Present health challenges in fresh and processed meat

Aktuelle helseutfordringer i ferskt og bearbeidet kjøtt

Philosophiae Doctor (PhD) Thesis

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Ås 2016

Thesis number 2016:28
ISSN 1894-6402
ISBN 978-82-575-1355-9

Preface

This thesis is submitted to the Department of Chemistry, Biotechnology and Food Sciences (IKBM) at the Norwegian University of Life Sciences (NMBU), Ås, Norway to attain the degree of Philosophiae Doctor (PhD). The present work was carried out at IKBM during the period of 2010-2015. The thesis consists of a theoretical introduction with three scientific papers and a manuscript.

Paper I studied a new modified method for measuring hydroperoxide value in meat products. Paper II, II and IV researched on how different additives effect on the lipid peroxidation in meat or processed meat. Calcium as an additive to sausages were studied in Paper III and Paper IV. The work has been under supervision of Professor Bjørg Egelandsdal, Professor Anna Haug and Dr. Marije Oostindjer.

DEDICATION

To my mother

Acknowledgements

First of all, I would sincerely like to thank my supervisor, Professor Bjørg Egelandsdal, who gave me the opportunity to join the group, started this Ph.D and guided me with all the immense knowledge, patience, enthusiasm, encouragement, motivation and supervisions. I would also like to thank my co-supervisors Professor Anna Haug and Dr. Marije Oostindjer for their help, comments and encouragement which widened my knowledge and perspectives. Thank you for all the suggestions, discussions and inspirations.

My co-authors and my group members Erik Slinde, Valadana Grabež, Milena Bjelanovic, Lene Ruud Lima, Kari Olsen, Nicole Frost Nyquist, Berit Nordvi, Kristin Saarem, Øyvind Langsrud, Linda Saga, Qing Wang, Jon Volden, Thomas Isaksson and Vinh Thanh Phung are thanked for their contributions and cooperation to this study. Thank Tove Devold to read through the last manuscript. Special thanks to the panelists who were involved in both sensory analysis. All the other colleagues at IKBM are thanked for a pleasant working environment.

My thanks also go to all my friends in China and Norway, for the sleepless nights, fun, chatting, sharing, gossiping, crying and laughing.

Thank all my families, for their continuous love, supports and encouragements. I am deeply grateful to my husband Danh and my daughter Vela, for always being there for me, for every moment together, for enlightening me in the desperate darkness and giving me the endless love and hope.

At last, I would like to thank my mother, for everything that I have gotten.

Gu Yi

Oslo, 10.02.2016

Summary

Processed meat intake is claimed to involve a higher risk of colon cancer. In particular, sausage intake is high and therefore has a potential for influencing consumers' health. In Norway, sausage consumption has been linked to colon cancer in females. Reactive oxygen molecules are chemically active pro-oxidant molecules which are involved in a variety of physiological and pathological processes and also suggested to elevate the risk of colon cancer. This thesis therefore has a focus on oxidation processes in processed meats and how different additives effect the hydroperoxide formation ability in sausages.

Specific food additives were tested for their pro- or/and antioxidant properties in minced meat. Selected Krebs cycle substrate combinations acted initially as pro-oxidants in both high and low oxygen conditions, but when oxygen was completely removed, the substrate appeared as antioxidants. A clove extract added to sausages acted as a strong antioxidant that counteracted the effect of myoglobin as a pro-oxidant. However, the clove had a recognizable clove flavor in the sensory test. Calcium addition appeared to reduce peroxide formation. The addition of nitrite and fish oil seemed to interact to support nitroso-myoglobin formation.

In **Paper I**, the ferric-xylenol orange (FOX) method was modified to measure the total peroxides in lean meat from beef, lamb, pork and chicken to investigate their hydroperoxide formation potential. The total peroxides of fresh comminuted raw meat were determined by analyzing protein-bound peroxides and hydroperoxide compounds in water-methanol and chloroform extracted phases. The amount of total peroxides was ranked as: beef > pork > lamb > chicken. Hydroperoxide formation was examined at different pH values and at different incubation times using beef and chicken samples. All peroxides were transient. Unpolar peroxide formation could largely be described by variation in fatty acid composition and hemin content of the meat while protein-bound peroxide variation was less well-explained by these variables.

In **Paper II**, the effect of Krebs cycle substrates (KCS) on the lipid oxidation in minced meat was studied. KCS combinations of succinate and glutamate increased peroxide forming potential (PFP) (12%) and TBARS (27%) under low oxygen storage conditions. Both succinate and

glutamate were metabolized. Moreover, under high oxygen storage conditions, KCS combinations of glutamate, citrate and malate increased PFP (5.7%) and TBARS (8%). Only glutamate was metabolized. The KCS combinations acted initially as pro-oxidants that promoted lipid oxidation in both high and low oxygen conditions, but when oxygen was completely removed, the substrate appeared as antioxidants.

In **Paper III**, difference additives' effect on the peroxidation formation in two different sausage systems was investigated. The effects of fish oil, myoglobin/meat juice, nitrite, clove extract and calcium sources on oxidation and sensory properties were examined. Two sausage systems (a model sausage and a more standard frankfurter type sausage) were stored in three different ways prior to testing: 1) frozen immediately at -80°C; 2) chilled stored for 2.5 weeks followed by fluorescent light illumination at 4°C for another two weeks before being stored at -80°C; 3) frozen at -20°C for 5 months and then stored at -80°C. The frozen group 3 showed the highest peroxide formation and TBARS for both sausage systems. Unpolar peroxides dominated in both systems. The clove extract acted as an antioxidant, but the clove flavor was recognized in the sensory test. Calcium appeared to reduce peroxide formation. The addition of nitrite and fish oil seemed to interact to support nitroso-myoglobin formation.

In **Paper IV** (manuscript), sausages were produced at three sodium levels (equivalent to 13, 15 and 17g NaCl/kg sausage) using four different milk ingredients (a dried skimmed milk powder, a calcium enriched milk mineral powder, a potassium enriched milk mineral powder, and a lactose rich powder). The sausages with added calcium and potassium enriched milk powders produced the hardest sausages when compared at the same sodium level. Milk mineral powder addition also yielded whiter and less red sausages. After 6 weeks at chill storage, no effect on rancidity was observed from adding milk minerals when compared with adding dried skimmed milk powder to the sausages. A significant advantage of using these milk mineral powders in sausages is that the Na:K ratio can be reduced from an unhealthy (36) to a far healthier ratio (~2) with limited or no taste changes. High additions of high calcium (6g/kg sausage) milk mineral had no influence on sensory bitterness or after-taste, which is typically observed with $CaCl_2$ addition. The addition of Ca to sausages is presumed to be healthier, helping to avoid calcium deficiency and presumably to reduce the risk of colon cancer.

