validation (leave out 1 sample) on centered data. The OCR responses were used as **X** variables and the WBSF peak values were responses (**Y**) in the multivariate regression. The PLS analysis actively connects the **X** and **Y** data matrices directly. Partial least squares models are interpreted through PLS loadings that expresses the relationship between the original **X** matrix and PLS scores. A multiple regression will be as follows: $\mathbf{Y} = \mathbf{XW} \times \mathbf{C}' + \mathbf{F}$; the multiple regression coefficients, again a matrix, are $\mathbf{B} = \mathbf{W} \times \mathbf{C}'$. **W** is a weight matrix for predictors (**X**) and **C′** presents a weight matrix for responses (Wold et al., 2001). **F** defines the matrix of residuals. To investigate the quality of the multivariate model for WBSF measurements based on OCR response, (y_i) can be plotted and inspected. The regression coefficient (b_k) of a given **X** variable (here the steps in the OCR measurements) provides some information regarding their relative importance. Partial least squares regression is particularly well suited when there is multicolinearity among **X** (Unscrambler X 10.1; CAMO).

RESULTS AND DISCUSSION

The Relation between Oxygen Consumption Rate and Warner–Bratzler Shear Force

The samples were collected over 4 wk in a commercial slaughterhouse. Partial least squares regression with full cross-validation was performed to investigate the relation between quality variables and OCR measurements. Using all samples (1–41) provided weak models to OCR (Table 1). The samples from group 2 (samples 21–41) provided better models. This was predominantly due to 2 outlier samples (sample 4 and sample 19) collected during the first and second weeks.

Statistics of the WBSF measurements (N/cm^2) are presented in Table 2. There were apparently less robust models between OCR and WBSF values for the group 1 (samples 1–20) collected. But these 2 sample groups were statistically similar, with the exception of the internal temperature and $pH_{4\ h}$, when the samples were acquired at the hot boning line (Table 2). Warner–Bratzler shear values had close to the same range and the same mean values for the samples collected the first 2 wk versus the latter 2 wk. A lower overall mitochondrial oxygen consumption for fresh permeabilized fibers was measured during the earlier sample collection period for group 1 (not shown). The first sample group (1–20) also had relatively less changes in OCR with time compared to the sample group 21–41 (not shown).

The difference between group 1 and 2, regarding relationship between OCR and WBSF, cannot be explained beyond possibly being caused by slower chilling (Table 2). The reason might also be because of larger analytical errors in OCR, especially during the first collection week.

Response on succinate was nominally greatest for all samples in the second group (21–41; Table 3). After 3 wk of storage, the activity of complex II was functionally the most important (21–41; Table 3), in agreement with Phung et al. (2013). Average ROX (21–41; Table 3) underwent no significant change with time (after 3–4 h the absolute values were between 0.6 and 2.4 (pmol O_2/s)/mg protein) for both groups.

The nominally highest correlation ($r = 0.75$) was found between WBSF values and the difference in $OCR_{3–4\ h}$ and $OCR_{3\ wk}$ (group 2; Table 1). A comparable high value was also found when OCR3–4 h (Table 1) was regressed to WBSF. This supports the interpretation that it was the early postmortem measurements that were the most important for the relation between OCR measurements and WBSF values. Figure 1 shows a clear differentiation in samples within the second animal group using the relation between their OCR profile and WBSF values with the greatest correlations. The poorer models (Table 1) showed little influence of ADP addition (Fig. 2), but to encompass all samples optimally, response to ADP addition had to be included. Furthermore, only Te samples were found with high response to ADP addition whereas low response to ADP addition was associated to a wide variation in tenderness.

Table 1. Partial least squares regression relationship between Warner–Bratzler shear force (WBSF) and oxygen consumption rate (OCR; (pmol O_2/s)/mg protein) expressed as regression coefficients (r) between predicted and measured WBSF values[1]

Samples	Variables	$OCR_{3-4\ h}$[2]	$OCR_{3\ wk}$[3]	$OCR_{3-4\ h} - OCR_{3\ wk}$
1–41[4]	WBSF	0.38 (2)*	0.42 (2)**	0.40 (2)*
21–41	WBSF	0.72 (3)***	0.43 (3)**	0.75 (6) ***

[1]The number of principal components obtained from partial least squares regression with full cross-validation is in parentheses.

[2]$OCR_{3-4\ h}$ = OCR measurement at 3 to 4 h postmortem.

[3]$OCR_{3\ wk}$ = OCR measurement after 3 wk chilled storage.

[4]Two samples were removed, no. 4 and no. 19, due to a very high residual variance of OCR values.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

The regression coefficients in PLS regression increase with the number of factors (see from 3 to 6 principal components; Fig. 2). This is typical of a method such as PLS, which attempts first to reduce the magnitudes of the regression coefficients. But as the number of PLS factors increases, the magnitudes of the regression coefficient will typically increase and they will become more comparable to those obtained in ordinary regression (De Jong, 1995; Goutis, 1996; Krämer, 2007).

The substrates shown in Fig. 2 were additives (except ROX) used in OCR measurements. The data set gave significant univariate relationships (animals 1–41, 2 outlier samples removed, and no validation) to ADP, glutamate, and octanoylcarnitine addition ($P = 0.029$, $P = 0.024$, and $P = 0.044$, respectively). In order to reach better correlation between WBSF values and OCR variables (Table 1), the additional protocol additives given in Fig. 2 (e.g., succinate, FCCP) needed to be included in a multivariate regression model. Complete separation of the tougher samples from the more tender samples required an adjustment towards relatively more weighting of the response to ADP addition (Fig. 2).

High WBSF peak values were inversely correlated to the response of succinate and FCCP addition and positively correlated to ROX (Fig. 2). Residual oxygen-consuming side reactions may possibly have an effect on oxidation of mitochondrial enzymes. However, because the variation in ROX was small, its relatively high β coefficient may not add much explanation. The OCR data explained up to 56% (from r^2; Table 1) of the variance in WBSF peak values. Furthermore, following full cross-validation, this high value for explained variance was not always achieved (Table 1). A more robust estimate is that OCR data can explain approximately 20% (from r^2) of the variation in WBSF values when samples were collected in a slaughterhouse operating under commercial conditions.

Only the sample group (21–41) with the higher initial mitochondrial enzyme activities and the better correlation to WBSF values was considered of interest for proteomic analysis on early postmortem isolated mitochondria (see below).

Proteomic Results for Tender vs. Tough Meat Samples

A subgroup of 8 animals (obtained from the second animal group, 21–41) was picked randomly for proteomic analysis, 4 with low (Te) and 4 with high (To) WBSF values. Tender samples belonged to 14- to 39-mo-old animals, carcass weight was 278 ± 46 kg (mean ± SD), and all belonged to Norwegian Red cattle. Tough samples were obtained from animals 14 to 45 mo old, with 189 ± 39 kg carcass weight, and 3 were Norwegian Red cattle and 1 was a Hereford. Selected samples belonged to 2 different groups that maximized differences in oxygen consumption properties and their relationship with WBSF (Fig. 1). However, no other variable was given any attention. Further analysis (t-test) revealed no difference between the 2 breeds (Norwegian Red cattle and Hereford) in the To group and breed differences were not discussed further.

Mitochondrial proteins isolated from Te and To samples were separated on 2-dimensional gels. Differences between spot intensities of Te and To sample groups were tested by Student t-test ($P < 0.05$) and 476 spots were identified. One Te sample had protein spots closer, as revealed from the statistical test, to a To sample than to the other 3 Te samples. At the

Table 2. Quality variables for semimembranosus muscle samples[1]

	$T_{3-4\ h}$,[2] °C	$pH_{4\ h}$[3]	$pH_{24\ h}$[4]	WBSF,[5] N/cm^2
Animal group	Mean ± SD			Minimum – maximum (mean ± SD)
1–20	33.1 ± 2.9	6.25 ± 0.23	5.56 ± 0.10	17.7–83.0 (40.2 ± 16.9)
21–41	29.9 ± 2.3***	6.49 ± 0.23***	5.54 ± 0.08	23.0–86.1 (40.6 ± 18.2)

[1]± standard deviation of the mean.

[2]$T_{3-4\ h}$ = temperature on the surface of the proximal end of the semimembranosus muscle at 3 to 4 h postmortem.

[3]$pH_{4\ h}$ = muscle pH at 4 h postmortem.

[4]$pH_{24\ h}$ = muscle pH at 24 h postmortem.

[5]WBSF = Warner–Bratzler shear force.

***$P < 0.001$ for values in different rows.

Table 3. Initial OCR measurements at 3 to 4 h postmortem ($OCR_{3–4\ h}$; (pmol O_2/s)/mg protein) and changes in permeabilized fibers from 3 to 4 h to 3 wk (fresh stored) for samples 21 through 41[1]

Animal group	Malate	Glutamate	ADP	OctanoylC[2]	Succinate	FCCP[3]	Rotenone	ROX[4]
$OCR_{3–4\ h}$	–0.73 ± 0.07[2]	3.35 ± 0.42	3.33 ± 0.40	1.43 ± 0.19	5.89 ± 0.62	5.79 ± 0.60	5.83 ± 0.73	1.23 ± 0.08
$OCR_{3–4\ h} – OCR_{3\ wk}$[5]	0.21 ± 0.12	3.49 ± 0.40	3.55 ± 0.40	1.69 ± 0.21	4.17 ± 0.60	4.17 ± 0.76	4.17 ± 0.61	0.09 ± 0.07

[1]± SEM.

[2]OctanoylC = octanoylcarnitine.

[3]FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.

[4]ROX = residual oxygen-consuming side reactions.

[5]$OCR_{3\ wk}$ = OCR measurement after 3 wk chilled storage

significance level ($P < 0.05$), the number of differently expressed proteins in the groups (Fig. 1) was reduced to 45. Identified proteins and peptide fragments (Fig. 3a and 3b) were classified according to cell function (Table 4). Theoretical Mw of the proteins were from 10 to 200 kDa with pI range between 4 and 9. Variation in experimental Mw and/or pI compared to theoretical values may occur due to posttransitional modification and protein degradation (proteolysis), indicating presence of fragments or isoforms of the parent protein.

Out of the 45 differently expressed protein spots, 12 were identified as structural proteins and were present in both groups of samples. The cytoskeletal proteins in To samples (actin alpha skeletal muscle) showed greater Mw than those in Te meat counterparts (apparent Mw were 50 and 44 kDa, respectively; Tables 4 and 5) as previously reported by Laville et al. (2009).

Effect of Mitochondria Isolation

A critical point in the methodology of mitochondria isolation is cocentrifugation of cytoskeletal proteins. These proteins have close connections with mitochondria in skeletal muscle architecture, and detection of these tissue-specific proteins in mitochondria pellets is acceptable (Taylor et al., 2003; Forner et al., 2006; Egan et al., 2011). In general, mitochondria are difficult organelles to isolate from the muscle system (O'Connell and Ohlendieck, 2009) and the presence of 44% mitochondrial proteins out of the 45 identified proteins as significantly different among Te and To samples (Table 4) was considered acceptable for a slaughter-line isolation method. Identified cytoskeletal proteins/fragments were either attached to mitochondria after treatment, precipitated with mitochondrial proteins upon trypsin exposure, or sedimented as myofibrillar fragments.

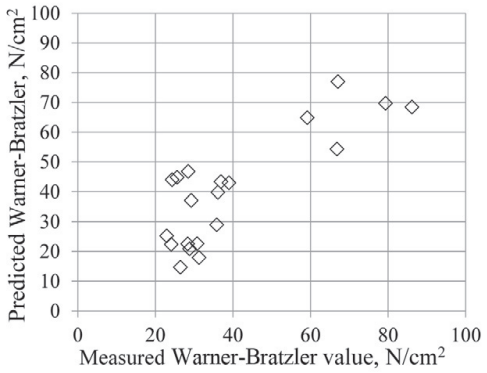

Figure 1. The figure shows the relation between measured and predicted Warner–Bratzler shear force (WBSF) peak values; the latter were obtained from the fully cross-validated partial least squares regression model between WBSF peak values (response) and the changes in oxygen consumption rate from 3 to 4 h to 3 wk as induced by protocol additives for the second group of animals (21–41).

Early Postmortem Effects of Electron Transport System

High response to succinate addition gave more Te samples (Fig. 2), suggesting prevention of proteases oxidation by mitochondrial respiration. The ETS activity is regarded as beneficial (in vivo), although it loses reactive oxygen species (**ROS**) when oxygen is reduced to water that have signaling role triggering

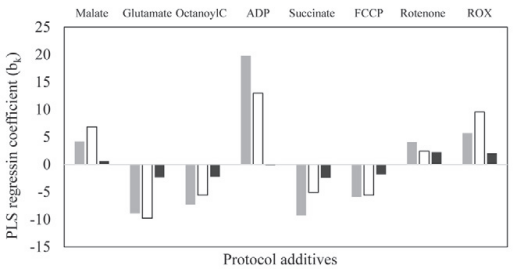

Figure 2. Regression coefficients (b_k; dimension $(N/m^2)/(pmolO_2/s)/mg$ protein)) after 6 partial least squares (PLS; grey bars), 7 PLS (white bars; **X** variables weighted with 1/SD), and 3 PLS (black bars) components obtained from the partial least squares regression. Regression models were between Warner–Bratzler shear force peak values and the changes in oxygen consumption rate from 3 to 4 h to 3 wk for each protocol additive (k) of second animal group (21–41). OctanoylC = octanoylcarnitine; FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; ROX = residual oxygen-consuming side reactions.

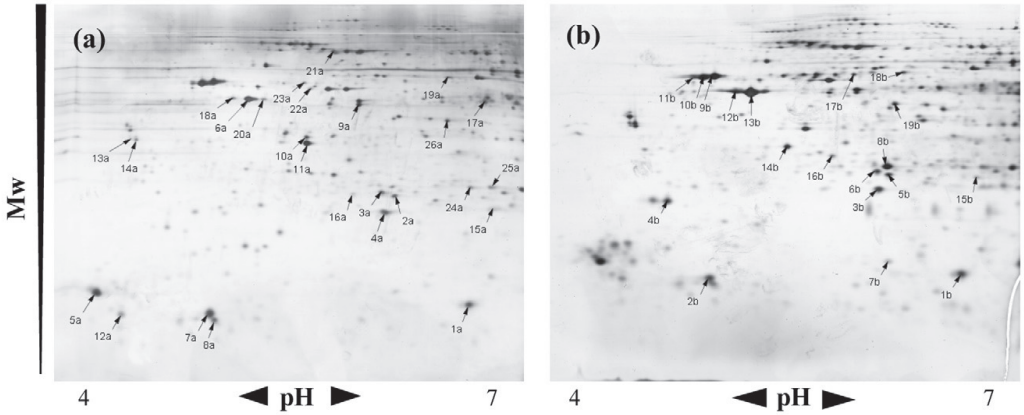

Figure 3. Silver stained 2-dimensional gels of mitochondrial proteins isolated from semimembranosus muscle of Norwegian Red cattle early postmortem. Protein spots marked with arrows were differently expressed in tender (a; Warner–Bratzler shear force [WBSF] = 19.9 N/cm^2) and tough (b; WBSF = 80.6 N/cm^2) samples (see also Table 4). Mw = molecular weight.

the apoptotic mechanism. We proposed that in vitro–activated antioxidants prevented ROS accumulation by prolonging ETS activity and protecting mitochondrial function. Positive correlation was found for octanoylcarnitine addition (Fig. 2) and more abundant Krebs enzymes (Table 4) in Te samples.

In To samples, mitochondrial respiration may also cease to remove O_2 earlier (lose electrochemical gradient across inner mitochondrial membrane), inducing a high concentration of O_2 in mitochondria and giving protein and lipid oxidation with fast inactivation of proteolytic enzymes.

Identified ETS proteins were more abundant in Te samples (Tables 4 and 5), suggesting that ETS was, relatively speaking, upregulated (as in well-fed animals) early postmortem. In addition, a reduced activity of complex I enzyme (NADH dehydrogenase) in To samples postmortem may be connected with impaired electron transfer, causing accumulation of reducing equivalents and promotion of ROS production (Turrens et al., 1991; Zeng et al., 2013). Bc1 complex is thermosensitive (Brasseur et al., 1997); therefore, structural modifications of this enzyme in To meat (Table 4; greater exp(Mw/pI) compared to parent protein) possibly decreased complex III activity. The neighboring complex IV also exhibited an activity deficiency, favoring the idea of its downregulation (not so well-fed animals) in To meat (see Table 5).

Based on the presented results, a lack of oxygen supply stimulated ATP production through the glycolytic pathway and donation of electrons through β-oxidation, providing energetic efficiency of the muscle system. The extended mitochondrial respiration positively affected activity of proteolytic enzymes and the tenderization processes.

Maintaining the ATP Production Postmortem

Activity of metabolic enzymes early postmortem presumptively had a significant effect on a final meat quality. In agreement with Jia et al. (2006), proteins identified in Te meat (Table 4) indicate prolonged ATP production after animal slaughtering. The glycolytic enzyme triosephosphate isomerase subunits and Krebs cycle enzymes (2 fragments of pyruvate dehydrogenase E1), presumably upregulated to fuel energy to the cell, were identified in Te meat. Isocitrate dehydrogenase [NAD] subunit alpha was identified both in Te and To meat but was more abundant in Te samples (see Table 5).

Four subunits of F_oF_1–adenosine triphosphatase structure identified in To samples were the full-length proteins that most probably hydrolyze available ATP early postmortem. Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, as key enzyme in redox regulation in Krebs cycle (Bunik et al., 2009), showed large shifts in Mw and pI in To meat. The fact that the To samples were from nominally smaller animals with both limited and substantial OCR response upon ADP addition may suggest also the presence of additional mechanisms in ATP reduction early postmortem (e.g., muscle cell and connective tissue differences, stress).

The Apoptosis/Necrosis Switch

There are 2 pathways of cell death: apoptosis, a strictly regulated cell death, and/or necrosis, an acute

Table 4. Differently expressed ($P < 0.05$) proteins between tender and tough bovine semimembranosus muscle identified by matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry or liquid chromatography/mass spectrometry after separation by 2-dimensional gel electrophoresis (see Fig. 3a and 3b)

Spot no.[1]	Identified protein (*Bos taurus*)	Accession number (source)[2]	M_r,[3] kDa exp/theory[3]	pI[4] exp/theory	Match peptides	Seq. cov.,[5] %	Mascot[6] score	Protein function
Proteins identified in tender meat								
Inner mitochondrial membrane proteins								
S1a	Chain F, structure of Bovine Heart Cytochrome C Oxidase At the Fully Oxidized State	gi|1942991	12.0 / 10.9	6.48 / 6.06	11	93	126	ETS[7]
S3a	NADH dehydrogenase 24 kDa subunit (AA6-217)	gi|1364245	26.0 / 23.8	6.16 / 5.71	15	66	147	ETS
S4a	ATP synthase subunit d, mitochondrial	gi|27807305	23.0 / 18.7	6.16 / 5.99	9	54	108	ATP synthesis
S7a	Chain E, structure of Bovine Hart Cytochrome C oxidase at the Fully Oxidized State	gi|1942990	12.0 / 12.5	4.84 / 5.01	6	57	76	ETS
S12a	Cytochrome b-c1 complex subunit 6, mitochondrial	P00126	11.0 / 10.9	4.30 / 4.45	5	23	24	ETS
S22a	Chain A, Cytochrome Bc1 Complex from Bovine	gi|4139392	47.0 / 49.9	5.96 / 5.46	24	64	322	ETS
Mitochondrial matrix proteins								
S9a	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	P41563	43.0 / 40.1	6.32 / 6.76	41	36	2,571	Krebs cycle
S10a	[8]Pyruvate dehydrogenase E1 component subunit beta, mitochondrial isoform 2	gi|426249337	35.0 / 37.5	5.83 / 5.51	8	26	90	Krebs cycle
S11a	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial precursor	gi|164420789	34.0 / 39.4	5.82 / 6.21	17	54	166	Krebs cycle
S15a	Superoxide dismutase [Mn], mitochondrial	P41976	24.0 / 24.8	6.85 / 8.70	11	22	139	Cell defense
S17a	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	gi|27806205	43.0 / 70.9	7.01 / 8.48	15	24	165	Lipid metabolism
S19a	Chain A, Aldehyde Dehydrogenase from Bovine Mitochondria	gi|2624886	50.0 / 54.9	6.84 / 6.05	15	34	195	Cell defense
S26a	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial precursor	gi|296488732	39.0 / 36.5	6.74 / 6.33	4	11	47	ETS
Cell structure proteins								
S6a	Actin, alpha skeletal muscle	gi|27819614	44.0 / 42.5	5.54 / 5.31	25	55	251	Muscle contraction
S18a	Actin, alpha skeletal muscle	gi|27819614	44.0 / 42.5	5.44 / 5.31	21	58	294	Muscle contraction
S20a	Actin, alpha skeletal muscle	gi|27819614	44.0 / 42.5	5.65 / 5.31	10	37	136	Muscle contraction
Contractile apparatus								
S5a	Myosin regulatory light chain 2, skeletal muscle isoform	Q0P571	14.0 / 19.1	4.19 / 4.91	33	7	1.199	Muscle contraction
S13a	Tropomyosin alpha-1 chain	Q5KR49	35.0 / 32.7	4.73 / 4.69	23	25	372	Muscle contraction
S14a	Tropomyosin alpha-1 chain	Q5KR49	35.0 / 32.7	4.76 / 4.69	23	39	957	Muscle contraction
S23a	Myosin-7	gi|41386711	48.0 / 223.9	5.93 / 5.58	12	4	66	Muscle contraction
Other proteins								
S2a	Thioredoxin-dependent peroxide reductase, mitochondrial precursor	gi|27806083	25.0 / 28.4	6.26 / 7.15	5	29	52	Cell defense
S8a	Thioredoxin	O97680	11.0 / 12.1	4.87 / 4.97	3	10	24	Cell defense and ETS
S16a	Thioredoxin-dependent peroxide reductase, mitochondrial precursor	gi|27806083	25.0 / 28.4	6.00 / 7.15	5	29	90	Cell defense
S21a	Stress-70 protein, mitochondrial	Q3ZCH0	59.0 / 74.0	6.08 / 5.97	193	62	3,374	Cell defense
S24a	Triosephosphate isomerase	Q5E956	27.0 / 26.9	6.74 / 6.45	68	47	216	Glycolytic pathway
S25a	Triosephosphate isomerase	gi|61888856	27.1 / 26.9	6.86 / 6.45	6	29	84	Glycolytic pathway
Proteins identified in tough meat								
Inner mitochondrial membrane proteins								
S1b	Chain F, structure of Bovine Heart Cytochrome C Oxidase At the Fully Oxidized State	gi|1942991	17.0 / 10.9	6.54 / 6.06	10	93	162	ETS
S2b	Chain E, structure of Bovine Hart Cytochrome C oxydase at the Fully Oxidized State	gi|1942990	16.0 / 12.5	4.96 / 5.01	7	57	112	ETS
S3b	Chain B, subcomplex of the stator of bovine mitochondrial ATP synthase	gi|110591027	28.0 / 18.6	6.20 / 6.02	9	51	115	ATP synthase
S6b	NADH dehydrogenase 24 kDa subunit (AA6-217)	gi|1364245	31.0 / 23.8	6.22 / 5.71	12	58	118	ETS

Table 4. Continued

Table 4. (Cont.)