Sammendrag

Inntak av bearbeidet kjøtt er påstått å involvere en større risiko for endetarmskreft. Konsumet av pølser er høyt i gruppen bearbeidet kjøtt og har derfor et stort potensial for påvirkning av forbrukers helse. Konsum av pølser har blitt knyttet til endetarmskreft blant kvinner i Norge. Reaktive oksygen molekyler er kjemisk aktive pro-oksidantiske molekyler som er involvert i en rekke fysiologiske og patologiske prosesser og er antydet til å øke risikoen for endetarmskreft. Denne avhandlingen fokuserer på oksideringsprosesser i bearbeidet kjøtt og hvordan ulike tilsetningsstoffer påvirker dannelse av peroxider i pølser.

Spesifikke tilsetningsstoffer ble testet for sine pro- og/eller antioksidative egenskaper i kjøttdeig. Kombinasjoner av substrater fra Krebs syklusen fungerte først som pro-oksidanter i både høy- og lav oksygen atmosfære, men da oksygenet var fullstendig fjernet fremsto tilsetningene som antioksidanter. Et kryddernellik ekstrakt tilsatt til pølser fungerte som en sterk antioksidant og motvirket myoglobinet aktivitet som pro-oksidant. Men kryddernelliken hadde en gjenkjennelig smak i den sensoriske testen. Kalsium tilsetning så ut til å redusere peroksid dannelse. Tilsetning av nitritt og fiskeolje førte til interaksjoner som økte nitroso-myoglobin dannelsen.

I **Paper I**, ferric-xylenol orange (FOX) metoden ble modifisert for å måle total peroksider i magert kjøtt fra okse, lam, svin og kylling og for å undersøke potensialet i hydroperoksid dannelse fra disse produktene. Total peroksider av fersk, malt, rått kjøtt ble bestemt ved å analysere protein-bundet peroksider og hydroperoksid komponenter i vann-metanol og kloroform ekstraherte faser. Mengden av total peroksider ble rangert slik: okse > svin > lam> kylling. Hydroperoksid dannelse var undersøkt ved ulike pH verdier og ulike inkuberingstider ved bruk av okse- og kyllingprøver. Alle peroksider var transitoriske. Dannelsen av upolare peroksider dannelse kunne i større grad beskrives av variasjon i fettsyre sammensetning og heme innholdet i kjøttet, mens protein-bundet peroksid variasjon var mindre godt forklart av disse variablene.

I **Paper II**, effekten av substrater fra Krebs syklusen (KCS) på lipid oksidering i hakkete kjøtt ble studert. KCS kombinasjoner av ravsyre og glutamat økte peroksid dannelsespotensialet (PFP)

(12%) og TBARS (27%) i lav oksygen lagringsomgivelser. Både ravsyre og glutamat ble metabolisert. Videre, under høy oksygen atmosfære, og med KCS kombinasjoner av glutamat, sitrat og malat økte PFP (5.7%) og TBARS (8%). Kun glutamat ble metabolisert. Substratkombinasjonene av KCS var derfor først pro-oksidanter som fremskyndet lipid oksidering i både høy og lav oksygen sfære, men når oksygenet var helt fjernet, kan substratene ansees som antioksidanter.

I **Paper III**, ulike tilsetningsstoffer som påvirker peroksid dannelse i to ulike pølser systemer ble kartlagt. Virkningene av ulike tilsetninger inkludert fiskeolje, myoglobin, nitritt, ekstrakt av kryddernellik og kalsium kilder på oksidasjon og sensoriske egenskaper ble undersøkt. To pølsesystemer (en testmodell pølse og en mer standard frankfurter type pølse) ble lagret på tre ulike måter før testing: 1) umiddelbart frosset ned ved -80°C; 2) lagret kjølig for 2.5 uker etterfulgt av belysning under fluoreserende lys ved 4°C i to uker før lagring ved -80°C; 3) frosset ved -20°C for 5 måneder og deretter lagret ved -80°C. Frosset gruppe 3 viste den høyeste peroksid dannelsen og TBARS for begge pølsesystemene. Upolare peroksider dominerte i begge systemene. Ekstrakt fra kryddernellik fungerte som en antioksidant, men kryddernellik smaken ble gjenkjent i den sensoriske testen. Kalsium så ut til å redusere peroksid dannelsen. Tilsetning av nitritt og fiskeolje førte til interaksjoner som støttet nitroso-myoglobin dannelse.

I **Paper IV** (manuskript), pølser ble produsert på tre natrium nivåer (tilsvarende 13, 15 og 17 g NaCl/kg pølse) ved bruk av fire ulike meieri ingredienser (tørket skummet melk, en kalsiumrik melkemineral pulver, en kaliumrikt melkemineral pulver og et laktoserikt pulver). Pølser med kalsium og kaliumrike melkepulver resulterte i de hardeste pølsene ved sammenligning med samme natrium nivå. Tilsetning av melkemineral pulver gav hvitere og mindre røde pølser. Etter 6 ukers kjøle lagring var det ingen effekt på harskhet knyttet til tilsetning av melkemineraler sammenlignet med å tilsette skummet melk pulver til pølsene. En klar fordel ved å bruke disse melkemineral pulverne i pølser er at Na:K forholdet kan bli redusert fra usunn (36) til et sunnere forhold (~ 2) med lite eller ingen smaksendring. Høy tilsetning av kalsium (6g/kg pølse) som melkemineral hadde ingen påvirkning på sensorisk bitterhet eller ettersmak, noe som er typisk for $CaCl_2$ tilsetning. Tilsetning av Ca til pølser er antatt til å være sunt for å unngå kalsium mangel og trolig for å redusere risikoen for endetarmskreft.