Spot no.[1]	Identified protein (*Bos taurus*)	Accession number (source)[2]	M_r[3] kDa exp/theory[3]	pI[4] exp/theory	Match peptides	Seq. cov.,[5] %	Mascot[6] score	Protein function
S17b	Chain A, Cytochrome Bc1 Complex from bovine	gi|4139392	56.0 / 49.9	6.28 / 5.46	29	69	309	ETS[7]
Intermembrane space proteins								
S7b	Superoxide dismutase [Cu-Zn]	P00442.2	18.0 / 15.8	6.09 / 5.85	6	28	37	Cell defense
Mitochondrial matrix proteins								
S19b	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor	gi|27807161	46.0 / 40.1	6.48 / 6.76	14	37	153	Krebs cycle
Cell structure proteins								
S12b	Actin, alpha skeletal muscle	gi|27819614	50.0 / 42.5	5.51 / 5.31	25	55	251	Muscle contraction
S13b	Actin, alpha skeletal muscle	gi|27819614	50.0 / 42.5	5.61 / 5.31	24	71	267	Muscle contraction
Contractile apparatus								
S4b	Myosin light chain 1/3, skeletal muscle isoform	gi|118601750	26.0 / 21.0	4.85 / 4.96	6	40	82	Muscle contraction
S8b	Type slow/beta myosin heavy chain	gi|4001708	32.0 / 11.5	6.30 / 4.93	7	49	84	Muscle contraction
S14b	Myosin-7	gi|41386711	36.0 / 223.9	5.71 / 5.58	15	6	52	Muscle contraction
Other proteins								
S5b	Thioredoxin-dependent peroxide reductase, mitochondrial isoform X1	gi|529009145	31.0 / 26.5	6.30 / 6.75	5	31	64	Cell defense
S9b	Chain D, Crystal Structure of Bovine F1-C8 Sub-Complex of ATP Synthase	gi|306991567	55.0 / 50.3	5.40 / 4.96	35	84	430	ATP synthesis
S10b	Chain D, Crystal Structure of Bovine F1-C8 Sub-Complex of ATP Synthase	gi|306991567	55.0 / 50.3	5.33 / 4.96	26	72	317	ATP synthesis
S11b	Chain D, Crystal Structure of Bovine F1-C8 Sub-Complex of ATP Synthase	gi|306991567	53.0 / 50.3	5.26 / 4.96	19	56	196	ATP synthesis
S15b	Protein-L-isoaspartate (D-aspartate) O-methyltransferase	P15246	30.0 / 24.7	6.84 / 7.07	8	25	38	Repair and/or degradation of damaged proteins
S16b	Tripartite motif-containing protein 72	gi|119917044	34.0 / 54.1	5.98 / 6.05	11	22	124	Signaling pathways
S18b	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	gi|115497112	57.0 / 49.3	6.60 / 9.11	8	16	79	Krebs cycle

[1]Spot no. as given in Fig. 3.

[2]Accession number from the SwissProt (www.ebi.ac.uk/uniprot) and National Center Biotechnology Information databases (www.ncbi.nlm.nih.gov/Database/).

[3]M_r exp/theory = molecular weight of the protein in gel/theoretical value.

[4]pI exp/theory = isoelectric point of the protein in gel/theoretical value

[5]Seq. cov. = Sequence coverage.

[6]http://www.matrixscience.com.

[7]ETS = electron transport system.

[8]Protein originates from *Ovis aries*.

metabolic cell disruption. Switching between these 2 processes is strongly dependent on the capability of mitochondria to recover ATP at anaerobic conditions (Kroemer et al., 1998; Lemasters et al., 2002; Kim et al., 2003; Tatsuta and Langer, 2008), leading to hypotesis of a prolonged apoptotic phase in Te meat.

Antioxidants, as a part of the stress regulatory mechanism, play important role in tenderness-related phenomena. Thioredoxin and thioredoxin-dependent peroxide reductase (**Prx III**), mitochondrial precursor were identified in Te meat samples. Oxidative stress and electron-transfer steps most probably caused the structural changes in local conformation of the active site and surface area changing protein's mobility. Thioredoxin/Prx III is a very efficient system when it comes to regulation of ROS production in mitochondrial respiration (Tanaka et al., 2002; Jia et al., 2006, 2007). In addition, ROS formed by complex I and III on the matrix side of mitochondria (Turrens et al., 1991; Oh-ishi et al., 1997; Egan et al., 2011) are removed by superoxide dismutase [Mn]. Thus, identified mitochondrial aldehyde dehydrogenase protects cells from the oxidative stress through the oxidation of toxic aldehyde derivates (Ohta et al., 2004). Stress-70

Table 5. The difference in intensity ($P < 0.05$) for proteins identified both in tender and tough samples of bovine semimembranosus muscle

Identified protein (*Bos taurus*)	Tender		Tough		Percentage difference, %
	Mean,[1] %	SE	Mean, %	SE	
Chain F, structure of Bovine Heart Cytochrome C Oxidase At the Fully Oxidized State	1.715	0.308	1.649	0.303	3.9
NADH dehydrogenase 24 kDa subunit (AA6-217)	0.831	0.118	0.740	0.071	10.9
Chain E, structure of Bovine Hart Cytochrome C oxidase at the Fully Oxidized State	1.370	0.454	0.981	0.231	28.4
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	0.238	0.028	0.209	0.026	12.2
Chain A, Cytochrome Bc1 Complex from Bovine	0.297	0.070	1.314	0.112	75.0
Myosin-7	0.257	0.074	0.212	0.045	17.5
Actin, alpha skeletal muscle	2.181	0.329	2.362	0.182	8.3
Actin, alpha skeletal muscle	0.892	0.260	1.824	0.156	51.1

[1]Average intensity of normalized expression amount for group of samples.

protein (HSPA9), a member of 70 kDa heat shock protein family, was identified in Te samples. Positive correlation of HSPA9 with tenderness (Guillemin et al., 2011) may be related to reparation or degradation of misfolded proteins and preventing protein aggregation ensuring proteolysis (Wagner et al., 1994).

On the other hand, superoxide dismutase (Cu-Zn) was identified in To meat. Antioxidant function of Cu/Zn superoxide dismutase is related to conversion of reactive O_2 species (O_2– and H_2O_2) into water and molecular oxygen (Scandalios, 1993). In agreement with previous studies (Dimmeler et al., 1999; Guillemin et al., 2011), we suggest that stronger oxidative stress caused ROS accumulation in To meat and reduced cell protection by Cu/Zn superoxide dismutase, because of enzyme's underexpression or overexpression with changes in Mw (18 vs. 15 kDa).

The cell function of Prx III, mitochondrial precursor X1, has not been reported, and in To samples, this precursor showed greater Mw (31 vs. 26 kDa) than the parent protein, suggesting the enzyme's modification.

During the early postmortem period, acute metabolic cell disruption and protein modification most probably activated different repairing pathways. Protein L-isoaspartate-(D-aspartate) *O*-methyltransferase in To samples played a role in reparation of damaged proteins and prevention of protein accumulation (DeVry and Clarke, 1999). Furthermore, tripartite motif-containing protein 72 identified in To samples, as mediator of signaling pathways (Kawai and Akira, 2011), was probably involved in removal of cell protein fragments accumulated under progressive apoptotic processes.

Conclusions

This study identified the level of protein modifications implicated in mechanical properties of beef meat based on OCR measurements. High mitochondrial respiration suggested lower meat shear force and can explain at least 15 to 20% of the variation in tenderness at slaughter conditions. Proteomic analysis on mitochondria from 2 groups (Te and To), based on measured and predicted WBSF, showed more abundant ETS protein in the Te group. In addition, glycolytic, β-oxidation, and Krebs cycle enzymes were significantly different in Te compared to To samples, supporting a relationship between greater OCR and lower WBSF in Te samples. We hypothesized that mitochondrial oxygen removal early postmortem may preserve proteases, reducing their oxidation, and thereby increase tenderness. High response to ADP addition early postmortem suggested more Te meat, which was, however, not the case vice versa. Antioxidant enzymes and chaperon identified in Te beef may explain ROS reduction, prolonged ETS activity, and apoptotic promotion.

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Paper II

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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.

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 $\varepsilon = 14{,}840$ 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 25,186g at 4 °C. After centrifugation, the absorption of the supernatant was measured by spectrophotometer at 532 nm using $\varepsilon = 156{,}000\ M^{-1}\ cm^{-1}$ (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-mercatopropionic (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 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).

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.

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

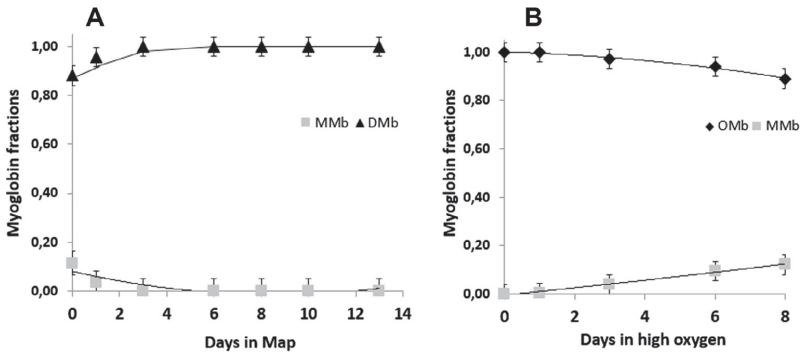

Fig. 1. Myoglobin fractions on surfaces of minces in two different packaging. (A) Myoglobin states with added succinate, glutamate and malate (molar ratio 50:37.5:12.5) in MAP; (B) myoglobin states with added glutamate, citrate and malate (molar ratio 56:25:19) in high oxygen. Total concentration of 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

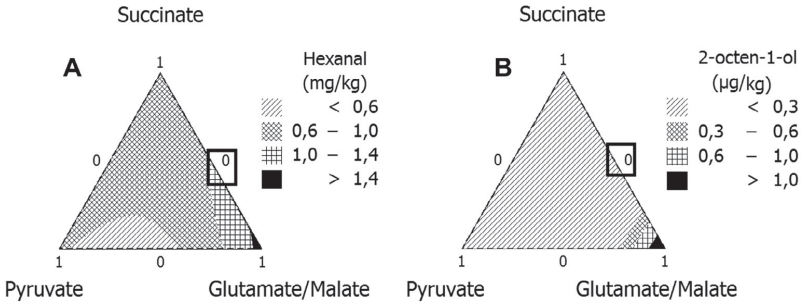

Fig. 2. Hexanal (A) and 2 octen-1-ol (B) concentration as a function of the composition of succinate, pyruvate and glutamate/malate (KCS, mean conc. = 0.075 mol/kg) for minces chill stored for 13 days in MAP. The slopes of the response surfaces were significant ($P < 0.05$). Square indicates the area 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.

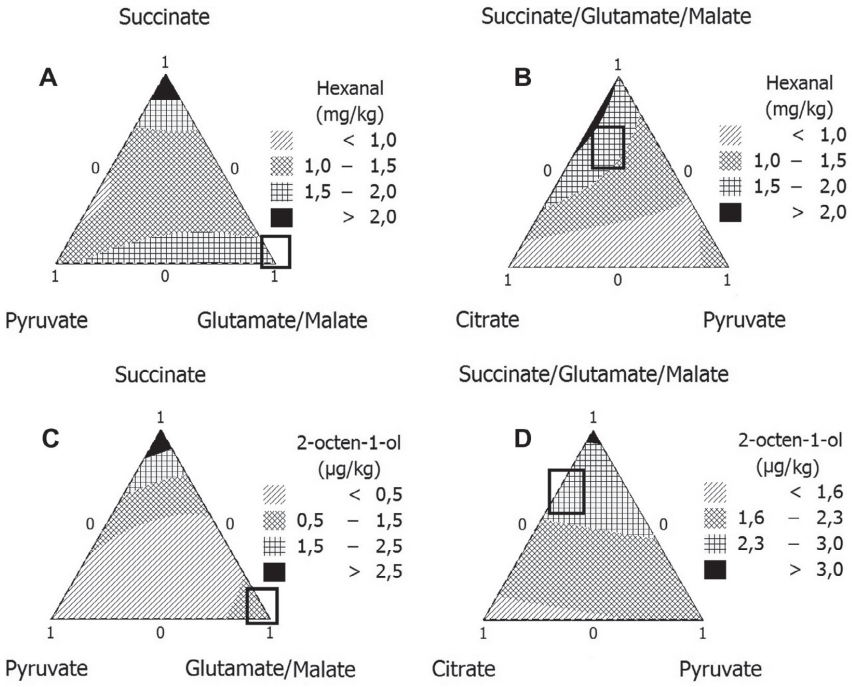

Fig. 3. Hexanal concentration (A) and 2 octen-1-ol (B) as a function of the composition of succinate, pyruvate and glutamate/malate (Krebs cycle substrates, mean conc. = 0.075 mol/kg) for minces chill stored for 8 days in high oxygen. The slopes of the response surfaces were significant ($P < 0.05$). 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

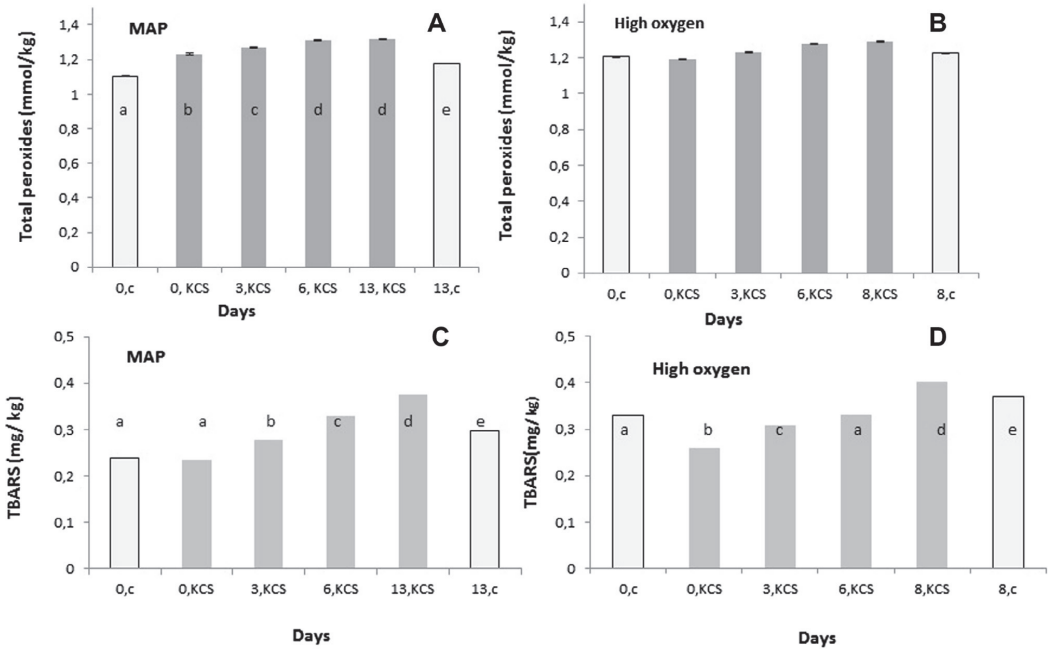

Fig. 4. (A) Total PFP over time for a succinate and glutamate mixture in MAP; (B) total PFP over time for a glutamate, citrate and malate mixture in high oxygen; (C) TBARS formed over time for a succinate and glutamate mixture in MAP; (D) TBARS formed with time for glutamate, citrate and malate mixture 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).

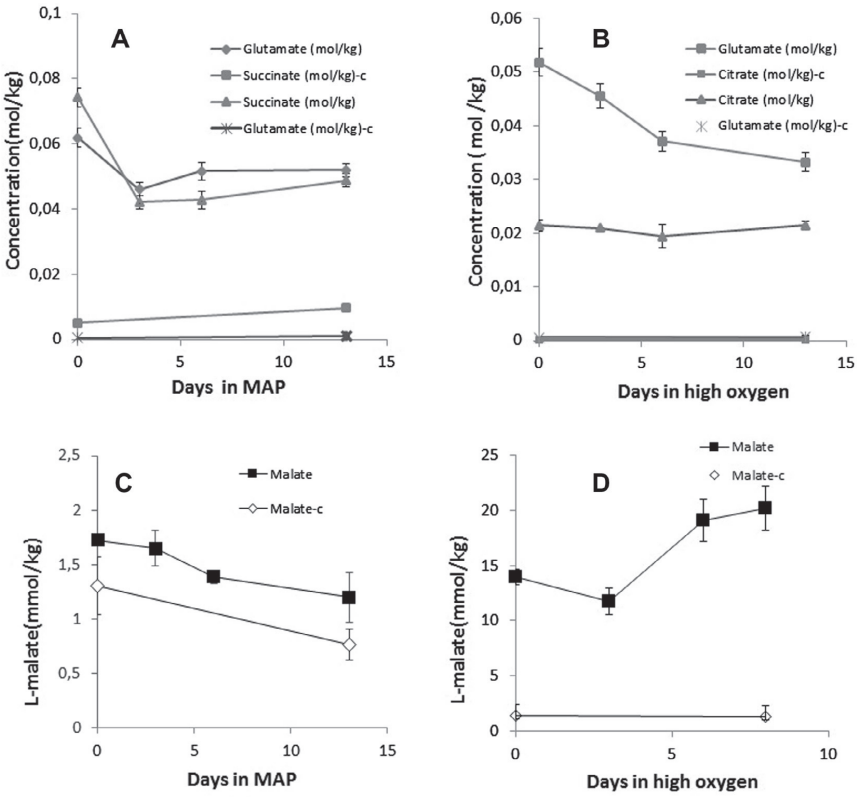

Fig. 5. (A) Succinate and glutamate concentrations (molar ratio 50:50, totally 0.1 mol KCS/kg) in minces stored in MAP; (B) glutamate, citrate and malate in high oxygen (molar ratio 56:25:19, totally 0.1 mol KCS/kg); (C and D) malate concentration in the same minces stored in MAP and in high oxygen,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

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Paper III

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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*).

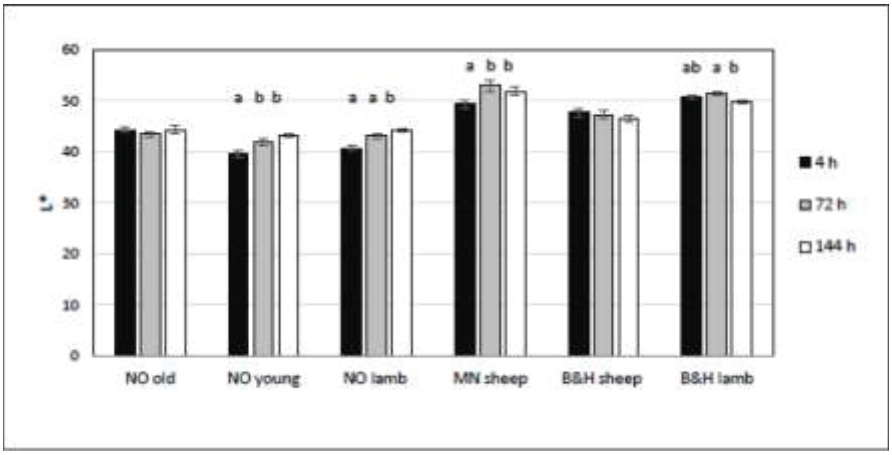

Figure 1a. The average changes in L* during aerobic incubation for different sheep/lamb groups and times. Different letters indicate significant (*P*<0.05) differences.

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.

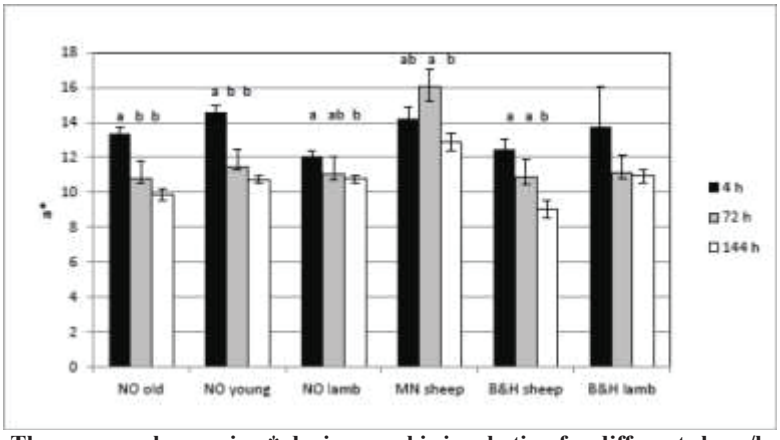

Figure 1b. The average changes in a* during aerobic incubation for different sheep/lamb groups and times. Different letters indicate significant (*P*<0.05) differences.

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)	$0.23(\pm0.05)^{a}$	$0.21(\pm0.04)^{ab}$	$0.15(\pm0.03)^{c}$	$0.24(\pm0.04)^{a}$	$0.21(\pm0.05)^{ab}$	$0.18(\pm0.03)^{bc}$
Water content*	$73.13(\pm0.6)^{b}$	$73.42(\pm1.0)^{b}$	$75.30(\pm0.9)^{b}$	$73.15(\pm0.4)^{b}$	$70.93(\pm0.6)^{c}$	$75.83(\pm0.4)^{a}$
Dry matter*	$26.87(\pm0.6)^{b}$	$26.58(\pm0.9)^{b}$	$24.69(\pm0.6)^{b}$	$26.85(\pm0.4)^{b}$	$29.07(\pm0.6)^{a}$	$24.17(\pm0.4)^{c}$
Protein content*	$21.38(\pm0.9)^{a}$	$21.61(\pm0.9)^{a}$	$20.56(\pm0.6)^{b}$	$17.12(\pm0.4)^{c}$	$20.49(\pm0.6)^{b}$	$20.63(\pm0.4)^{b}$
Fat content*	$3.88(\pm0.5)^{b}$	$3.38(\pm0.2)^{b}$	$2.58(\pm0.6)^{c}$	$7.46(\pm0.8)^{a}$	$7.39(\pm0.4)^{a}$	$2.35(\pm0.1)^{c}$
Vitamin E (mg/100g)	$0.23(\pm0.04)^{ab}$	$0.12(\pm0.05)^{c}$	$0.09(\pm0.07)^{c}$	$0.29(\pm0.15)^{a}$	$0.22(\pm0.08)^{ab}$	$0.16(\pm0.06)^{bc}$
Vitamin E/Fat (mg/100g)	$0.07(\pm0.05)^{ab}$	$0.03(\pm0.03)^{b}$	$0.05(\pm0.04)^{b}$	$0.04\ (\pm0.02)^{b}$	$0.04(\pm0.02)^{b}$	$0.11(\pm0.05)^{a}$
DPPH (total antioxidant)*	$66.2(\pm5.2)^{b}$	$66.5(\pm3.3)^{b}$	$66.3(\pm4.8)^{b}$	$70.9(\pm2.6)^{a}$	$68.7(\pm3.7)^{ab}$	$72.7(\pm3.7)^{a}$
Cathepsin B**	$0.33(\pm0.07)^{ns}$	$0.33(\pm0.04)^{ns}$	$0.30(\pm0.03)^{ns}$	$0.32(\pm0.05)^{ns}$	$0.31(\pm0.04)^{ns}$	$0.32(\pm0.03)^{ns}$
TBARS***	$0.33(\pm0.13)^{ab}$	$0.33(\pm0.21)^{ab}$	$0.22(\pm0.05)^{b}$	$0.47(\pm0.25)^{a}$	$0.23(\pm0.23)^{b}$	$0.43(\pm0.03)^{a}$

* 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 IV

Volatile profiles of adipose tissue, metabolites of lean meat and sensory attributes of lamb/sheep meat from different production systems

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Abstract

Flavour characteristics of female lamb and sheep meat from Norway (NW; n=47), Bosnia and Herzegovina (BH; n=30), Montenegro (MN; n=15) were investigated. Sensory analysis, Gas Chromatography (GC)/Mass Spectrometry analysis were used on heated adipose tissue (headspace, HS) and extracted lean meat were conducted. HS-GC differentiated NW lambs (*e.g.* 5-ethyl-3-nonanal, 2-heptadecanone) from 4 y NW sheep (*e.g.* 2-ethylfuran). NW 4 y sheep were more gamy and grassy but less acidic than NW lambs. HS-GC analysis differentiated BH lamb (*e.g.* dimethyl sulfone) from sheep adipose tissue (*e.g.* β-caryophyllene). Lean meat from BH sheep had higher prevalence of free amino acids than lambs. BH lamb meat was more bitter and metallic than BH sheep. Sheep from NW, BH and MN had distinct flavour profiles. No single sensory attribute separated all 3 sheep meat. BH lambs differed from NW lambs by their higher taste intensity and different flavour profile.

Keywords: sheep; adipose tissue volatiles; lean meat metabolites; sensory attributes; principal component analysis; partial least squares regression

1. Introduction

Several ante-mortem factors, *i.e.* breed, age, diet and production system contribute to sensory properties of lamb/sheep meat (Ekiz, Yilmaz, Ozcan, Kaptan, Hanoglu, Erdogan, et al., 2009; Resconi, Escudero, & Campo, 2013; Watkins, Frank, Singh, Young, & Warner, 2013). Meat flavour is an important quality criterion with a key role in the overall lamb/sheep meat acceptability (Wood, Enser, Fisher, Nute, Richardson, & Sheard, 1999). In general, the effect of genetics on lamb/sheep flavour development still remains unresolved. Although some authors emphasize the breed effects (Ekiz et al., 2009; Hoffman, Muller, Cloete, & Schmidt, 2003), the conclusions differ significantly with regard to genetic effect on lamb and sheep meat flavour. The effects of the production system and diet on flavour constituents, chemical composition and overall ruminant meat acceptability are not fully understood, even though some studies suggested diet as a powerful tool in interfering with sensory attributes of lamb meat (Juárez, Horcada, Alcalde, Valera, Polvillo, & Molina, 2009; Resconi, Campo, Furnols, Montossi, & Sañudo, 2009). However, the animals' diet can induce a different degree of carcass fatness (Aurousseau, Bauchart, Faure, Galot, Prache, Micol, et al., 2007) and fatty acid composition (Dewhurst, Scollan, Lee, Ougham, & Humphreys, 2003; Resconi et al., 2009). Variations in meat flavour have been associated with pasture differences, and sheep fed with white clover, lucerne, phalaris and rape (*Brassica*) had an unacceptable meat flavour (Watkins et al., 2013). The metabolites obtained through the diet or metabolized by animals and deposited in the muscle cells or adipose tissue have been described as compounds that contribute to flavour (Watkins et al, 2013).

Two types of the indigenous breed Pramenka (Vlašićka and Pivska Pramenka) from the Western Balkans region were studied. Vlašićka Pramenka is a dominant sheep in the mountain region Vlašić in Bosnia and Herzegovina (Sinanović, Katica, Varatanović, & Ališah, 2011). In the Vlašić region, grasses (*Poa pratensis, Bromus racemosus, Dactylis glomerata*, and *Briza media*) as well as some cloves (*Lotus corniculatus*, *Trifolium pratense, Trifolium repens*, and *Vicia sativa*) are dominant (Rogić, personal communication). The lambing period for Vlašićka Pramenka is early winter. During the winter months sheep herds return to lowland areas. In the period from November until the end of April the feeding system mostly consists of hay and corn.

Pivska Pramenka is a characteristic Pramenka type for Northern Montenegro for having a cold, mountain climate and a long winter period. Ljubišnja grazing area is characterized by *Poa violacea*, *Festuca ovina, Festuca rubra*ssp. *fallax, Festuca valesiaca, Nardus stricta*, and *Brometum erectistrictae* as the most dominant species (Dubljević, 2009). In the period from May until the middle of October the feeding system of Pivska Pramenka is based on semi-natural mountain pasture, fertilized areas (moving sheepfold), and during other periods of the year animals are fed with hay. From late December until the end of March, diet is supplemented with concentrate.

Norwegian White sheep, a crossbreed composed of Dala, Rygja, Steigar, and Texel breeds, is the dominant breed in Norway (Bjelanović, Grabež, Vučić, Martinović, Lima, Marković, et al., 2015). Typical production systems consist of mountain pasture from May to September/October, grazing on fenced areas during spring and autumn. According to common practice, lambs with lower live weight are finished on rich pasture. The botanical composition of meadows is a mix of mainly *Phleum pratense, Festuca pratensis, Poa pratensis, Trifolium*

sp., and *Elymus repens* (Lunnan & Tondem, 2014). During winter, lamb and sheep are indoor and fed a diet based on roughage and concentrate supplements (Asheim, & Mysterud, 1999). The lambing period occurs in early spring when sheep and lamb are still housed. Lambs are pasture-fed, grown under conventional and ecological production systems.

In general, Wong, Nixon, & Johnson (1975) define sheep flavour as sweaty, oily, acidic, acrid, urinary, fecal, barnyard and sharp. The mutton-like flavour was associated with branched chain fatty acids (BCFA; $C_8 - C_{10}$), more abundant in adipose tissue of aged animals (Watkins et al., 2013). However, discrimination of lamb from sheep meat according to BCFA concentration was not possible (Watkins, Rose, Salvatore, Allen, Tucman, Warner, et al., 2010). Pastoral sheep flavour, defined as animal, barnyard, grassy, sheep-like, gamey, milky, and fecal, was related to skatole, indole and phenols (Priolo, Cornu, Prache, Krogmann, Kondjoyan, Micol, et al., 2004; Young, Lane, Priolo, &Fraser, 2003). According to our knowledge, compounds responsible for increased odour intensity in aged sheep, for identical or similar sheep breeds have not been studied yet. Detailed comparison of production systems (ecological vs. conventional production) and age-related unique compounds with the concentration changes are particularly important for understanding changes in lamb and sheep flavour and odour. In addition, flavour development and sensory characteristics of Vlašićka and Pivska Pramenka have not been described so far. To define representative biomarkers, flavour analysis should be repeated due to seasonal changes.

In order to understand the variation in flavour profiles among different lamb and sheep meat, volatile compounds and metabolites from adipose tissue and lean meat were studied, plus sensory attributes of 2 types of lamb and 4 different sheep meat. Meat from sheep and lamb is also used for production of dry-cured hams and it was also of interest to elucidate flavour

differences that may be relevant after dry-curing (Stojković, Grabež, Bjelanović, Mandić, Vučić, Martinović, et al., 2015). The aim of our study was to: 1.) Identify and quantify volatiles and metabolites as constituents of lamb/sheep meat flavour; 2.) Evaluate sensory properties of different animal groups; 3.) Elucidate how volatiles and metabolites correlated with sensory attributes.

2. Materials and methods

2.1. Experimental design

Ninety-two animals used in the study were classified into seven animal groups. To reflect consumption and cultural habits typical for each country, the studied animals were: lambs (5−6 months), young sheep (~2 years) and old sheep (4−5 years). All animals were females and representative of the production system of the country of origin (Bosnia and Herzegovina, Montenegro and Norway). The relatively small sheep production in Montenegro could not provide an optimal number of 5−6 months old lambs of Pivska Pramenka from the same herd, therefore this group was not examined in this study (for more information see Bjelanović et al., 2015).

Bosnia and Herzegovina: Sheep (BH 4y) and lambs from conventional production (BH lamb; 15 animals each) belonged to two flocks of Vlašićka Pramenka. Lambs, born in December, were raised on maternal milk with access to pasture, and stayed with sheep until April when they were slaughtered. Sheep were slaughtered in November directly from the pasture.

Montenegro: Fifteen sheep of Pivska Pramenka (MN 4y) were included in this experiment. Sheep were slaughtered in November directly from the pasture.

Norway: Lamb (ecological−NW eco and conventional−NW ord; later presented as a joined group NW lamb), young (NW 2y) and old sheep (NW 4y) belonged to the Norwegian White. Lambs from ecological and conventional production (9 animals each), and young sheep (15 animals) were transported directly from the rangeland and slaughtered in October. Old sheep (14 animals) were indoor-finished (hay + concentrate supplemented) for a period of 3 months before slaughtering. This is a tradition in Norway since the number of older sheep in Norway is regulated in January. All animals were from the Hallingskarvet mountain region in Southern Norway, *i.e.* areas with origin labeling and signaling a special meat quality that is not well documented.

2.2. Tissue sampling

All animals were slaughtered in the country of origin as described by Bjelanović et al. (2015). The *M. longissimus thoracis et lumborum* from both sides of carcass were removed and adipose tissue above the muscle was excised within 24 h *post mortem*, vacuum-packed and stored at -80 °C. Vacuum-packed meat, refrigerated for 7 days (at 4 °C), was divided into 5 cm slices, vacuum-packed and stored at -80 °C for sensory and GC/MS analysis.

2.3. Quality measurements

160 *2.3.1. Headspace gas chromatography-mass spectrometry (HS-GC/MS) analysis of volatile*
161 *compounds*
162 Frozen adipose tissue was homogenized with a crushing machine (IKA® A11 Basic Analytical
163 Mill, Staufen, Germany) to a fine powder. Four grams of homogeneous powder were placed in
164 a glass vial (50 mL) and stored at -80 °C until the next preparation step. In order to increase
165 the volatile compounds extraction and generate representative volatile profiles, the
166 homogenized sample was heated at 75 °C in water bath for 30 min on the day of analysis. The
167 liquid fat phase (1 g) was transferred to a clean glass vial and kept at 4 °C for ~ 4 h before
168 measured. All samples were analyzed in two replicates.
169 A mixture of five compounds in Mygiol (AXO INDUSTRY, Warve, Belgium) was used as a
170 control sample. These compounds were: butanal (99%), *cis*-2-penten-1-ol (95%), 2-
171 undecanone (99%), and dimethyl sulfone (98%) (Sigma-Aldrich Chemie GmbH, Schnelldorf,
172 Germany), and acetic acid (100%, VWR, Fontenay-saus-Bois, France).
173 HS-GC/MS analysis was performed according to a modified method by Volden et al. (2011).
174 Fat volatiles were extracted by dynamic headspace analyzer Teledyne Tekmar HT3 (Teledyne
175 Tekmar, Ohio, USA) coupled to an Agilent gas chromatograph 6890N (Agilent Technologies,
176 Santa Clara, CA, USA). The DB - WAXetr fused silica capillary column (30 m × 0.25 mm
177 i.d., 0.50 µm film thickness; J&W Sientific, USA) was connected to the ion source (230 °C)
178 of a Agilent 5975 (Agilent Technologies, Santa Clara, USA) quadruple mass spectrometer
179 (interface line 250 °C). The carrier gas was He with a flow rate of 1.0 mL/min. Samples were
180 heated at 150 °C in the headspace. The temperature program for GC was: 35 °C for 10 min,
181 ramped 1.5 °C/min up to 40 °C, ramped 4.0 °C/min up to 70 °C, ramped 7.5 °C/min up to 230
182 °C, and 1 min at 230 °C. Analysis time was 54.62 min, and recorded mass range was *m/z*

33−300. Volatiles were tentatively identified by computer-matching of generated mass spectra with NIST05 database (National Institute of Standards and Technology/Gaithersburg, MD, USA). Identified volatile compounds are presented in Table S-1 as % of total area in a specific chemical group.

2.3.3. Extraction, derivatization, and GC/MS analysis of meat metabolites

One gram of lean meat was transferred into a 15 mL tube, and 5 mL of a water: methanol: chloroform (1 : 2.5 : 1) mixture with internal standard ribitol (66 μg/mL) was added. The sample was incubated at 60 °C for 60 min in sonication bath and centrifuged for 10 min at 3 000 rpm at 4 °C. An aliquot of 1 mL was transferred into a 1.5 mL Eppendorf tube, dried in a SpeedVac (Thermo Scientific, Waltham, MA, USA) overnight and stored at -80 °C. The dried residues were resuspended in 80 μL methoxyamine hydrochloride with pyridine (20 mg/mL) at 30 °C for 60 min and sonicated at 30 °C for 30 min. Finally, samples were treated with 80 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide at 37°C for 30 min.

GC/MS analyses were performed according to Sissener et al. (2011). Derivatized sample in the amount of 1 μL was analyzed on an Agilent 6890 GC connected with an Agilent 5975 MS detector. A HP-5MS capillary column (i.d. 30 m × 0.25 mm, film thickness 0.25 μm) was used. The carrier gas (He) flow rate through the column was 1 mL/min. The GC temperature program: 70 °C for 5 min, ramped at 5 °C/min until 310°C. Analysis time was 60 min. The MS operated at 230 °C, and the recorded mass range was *m/z* 50−700.

MS files from Agilent ChemStation (Agilent Technologies, Waldbronn, Germany) were exported in the netCDF format (OPENChrom, Eclipse Public License 1.0) to MetAlign (version 041012, RIKILT Wageningen UR, Plant Research International) for data pre-

processing and alignment. Metabolites were identified with 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 database (Max-Planck Institute for Molecular Plant Physiology, Golm, Germany). Normalization of the peak area was performed on the internal standard ribitol and expressed as mg/kg of meat. Samples were run randomized. Metabolites are presented in a Table S-2. All samples were analyzed in the same laboratory.

2.4. Sensory analysis

For sensory testing meat samples were defrosted at 4 °C overnight. The 2.5 cm slices of lean meat were heated in water bath set to 80 °C until internal temperature of 71 °C was achieved (AMSA, 1995). A panel consisting of 8 trained (ISO 8586–1:1993) assessors (4 females and 4 males 30−59 years old) was selected for the sensory analysis. The laboratory for sensory analysis at Faculty of Technology in Novi Sad was designed according to ISO 8589:2007. During the evaluation, water and bread were served to assessors to cleanse their palate between samples. All samples were randomly served. Sensory traits of lamb/sheep meat were evaluated by the quantitative-descriptive analysis (Lawless & Heymann, 2010), using a scale from one (none) to nine (very intense) according to ISO 4121:2003. Assessors were asked to evaluate the following: odour (gamy, grass, rancid), taste (acidic, bitter, metallic), as well as tenderness, fattiness, and juiciness. These attributes were selected as they have been observed to distinguish Norwegian lamb samples earlier (Lind, Berg, Eilertsen, Hersleth, & Eik, 2011). All samples were analyzed in the same sensory laboratory.

229 *2.5. Statistical analysis*

230 Identified instrumental variables (volatile compounds and metabolites) were tested using One-
231 way ANOVA (Minitab, version 16 from Minitab Inc., State College, PA, USA). Means were
232 separated by the Tukey's test at $P < 0.001$ to determine the significance level of each
233 instrumental variable for each animal group.

234 Principal component analysis (PCA) models were performed to visualize the complete data set
235 in reduced dimension plot. The first PCA model included all volatile compounds identified in
236 adipose tissue in six animal groups corresponding to the Fig. 2a, while the second PCA model
237 (Fig. 2b) included only four animal groups that showed poor separation in the first PCA
238 model. Seventy-six volatile compounds were included in each case. The third PCA model
239 included 69 metabolites identified in six animal groups (Fig. 3). Sample names were coded as
240 described in the Experimental design section.