List of papers

I. Yi, G., Haug, A., Nyquist, N. F., & Egelandsdal, B. (2013). Hydroperoxide formation in different lean meats. *Food Chemistry, 141(3), 2656-65.*

II. Yi, G., Grabež, V., Bjelanovic, M., Slinde, E., Olsen, K., Langsrud, O., Egelandsdal, B. (2015). Lipid oxidation in minced beef meat with added Krebs cycle substrates to stabilise colour. *Food Chemistry, 187, 563–571.*

III. Yi, G., Haug, A., Nordvi, B., Saarem, K., Oostindjer, M., Langsrud, Ø., & Egelandsdal, B. (2015). Compositional Factors that Influence Lipid Peroxidation in Beef Juice and Standard Sausages. *Journal of Food Science, 80(12), C2692-2700.*

IV. Engeloug, A. M., Yi, G., Egelandsdal, B., Haug, A., & Nordvi, B. (2016). Commercial Mineral Enhanced Dairy By-products as Sodium Replacers, Antioxidants and Calcium Fortifiers in Sausages. *Manuscript.*

Abbreviations

ALA	α-linolenic acid
ANOVA	Analysis of variance
ARA	Arachidonic acid
ATP	Adenosine triphosphate
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DMb	Deoxymyoglobin
DPA	Docosapentaenoic acid
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
ETS	Electron transport system
$FADH_2$	Flavine adenine dinucleotide
FAME	Fatty acid methyl esters
FDA	Food and Drug Administration
GC-MS	Gas chromatography-mass spectometry
GLM	General Linear Model
Hb	Hemoglobin
KCS	Krebs cycle substrates
LA	Linoleic acid
MANOVA	Multivariate analysis of variance
MAP	Modified atmosphere packaging
Mb	Myoglobin
MDA	Malondialdehyde
MM-A	Milk mineral A
MM-B	Milk mineral B
MM-C	Milk mineral C
MSG	Monosodium glutamate

MUFA	Monounsaturated fatty acid
NADH	Nicotinamide adenine dinucleotide
NOS	Nitroso compounds
OMb	Oxymyoglobin
PCA/PCR	Principal component analysis/regression
PFP	Peroxide formation potential
PLS	Partial least square
PUFA	Polyunsaturated fatty acid
QDA	Quantitative Descriptive Analysis
ROS	Reactive oxygen species
SFA	Saturated fatty acid
SKM	Skimmed milk
TBARS	Thiobarbituric acid reactive substances
WP	Whey permeate

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1 Theory

1.1 Benefits of eating meat

Meat consumption is high in postindustrial and high-income countries and is also increasing in some developing countries due to their growing economies (Walker, Rhubart-Berg, McKenzie, Kelling, & Lawrence, 2003). Although meat is not the most essential component in the human diet, it is rich in nutrients and high quality proteins. Meat has many healthy compounds such as minerals (*e.g.* iron, zinc and selenium), vitamins (*e.g.* vitamin A, B_6, B_{12} and E), some essential amino acids and fatty acids (Biesalski, 2005). Iron is part of the hemoglobin molecule that can transport oxygen all over the body. Zinc helps in the formation and metabolism of tissues, and selenium aids in the production of antioxidant enzymes and protects our body after vaccinations. Meat is rich in vitamin A, which helps to maintain good vision, while vitamin B helps with healthy skin and hair. Protein, as one of the important components in meat, improves the overall health of the body including building and repairing body tissues, producing antibodies, and strengthening the immune system (Agarwal, Fulgoni, & Berg, 2015). Some essential fatty acids in meat, such as stearic acid (C18:0), have been shown to neutralize cholesterol concentrations and myristic (C14:0), palmitic (C16:0) and oleic (C18:1) fatty acids have been shown to reduce cholesterol among other positive attributes including a lower risk of stroke and a significant decrease in blood pressure (Kris-Etherton, 1999; S. Yu, Derr, Etherton, & Kris-Etherton, 1995).

1.2 Negative health aspects of eating meat

1.2.1 Fresh red meat

Red meat is normally red when raw and not pale/grey as after cooking. In this thesis, the term "red meat" is used for pork, lamb and beef meat. However, there are also many negative aspects of meat diets. Compared to a meat diet, a vegetarian diet which does not include eggs, milk and fatty dairy products can also provide nutritional requirements and such a diet could result in a lower risk of chronic diseases (Singh, Sabaté, & Fraser, 2003). Industrial animal feeds contain high amounts of grains, which are rich in n-6 PUFAs, and therefore result in red meat high in n-6 and low in omega-3 (n-3). Many studies have shown that a high n-6/n-3 ratio contributes to a

higher risk of many diseases, *e.g.* heart disease and autoimmune disorders (M. Yu et al., 2013). A high percentage of saturated fat in meat is highly correlated to an increased risk of diseases such as high blood cholesterol level, blood pressure, hypertension, type 2 diabetes, cardiovascular disease (CVD), colon and prostate cancer (Ascherio, Willett, Rimm, Giovannucci, & Stampfer, 1994; Mozaffarian, Micha, & Wallace, 2010). High heme levels in red meat can induce DNA damage and hyperproliferation of epithelial cells in colon via hydroperoxide produced by heme oxygenase and is associated with a higher risk of colon cancer (Ishikawa, Tamaki, Ohata, Arihara, & Itoh, 2010; Oostindjer et al., 2014).

1.2.2 Processed meat

Processed meat is often smoked, cured, salted/or with added chemical preservatives and is often packaged for a longer shelf life. Processed meat in this thesis is used to describe preserved poultry, pork, lamb and beef meat. Substantial amounts of sodium used in processed meat have increased the risk of many diseases, *e.g.* high blood pressure and heart disease (Joosen et al., 2009; Sinha, Cross, Graubard, Leitzmann, & Schatzkin, 2009). Sodium nitrite is often used to give a pink color to processed meat, as the color of meat is an important parameter for consumers. However, the addition of nitrite partly promotes the formation of nitroso compounds either in the meat products or in the stomach and is therefore considered to be carcinogenic. Such nitrosamines can, in particular, cause harm to the pancreas and liver (Santarelli et al., 2010). Among all the processed meat products consumed in Norway, the intake of sausages is among the highest. Substantial amounts of sodium and pork fat with a high n-6/n-3 ratio, as well as high arachidonic acid content in sausages, are associated with a higher risk of many diseases (Araujo de Vizcarrondo & Martín, 1997; Sinha et al., 2009; Solakivi, Kunnas, Karkkainen, Jaakkola, & Nikkari, 2009). Parr and others (2013) suggested that when the consumption of sausages is above 30 g/day, there is an increased risk of proximal colon, distal colon and rectal cancer in the Norwegian population. Norwegian women have the highest incidence of colon cancer in the world, and sausages were implicated as a product with increased risk of colon cancer (Santarelli et al., 2013).

1.3 Lipids in meat

Any group of naturally organic molecules that contains fats, waxes, sterols, fat-soluble vitamins, and triglycerides can be called lipids (Breslow, 2006). The major functions of lipids include storage and supplement of energy, signaling specific cell responses, and maintaining cell membrane integrity (Cooke, Evans, Dizdaroglu, & Lunec, 2003). Although most of the lipids can be broken down and synthesized by the body, some essential lipids must be obtained from the diet. The term lipid is often confused with fat, but fat is actually a subgroup of lipids called triglycerides. Fat is found in most meat products, and meat fat normally provides many different fatty acids, both saturated and unsaturated.

1.3.1 Triglyceride

When three fatty acid molecules react with a glycerol molecule to yield a triglyceride and three water molecules, fat or oil is formed (Nelson & Cox, 2000). The glycerol molecule has three hydroxyl groups and each fatty acid has one carboxyl group. Triglycerides are the main composition of vegetable oil and fish oil (normally unsaturated, liquid in room temperature) and animal fat (more saturated, solid in room temperature). Triglycerides, also known as triacylglycerols or fat, make up 90% of dietary lipids and are the major form of energy storage in the body (Nelson & Cox, 2000).

1.3.2 Phospholipid

Phospholipids consist of a hydrophilic head and hydrophobic tail and are the major structural lipid that form cell membranes. Most phospholipid molecules are composed of a diglyceride; fatty acids, phosphate and glycerol (Lucas et al., 2011). Due to their emulsification properties, phospholipids are often used as food additives, such as egg yolk and soybean lecithin.

1.3.3 Fatty acid

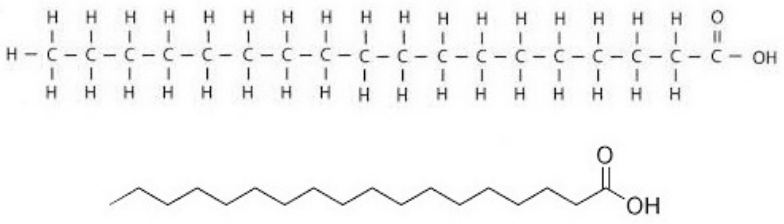
Fatty acids are usually derived from triglycerides or phospholipids. Most common fatty acids have a chain with an even number of carbon atoms with a methyl group at one end and a carboxyl group at the other end of the molecule (Rustan & Drevon, 2005). Fatty acids include free fatty acids or a part of triglycerides and phospholipids. They play major roles in energy storage, energy transport, gene regulation, and keeping the structure of the membrane (Neuringer, Anderson, & Connor, 1988).

1.3.3.1 Saturated fatty acids (SFAs)

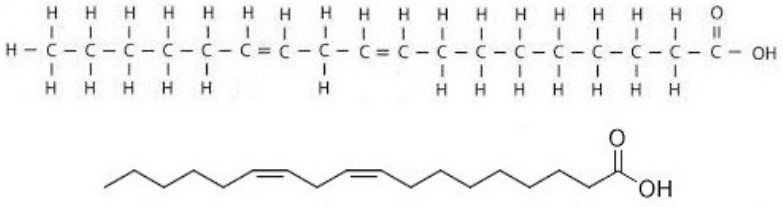
Saturated fatty acids are 'filled' with hydrogen, which means there are no double bonds between the carbon atoms of the fatty acid chain. Most of the saturated fatty acids have an even number of carbon atoms on straight hydrocarbon chains and contain between 12 to 22 atoms (Rustan & Drevon, 2005). Most of the saturated fatty acids found in animal fats contain 14, 16 or 18 carbon atoms. Compared to unsaturated fatty acids, SFAs are more stable in room temperature with the least chemical reactivity and the melting point increases with the carbon chain length (Wood et al., 2002).

1.3.3.2 Unsaturated fatty acids

Due to the number of double bonds present in the hydrocarbon chain, unsaturated fatty acids are divided into monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). MUFAs contain one carbon-carbon double bond, which can occur in different positions, while PUFAs have two or more double bonds that are separated by a single methylene grouping. If the first double bond occurs between the third and the fourth carbon atom from the methyl end, they are called n-3 fatty acids; if the first double bond occurs between the sixth and the seventh carbon atom, then these are called n-6 fatty acids.



Stearic 18:0, an example of a saturated fatty acid



Linoleic acid 9c, 12c-18:2 (18:2 n-6), an example of a unsaturated fatty acid

Figure 1. A saturated fatty acid and an unsaturated fatty acid.

In most of the naturally occurring unsaturated fatty acids, the double bond is in the *cis* configuration. A double bond with the *cis* configuration means that the hydrogen atoms on either side of the double bond are in the same direction. If the hydrogen atoms are in the opposite direction, the configuration is called *trans.* The presence of this double bond restricts the mobility of the acyl chain at this point in *trans* fatty acids. Compared to *trans* fatty acids, *cis* fatty acids have lower melting points. The *cis* configuration has a kink in the molecular shape and is thermodynamically more unstable than the *trans* configuration (Wood et al., 2002).

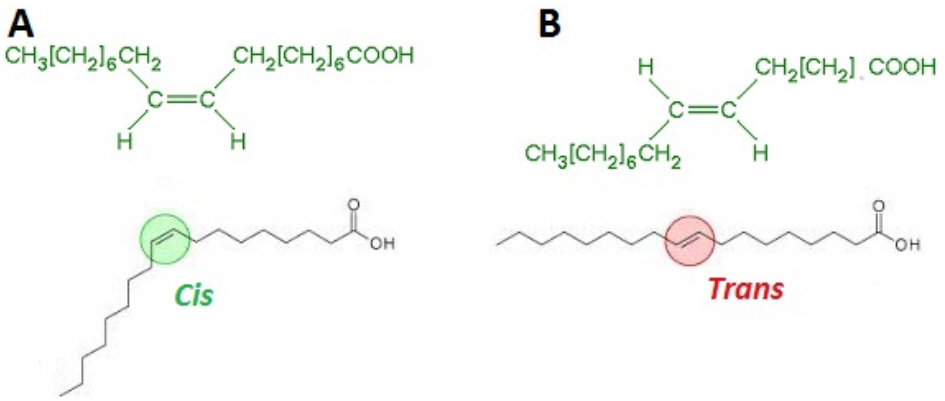


Figure 2. *Cis*-configuration and *Trans*-configuration. A. Oleic acid; B. Elaidic acid.