241 The correlation between instrumental data (X-matrix) and each sensory attribute (Y-matrix)
242 was evaluated by partial least squares regression (PLSR). Models were developed using
243 Unscrambler, version X10.1 software (Camo, Trondheim, Norway). Instrumental variables
244 were mean-centered and weighted using the inverse of the standard deviation of each variable
245 to have unit variance and zero mean before applying the PLSR analysis. Sensory variables
246 were not centered, the same category scoring scheme was used for measurements. Validation
247 of regression models was performed by full cross-validation. Regression coefficients (b_k) for
248 volatile compounds ($b_k \geq 0.03$) and metabolites ($b_k \geq 0.06$) obtained from PLSR analysis were
249 used in linear regression analysis to indicate their significance for each sensory attribute. In
250 addition, significant ($P < 0.01$) instrumental data were selected and related to odour, taste and
251 texture attributes.

3. Results and discussion

The variations in muscle composition were a result of interactions between breed, diet, age and production systems, but represent typical productions from the regions. These variations resulted in differences in fatty acids, chemical composition and sensory traits.

3.1. Characterization of intramuscular fatty acids

Intramuscular fatty acids were analyzed for the seven animal groups, two groups from Bosnia and Herzegovina (BH lamb and BH 4y), Montenegro (MN 4y), and four groups from Norway (NW eco, NW ord, NW 2y, and NW 4y). According to Bjelanović et al. (2015), the content of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) was highest in the intramuscular fat of MN 4y sheep (47.4 mg/100 g of tissue, 36.8 mg/100 g of tissue, and 4.7 mg/100 g of tissue, respectively) compared with other animal groups (Fig. 1).

Figure 1

3.1.1. Differences between Norwegian White lambs from ecological and conventional production

The concentration levels in SFA (9.2 and 8.1 mg/100 g of tissue), MUFA (7.2 and 6.3 mg/100 g meat) and PUFA (2.1 and 2.7 mg/100 g tissue) content were, however, not significantly different between the two groups of NW lamb, from ecological (NW eco) and conventional (NW ord) production (Fig. 1; Bjelanović, personal communication). Angood, Wood, Nute,

Whittington, Hughes, & Sheard (2008) found no significant differences in MUFA and PUFA content among organic and conventional lamb meat, while SFA was higher ($P < 0.05$) in organically produced lamb.

The headspace analysis of heated adipose tissue trimmed above *M. longissimus thoracis et lumborum* muscle showed significant difference ($P < 0.05$) in thirteen volatile compounds between the two NW lambs. NW eco was characterized by a higher content of propanal, hexanal, heptanal, hexane, 1-octen-3-ol, (*E, Z*)-2,4-heptadienal, 1-pentanol and dimethyl sulfone. On the other side, the content of volatile compounds such as (*E*)-2-hexenal, (*E*)-2-octenal, 2-pentadecanone, γ-butyrolactone and benzonitrile was higher in adipose tissue of conventionally produced lamb. Most of the identified volatile compounds were degradation products of different unsaturated fatty acids. In addition, NW eco had significantly ($P < 0.05$) higher content of ten metabolites (arginine, β-alanine, fructose-6-phosphate, glycerol-3-phosphoethanolamine, glycerol-3-phosphate, fumaric acid, malic acid, pyroglutamic acid, succinic acid, and thymine), identified in both NW lamb groups. Schønheyder & Lyngbye (1962) suggested that better fed animals and longer starvation period before slaughtering increased the abundance of metabolites identified in NW lamb meat. The presence of specific amino acids, organic acids, and many other metabolite compounds may contribute to the overall meat flavour, *i.e.*β-alanine and sugar phosphates contributed to the sweet taste of meat (Watkins et al., 2013). The concentration of arginine (bitter/sweet) in NW eco samples (0.83 mg/kg) was far below the taste detection threshold reported by Chen & Zhang (2007). Fumaric acid may contribute to acidic taste while succinic acid appears to have a matrix dependent taste from sweet/umami taste (Watkins et al., 2013; Schlichtherle-Cerny & Grosch, 1998) to seashell (Yi, Grabež, Bjelanovic, Slinde, Olsen, Langsrud, et al., 2015). Differences

298 in the concentration of free amino acids and organic acids may suggest a more desirable
299 flavour of lamb meat from ecological production, although the panelists indicated no
300 significant differences in sensory attributes among the two groups of NW lambs.
301 Considering the significant differences in abundance of volatile compounds from heated
302 adipose tissue and metabolites of lean meat between NW eco and NW ord, on one side and no
303 significant difference in fatty acid composition and sensory attributes on the other side, the
304 two lamb groups were merged into one group (NW lamb) below, to make graphs easier to
305 read.
306
307 *3.2. Volatile profile of heated adipose tissues*
308 Adipose tissues of lamb and sheep from the Western Balkans (BH and MN) and Norway were
309 subjected to HS-GC/MS analysis simulating oven-roasting temperature effect (150°C).
310 Seventy-six volatile compounds were identified as significant ($P \leq 0.05$) representing flavour
311 profiles of adipose tissues (see Table S-1). In order to define a relationship between identified
312 volatile compounds and the animals that belonged to different groups defined by breed, age
313 and production systems, two PCA models were run. The first PCA included all VOCs and
314 animal groups (Fig. 2a) showing a clear separation of BH 4y and MN sheep's volatile profiles.
315 The magnified graph (Fig. 2b) included animal groups that apparently showed poor separation
316 in Fig. 2a, *i.e.* BH lamb and NW animals. The results may reflect differences in rumen
317 microbial communities of different breeds (Pramenka vs. Norwegian White), large variations
318 in fatty acid composition, annual variation in a quality and availability of mountain vegetation.
319 Approximately 50% of all identified volatile compounds were products of thermal oxidation
320 processes of long-chain fatty acids. Thus, high temperatures (150−200°C) increased formation

of aldehydes and ketones with reduction in hydrocarbons and furans in agreement with Almela, Jordán, Martínez, Sotomayor, Bedia, & Bañón (2010).

According to Fig. 2a, it can be generally concluded that typical diet tracers, β-caryophyllene and γ-butyrolactone, discriminated the volatile profile of BH 4y sheep adipose tissue from MN 4y. Sesquiterpenes, such as β-caryophyllene, are almost exclusively synthesized by plants and found in high concentration in adipose tissue of lamb raised and finished on grass (Priolo et al., 2004). The absence of terpenes in adipose tissues of other pasture-fed animal groups on one hand and very minute amounts in BH 4y sheep samples on the other hand, could be due to: (i) lack of sensitivity of the dynamic headspace method; (ii) low concentrations of these compounds compared to other volatile compounds; (iii) terpenes were metabolized during the period animals spent in lairage; (iv) BH 4y sheep were slaughtered directly from the pasture. Plant diversity of grazing region may affect the absence of terpenes in analyzed samples. Mariaca, Berger, Gauch, Imhof, Jeangros, & Bosset (1997) reported a low terpene content in pasture more rich in *Gramineae* than *Dicotyledons*. However, terpenes allow discrimination between pasture and concentrate feeding systems, but their impact on sensory characteristics is not clear yet (Resconi et al., 2013). γ-butyrolactone was the most abundant volatile in adipose tissue of BH 4y sheep. Lactones are derivates of the thermal oxidation of lipids in the rumen partly reflecting animal diet (Resconi et al., 2013). Sivadier, Ratel, Bouvier, & Engel (2008) reported presence of butyrolactone in adipose tissue of pasture fed lambs. Dominant volatile compounds in MN adipose tissue were lipid degradation products, *i.e.* alkanes, alkenes, alcohols, and esters. Although the lean meat of MN sheep had a higher vitamin E content and total antioxidant capacity (Bjelanović et al., 2015), it did not maintain oxidative stability of adipose tissue probably due to a higher content of unsaturated fatty acids (see Fig.

1). Furthermore, 3-methylphenol was a significant ($P < 0.001$) contributor to MN volatile profile. The botanical composition of the MN pastures clearly influenced the accumulation of a phenolic compound in adipose tissue. Various phenolic compounds present in plants are transferred to the meat in ruminants due to fermentation of dietary phenolic precursors, such as lignin (Almela et al., 2010).

Figure 2b outlines a clear separation of BH lamb and NW animals (lamb, 2y sheep, and 4y sheep). Dimethyl sulfone was the most abundant and significant ($P < 0.001$) compound of the flavour profile of BH lamb adipose tissue. In general, pasture feeding system increases the concentration of sulphur compounds, such as dimethyl sulfone (Tansawat, Maughan, Ward, Martini, & Cornforth, 2013) that contributes to the sulphur odor and meat flavour. Tansawat et al. (2013) reported a correlation between bitterness and higher concentrations of dimethyl sulfone in pasture-fed beef. The production systems and digestive activity contributed to different lamb flavour profiles (Fig. 2b); BH lamb adipose tissue had a low abundance of lipid degradation products. Although the production systems were presumed to be basically different, the fatty acid composition of lamb groups was not significantly affected (see Fig.1). In general, the age groups of NW animals (lamb, 2y, and 4y sheep) yielded small differences in volatile profiles due to differences in fatty acid content. The existence of a number of aldehydes in NW lamb adipose tissue indicated the progression of lipid oxidation in adipose tissue of young animals. Apparently, significant ($P < 0.001$) and abundant oxidation markers such as propanal, 2-propenal and (*Z*)-2-nonenal present in NW lamb suggested lower oxidative stability for unsaturated lipids. Jo & Ahn (2000) reported that 2-propenal generates an unpleasant odour. Compared to NW lamb, there were no specific volatiles for NW 2y sheep. Thus, NW 2y volatile profile was close to BH lambs. On the other side, 2-ethylfuran

belonged significantly ($P < 0.01$) to the NW 4y animal group. A low number of significant compounds impeded a more detailed characterization of NW 4y sheep volatile profiles. It is reasonable to state that breed combined with production system had different volatile profiles (Fig. 2a and 2b) leading to the identification of local, specific meat volatile profiles.

Figure 2a

Figure 2b

3.3. Metabolites (flavour precursors) identified in raw lean meat

In total, 69 metabolites were separated and identified in the lean meat of six animal groups using GC/MS analysis (Table S-2). Thirty-five significantly different ($P \leq 0.001$) metabolites among animal groups are presented in Figure 3. The first two principal components separated the Western Balkans animal groups (BH lamb, BH 4y, and MN 4y) and NW animals (NW lamb, NW 2y, and NW 4y). BH lamb were characterized by compounds with antioxidant function. Prevalence of ascorbic acid and threonic acid, as a metabolic product of ascorbic acid catabolism (Kagawa, 1962), may reduce formation of volatile compounds originated from lipid degradation (Fig. 2b). Furthermore, hypotaurine levels were also significantly higher ($P \leq 0.001$) in the BH lamb metabolite profile. The metabolic precursor of taurine, hypotaurine has an antioxidant function *in vivo* removing highly reactive hydroxyl radicals (Aruoma, Halliwell, Hoey, & Butler, 1988). Amino acids were the most abundant metabolites for BH 4y. The broad variation among animal groups can be attributed to differences in metabolic activity, breed, age, and production system (diet, environmental conditions).

390 Differences in amounts of free amino acids could be related to differences in enzyme activities
391 (Koutsidis, Elmore, Oruna-Concha, Campo, Wood, & Mottram, 2008), although Bjelanović et
392 al. (2015) reported no significant difference in cathepsin B among studied animal groups.
393 Glutamic acid content was the most abundant amino acid (see Table S-2), as previously
394 reported for ruminants (Williams, 2007). An interesting result was the significantly higher
395 level of essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine,
396 threonine, tryptophan, and valine) for BH 4y. The amount of essential amino acids may relate
397 to the protein metabolism in the rumen, availability and efficiency in the use of nutrients by
398 rumen bacteria (Bach, Calsamiglia, & Stern, 2005). Amino acids can directly or indirectly
399 affect flavour due to generation of different volatiles through Maillard and Strecker reactions,
400 which are thermal degradation reactions. Abundant metabolites in MN 4y were free fatty acids
401 (C17:0, C18:1 cis-9 and C18:3) and Krebs cycle intermediates and acids (pyruvic, citric, and
402 ribonic acid). Utilization of glucose stimulates ATP production in Krebs cycle. Abundance of
403 pyruvic acid in MN 4y animals can indicate well-fed animals and/or exposure to stress.
404 Furthermore, a significant effect of fatty acids on MN 4y metabolite profile is in agreement
405 with high fatty acid content (Fig. 1). Some of the PUFA and intermediate products ingested by
406 ruminants may escape biohydrogenation to their saturated counterparts by direct incorporation
407 in body fat (Dervishi, Serrano, Joy, Serrano, Rodellar, & Calvo, 2010). Thus, we expect that
408 MN 4y animals have more fiber type I, more mitochondria and intramuscular fat to be used
409 for cell energy production. Apparently, in lean meat of all animal groups, small amounts of
410 sucrose were identified, being the most abundant in MN 4y samples. Animal groups differed
411 in the abundance of carbohydrates, presented in Table S-2. Quantitatively, glucose and sugar
412 phosphates (glucose-6-phosphate, fructose-6-phosphate, and mannose-6-phosphate) were

most abundant in NW lamb. Koutsidis et al. (2008) reported higher concentration of glucose, glucose-6-phosphate, fructose-6-phosphate, and mannose-6-phosphate in concentrate-fed steers. The carbohydrate content is directly related to feed availability and the level of stress animals were exposed to. Animals finished on high energy diet compared to low energy diet contained higher glycogen level (Immonen, Ruusunen, Hissa, & Puolanne, 2000). Elevated glucose concentration gave a higher level of glucose-6-phosphate. Although NW lamb group showed a different metabolite pattern characterized by carbohydrates, again, differences among NW 2y and NW 4y animals were relatively small. Increased abundance of urea in NW 4y may be a result of pre-slaughter stress that affected the physiological variables (Knowles & Warriss, 2000). The six animal groups (BH lamb, BH 4y sheep, MN 4y sheep, NW lamb, NW 2y sheep, and NW 4y sheep) showed clear separation related to specific dominant metabolites.

Figure 3

3.4. Sensory attributes

The sensory attributes, odour, taste and texture for all animal groups are presented in Figure 4. MN 4y had the strongest gamy and grassy odour, affected by pasture, environmental conditions, breed type and lipid degradation products. Thus, NW 4y animals had a high score for grassy flavour, as expected for an animal breed towards increased growth. On the other side, NW 4y had the lowest intensity of acidic taste among the examined animal groups. Furthermore, bitterness and metallic taste were highest for BH lamb. Panelists did not identify a rancid odour. The sensory evaluation showed consistency for tenderness measured by the Warner-Bratzler shear force (Bjelanović et al., 2015). Tenderness is often associated with

436 meat of younger animals. According to the panelists, BH animals (lamb and sheep) and NW
437 lamb were the most tender, while MN sheep was the toughest. The difference in juiciness
438 between lamb and sheep could be due to the age effect, because juiciness was the highest in
439 lamb, both BH and NW lamb. Sensory fattiness had no clear relation to fat content
440 (Bjelanović et al., 2015) or to the age of examined animals, suggesting different fat
441 distribution. Samples of NW 2y were rated as fattiest, followed by NW 4y and BH animals.
442
443 **Figure 4**
444
445 *3.5. Correlation among volatile compounds, metabolites and sensory attributes*
446 PLS1 regression models were developed to correlate volatile compounds and metabolites with
447 sensory attributes (Fig. 5). Positive correlation ($P < 0.01$) was found between gamy flavour
448 and several alkanes, alkenes, alcohols, phenol (3-methylphenol), amino acid and acids. The
449 relation of 3-methylphenol with lamb pastoral flavour has been noted previously by Young et
450 al. (2003). The taste active aspartic acid has been associated with acidity (Watkins et al.,
451 2013), possibly together with citric, gluconic and pyruvic acid, all indirectly contributing to
452 the gamy flavour perception. Glycine and succinic acid were negatively correlated ($P < 0.01$)
453 to the gamy odour together with six volatile compounds. Glycine was characterized as a sweet
454 compound (Watkins et al., 2013). (*Z*)-2-nonenal with plastic/chlorine odour (Watkins et al,
455 2013) showed negative correlation both to gamey and grass odour. Jo et al. (2000) reported 2-
456 propenal as a generator of unpleasant odour, and here it had a negative effect on grass odour.
457 Bitter and metallic taste showed positive correlation to hexane and dimethyl sulfone (sulfur
458 odour). Thus both taste attributes had negative correlation to heptanal and (*E, Z*)-2,4-

heptadienal. Tenderness was positively associated with higher levels of metabolites in lean meat such as ascorbic acid, β-alanine, 2-amino-butyric acid, 4-hydroxybutyric acid, succinic acid, and uridine. Oxidative-stress induced changes in energy metabolism, affected enzymes of Krebs cycle and increased formation of succinate and 4-hydroxybutyric acid. In addition, ascorbic acid as a scavenger of free radicals protects muscle tissue against reactive oxygen species (ROS). Antioxidants support the activity of respiratory mitochondrial chain and energy formation processes (Kanunnikova, Bashun, & Moiseenok, 2012). Mechanical properties of meat, *i.e.* tenderness, depend on mitochondrial activity, prolonged enzyme activity and apoptotic promotion early *post mortem* (Grabež, Kathri, Phung, Moe, Slinde, Skaugen, et al., 2015). A negative correlation was found between tenderness and higher levels of citric acid, gluconic acid, and pyruvic acid content, either related to a pro-oxidative function in the cell or to the role of enzyme inhibitors.

Figure 5

4. Conclusion

Sheep from Bosnia and Herzegovina had more complex lipid degradation products, diet tracers (β-caryophyllene and γ-butyrolactone) and were abundant in essential amino acids. Typical sulphur compounds (dimethyl sulfone) found in BH lamb contributed to the bitter and metallic taste attributes. Furthermore, sheep meat from Montenegro showed a higher content of unsaturated fatty acids, responsible for the occurrence of many lipid degradation products and had a gamy and grassy odour. Also, organic acids and diet tracers (3-methylphenol)

482 characteristic for MN 4y sheep were identified. The volatile active compounds of BH and MN
483 adipose tissue were influenced by different feeding systems. In addition, a clear separation
484 among volatile profiles of NW animals and higher content of lipid degradation products in
485 NW lamb suggested lower antioxidant stability of younger animals. An abundance of
486 carbohydrates observed for NW lamb was associated with better-fed animals with less intense
487 flavour compared to BH lambs. In general, the Norwegian samples typically scored lower for
488 taste and odour attributes except for the grassy odour.
489 The present study provided an aroma signature for two types of Pramenka breed. NW lamb
490 had a unique volatile profile compared to NW sheep.
491

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493

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639

640 **Figure Captions**

641

642 **Fig. 1.** Means of fatty acids (mg/100 g of tissue) of intramuscular fat of *M. longissimus*
643 *thoracis et lumborum*: BH lamb – Bosnia and Herzegovina lambs from conventional
644 production; BH 4y – Bosnia and Herzegovina 4y old sheep; MN 4y − Montenegro 4y old
645 sheep; NW ord – Norwegian White lambs from conventional production; NW eco –
646 Norwegian White lambs from ecological production; NW 2y – Norwegian White 2y old
647 sheep; NW 4y – Norwegian White 4y old. Animals are from typical production systems of
648 three countries. Saturated fatty acids (SFA) include 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0 and
649 20:0. Monounsaturated fatty acids (MUFA) include 16:1 and 18:1. Polyunsaturated fatty acids
650 (PUFA) include 18:2, 18:3, 20:2, 20:3, 20:4, 20:5, 22:5 and 22:6. Error bars indicate standard
651 error of means. Significant differences between mean values at the $P \leq 0.001$ level are
652 indicated using different letters.

653

654 **Fig. 2.** Principal component analysis of significant ($P < 0.001$) volatiles identified in heated
655 adipose tissues. The components 1 and 2 discriminated volatile profiles of: (a) six animal
656 groups (BH lamb, BH 4y, MN 4y, NW lamb, NW 2y, and NW 4y) and (b) four animal groups
657 (BH ord, NW lamb, NW 2y and NW 4y). Volatiles extracted from adipose tissue of all animal
658 groups are summarized in Table S-1.

659

660 **Fig. 3.** Principal component analysis of metabolites extracted from *M. longissimus thoracis et*
661 *lumborum*. Bi-plot of the first two principal components discriminated six animal groups
662 according to significant metabolites ($P \leq 0.001$) presented in Table S-2.

663 **Fig. 4.** Sensory quality profile (evaluated on a 1−9 scale) assessed by trained assessors on lean
664 meat of six animal groups. Mean scores for each attribute were tested at significance level $P <$
665 0.001 using one-way ANOVA (n = 92; eight trained panelists tested each sample in all animal
666 groups). Different letters (a−d) define significant differences ($P < 0.05$).
667
668 **Fig. 5.** Estimated regression coefficients (b_k; dimension (1/area) for VOC and (1/(mg/kg)) for
669 metabolites) obtained from Partial Least Squares Regression (PLS1) analysis of instrumental
670 (X) and sensory attributes (Y). Values for $b_k \geq 0.03$ and $b_k \geq 0.06$ were used for relation
671 between sensory attributes and volatiles and between sensory attributes and metabolites,
672 respectively.