1.4 Reactive oxygen species

Reactive oxygen species (ROS) are chemically reactive compounds containing oxygen. The reduction of the oxygen molecule (O_2) by way of a one-electron reduction process, can produce highly reactive and short-lived ROS. ROS is a term that includes both oxygen radicals, *e.g.* superoxide ($O_2^{\cdot -}$), hydroxyl ($^{\cdot}OH$) and hydroperoxyl ($^{\cdot}HO_2$) radicals, and non-radical oxidizing molecules that can be converted to radicals easily, *e.g.* hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3), as well as iron-oxygen complexes. These compounds may either directly or indirectly lead to lipid oxidation in meat (Min & Ahn, 2005).

1.5 Lipid oxidation

Lipid oxidation is a free radical chain reaction, and the presence of oxygen is the most important factor in promoting lipid oxidation in meat. Due to the thermodynamic constraints, ground state oxygen molecules do not interact with PUFAs. They only become highly reactive and primarily initiate lipid oxidation once the oxygen molecules are converted to ROS. Iron as a major catalyst plays an important role in lipid oxidation.

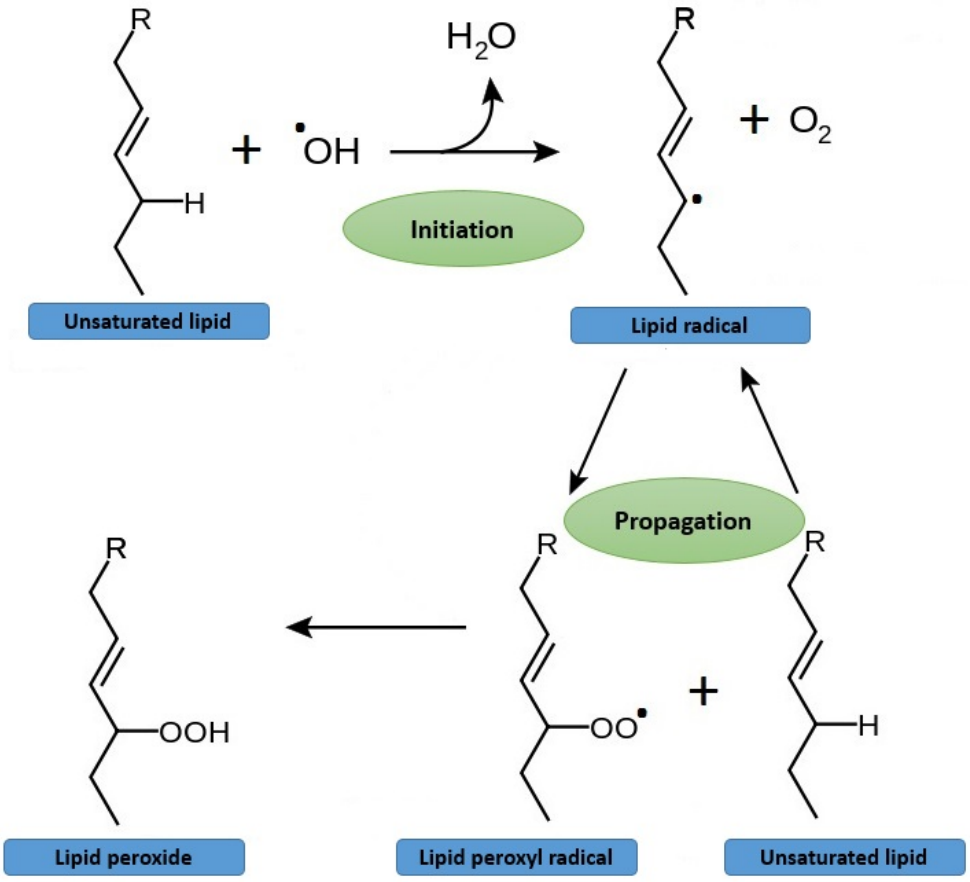


Figure 3. Radical chain reaction mechanism of lipid oxidation.

Lipid oxidation is an oxidation degradation process of lipids which includes initiation, propagation, and termination. The most common initiators in living cells are ROS, such as the hydroxyl radical ($OH^{\cdot}$) and hydroperoxyl radical ($HO_2^{\cdot}$). This initiation occurs by attacking any sufficient reactive species, *e.g.* unsaturated fatty acids, to form water and lipid radicals. In the propagation step, the unstable lipid radicals react with oxygen molecules to form lipid peroxyl radicals. However, these lipid peroxyl radicals are also unstable species that are able to react with other lipid molecules, such as surrounding unsaturated fatty acids, to produce other fatty acid radicals and lipid peroxides. Lipid peroxides can also undergo further reactions such as combination, intermolecular addition, and rearrangement, or react with other oxygen molecules to form cyclic peroxides (Min & Ahn, 2005). These reactions continue to produce new fatty acid radicals that react in the same way (Fig. 2), until the last step of the process: termination. When a lipid radical reacts with another, it always produces another radical; therefore, it is called a

chain reaction. Lipid radicals react with each other and/or perform self-destruction in order to produce non-radical species. Vitamin E and some antioxidants produced within the living organism can speed up termination by catching free radicals and protecting the cell membrane.

1.5.1 Factors that influence lipid oxidation in meat

Lipid oxidation is one of the main causes of quality deterioration in meat. Lipid oxidation probably starts immediately after slaughtering. The biochemical changes during the process of changing muscle into meat cause an imbalance between antioxidant and pro-oxidant levels and therefore initiate lipid oxidation (Min & Ahn, 2005). Pre-slaughtering events such as stress and physical damage and post-slaughtering events such as electrical stimulation, early post-mortem events and pH decline can all effect the degree of muscle degradation and effect lipid oxidation (Morrissey, Sheehy, Galvin, Kerry, & Buckley, 1998). In processed meat products, lipid oxidation is influenced by factors such as meat type, percentage of raw meat, size reduction process, cooking time, additives, and storage conditions (Kanner, 1994).

1.5.2 Consequences of lipid oxidation

1.5.2.1 Lipid oxidation and degradation in products (sensory aspects)

To both the food industry and consumers, lipid oxidation is one of the factors that limits shelf life and affects meat quality. Lipid oxidation is the primary cause of quality deteriorations such as appearance, color, texture and flavor, which are the main attributes that affect consumers' acceptance of meat (Addis, 1986). Lipid oxidation causes changes in the following three aspects:

1) Lipid oxidation increases the flavor quality loss, produces a rancid flavor and changes color and texture, which affect the consumers' acceptance, and leads to economic losses.
2) Lipid oxidation increases the nutritional quality loss of foods that contain lipids by decreasing the composition of some essential PUFAs and vitamins.
3) Lipid oxidation produces toxic compounds such as free radicals that may participate in the development of many diseases, *e.g.* atherosclerosis and cancer (Stocker & Keaney Jr,

2004). Hence, lipid oxidation produces compounds that are potentially unhealthy for consumers.

Fatty acids autoxidation can form monohydroperoxides that eventually break down to volatile products such as aldehydes, ketones, alcohols, acids, hydrocarbons, furans,

and lactones (Mottram, 1998). Most of these volatile hydroperoxide degradation products have low odor thresholds; therefore, even at very low concentration, they can still impair the sensory properties of meat. Volatiles such as hexanal, 2-nonenal, 1-octen-3-ol, and 4-hydroxy-2-trans-nonenal are degraded from n-6 fatty acids and volatiles such as propanal, 2-hexenal, 4-heptenal, 2,4-heptadienal, 2,4,7-decatrienal, 1,5-octadien-3-ol are degraded from n-3 fatty acids (Frankel, 1993). Aldehydes generated from lipid oxidation are some of the most reactive volatiles that are regarded as second toxic messengers to disseminate free radical reactions and deteriorate the protein instability and dysfunctionality (Echarte, Ansorena, & Astiasaran, 2001; K. Eder, 1999). Aldehydes are related to the deterioration of meat flavor and color because they increase pro-oxidant activity and oxymyoglobin oxidation and reduce metmyoglobin (Lynch & Faustman, 2000). Among aldehydes, hexanal is the most prevailing volatile that is generated from cooked meat products. Because its concentration increases more quickly than any other aldehyde, it has been suggested as an index of meat flavor deterioration during early storage of cooked meat products.

1.5.2.2 Consequences of ingesting lipid peroxides

The positive nutritional and negative toxicological effects of lipid oxidation in food have attracted much attention. High amounts of lipid peroxides exist in the human diet, such as fatty fish (*e.g.* salmon), fish oil, deep frying fat food, pre-cooked frozen food, and some other foods that have the potential to be oxidized (Kubow, 1990). Fatty acid peroxides, which are the primary products of lipid oxidation, may not be readily absorbed from the gut. Fatty acid peroxides accelerate all three phases of atherosclerosis and they decompose to low molecular compounds that are absorbed into the blood circulation and access other organs and tissues (Morrissey et al., 1998). Lipid peroxides, as the secondary products of lipid oxidation, especially malondialdehyde, are suggested as the main components degrading the gastrointestinal mucosa (Kubow, 1990). Malondialdehyde is highly reactive in cross-linking reactions with DNA and

proteins, while oxidized cholesterols are strong cytotoxic atherogenic agents *in vivo* and *in vitro* (Kubow, 1990). Lipid or lipid derived peroxides are also major sources of dietary pro-oxidants speculated to be of toxicological importance and risk factors to colon cancer (Halliwell & Chirico, 1993). In summary, lipid oxidation products are not single products but are rather a mixture of the above groups in the daily diet; therefore, it is necessary to minimize their concentration and eliminate their negative deleterious effects.

1.6 Protein oxidation

There are a variety of mechanisms that contribute to the protein oxidation process, such as formation of disulfide cross-links and glycoxidation adducts, nitration of tyrosine residues and carbonylation of specific amino acid residues (Davies, 2005; Oracz et al., 2007). Several studies show that the basic principles of the oxidation of proteins are governed by reactive oxygen species (ROS) (Garrison, 1987; Garrison, Jayko, & Bennett, 1962; Swallow & Charlesby, 1960). This demonstrates that protein modification if initiated mainly by reactions with $OH^{\cdot}$ (Berlett & Stadtman, 1997), however the availability of O_2 ,O_2^{-} or $HO_2^{\cdot}$ also determined the oxidation process (Berlett & Stadtman, 1997).

The oxidative modified forms of proteins result in protein fragmentation or aggregation and decrease protein solubility, thus affecting many biological modifications, such as the quality of meat and meat products (Decker et al. 1993). A study by Starke-reed and Oliver (1989) showed that the oxidation process might be effective in controlling the proteolytic activity of enzymes, and could also be linked to meat tenderness (Mercier, Gatellier, & Renerre, 2004). The balance between pro- and antioxidants and the concentration of polyunsaturated fatty acids, play an important role in the oxidative stability of meat. Higher levels of poly-unsaturated rather than saturated fatty acids in meat are more likely to lead to oxidation.ø-.o but other cations (*e.g*. K, Ca and Mg) vary through the addition of dairy by-productsk

1.7 DNA oxidation

DNA oxidation is the oxidative damage process on deoxyribonucleic acid (DNA). Reactive oxygen species can be generated during normal cellular metabolism and/or under certain exogenous conditions, *e.g.* by ionizing, ultraviolet radiation, and chemicals. Irrespective of their producing origin, ROS may interact with DNA and potentially lead to modification and mutation of the DNA sequence and damage of the cells (Cooke et al., 2003). There are more than 20 oxidatively damaged DNA lesions that have been identified, and among these lesions 8-oxo-2'deoxyguanosine has received the most attention. This lesion has higher oxidation potential when compared to cytosine, thymine, and adenine and the high oxidation potential is widely believed to be related to the risk for many diseases (Cooke et al., 2003).