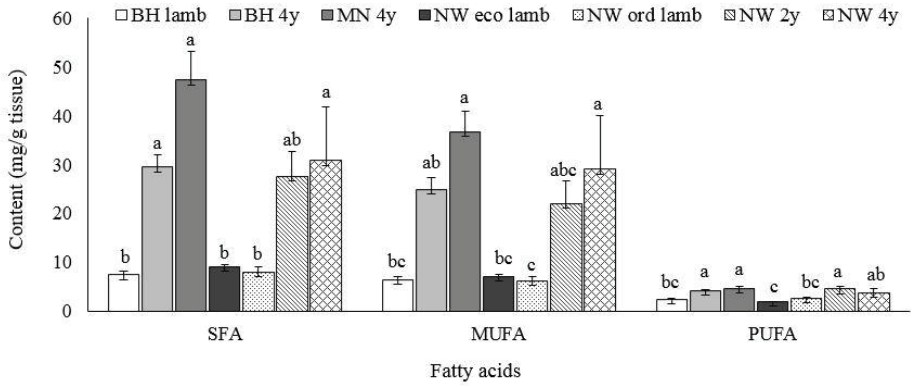

BH lamb
BH 4y
MN 4y
NW eco lamb
NW ord lamb
NW 2y
NW 4y
Content (mg/g tissue)
60
50
40
30
20
10
0
a
a
b
b
b
ab
a
bc
ab
a
bc
c
abc
a
bc
a
a
c
bc
a
ab
SFA
MUFA
PUFA
Fatty acids

Fig. 1.

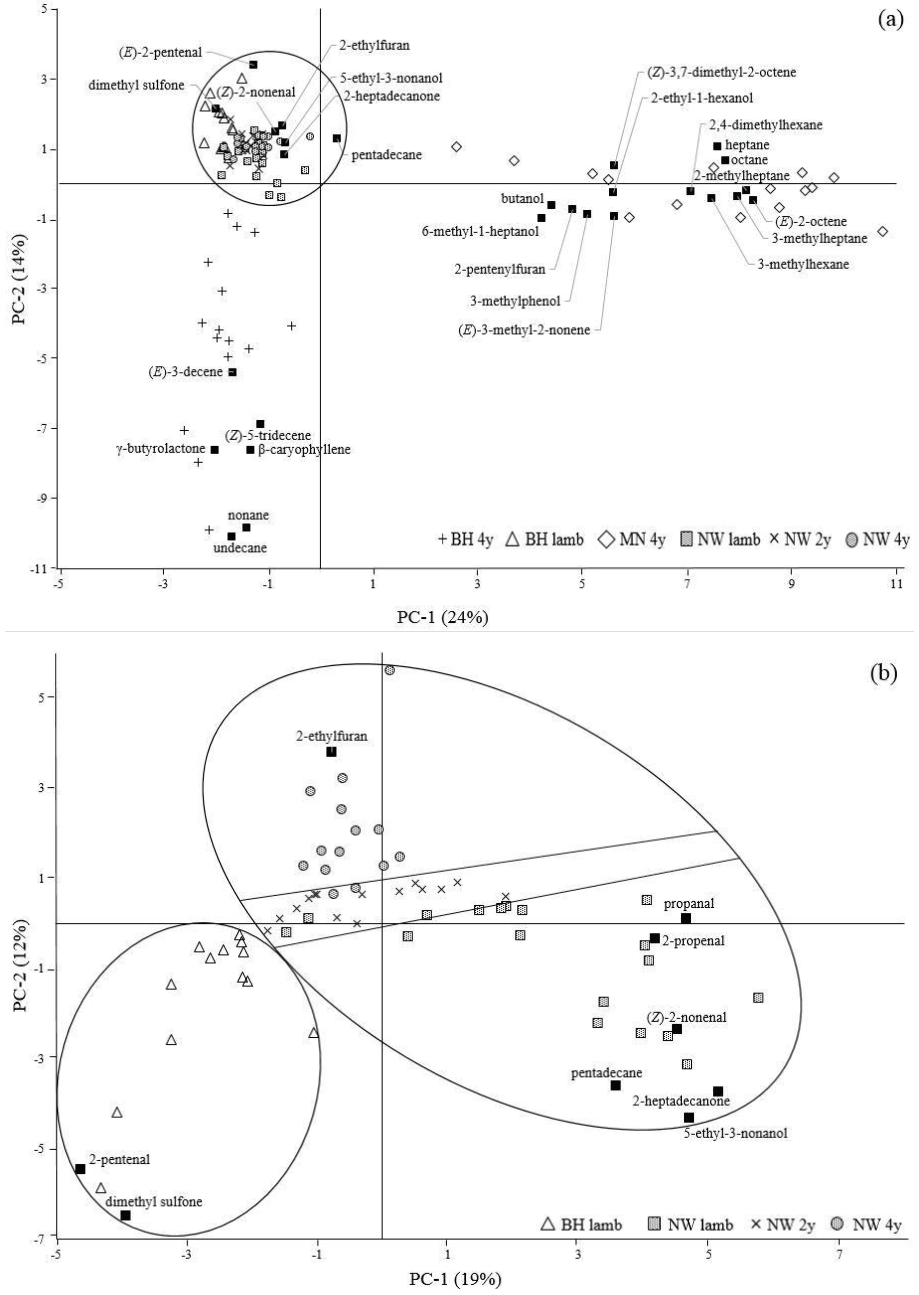

(a)
(E)-2-pentenal
dimethyl sulfone
(Z)-2-nonenal
2-ethylfuran
5-ethyl-3-nonanol
2-heptadecanone
pentadecane
(Z)-3,7-dimethyl-2-octene
2-ethyl-1-hexanol
2,4-dimethylhexane
heptane
octane
2-methylheptane
butanol
6-methyl-1-heptanol
2-pentenylfuran
3-methylphenol
(E)-3-methyl-2-nonene
(E)-2-octene
3-methylheptane
3-methylhexane
(E)-3-decene
(Z)-5-tridecene
γ-butyrolactone
β-caryophyllene
nonane
undecane
+ BH 4y △ BH lamb ◇ MN 4y ▨ NW lamb × NW 2y ◍ NW 4y
PC-2 (14%)
PC-1 (24%)
(b)
2-ethylfuran
propanal
2-propenal
(Z)-2-nonenal
pentadecane
2-heptadecanone
5-ethyl-3-nonanol
2-pentenal
dimethyl sulfone
△ BH lamb ▨ NW lamb × NW 2y ◍ NW 4y
PC-2 (12%)
PC-1 (19%)

Fig. 2.

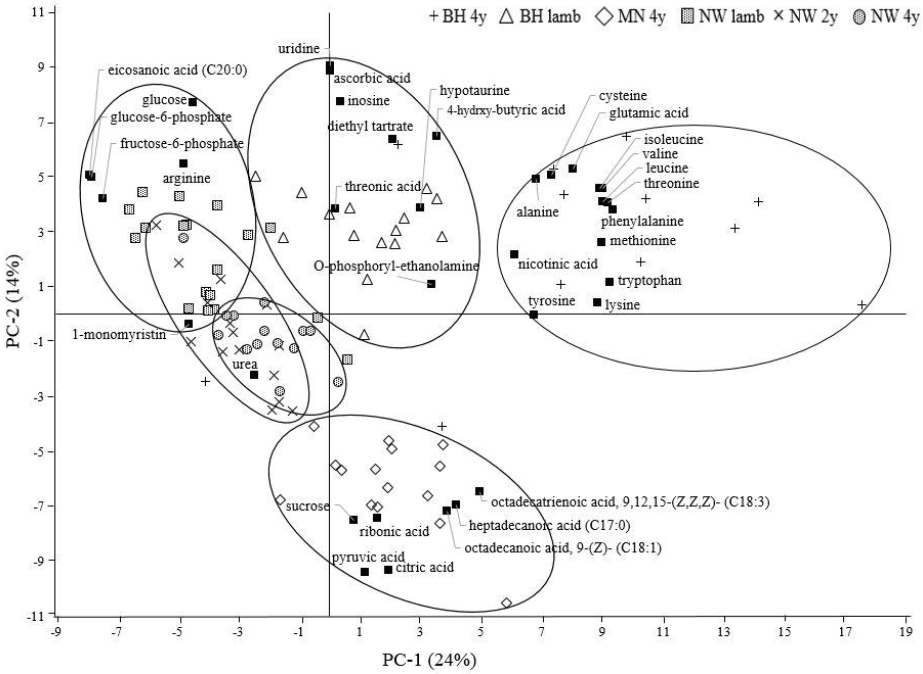

+ BH 4y
△ BH lamb
◇ MN 4y
NW lamb
× NW 2y
NW 4y
uridine
eicosanoic acid (C20:0)
ascorbic acid
hypotaurine
cysteine
glucose
inosine
4-hydrxy-butyric acid
glutamic acid
glucose-6-phosphate
diethyl tartrate
isoleucine
fructose-6-phosphate
valine
arginine
leucine
threonine
threonic acid
alanine
phenylalanine
methionine
O-phosphoryl-ethanolamine
nicotinic acid
tryptophan
tyrosine
lysine
1-monomyristin
urea
sucrose
octadecatrienoic acid, 9,12,15-(Z,Z,Z)- (C18:3)
heptadecanoic acid (C17:0)
ribonic acid
octadecanoic acid, 9-(Z)- (C18:1)
pyruvic acid
citric acid
PC-2 (14%)
PC-1 (24%)

Fig. 3.

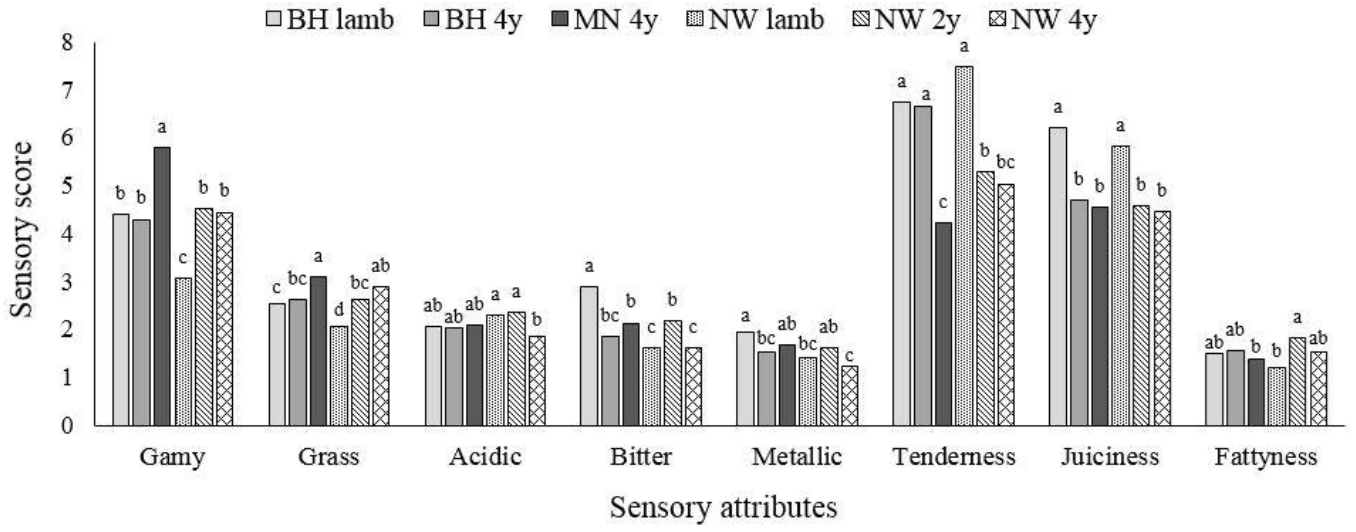
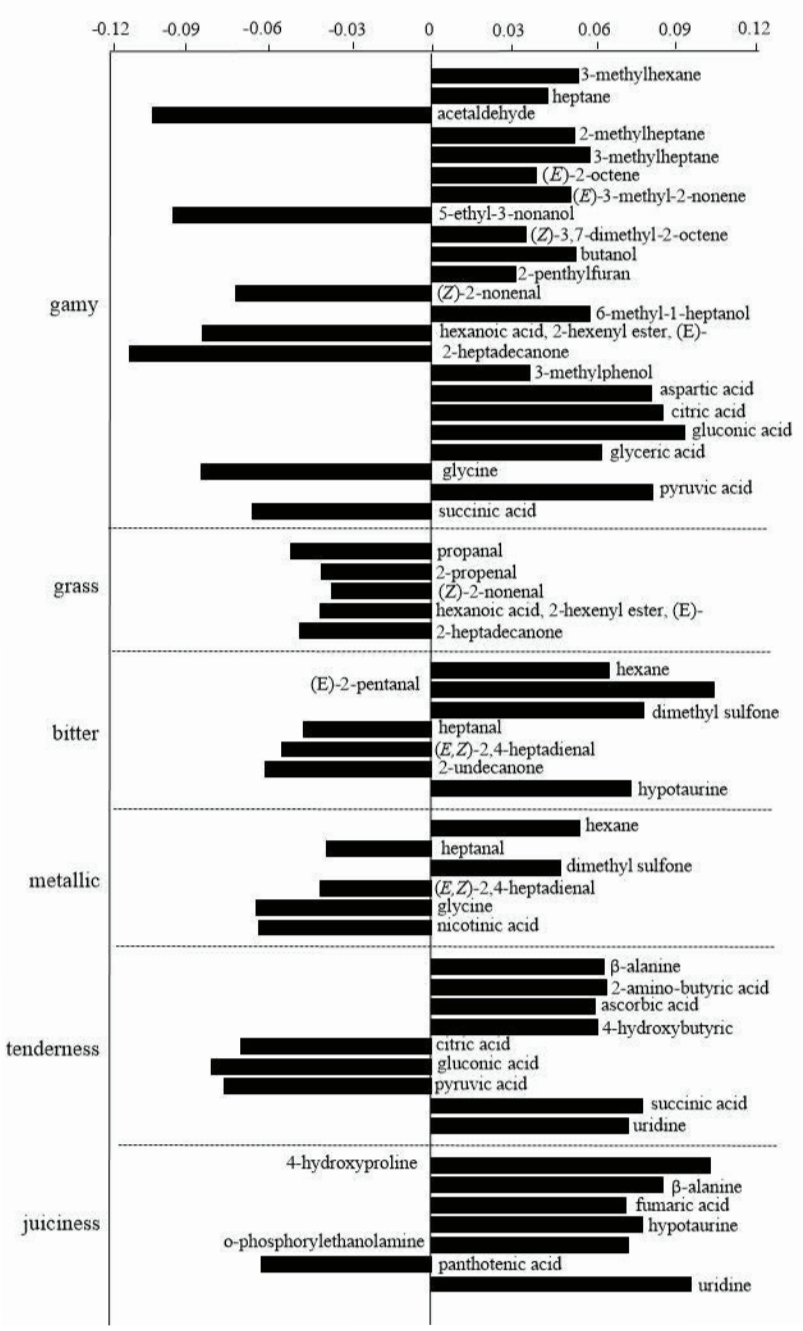

BH lamb
BH 4y
MN 4y
NW lamb
NW 2y
NW 4y
Sensory score
0
1
2
3
4
5
6
7
8
Gamy
Grass
Acidic
Bitter
Metallic
Tenderness
Juiciness
Fattyness
Sensory attributes

Fig. 4.

Fig. 5.


-0.12
-0.09
-0.06
-0.03
0
0.03
0.06
0.09
0.12
gamy
grass
bitter
metallic
tenderness
juiciness
3-methylhexane
heptane
acetaldehyde
2-methylheptane
3-methylheptane
(E)-2-octene
(E)-3-methyl-2-nonene
5-ethyl-3-nonanol
(Z)-3,7-dimethyl-2-octene
butanol
2-penthylfuran
(Z)-2-nonenal
6-methyl-1-heptanol
hexanoic acid, 2-hexenyl ester, (E)-
2-heptadecanone
3-methylphenol
aspartic acid
citric acid
gluconic acid
glyceric acid
glycine
pyruvic acid
succinic acid
propanal
2-propenal
(Z)-2-nonenal
hexanoic acid, 2-hexenyl ester, (E)-
2-heptadecanone
(E)-2-pentanal
hexane
dimethyl sulfone
heptanal
(E,Z)-2,4-heptadienal
2-undecanone
hypotaurine
hexane
heptanal
dimethyl sulfone
(E,Z)-2,4-heptadienal
glycine
nicotinic acid
β-alanine
2-amino-butyric acid
ascorbic acid
4-hydroxybutyric
citric acid
gluconic acid
pyruvic acid
succinic acid
uridine
4-hydroxyproline
β-alanine
fumaric acid
hypotaurine
o-phosphorylethanolamine
panthotenic acid
uridine

Appendix A. Supplementary data

Table S-1. The volatile compounds identified in aliquot of 1g lamb/sheep fat by HS-GC/MS.

RT [1] (min)	RI [2]	Compounds	% of total area						
			BH lamb [3] (n = 15)	BH 4y [3] (n = 15)	MN 4y [3] (n = 15)	NW eco [3] (n = 9)	NW ord [3] (n = 9)	NW 2y [3] (n = 15)	NW 4y [3] (n = 14)
Alkanes									
2.09	654	hexane***	0.796 [a, 4]	n.d.[5]	n.d.	0.482[b]	0.107[b]	0.726[a]	0.176[b]
2.27	674	3-methylhexane***	n.d.	n.d.	0.039	n.d.	n.d.	n.d.	n.d.
2.39	687	heptane***	0.047[b]	n.d.	0.097[a]	0.289[b]	0.089[b]	0.086[b]	0.056[b]
2.52	700	2,4-dimethylhexane***	n.d.	n.d.	0.087	n.d.	n.d.	n.d.	n.d.
2.83	732	2-methylheptane***	n.d.	n.d.	0.179[a]	n.d.	n.d.	n.d.	0.038[b]
2.93	741	3-methylheptane***	n.d.	n.d.	0.360	n.d.	n.d.	n.d.	n.d.
2.98	746	methylcyclohexane**	n.d.	n.d.	n.d.	n.d.	0.235	n.d.	n.d.
3.25	771	octane***	0.015[c]	0.040[c]	0.225[a]	0.216[bc]	0.517[b]	0.100[bc]	0.440[bc]
5.25	914	nonane***	n.d.	0.033[a]	n.d.	n.d.	n.d.	n.d.	n.d.
8.44	1022	2-methyldecane**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.286
17.34	1080	undecane***	0.142[b]	0.927[a]	0.008[b]	n.d.	n.d.	0.088[b]	n.d.
25.83	1187	2-propyl-1,1,3-trimethyl-cyclohexane*	n.d.	n.d.	0.004	n.d.	n.d.	n.d.	n.d.
32.62	1393	tetradecane**	n.d.	n.d.	n.d.	0.014[a]	0.005[ab]	n.d.	n.d.
34.96	1488	pentadecane***	n.d.	n.d.	0.001[b]	n.d.	0.047[a]	n.d.	n.d.
37.06	1584	hexadecane**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.003
		Total area	2,23E+08	1,28E+08	1,20E+09	7,08E+07	1,43E+08	2,15E+08	1,16E+08
Alkenes									
2.20	666	(E)-2-hexene**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000
4.60	875	(*E*)-2-octene***	0.100[b]	n.d.	0.998[a]	n.d.	n.d.	n.d.	n.d.
11.23	1043	(*E*)-3-decene**	n.d.	0.545	n.d.	n.d.	n.d.	n.d.	n.d.
12.73	1044	(*Z*)-5-tridecene***	n.d.	0.154	n.d.	n.d.	n.d.	n.d.	n.d.

14.87	1050	(*Z*)-3,7-dimethyl-2-octene***	n.d.	n.d.	0.002	n.d.	n.d.	n.d.	n.d.
14.77	1050	(*E*)-3-methyl-2-nonene***	n.d.	n.d.	0.002	n.d.	n.d.	n.d.	n.d.
26.76	1209	(*E*)-3-dodecene***	n.d.	0.138	n.d.	n.d.	n.d.	n.d.	n.d.
40.63	1772	[R-[R*,R*-(*E*)]]-3,7,11,15-tetramethyl-2-hexadecene**	n.d.	0.163^{a}	n.d.	0.100^{ab}	0.100^{ab}	n.d.	n.d.
		Total area	3,22E+07	4,68E+07	2,15E+09	4,84E+06	1,80E+06	7,20E+05	4,92E+06
Alcohols									
7.36	998	ethyl alcohol*	n.d.	0.923^{ab}	0.940^{ab}	n.d.	0.957^{a}	n.d.	n.d.
16.16	1064	5-ethyl-3-nonanol***	n.d.	n.d.	n.d.	0.010^{a}	0.004^{b}	n.d.	n.d.
22.57	1126	butanol***	n.d.	n.d.	0.004	n.d.	n.d.	n.d.	n.d.
23.59	1143	1-penten-3-ol*	n.d.	0.035^{a}	0.017^{ab}	0.370^{a}	0.010^{ab}	0.336^{ab}	0.282^{ab}
25.83	1187	2-heptyn-1-ol*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.050
28.36	1251	1-pentanol**	n.d.	0.022^{ab}	0.020^{ab}	0.432^{a}	0.020^{a}	0.660^{ab}	0.524^{a}
31.74	1360	hexanol*	n.d.	0.009^{a}	n.d.	0.106^{a}	0.005^{a}	n.d.	0.070^{a}
34.25	1458	1-octen-3-ol***	n.d.	0.012^{a}	0.011^{a}	0.082^{ab}	0.003^{ab}	n.d.	0.054^{ab}
35.01	1490	2-ethyl-1-hexanol***	n.d.	n.d.	0.004	n.d.	n.d.	n.d.	n.d.
36.56	1561	6-methyl-1-heptanol***	n.d.	n.d.	0.001	n.d.	n.d.	n.d.	n.d.
36.57	1561	1-octanol*	n.d.	n.d.	0.002^{ab}	n.d.	0.002^{a}	n.d.	0.020^{ab}
42.69	1893	benzyl alcohol*	n.d.	n.d.	0.001	n.d.	n.d.	n.d.	n.d.
		Total area	1,08E+06	4,51E+08	5,69E+08	5,20E+07	1,11E+09	2,51E+07	3,97E+07
Aldehydes									
2.58	707	acetaldehyde**	0.163^{ab}	0.389^{ab}	n.d.	0.052^{b}	0.339^{a}	0.084^{b}	0.278^{ab}
3.37	782	propanal***	n.d.	0.041^{b}	n.d.	0.084^{a}	0.013^{b}	0.034^{b}	n.d.
3.41	785	butanedial**	n.d.	0.031^{ab}	n.d.	0.011^{ab}	0.031^{a}	n.d.	0.026^{ab}
4.20	848	2-methylpropanal*	n.d.	n.d.	n.d.	n.d.	n.d.	0.031	n.d.
4.38	860	2-propenal***	n.d.	0.035^{b}	n.d.	0.077^{a}	0.019^{b}	n.d.	n.d.
5.01	900	butanal*	n.d.	0.023	n.d.	n.d.	n.d.	n.d.	n.d.

6.33	964	3-methylbutanal*	0.147^{ab}	0.048^{ab}	n.d.	0.033^{ab}	0.037^{a}	0.096^{ab}	n.d.
6.88	984	pentanal**	0.033^{a}	0.023^{ab}	n.d.	0.001^{ab}	0.047^{ab}	0.026^{ab}	n.d.
14.20	1047	2-butenal**	n.d.	n.d.	0.111	n.d.	n.d.	n.d.	n.d.
17.37	1080	hexanal***	0.304^{a}	n.d.	0.258^{ab}	0.243^{a}	0.196^{a}	0.196^{a}	0.198^{ab}
21.23	1108	(*E*)-2-pentenal ***	0.256^{a}	n.d.	0.005^{b}	n.d.	n.d.	n.d.	n.d.
24.89	1167	heptanal***	n.d.	0.254^{b}	0.507^{ab}	0.288^{a}	0.183^{ab}	0.311^{ab}	0.230^{b}
27.90	1238	(*E*)-2-heptenal*	n.d.	0.011^{a}	n.d.	0.003^{ab}	0.003^{ab}	n.d.	0.004^{ab}
26.77	1209	(*E*)-2-hexenal**	n.d.	0.025^{a}	0.030^{ab}	0.010^{ab}	0.013^{ab}	n.d.	0.022^{ab}
29.61	1288	octanal*	0.023^{ab}	n.d.	0.019^{ab}	0.032^{ab}	0.018^{ab}	0.050^{a}	0.062^{ab}
32.79	1399	nonanal**	0.074^{ab}	0.048^{ab}	0.057^{a}	0.099^{a}	0.065^{ab}	0.120^{a}	0.101^{ab}
33.82	1440	(*E*)-2-octenal**	n.d.	0.013^{abc}	0.009^{bc}	0.008^{abc}	0.014^{a}	0.014^{ab}	0.011^{abc}
34.76	1479	(*E,Z*)-2,4-heptadienal***	n.d.	0.058^{a}	0.085^{a}	0.050^{a}	0.020^{ab}	0.038^{a}	0.067^{a}
36.24	1546	(*Z*)-2-nonenal***	n.d.	n.d.	n.d.	0.013^{ab}	0.028^{a}	n.d.	n.d.
		Total area	2,69E+08	2,60E+08	1,53E+08	4,04E+08	4,62E+08	3,17E+08	2,39E+08
Ketones									
4.16	845	acetone*	0.481^{a}	0.041^{ab}	n.d.	0.045^{ab}	0.102^{ab}	0.039^{ab}	0.008^{b}
29.61	1288	3-hydroxy-2-butanone**	n.d.	0.333^{a}	n.d.	n.d.	n.d.	n.d.	0.246^{ab}
30.88	1330	2,3-octanedione**	0.519^{b}	0.595^{ab}	0.944^{ab}	0.926^{a}	0.797^{ab}	0.955^{a}	0.746^{ab}
37.44	1603	2-undecanone***	n.d.	0.005^{ab}	0.015^{a}	0.009^{a}	0.029^{a}	0.005^{ab}	n.d.
38.83	1674	acetophenone**	n.d.	0.013	n.d.	n.d.	n.d.	n.d.	n.d.
44.78	2026	2-pentadecanone***	n.d.	0.022^{a}	0.041^{a}	0.017^{a}	0.057^{a}	n.d.	n.d.
48.01	2252	2-heptadecanone***	n.d.	n.d.	n.d.	0.003^{ab}	0.016^{a}	n.d.	n.d.
		Total area	9,34E+07	4,54E+08	3,10E+08	5,39E+08	1,54E+08	5,10E+08	2,35E+08
Acids									
35.72	1522	formic acid**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.422
36.84	1574	2-methylpropanoic acid*	n.d.	0.175^{a}	0.156^{a}	0.061^{a}	0.122^{a}	n.d.	n.d.

38.01	1631	butanoic acid**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.219
45.38	2066	octanoic acid**	n.d.	0.089^{ab}	n.d.	0.126^{ab}	0.254^{a}	n.d.	0.184^{ab}
48.50	2288	decanoic acid*	1.000^{ab}	0.736^{ab}	0.844^{ab}	0.813^{a}	0.625^{ab}	n.d.	0.174^{ab}
		Total area	7,30E+06	1,32E+07	1,11E+07	1,50E+07	1,64E+07	4,50E+05	1,21E+07
Lactone									
38.49	1656	γ-butyrolactone***	n.d.	5.36E+06^{a}	n.d.	9.94E+05^{b}	3.15E+06^{ab}	3.55E+05^{b}	1.36E+06^{b}
Terpene									
37.32	1597	β-caryophyllene***	n.d.	3.00E+06	n.d.	n.d.	n.d.	n.d.	n.d.
Sulphur compound									
43.33	1933	dimethyl sulfone***	1.89E+07^{a}	4.55E+06^{b}	1.71E+06^{b}	2.58E+06^{b}	6.29E+06^{b}	n.d.	1.96E+06^{b}
Phenol									
45.91	2103	3-methylphenol***	n.d.	n.d.	1.66E+06	n.d.	n.d.	n.d.	n.d.
Esters									
38.32	1647	hexanoic acid, 2-hexenyl ester, (E)-***	n.d.	4.69E+05^{b}	n.d.	1.53E+06^{a}	2.21E+05	n.d.	n.d.
Others									
7.89	1011	2-ethylfuran**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7,64E+06
13.51	1045	toluene**	n.d.	n.d.	4.13E+06	n.d.	n.d.	n.d.	n.d.
28.76	1263	1-methyl-4-(1-methylethyl)-benzene **	8.35E+06^{a}	n.d.	4.11E+06^{ab}	n.d.	n.d.	n.d.	n.d.
30.14	1305	2-pentenylfuran***	n.d.	n.d.	1,97E+06	n.d.	n.d.	n.d.	n.d.
37.93	1627	benzonitrile**	2.14E+06^{b}	7.03E+06^{ab}	9.31E+06^{a}	9.92E+06^{a}	5.44E+06^{ab}	4.41E+06^{ab}	6.02E+06^{ab}

[1] RT = Retention time

[2] RI = Retention index in DB - WAXetr column

[3] Adipose tissues of seven animal groups: BH lamb – Bosnia and Herzegovina lambs from conventional production; BH 4y – Bosnia and Herzegovina 4y old sheep; MN 4y − Montenegro 4y old sheep; NW ord – Norwegian White lambs from conventional production; NW eco – Norwegian White lambs from ecological production; NW 2y – Norwegian White 2y old sheep; NW 4y – Norwegian White 4y old.

[4] *abcd* Means of area values (%) with different letters in the same row indicate statistically significant differences

[5] n.d. = Not detected in the sample

* = $P < 0.05$

** = $P < 0.01$

*** = $P < 0.001$

Table S-2. List of metabolites detected in lamb/sheep *M. longissimus thoracis et lumborum* and identified by GC/MS.

RI[1]	Metabolites	Concentration (mg/kg)						
		BH lamb [2] (n = 15)	BH 4y [2] (n = 15)	MN 4y [2] (n = 15)	NW eco [2] (n = 9)	NW ord [2] (n = 9)	NW 2y [2] (n = 15)	NW 4y [2] (n = 14)
	Amino acids							
1090.70	alanine	174.51^{ab}	196.91^{a}	146.68^{bc}	163.77^{ab}	136.26^{bcd}	108.04^{d}	122.05^{cd}
1162.23	2-aminobutyric acid	0.86^{abc}	1.16^{a}	0.62^{c}	1.08^{ab}	0.95^{abc}	0.70^{bc}	0.67^{c}
1526.53	4-aminobutyric acid	8.36^{ab}	10.50^{a}	5.11^{bc}	4.89^{bc}	5.03^{bc}	3.16^{c}	3.58^{c}
1814.43	arginine	0.64^{ab}	0.32^{c}	0.52^{bc}	0.83^{a}	0.59^{abc}	0.41^{bc}	0.44^{bc}
1666.44	asparagine	1.58^{b}	2.72^{a}	1.20^{b}	1.37^{b}	1.42^{b}	0.86^{b}	1.09^{b}
1509.77	aspartic acid	5.98^{b}	10.95^{a}	15.40^{a}	2.36^{b}	2.87^{b}	2.53^{b}	5.27^{b}
1424.73	β-alanine	29.48^{ab}	13.89^{cd}	11.28^{d}	39.31^{a}	23.34^{bc}	11.36^{d}	12.42^{d}
1140.39	butyro-1,4-lactam	0.16^{c}	0.88^{a}	0.57^{ab}	0.11^{bc}	0.30^{bc}	0.18^{bc}	0.33^{bc}
1366.92	cyclo-leucine	5.29^{ab}	6.56^{a}	6.32^{a}	3.86^{bc}	3.52^{c}	3.92^{bc}	4.97^{abc}
1549.83	cysteine	4.80^{b}	7.38^{a}	2.10^{c}	2.07^{c}	2.50^{bc}	1.88^{c}	2.59^{bc}
1615.40	glutamic acid	86.53^{b}	228.66^{a}	28.10^{b}	56.56^{b}	55.35^{b}	40.80^{b}	53.09^{b}
1304.47	glycine	184.42^{bc}	220.23^{ab}	180.56^{bc}	230.10^{ab}	266.70^{a}	139.73^{c}	176.73^{bc}
1661.30	homocysteine	0.51^{ab}	0.67^{a}	0.60^{a}	0.47^{ab}	0.31^{b}	0.41^{b}	0.52^{ab}
1518.00	4-hydroxyproline	8.57^{a}	2.36^{b}	2.66^{b}	6.16^{a}	6.10^{a}	2.38^{b}	2.32^{b}
1605.89	hypotaurine	2.72^{a}	0.94^{b}	0.90^{b}	0.55^{b}	0.48^{b}	0.39^{b}	0.42^{b}
1288.60	isoleucine	42.63^{b}	69.03^{a}	32.66^{bc}	27.82^{bc}	25.69^{bc}	22.43^{c}	25.75^{c}
1264.43	leucine	69.68^{b}	123.60^{a}	56.73^{bc}	42.97^{bc}	40.47^{bc}	36.77^{c}	41.55^{bc}
1912.30	lysine	15.97^{bc}	35.06^{a}	22.27^{b}	14.43^{bc}	12.58^{c}	12.61^{c}	15.20^{bc}
1514.50	methionine	18.24^{b}	29.64^{a}	17.35^{bc}	9.96^{c}	10.77^{bc}	10.45^{c}	13.26^{bc}
1809.10	ornithine	5.89^{b}	12.60^{a}	4.86^{b}	2.45^{b}	2.43^{b}	3.24^{b}	3.16^{b}
1629.33	phenylalanine	45.36^{b}	83.91^{a}	41.87^{bc}	29.24^{bc}	27.78^{bc}	24.88^{c}	30.66^{bc}
1519.43	pyroglutamic acid	64.31^{b}	90.09^{a}	44.33^{b}	62.91^{ab}	41.23^{b}	49.75^{b}	54.83^{b}
1379.43	threonine	32.19^{b}	63.04^{a}	29.89^{bc}	28.56^{bc}	22.33^{bc}	19.11^{c}	20.83^{bc}
2214.74	tryptophan	6.94^{bc}	12.89^{a}	7.50^{b}	3.32^{c}	3.51^{bc}	3.72^{c}	5.34^{bc}
1932.60	tyrosine	24.25^{bc}	64.06^{a}	44.21^{ab}	24.72^{bc}	25.28^{bc}	24.23^{c}	30.06^{bc}
1208.77	valine	52.13^{b}	98.43^{a}	42.19^{bc}	35.62^{bc}	32.06^{bc}	28.48^{c}	30.47^{bc}
	Total concentration	892.00	1386.47	746.46	795.47	749.83	552.43	657.60
	Acids							
1937.87	ascorbic acid	0.89^{a}	0.76^{ab}	0.17^{c}	0.74^{ab}	0.77^{ab}	0.39^{bc}	0.38^{bc}

1804.57	citric acid	4.84^{b}	2.04^{bc}	23.09^{a}	2.08^{bc}	2.36^{bc}	1.92^{c}	2.72^{bc}
2010.00	diethyl tartrate	1.06^{a}	0.86^{ab}	0.56^{b}	0.90^{ab}	0.56^{ab}	0.42^{b}	0.39^{b}
1346.23	fumaric acid	9.79^{a}	3.84^{c}	4.30^{c}	9.15^{ab}	5.91^{abc}	4.91^{bc}	8.51^{ab}
1980.49	galactonic acid	0.11^{c}	0.31^{abc}	0.24^{bc}	0.57^{a}	0.51^{a}	0.47^{a}	0.46^{ab}
1989.07	gluconic acid	6.16^{b}	2.27^{b}	51.44^{a}	2.16^{b}	2.62^{b}	2.46^{b}	3.38^{b}
1927.57	glucuronic acid	0.44^{a}	0.49^{a}	0.09^{b}	0.21^{b}	0.20^{b}	0.16^{b}	0.11^{b}
1321.67	glyceric acid	32.34^{ab}	6.85^{c}	42.72^{a}	27.78^{b}	19.94^{bc}	26.39^{b}	22.95^{b}
1149.20	3-hydroxybutyric acid	8.42^{ab}	12.09^{a}	5.01^{b}	3.08^{b}	2.96^{b}	2.50^{b}	2.93^{b}
1228.67	4-hydroxybutyric acid	2.95^{a}	2.59^{ab}	1.13^{d}	2.29^{abc}	1.68^{bcd}	1.08^{d}	1.36^{cd}
1477.30	malic acid	17.32^{abc}	11.60^{bcd}	22.50^{a}	22.59^{ab}	15.06^{abcd}	7.07^{d}	10.05^{cd}
1037.80	pyruvic acid	0.84^{b}	0.39^{b}	8.57^{a}	0.54^{b}	0.42^{b}	0.74^{b}	0.43^{b}
1750.53	ribonic acid	1.16^{b}	1.05^{b}	7.00^{a}	0.84^{b}	0.97^{b}	0.77^{b}	1.07^{b}
1310.17	succinic acid	228.61^{ab}	273.02^{a}	94.95^{e}	207.67^{bc}	173.95^{cd}	141.96^{d}	172.92^{cd}
1546.43	threonic acid	1.01^{a}	0.63^{b}	0.81^{ab}	0.85^{ab}	0.70^{b}	0.60^{b}	0.66^{b}
	Total concentration	315,96	318,79	262,58	281,45	228,61	191,85	228,32
	Alcohols							
1707.80	arabitol	5.71^{c}	8.29^{a}	1.67^{d}	5.98^{abc}	5.81^{bc}	5.12^{c}	7.70^{ab}
1031.00	2,3-butanediol	8.09^{b}	28.17^{a}	4.47^{b}	6.68^{b}	7.32^{b}	11.71^{b}	14.20^{b}
1927.45	galactitol	24.43^{b}	34.08^{a}	11.13^{c}	14.90^{bc}	13.18^{c}	14.47^{bc}	12.00^{c}
2083.87	*myo*-inositol	319.19^{a}	326.71^{a}	206.81^{b}	327.69^{a}	313.34^{ab}	315.73^{a}	206.34^{b}
	Total concentration	357,42	397,24	224,08	355,26	339,65	347,03	240,24
	Sugars							
1856.23	fructose	237.09^{a}	130.63^{bc}	114.28^{c}	291.32^{a}	212.06^{abc}	186.38^{ab}	228.33^{a}
1897.67	galactose	607.04^{a}	502.74^{ab}	330.67^{b}	630.09^{a}	605.67^{a}	570.28^{a}	634.11^{a}
1878.20	glucose	926.08^{ab}	676.39^{bc}	454.17^{c}	1112.88^{a}	982.31^{ab}	789.82^{ab}	867.94^{ab}
2629.63	sucrose	0.16^{b}	0.11^{b}	2.20^{a}	0.39^{b}	0.64^{b}	0.41^{b}	0.16^{b}
	Total concentration	1770.37	1309.86	901.32	2034.68	1800.69	1546.89	1730.54
	Phosphates							
2292.77	fructose-6-phosphate	216.99^{bcd}	165.94^{cd}	163.96^{d}	391.42^{a}	285.84^{abc}	299.11^{ab}	328.34^{a}
2329.63	glucose-6-phosphate	720.40^{bc}	530.50^{c}	537.61^{c}	1296.92^{a}	1010.43^{ab}	909.11^{ab}	1020.33^{ab}
2236.42	glycero-3-phosphoethanolamine	5.11^{bc}	7.13^{ab}	5.57^{bc}	8.62^{a}	4.86^{bc}	4.93^{bc}	4.12^{c}
1748.53	glycerol-3-phosphate	25.00^{bc}	58.07^{a}	36.44^{abc}	44.71^{ab}	24.83^{bc}	40.63^{abc}	16.79^{c}
2307.10	mannose-6-phosphate	37.19^{bc}	21.92^{c}	26.73^{c}	84.68^{a}	61.44^{ab}	47.06^{b}	52.05^{b}

1779.12	O-phosphoryl-ethanolamine	4.29^{a}	2.67^{b}	2.86^{b}	2.12^{b}	1.88^{b}	1.73^{b}	1.63^{b}
	Total concentration	1008,98	786,24	773,17	1828,47	1389,28	1302,56	1423,27
	Lipids							
2451.63	eicosanoic acid (C20:0)	3.60^{cd}	2.21^{d}	2.68^{d}	8.16^{a}	5.98^{ab}	4.52^{bc}	5.03^{bc}
2144.58	heptadecanoic acid (C17:0)	0.47^{b}	0.66^{ab}	0.85^{a}	0.32^{b}	0.39^{b}	0.48^{b}	0.58^{ab}
2382.00	1-monomyristin	0.18^{b}	0.12^{b}	0.26^{ab}	0.48^{a}	0.33^{ab}	0.24^{b}	0.28^{ab}
2208.23	octadecadienoic acid, 9,12-(*Z*,*Z*)- (18:2)	1.11^{abc}	2.15^{a}	1.77^{ab}	0.55^{c}	0.64^{bc}	0.76^{bc}	0.93^{bc}
2214.83	octadecanoic acid, 9-(*Z*)- (C18:1)	4.48^{b}	7.62^{ab}	14.92^{a}	2.35^{b}	3.20^{b}	3.89^{b}	5.61^{b}
2217.41	octadecatrienoic acid, 9,12,15-(*Z*,*Z*,*Z*)- (C18:3)	0.06^{c}	0.31^{ab}	0.36^{a}	0.08^{bc}	0.06^{bc}	0.13^{bc}	0.13^{bc}
	Total concentration	9,90	13,08	20,84	11,94	10,60	10,00	12,56
	Other N-containing compounds							
1135.77	3-hydroxypyridine	1.22^{a}	0.85^{b}	1.14^{a}	1.41^{a}	1.33^{a}	1.14^{a}	1.28^{a}
2577.30	inosine	215.92^{a}	180.22^{ab}	154.39^{b}	192.75^{ab}	165.69^{b}	140.01^{b}	171.29^{b}
1274.00	nicotinic acid	1.73^{b}	3.06^{a}	2.15^{b}	2.15^{b}	2.11^{b}	1.87^{b}	2.02^{b}
1984.50	panthotenic acid	0.51^{ab}	0.78^{a}	0.39^{b}	0.24^{b}	0.28^{b}	0.66^{a}	0.78^{a}
1396.91	thymine	1.97^{a}	2.17^{a}	1.38^{ab}	1.26^{ab}	0.88^{b}	1.10^{b}	1.40^{ab}
1910.63	tyramine	46.78^{a}	58.68^{a}	3.70^{b}	1.00^{b}	0.69^{b}	0.58^{b}	0.85^{b}
1235.60	urea	70.57^{c}	75.27^{bc}	114.07^{ab}	117.21^{ab}	111.66^{abc}	115.68^{a}	68.20^{c}
2463.67	uridine	6.84^{a}	4.31^{bc}	2.90^{d}	4.99^{b}	4.87^{b}	3.49^{cd}	3.28^{cd}
	Total concentration	345.54	325.33	280.11	321.01	287.52	264.53	249.09

[1] RI = Retention index

[2] *M. longissimus thoracis et lumborum* of seven animal groups: BH lamb – Bosnia and Herzegovina lambs from conventional production; BH 4y – Bosnia and Herzegovina 4y old sheep; MN 4y − Montenegro 4y old sheep; NW ord – Norwegian White lambs from conventional production; NW eco – Norwegian White lambs from ecological production; NW 2y – Norwegian White 2y old sheep; NW 4y – Norwegian White 4y old.