1.8 Iron in meat

Generally, there are two types of iron in food: heme iron and non-heme iron. Heme iron is mainly derived from myoglobin and hemoglobin in animal protein sources such as meat, fish and poultry, whereas non-heme iron is commonly found in plants and vegetables. Heme iron is more easily absorbed due to the porphyrin ring and is not affected by the iron-binding inhibitory factors in the daily diet. Non-heme iron enters an exchangeable pool, which is strongly influenced by inhibitory iron-binding factors (West & Oates, 2008). One of the largest dietary sources of heme iron is red meat and the overall absorption of iron from meat may be at least 20-25% (Carpenter & Mahoney, 1992; Layrisse et al., 1969). The data for the heme iron concentration in meats is limited in food composition databases, since this type of analytical work has not been prioritized. Heme measurement levels are affected by various factors, including types of meats, methods of preparation of meat products, as well as analytical methodology (Carpenter & Clark, 1995; Kongkachuichai, Napatthalung, & Charoensiri, 2002; Lombardi-Boccia, Martinez-Dominguez, & Aguzzi, 2002). Studies have shown that both non-heme and heme iron are able to catalyze the formation of ROS, thus inducing oxidative DNA damage (Glei et al., 2002; Tappel, 2007).

1.9 Biochemistry of iron

Heme iron has numerous important biological roles within the body, such as oxygen transport, DNA synthesis, and energy metabolism. This biometal exists in two oxidation states: the reduced ferrous form (Fe^{2+}), which is found at acidic pH, and the oxidized ferric form (Fe^{3+}), which is favored under conditions of neutral or alkaline pH. Due to its redox reactivity and flexible coordination chemistry, iron is able to associate with proteins and bind to oxygen, and functions as an efficient catalyst for electron transfer (Aisen, Enns, & Wessling-Resnick, 2001). However, iron in an unbound state, *e.g.* not bound to protein or other organic molecules, is potentially toxic, because it catalyzes the generation of ROS and highly reactive radicals (hydroxyl radical) and nitro compounds (Kubicova, Hadacek, Weckwerth, & Chobot, 2015).

1.9.1 Heme proteins (including hemoglobin and myoglobin)

The crystal structures of the heme proteins, myoglobin (Mb) and hemoglobin (Hb), were solved at high resolution over 50 years ago (Kendrew et al., 1960; Perutz et al., 1960). Heme proteins are a group of proteins that have heme as the prosthetic group. These proteins are widespread in biological systems and play many important biological activities, such as electron transfer and storage by myoglobin and hemoglobin, ion channel chemosensing (Tang et al., 2003), circadian clock control (Kaasik & Lee, 2004) and microRNA processing (Faller, Matsunaga, Yin, Loo, & Guo, 2007).

The human blood stream contains approximately 150 g/L of hemoglobin, making the protein an effective oxygen carrier. Once the Hb-O_2 complex reaches tissues that consume oxygen, the O_2 molecules are transferred to myoglobin for the storage and use of oxygen in tissues. The oxygen molecule binds to the sixth coordination site of the heme of both hemoglobin and myoglobin. A nitrogen atom from a histidine side chain occupies the fifth coordination site, whereas nitrogen atoms from a planar porphyrin ring occupy the remaining four coordination sites, while the Fe (II) atom can be found at the center of the heme (Figure 4).

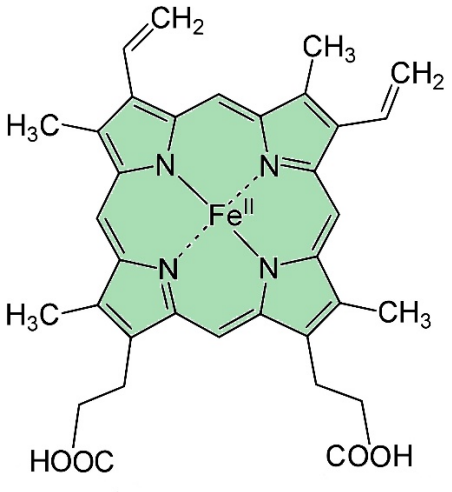


Figure 4. The hemoglobin B molecule with Fe II at the center.

Although the main function of Hb is to transport oxygen to tissues, the protein also interacts with carbon dioxide (CO_2), carbon monoxide (CO) and nitric oxide (NO) gasses (Schechter, 2008). In addition to its function as a storage for oxygen molecules, myoglobin has been described as an NO scavenger and hypoxic nitrite reductase (Rassaf et al., 2007; Shiva et al., 2007).

1.9.2 Function of iron/heme

Iron is an important component of hemoglobin in red blood cells, which transport oxygen around the body, form myoglobin and help muscles to store and use oxygen. The oxygen-carrying protein hemoglobin can release oxygen in different tissues and is used in oxidative metabolism. Hemoglobin can bind to carbon dioxide in the body and transport it to the lungs, where it is exhaled. Iron is also an important component of iron-sulfur complexes present in enzymes that are needed for electron transport chain and energy generation in mitochondrial respiration and the Krebs cycle. Iron is mainly present in hemoglobin in circulating red blood cells and in muscles. The remaining iron can primarily be found in the liver and exists as ferritin and hemosiderin in the reticulo-endothelial system (Geissler & Singh, 2011).

1.9.3 The influence of heme protein on lipid oxidation

Lipid oxidation is a main cause of meat quality deterioration, which develops off-odors and off-flavors (Kanner, 1994). Iron has been regarded as the primary catalyst of lipid oxidation, while heme-proteins such as hemoglobin (Hb) and myoglobin (Mb) are pro-oxidants of lipid oxidation (Johns, Birkinshaw, & Ledward, 1989). They are also regarded as major catalysts for initiation of oxidation. Iron-oxygen complexes are considered as initiators of lipid peroxidation in meat (Johns et al., 1989). Iron is a transition metal which can readily accept and donate electrons, which makes it function as an oxidant or reductant in many biochemical reactions. The ferrous iron Fe (II) and the ferric iron Fe (III) are the most abundant transitional metals and dominate in biological systems. Iron-binding protein molecules are normally classified by the functional role of metal ion, *e.g.* structural, transport, dioxygen binding, and catalytic protein (Johns et al., 1989). Iron can catalyze the detrimental oxidation of different biomolecules such as protein, lipid or DNA. Therefore, iron-binding protein can regulate the iron metabolism which can ensure the absence of free iron molecules (Min & Ahn, 2005).

1.10 Additives in processed meat products and their function

The Food and Drug Administration (FDA) defines "food additive" as any added substance that directly or indirectly affects the characteristics of any food. The definition covers any substances used in the production, treatment, processing, packaging, transportation or storage of food. Food additives that are added to a food for specific purposes are defined as "direct" food additives, whereas foods with "indirect" food additives are described as foods that have these additives due to the packaging, storage or other handling methods. The use of food additives is necessary to maintain or improve safety, freshness, nutritional taste, texture and appearance (Faia).