^{abcde} Mean values with different letters indicate significant difference at ($P \leq 0.001$).

Paper V

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Production process and quality of two different dry-cured sheep hams from Western Balkan countries

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ABSTRACT

Differences in the production process, the composition of volatile compounds (VOCs), physicochemical parameters and sensory properties were studied in *Stelja* sheep ham, produced in Bosnia and Herzegovina (B&H) and Montenegro (MN) using different technologies. Gas Chromatography–Mass Spectrometry was used for the analysis of volatile compounds. MN sheep hams were featured with more intense smoke flavour, relatively higher salt content (6.4% w/w) and a one week salting period. The most prominent smoke compounds identified in MN hams were furans and phenols. Furthermore, lipid degradation compounds (butanal, hexanal, heptanal, 2,3-pentanedione, and 1-hydroxy-2-propanone) differed among the two ham productions, being more abundant in MN hams, yet the products were not evaluated as rancid. B&H hams were less salty (4% w/w after 3 weeks of salting), with a distinct garlic and metallic flavour and a more acidic taste compared to MN hams. Metabolites of the B&H hams implied that, due to the longer salting step, fermentation by microorganisms could have occurred. Differences in process technology significantly influenced the composition of volatile compounds and sensorial properties of these products produced in neighboring geographical areas.

1. Introduction

Dry-cured sheep meat traditionally produced in the Western Balkan countries is often made using sheep aged between 1 and 6 years. The name *Pastrma* refers to salted, smoked and dried sheep carcasses (Stamenković & Dević, 2006). Užice sheep *Pastrma* (Serbia) consists of the whole carcass after cutting along sternum and pelvic and removing head, organs and spinal cord from the inside of the carcass. Kidneys with fat are kept on the carcass. Leg muscles are removed for ham production. Salted and dried sheep meat without bones is called *Stelja*. In Zlatibor (Serbia) mountain region, sheep ham and *Stelja* were produced by using nitrite and nitrate during a 28–42 days period (Troeger, Vesković-Moračanin, Turubatović, Ristić, & Dederer, 2009). In B&H *Stelja*, ham and shoulder are typical sheep products (Ganić, Čaušević, Karahmet, Stojković, & Ratković, 2013; Operta, Smajić, Tahmaz, & Ganić, 2010). In B&H garlic and pepper are added in some regions with salt to achieve characteristic aroma to the product (Ganić et al., 2013). The eastern part of B&H uses dry spruce branches during the first few days of smoking to obtain gold-yellow colour and specific aroma (Ganić, 2012). The former Yugoslav Republic of Macedonia has its own *Pastrma* produced from deboned sheep carcass using characteristic processing steps like wrapping of the product in sheep skin or sprinkling it with corn flour (Džinleski, 1969). *Kastradina* (Croatia) is produced during 43 and 73 days from sheep haunch, shoulder, and “kora” (remaining part of carcass) using salting (sea salt, with or without spices), washing, drying-smoking, and ripening (Krvavica, Friganović, Đugum, & Kegalj, 2009; Krvavica et al., 2011).

Mediterranean countries consider dry-salted and dried sheep meat as a regional, traditional product (Villalobos-Delgado et al.,

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2014). However, North–Europe has also traditionally produced dried sheep and lamb in the Faeroe Islands, Iceland and Norway (Håseth, Thorkelsson, Puolanne, & Sidhu, 2014). The Faeroe Islands have a unique air-dried and unsalted lamb meat product named *Skerpikjøt*. Norway has a protected product from dry-cured lamb or sheep leg called *Fenalår*. The production varies in salt content, the use of nitrite, smoke, and flavour additives, and the entire processing takes 90 days (Håseth et al., 2014).

The number of publications related to Western Balkan lamb and sheep dry-cured ham is small (Ganić, 2012; Ganić et al., 2013; Krvavica et al., 2009, 2011; Operta et al., 2010; Stamenković & Dević, 2006; Troeger et al., 2009) as for other parts of Europe (Håseth et al., 2014; Villalobos-Delgado et al., 2014). Chemical characterization of sheep hams is useful for understanding and comparing sensory properties of specific regional products, but is largely missing in the literature.

The present study focuses on *Stelja* production from two regions of Western Balkan, *i.e.* from B&H and Montenegro. From B&H the dry-cured product studied was typical for the Vlašić region while Montenegro has only one production process. Both products are produced by the local sheep Pramenka. The dry-cured sheep meat in B&H and MN is mostly produced in local butchers' during the winter and spring season (Stamenković & Dević, 2006) in small quantities. Nevertheless, larger scale production has been established during the last decade mimicking the old traditional production processes.

This study was designed to describe the differences in salt content, development of volatile compounds, organic acid profiles and sensory attributes of traditional, dry cured sheep ham produced in different regions of Western Balkan (B&H and MN).

2. Materials and methods

Fifteen sheep of the Vlašićka Pramenka from mountain Vlašić (B&H) and 15 sheep of Pivska Pramenka from Pljevlja area (MN) were obtained. The animals were approximately 5 years old, taken from the same herd and production system.

2.1. Raw materials and processing

After slaughtering (at 10 °C), the carcasses were chilled at +4 °C for 24 h. Carcasses were deboned, but shin bones (*Os tibia et Os fibula*) were kept for hanging the carcass in a smoke house. The pH was measured 24 h *post mortem* in *M. semimembranosus* using pH meters: HANNA 99161 (Cluj-Napoca, Romania) for B&H animals and Knick Portamess 913 (Berlin, Germany) for MN animals.

2.1.1. Bosnia and Herzegovina

The weight of sheep carcasses used in B&H ham production was 25.0 ± 3.1 kg with EU fat scores in a range 8–12 (scale 1–15 points). Average $pH_{24\ h}$ of the thigh was 5.87. The ham was cut by a butcher. Salting was done by rubbing coarse salt (no nitrite) on the surface (35 g NaCl/kg) with peppercorn (0.3% w/w) and crushed garlic (~0.3% w/w). Salted carcasses were placed horizontally in plastic containers, pressed with 100 kg during the first week, up to 200 kg during the second week, and to 300 kg during the third week to facilitate removal of blood and brine. Carcasses were left in their own brined juice for 21 days (at 4–10 °C, RH 85–90%). Drying and cold smoking with beech wood and sometimes sawdust was conducted in a traditional smoke house (12–18 °C, RH 70–80%), approximately 2 h per day for 14 days. The distance between stokehole and meat was about 2–3 m. Ripening in an aerated room without smoke (7–10 °C, RH 70–80%) lasted 7 days. The *Stelja* production process lasted for 42 days. The weight loss was 14.73%.

2.1.2. Montenegro

Sheep carcasses for MN ham production were 27.3 ± 3.6 kg with EU fat score in the range 6–11 (scale 1–15 points). Average $pH_{24\ h}$ was 5.58. The production took place in an industrial facility, following the same steps that are used in the traditional production. Carcasses were covered by salt (35–40 g NaCl/kg) and kept in plastic containers for 7 days (4–8 °C, RH 85–90%). Surplus salt was removed by quick immersion into water, followed by drying for 8 h (13–15 °C, RH 70%). The cold smoking process, regulated with airflow, was conducted 4 h/day for 7 days with controlled parameters: 15–18 °C, RH 75–80%. Smoke was generated by glowing beech wood in a smoke generator. The ripening phase continued in a chamber with controlled conditions (10 °C, RH 65–70%) for additional 15 days. Industrial production of *Stelja* lasted 29 days and the weight loss was 28%.

2.2. Chemical and sensory analysis of the products

Sheep ham was removed from dry-cured carcass called *Stelja* and sample of *M. Semimembranosus* was vacuum-packed in plastic bags and stored (+4 °C, in the dark, up to 2 months). The samples were than subjected to sensory analysis while the samples for volatile and chemical analysis were manually cut (2 × 2 × 2 mm^3 pieces) and frozen at −80 °C until analysis.

Chloride content was determined using the methodology suggested by Håseth, Egelandsdal, Bjerke, and Sørheim (2007). The obtained results are presented as % w/w NaCl.

Samples for HPLC quantitative analysis of organic acids were prepared and analysed by the method described by Narvhus, Østeraas, Mutukumira, and Abrahamsen (1998).

Volatile compounds were analysed using Gas Chromatography–Mass Spectrometry (GC–MS) using 2 g of the sample, in three replicates. The samples were analysed by a dynamic headspace collector (Teledyne Tekmar HT3, Ohio, USA) coupled with gas chromatograph 6890 and mass spectrometer 5975 (Agilent technologies Santa Clara, CA, USA). Volatiles were separated using a DB-water fused silica capillary column (J&W Scientific; 30 m; 0.25 mm i.d., film thickness 0.50 μm). Helium flow was 1 mL/min. The temperature program was as follows: 30 °C for 10 min, ramped 1 °C/min to 40 °C than 3 °C/min to 70 °C, and 6.5 °C/min to 230 °C, holding 5 min at 230 °C. GC/MS interface was set on 250 °C. Mass spectrum was obtained by electronic impact at 70 eV with recorded mass range 30–550 *m/z*. The volatiles were identified comparing the obtained mass spectrum with the mass spectrum in the NIST 05 (Mass Spectral Library, Agilent technologies Santa Clara, CA, USA). If the probability of correct identification of each volatile was below 60%, the component was discarded.

To exclude contamination from GC data set, GC–MS analysis were performed with samples of plastic bags in laboratory air under the same conditions as volatile analysis. Semi-quantitative amounts of volatiles were calculated using pentanal run routinely as an external standard at low and high concentrations. Other chemical groups were also run as external standards but pentanal had, in retrospective, the most suitable dynamic range.

The slices (1.5 mm thick) of *M. Semimembranosus* were sensory evaluated by 8 trained panellists in a room equipped with fluorescent lighting (at 25 °C). The evaluators were provided with 50 mL of water and 20 g of unsalted bread to rinse their palate. The panel was trained on different sensory attributes of *Stelja*, such as, appearance (fat yellowness, redness, marbling), texture (fat firmness, hardness, dryness, juiciness), flavour (smoke, garlic, saltiness, bitterness, acidity, mature, cured, metallic, rancid, and soapy), and aroma intensity. The sensory evaluation of sheep ham was carried out by a 9 point structured scale, using quantitative-descriptive analysis (Hootman, 1992).

2.3. Statistical analysis

Differences in sensory properties between B&H and MN samples were calculated using t-test (Minitab 16, Minitab Ltd., Coventry, United Kingdom).

VOCs present in only 1/3 of replicates were removed. VOCs present at the same level in all samples were also removed since the study focused on characteristic volatiles from two hams. Fifty-three VOCs were significantly different ($p < 0.05$) between B&H and MN samples as assessed from one-way ANOVA with Tukey's comparison test (Minitab 16). Results were expressed as % of total amount in a specific chemical group (*e.g.* acids).

Principal component analysis (PCA) was used for explorative examination of the data, as well as to link the set of samples with sensory attributes and volatile data. Furthermore, correlations between volatiles and sensory attributes were obtained through Partial Least Square Regression (PLSR). PLS1 models showed correlation between individual sensory attributes (2 matrices) that were significantly different ($p < 0.001$) among two dry-cured sheep hams. The volatile variables were weighed by their standard deviation before subjected to PCA and PLS analysis. Estimated regression coefficients from PLS regression models were analysed by jack-knife uncertainty test.

The PCA and PLSR calculations were done using Unscrambler X10.1 (Camo, Trondheim, Norway).

3. Results and discussion

3.1. Sensory properties

Fig. 1 shows the average values obtained for sensory properties of the two different sheep hams. The sheep hams were characterized by high redness, yellow fat, dry but juicy texture, high aroma intensity and a smoked, salty, mature and cured flavour. Existing literature has described sheep ham from Serbia as brownish, with soft elastic texture, slightly marbled with yellow fat tissue, and having smoke aroma, moderately to distinctly salted and dried, a slight acidic taste with smell of fermented sheep cheese (Stamenković & Dević, 2006). Troeger et al. (2009) reported that 25% of Serbian sheep hams randomly sampled at an exhibition, had intensive smoke flavour and some were very salty (measured as 6.2–6.7% w/w of salt). Sheep ham from B&H was more red, more marbled, with more yellow fat compared to MN's ham (Figs. 1 and 2). No significant differences were found between B&H and MN hams in terms of dryness ($p = 0.38$), juiciness ($p = 0.47$), aroma intensity ($p = 0.21$), and mature flavour ($p = 0.14$). Intensively smoked flavour of MN production presumed due to a more intense smoking of a shorter period, compared to the production in B&H. Garlic addition had a significant contribution for the flavour of B&H hams. Saltiness, cured flavour and hardness were significantly higher ($p < 0.001$) in MN sheep hams compared to the B&H hams. The higher weight loss (28% w/w) and salt content (6.3% w/w) lead to higher saltiness and hardness of the MN hams. Bitterness, acidity and metallic taste were more intense in B&H sheep hams (Figs. 1 and 2). Bitter and metallic taste is generally less desirable, at least in pork hams (Morales, Guerrero, Aguiar, Guàrdia, & Gou, 2013). In agreement with Arnau (2000), the shorter ripening period developed stronger metallic taste in B&H sheep hams (Figs. 1 and 2). The assessors did not identify any soapy and rancid flavour.

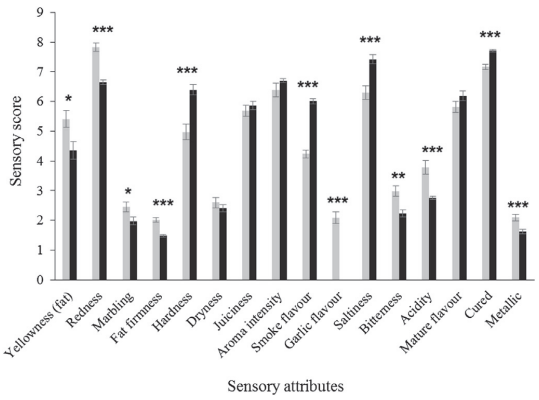

Fig. 1. Sensory attributes of 15 sheep ham samples from B&H (grey bars) and 15 samples from MN (black bars); *** = $p < 0.001$; ** = $p < 0.01$; *$p < 0.05$. Error bars are standard errors.

3.2. Volatile profiles

GC–MS analysis of sheep ham from two Western Balkan regions (B&H and MN) resulted in 53 volatiles (Table 1) significant for different production processes.

Principal component analysis (PCA) was carried out on all 53 identified volatile components of the 30 sheep hams. The bi-plot in Fig. 2 reveals relations between VOCs and sensory attributes of the sheep hams from the two production systems. Sheep hams from B&H and MN were separated along PC1 (Fig. 2). B&H samples are on the left and MN on the right side of the plot. There was, however, more variation in the sheep ham produced in Montenegro than among B&H samples.

Volatile profiles of B&H sheep hams were defined by sulphur compounds and ketones (Fig. 2). Allyl methyl sulphide, allyl methyl disulphide, diallyl disulphide and methyl thiirane in B&H hams originated from garlic (Wagner, da Silva, & Franco, 2008). These VOCs were not present in MN hams since garlic was not used during the salting stage. Garlic addition in B&H serves primarily as a flavour and secondly as an antimicrobial agent. Furthermore, ketones (2-pentanone, 1-hydroxy-2-butanone, 3-hydroxy-2-butanone, 2,3-dimethyl-2-cyclopenten-1-one, and 2-methyl-2-cyclopenten-1-one) were the second dominant group of volatiles in B&H ham (for identified compounds see Table 1). 2-pentanone can reflect lipid degradation or mould metabolism (Muriel, Antequera, Petrón, Andrés, & Ruiz, 2004), while 3-hydroxy-2-butanone may be formed via microbial glycolysis (Kandler, 1983). Among other volatiles, pentanal and 1-pentanol were significantly related to the profile of B&H hams. Pentanal's prevalence may reflect differences in fatty acid composition between B&H and MN ham or process differences, since B&H hams had shorter ripening period.

Dominant volatiles in MN sheep hams were smoke components (furans and phenols), acids, aldehydes, alcohols, and esters (Fig. 2). The smoke components (furan, furfural, 3-furaldehyde, 1-(2-furanyl)-ethanone, 2-furanmethanol, 5-methyl-2(5H)-furanone, and 4-ethyl-2-methoxy-phenol) played a significant role in the volatile profile. The higher concentration of smoke components identified in MN sheep ham compared to B&H was unexpected since the smoking process can be better controlled in an industrial facility. Possibly the preference for smoked flavour is, by tradition, more pronounced in MN.

Furans and derivates are classified by the International Agency for Research on Cancer (IRAC, 1997) as possible carcinogenic compounds. Increased attention is paid to their derivates (furan, 2-furfural, furfuryl alcohol, and penthylfuran), which are considered to be toxic for human and animal health (Pérez-Palacios, Petisca, Pinho, & Ferreira, 2012). According to GC–MS analysis furfural content (see Table 1) in MN ham was 13.8 mg/kg while in B&H

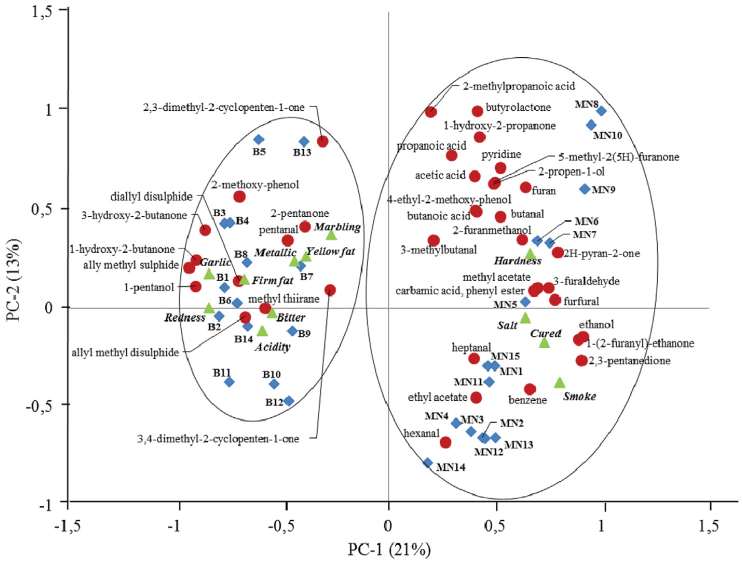

Fig. 2. Principal component analysis bi-plot with volatiles compounds and sensory attributes as well as the different samples used (blue diamonds are individual hams; red spots are volatile compounds; green triangles are sensory attributes). B stands for B&H hams and MN denotes Montenegro hams. Volatile compounds were removed for improved graphical clarity (see Table 1 for other significantly different compounds). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples it was much lower (1.7 mg/kg). Despite the distinct smoked flavour, these smoke components were at the safe level. Furthermore, four polycyclic aromatic hydrocarbons (PAH4) of concern in smoked meat and meat products are benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene (European Commission, 2014). According to Bjelanovic (personal communication), PAH4 concentration in dry-cured pork and beef meat, traditionally and industrially produced in B&H and MN, was below maximum acceptable level of 30 μg/kg.

Acids were important volatiles for MN products. Acetic acid, as a derivate of microbial metabolism of glucides (Pugliese et al., 2009) and wood degradation product, was higher for MN hams (32.17 mg/kg) compared to B&H samples (17.64 mg/kg; see Table 1). Higher concentration of acetic acid most likely reflected the intensive smoking of MN hams. Presence of alcohols (ethanol and 2-propen-1-ol) in MN sheep ham is possibly related to lipolysis, proteolysis, and microbial activity. Esters also contributed to the MN volatile profile. Methyl derivatives of short chain esters are particularly important for flavour, *i.e.* methyl acetate. Formation of ethyl esters can be connected to the presence of ethanol and can be result of the microbial enzymatic mechanisms (*e.g.* esterification) reported in species belonging to Micrococcaceae and other bacterial species often found in meat (Lori, Grisenti, Parolari, & Barbuti, 2005; Montel, Reitz, Talon, Berdagué, & Rousset- Akrim, 1996).

The following aldehydes contributed to MN sheep ham volatile profile: butanal, 3-methyl-butanal, hexanal and heptanal (see MN3 & MN4 and hexanal as an example; Fig. 2). Aldehydes are products of fatty acid oxidation, particularly monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Sheep meat from MN had the larger fraction of MUFA and PUFA, with a significantly lower ratio of n-6/n-3 fatty acids (Bjelanovic et al., 2015). Hexanal, which comes from linoleic acid is characterized by a rancid odour (Pugliese et al., 2009). The fact that assessors did not identify rancid flavour as sensory attribute can be related to the strong smoke flavour of MN ham that may camouflage rancid odour.

Branched aldehydes (i.e. 3-methylbutanal) are often found in dry and fermented sausages and are generated by microbial activity (Herranz, de la Hoz, Hierro, Fernández, & Ordóñez, 2005).

3.2.1. *The raw material's sensorial and volatile differences*

The raw material used here for ham production has been examined in detail (Bjelanovic et al., 2015; Grabež, personal communication). Fresh meat from MN was more bitter than fresh meat from B&H. However, no significant differences in juiciness ($p = 0.70$), acidity ($p = 0.66$) and metallic ($p = 0.42$) taste were found between B&H and MN samples (Grabež, personal communication).

The volatile compounds identified in the raw materials reflected lipid degradation products of the dry-cured sheep hams to some extent. Only nine volatiles (3-methyl-butanal, pentanal, hexanal, heptanal, 3-hydroxy-2-butanone, ethanol, 1-pentanol, 2-methylpropanoic acid, butanoic acid) isolated from fresh adipose tissue remained in the volatile profile of dry-cured B&H and MN sheep hams. Furthermore, 3-methylbutanal and 3-hydroxy-2-butanone were detected only in fresh B&H samples, while hexanal was only observed in fresh adipose tissue from MN. Only bitterness for B&H hams seemed to be an obvious raw material flavour that was also observed in the final dry-cured ham.

3.3. *Relationship between volatile compounds and sensory attributes*

Fig. 3 shows the results of PLS2 regression analysis used to reveal relationships between volatiles from the two dry-cured sheep hams (X-matrix; Table 1) and their sensory attributes (Y-matrix; Fig. 1). Sensory attributes were separated along PC1 with acidity, bitterness, metallic and garlic taste on a right side and smoke, cured and salt on the left side of the plot.

The following compounds 1-(2-furanyl)-ethanone, 2-methoxy-phenol, allyl methyl sulphide, pentanal, 1-hydroxy-2-butanone, 3-hydroxy-2-butanone, 2,3-pentanedione, benzene, ethanol, 1-

Table 1
Volatile compounds in sheep ham of *Stelja* produced in the traditional way in B&H ($n = 15$) and industrial conditions in MN ($n = 15$); identified by HS-GC/MS in both types.

RT[a]	Volatile compound	B&H	MN	Assumed origin
		% Of total amount		
	Furans			
3.5	Furan**,[b]	n.d	0.005 ± 0.014[c]	Maillard; pyrolysis of wood/pentose and cysteine reaction
34.9	Furfural***	0.570 ± 0.718	0.650 ± 0.418	Maillard; pyrolysis of wood/pentose and cysteine reaction
38.9	2-Furanmethanol	n.d	0.010 ± 0.282	Maillard; pyrolysis of wood/pentose and cysteine reaction
34.0	3-Furaldehyde***	n.d	0.010 ± 0.014	Maillard; pyrolysis of wood/pentose and cysteine reaction
37.3	5-Methylfurfural	0.120 ± 0.060	0.140 ± 0.031	Maillard; pyrolysis of wood/pentose and cysteine reaction
41.0	5-Methyl-2(5H)-furanone*	0.060 ± 0.046	0.020 ± 0.039	Maillard; pyrolysis of wood/pentose and cysteine reaction
40.4	4-Methyl-5H-furan-2-one	0.007 ± 0.010	0.003 ± 0.008	Maillard; pyrolysis of wood/pentose and cysteine reaction
35.8	1-(2-furanyl)-ethanone***	n.d	0.080 ± 0.095	Maillard; pyrolysis of wood/pentose and cysteine reaction
38.6	Butyrolactone	0.260 ± 0.105	0.060 ± 0.068	Lipid oxidation/Maillard reactions
Amount (B&H = 3.0 mg/kg ± 3.4 mg/kg; MN = 21.4 mg/kg ± 11.5 mg/kg)				
	Phenols			
42.3	2-Methoxy-phenol**	0.500 ± 0.347	0.710 ± 0.405	Degraded wood/smoke
44.1	4-Methyl-2-methoxy-phenol	0.260 ± 0.046	0.140 ± 0.063	Degraded wood/smoke
45.3	4-Ethyl-2-methoxy-phenol*	0.240 ± 0.126	0.140 ± 0.087	Degraded wood/smoke
Amount (B&H = 0.20 mg/kg ± 0.04 mg/kg; MN = 0.89 mg/kg ± 0.50 mg/kg)				
	Sulphur compounds			
8.1	Allyl methyl sulphide***	0.480 ± 0.217	n.d	Allicin originated from garlic, in Strecker degradation
29.1	Allyl methyl disulphide**	0.060 ± 0.081	n.d	Allicin originated from garlic, in Strecker degradation
35.1	Diallyl disulphide**	0.360 ± 0.532	n.d	Allicin originated from garlic, in Strecker degradation
5.4	Methyl thiirane**	0.110 ± 0.169	n.d	Metabolite derived from methionine, cysteine and cystine in Strecker degradation
Amount (B&H = 8.7 mg/kg ± 12.5 mg/kg; MN = n.d.)				
	Aldehydes			
3.7	Butanal	0.110 ± 0.162	0.130 ± 0.111	Lipid degradation
6.5	3-Methyl-butanal*	0.610 ± 0.675	0.630 ± 0.799	Strecker degradation of valine, isoleucine &leucine
6.3	Pentanal*	0.013 ± 0.016	0.003 ± 0.002	Lipid degradation/oxidation
17.6	Hexanal	0.069 ± 0.136	0.055 ± 0.054	Degradation of n-6 fatty acid
6.9	Heptanal*	0.200 ± 0.011	0.180 ± 0.034	Lipid degradation
Amount (B&H = 0.81 mg/kg ± 0.43 mg/kg; MN = 0.52 mg/kg ± 0.45 mg/kg)				
	Ketones			
32.7	1-Hydroxy-2-butanone***	0.010 ± 0.030	0.210 ± 0.216	Decarboxylation β-keto acids or β-oxidation fatty acids
30.1	3-Hydroxy-2-butanone**	0.290 ± 0.399	n.d.	Microbial fermentation of glucides
10.0	2-Pentanone	0.050 ± 0.107	n.d	Decarboxylation β-keto acids or β-oxidation fatty acids
16.5	2,3-Pentanedione***	0.007 ± 0.016	0.028 ± 0.008	Decarboxylation β-keto acids or β-oxidation fatty acids
30.7	1-Hydroxy-2-propanone*	0.640 ± 0.426	0.750 ± 0.743	Decarboxylation β-keto acids or β-oxidation fatty acids
40.2	4,5-Dimethyl-4-hexen-3-one	0.003 ± 0.008	0.002 ± 0.007	Lipid oxidation
Amount (B&H = 7.6 mg/kg ± 0.85 mg/kg; MN = 12.3 mg/kg ± 11.5 mg/kg)				
	Ketones-cyclic			
25.1	Cyclopentanone*	0.270 ± 0.623	0.470 ± 0.422	Lipid oxidation
36.7	2,3-Dimethyl 2-cyclopenten-1-one***	0.043 ± 0.106	0.009 ± 0.131	Lipid oxidation
31.5	3,4-Dimethyl-2-cyclopenten-1-one*	0.070 ± 0.090	0.040 ± 0.107	Lipid oxidation
42.0	3-Methyl-2-hydroxy-2-cyclopenten-1-one	0.590 ± 0.127	0.450 ± 0.275	Lipid oxidation
43.1	3-Ethyl 2-hydroxy-2-cyclopenten-1-one	0.033 ± 0.054	0.036 ± 0.083	Lipid oxidation
Amount (B&H = 0.90 mg/kg ± 0.5 mg/kg; MN = 0.85 mg/kg ± 0.34 mg/kg)				
	Hydrocarbons-cyclic			
40.7	Naphthalene*	0.080 ± 0.088	0.150 ± 0.120	Lipid degradation
11.9	Benzene**	0.004 ± 0.003	0.073 ± 0.125	Lipid degradation
35.3	1-Ethynyl-4-methylbenzene	0.120 ± 0.308	0.170 ± 0.103	Lipid degradation
32.3	2-Methyl-2-cyclohexen-1-one	0.060 ± 0.175	0.040 ± 0.158	Lipid degradation
32.5	2-Methyl-2-cyclopenten-1-one	0.730 ± 0.426	0.560 ± 0.494	Lipid degradation
Amount (B&H = 0.69 mg/kg ± 0.52 mg/kg; MN = 0.65 mg/kg ± 0.4 mg/kg)				
	Alcohols			
7.6	Ethanol***	0.040 ± 0.158	0.990 ± 0.982	Lipolysis and proteolysis
28.7	1-Pentanol***	0.960 ± 0.836	0.002 ± 0.004	Lipolysis and proteolysis
23.7	2-Propen-1-ol*	0.006 ± 0.006	0.008 ± 0.014	Lipolysis and proteolysis
Amount (B&H = 0.45 mg/kg ± 0.34 mg/kg; MN = 2.6 mg/kg ± 2.0 mg/kg)				
	Acids			
34.4	Acetic acid*	0.910 ± 0.881	0.917 ± 0.875	Microbial metabolism of glucides
36.9	2-Methylpropanoic acid	0.004 ± 0.006	0.003 ± 0.004	Hydrolysis of triglycerides&phospholipids/lipid oxidation
36.4	Propanoic acid	0.057 ± 0.092	0.060 ± 0.090	Hydrolysis of triglycerides&phospholipids/lipid oxidation
38.1	Butanoic acid*	0.029 ± 0.028	0.020 ± 0.024	Hydrolysis of triglycerides&phospholipids/lipid oxidation
Amount (B&H = 19.1 mg/kg ± 19.3 mg/kg; MN = 35.4 mg/kg ± 27.5 mg/kg)				
	Esters			
4.1	Methyl acetate***	0.940 ± 0.923	0.920 ± 0.848	Esterification of carboxylic acids and alcohols
9.9	Ethyl acetate**	0.060 ± 0.077	0.080 ± 0.147	Esterification of carboxylic acids and alcohols
33.8	Carbamic acid, phenyl ester**	n.d.	0.003 ± 0.005	Esterification of carboxylic acids and alcohols
Amount (B&H = 0.78 mg/kg ± 0.77 mg/kg; MN = 5.31 mg/kg ± 0.44 mg/kg)				
	Nitrogen-compounds			
26.1	Pyridine	0.190 ± 0.663	0.370 ± 0.455	Amino acids and aldehyde reaction/Maillard
37.6	3-Methoxy-pyridine	0.070 ± 0.146	0.100 ± 0.180	Amino acids and aldehyde reaction/Maillard

(*continued on next page*)

Table 1 (*continued*)

RT[a]	Volatile compound	B&H	MN	Assumed origin
		% Of total amount		
41.2	2H-pyran-2-one***	0.670 ± 0.055	0.240 ± 0.136	Amino acids and sugar reaction/Maillard
33.6	Dihydro-2H-pyran-3(4H)-one*	n.d	0.150 ± 0.186	Amino acids and sugars reaction/Maillard
30.3	3-Methyl-pyridine	0.060 ± 0.136	0.140 ± 0.042	Amino acids and aldehyde reaction/Maillard
Amount (B&H = 0.11 mg/kg ± 0.08 mg/kg; MN = 1.9 mg/kg ± 0.2 mg/kg)				
	Others			
5.9	Ethoxy-ethene**	6.67E+05 ± 5.67E+05	4.30E+06 ± 4.27E+06	
Amount (B&H = 17 μg/kg ± 15 μg/kg; MN = 11 μg//kg ± 1 μg/kg)				

[a] RT = retention time.
[b] Compounds that were significantly different between B&H and MN are labelled; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.
[c] Standard deviation.

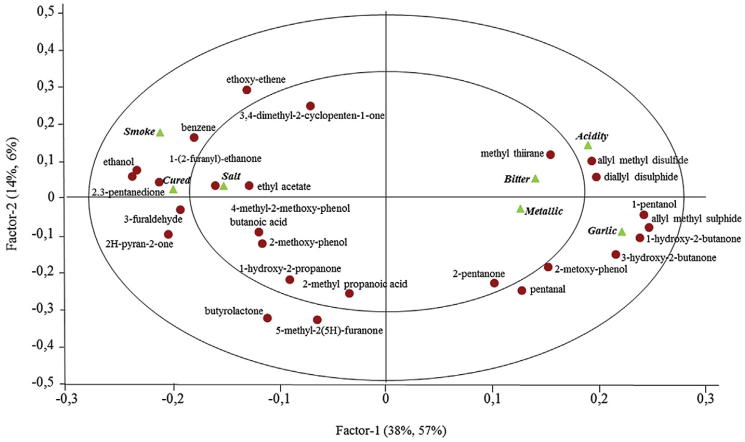

Fig. 3. Plot of Partial Least Square Regression (PLSR) analysis of the volatile compounds (X-matrix) and seven significantly different sensory attributes (Y-matrix) found for two types of sheep ham. Ellipses represent $r^2 = 0.5$ and 1.0, as defined by the PLSR model. Volatiles in a plot are listed in a Table 1; red spots are volatiles and green triangles are sensory attributes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pentanol, butanoic acid, 2H-pyran-2-one, and ethoxy-ethene were significantly ($p < 0.05$, jack-knife uncertainty test used) related with one or more sensory attributes of sheep hams.

PLS1 regression analysis used to identify volatiles were significantly ($p < 0.05$, jack-knife uncertainty test) correlated to ham flavour (salt, bitter, acidity, cured, metallic, smoke, and garlic flavour) and aroma. The smoke flavour was positively correlated with ethyl acetate, ethoxy-ethane, ethanol, benzene, 2,3-pentanedione, 1-(2-furanyl)-ethanone, 3,4-dimethyl-2-cyclopenten-1-one, 2H-pyran-2-one, and 4-methyl-2-methoxy-phenol. Allyl methyl sulphide, pentanal, 2-pentanone, 1-pentanol, 3-hydroxy-2-butanone, 1-hydroxy-2-propanone, 1-hydroxy-2-butanone, 2-methylpropanoic acid, butyrolactone, 5-methyl-2(5H)-furanone, and 2-methoxy-phenol had negative correlation with smoke flavour. The compounds allyl methyl sulphide, 1-pentanol, 3-hydroxy-2-butanone, and 1-hydroxy-2-butanone related positively, while ethoxy-ethene, ethanol, benzene, 2,3-pentanedione, 3-furaldehyde, 1-(2-furanyl)-ethanone, and 2H-pyran-2-one related negatively with garlic flavour. Saltiness correlated negatively with allyl methyl sulphide and 1-pentanol. Bitterness was positively correlated with allyl methyl sulphide and negatively with benzene. The acidity attribute had positive correlation with sulphur compounds and negative with ethyl acetate, 2-pentanone and benzene. Cured flavour was positively associated with ethanol and benzene. Allyl methyl sulphide, 3-hydroxy-2-butanone, 1-hydroxy-2-butanone, allyl methyl disulphide, and diallyl disulphide were compounds with negative effect on cured flavour. Allyl methyl sulphide, benzene and 2-methylpropanoic acid showed positive correlation with metallic taste, while ethoxy-ethene and ethanol were negatively correlated. However, these correlations may not reflect causal relationship; *i.e.* benzene (sweet smell) and bitterness seem unrelated. But other compounds co-vary with the causal compound that has smoke flavour (*i.e.* 4-methyl-2-methoxy-phenol).

3.4. Salt content, safety and sensory properties

The lower salt content (4% w/w) in B&H ham had no influence on the red colour of ham (Fig. 1) and the level of lipid degradation products (Table 1). All sheep hams were appreciated as acidic, despite the fact that higher salt and smoke content may mask acidity. Acidity was a more pronounced sensory attribute for B&H sheep ham than for MN hams, but had no correlation with the amount of acetic acid or with total amount of acids (see Table 1, Fig. 4).

The final content of 4.5% NaCl (or lower) in sheep ham is challenging in terms of unwanted bacterial growth, but preferable regarding health recommendations. Salt content varies depending on production routines; for Spanish lamb ham Villalobos-Delgado et al. (2014) reported 7.96% NaCl and water activity (a_w) of 0.88, and for Norwegian *Fenalår* 5–10% (Håseth et al., 2014). *Skerpikjøt*, with a_w value 0.90 has no salt addition (Håseth et al., 2014). Three incidences of botulism in 2007 were linked directly to the

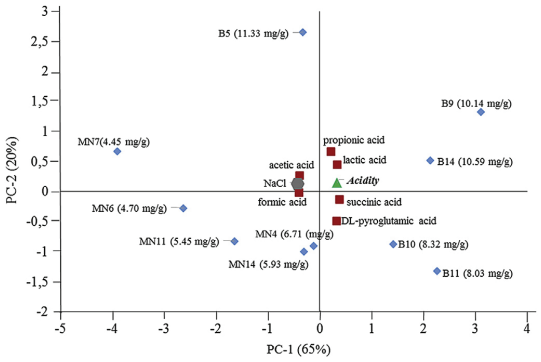

Fig. 4. Principal component analysis with samples that present extremes in acidic taste and organic acids (blue diamonds are B – B&H hams and MN – Montenegrin hams; red squares are organic acids; grey hexagon is salt; green triangle is acidity as taste attribute; amount of lactic acid in each analyzed sample in parenthesis). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

consumption of *Skerpikjøt*, and there was also a total of six incidents (two deadly) in the years 1988, 1989, and 1997 (Annual Report from the Chief Medical Officer in the Faroes), in 1989–1990 (ISSN 0903-7772), 1997 (ISSN 0903-7772) and 2007 (ISSN 0903-7772).

The concentrations of lactic acid in green hams was 89.36 mg/kg for B&H and 85.83 mg/kg for MN (Grabež, personal communication). A significantly higher level of lactic acid in the final product suggests the presence of lactic acid bacteria during ham production. Low salt B&H sheep ham with higher acidity also contained higher amount of lactic acid (9.68 g/kg) compared to MN samples (5.45 g/kg; see Fig. 4). Microbial growth was also supported by some possible microbial volatiles being more abundant in B&H hams, *i.e.* ethanol, and 1-hydroxy-2-butanone (not shown). The sensory threshold of lactic acid depends on food matrix (Korkeala, Alanko, Mäkelä, & Lindroth, 1990; Vermeiren, Devlieghere, & Debevere, 2004) so it is unclear if the increased acidity in low salt ham is due to increased lactic/propionic acid (Fig. 4) content. Troeger et al. (2009) reported that a certain fraction of sheep ham from Serbia would be rejected because of sourness. However, microbial activity could also be a desirable trait, provided the product is safe, depending on consumer's preferences.

Low salt sheep hams produced in butcher facilities deserve further investigation regarding the technology applied in the salting phase since it may rely on a suitable house flora. Managing the salting phase is important for maintaining a low salt content, microbial safety and a more robust ham flavour. The characteristic sensory properties of the traditional B&H product should be maintained without compromising the product's safety.

4. Conclusions

Significant differences in VOCs content and sensory properties between sheep ham produced in B&H, and those produced in MN, were found. B&H production differed from MN in: duration of smoking (14 versus 7 days), ripening time in air (7 versus 14 days) and additives; B&H hams were added garlic and peppercorn.

Sheep ham from MN had a strong smoke flavour (from furans and phenols) and salty taste. B&H sheep hams were rich in sulphuric compounds due to added garlic, with less salty taste.

The salting phase of the B&H ham seemed to involve fermentation and may be of critical importance for the safety of low salt dry cured ham production.

Acknowledgement

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