Food additives have a variety of functions in foods. All additives must be approved by the FDA and the U.S. Department of Agriculture or by the European Food Safety Authority (EFSA) prior to addition in meat. Approximately 2,800 food additives exist. However, only a fraction of approved additives is used in processed products. These additives have important functions, such as "curing" meat products, in addition to preventing the growth of bacteria. Here are some of the

most common additives used in processed and cured meat products:

- Proteins: milk protein, whey protein, casein and various hydrolyzed proteins.
- Flavor enhancers from milk proteins that have been broken into amino acids; Flavor enhancers such as glutamic acid, monosodium glutamate (MSG).
- Modified food starch that is separated from its original protein source and used as a thickener.
- Salt, an essential ingredient in both processed and cured meat. It adds flavor, texture, protects against bacteria, and extends shelf life.
- Sodium nitrite functions as an anti-oxidant and a curing ingredient, giving cured meat a characteristic pink color and taste. Nitrite is exclusively used to cure meats, which is, at high concentration, an effective way of preventing the growth of bacteria.
- Sodium ascorbate is one of the mineral salts of ascorbic acid and is often used as an antioxidant and an acidity regulator. Sodium ascorbate can delay the disappearance of color in meat and stabilize the aroma by its antioxidative action.
- Disodium phosphate and trisodium phosphate are used in conjunction as food additives in meat products to enhance juiciness and tenderness by retaining the moisture in meat products.

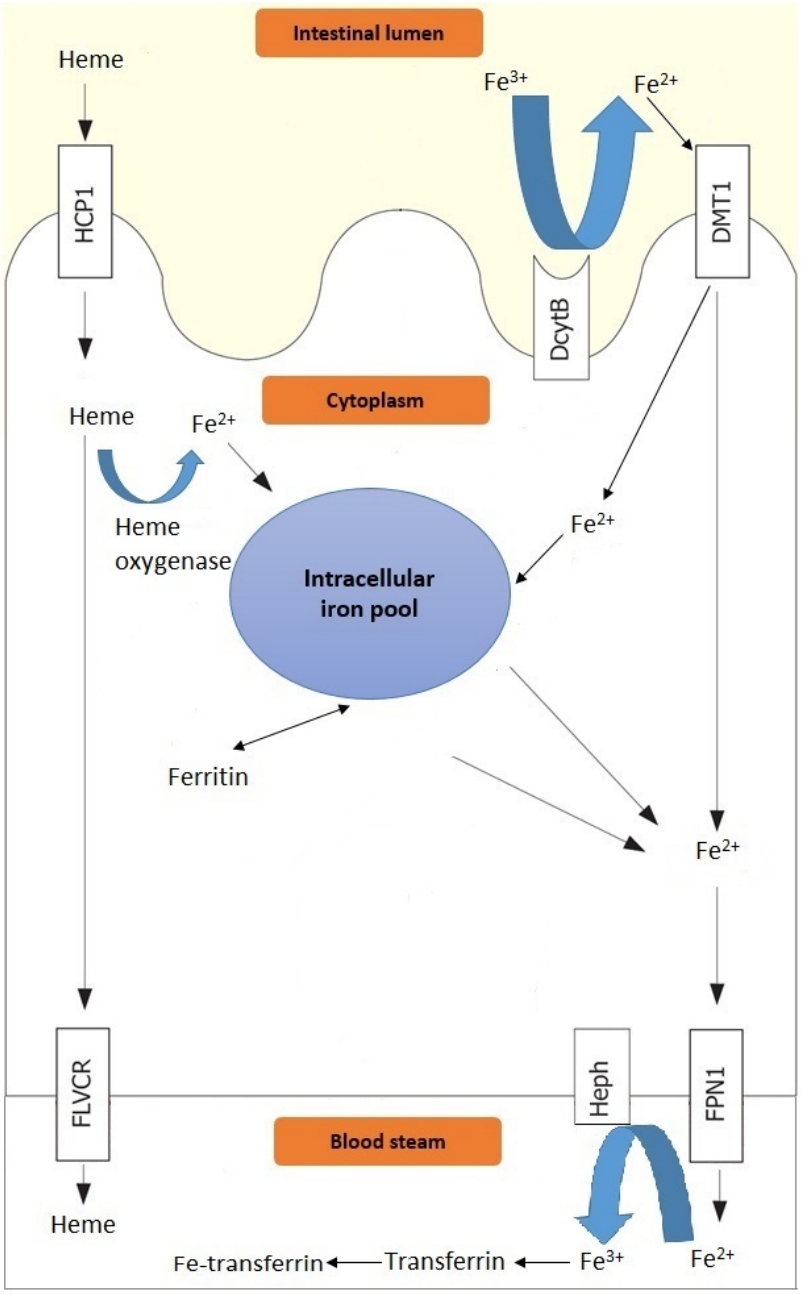


Figure 5. Iron absorption mechanisms in the intestinal lumen. Non-heme iron is taken up from the intestinal lumen by iron receptor DMT1 on the microvillus membrane before joining the introcellular iron pool in the cytoplasm. Heme iron is taken up by receptor (HCP1) mediated endocytosis.

1.10.1 Antioxidants in processed meat

The most important property of antioxidant molecules is to prevent or slow down the oxidation procedure of other molecules. Oxidation reactions include either an increasing oxidation state or the loss of electrons which produce free radicals at the end. Oxygen is one of the most essential molecules for life, but oxygen is also a highly active atom which can increase oxidation reactions and produce free radicals. The unstable free radicals contain an unpaired electron that can get electrons from other molecules in order to neutralize themselves, but this process can produce more free radicals and initiate a chain reaction. Free radicals are regarded as initiators of cytotoxic response and lead to cell death. Free radicals have also been reported to be major contributors to many diseases such as cancer, heart and brain disease and decline of the immune system (Kim, Lee, Song, & Kang, 2015; Mathew, Abraham, & Zakaria, 2015; Valko, Jomova, Rhodes, Kuča, & Musílek, 2015).

Antioxidants are associated with many cellular functions such as vascular disease, inflammatory damage and cancer (Padayatty et al., 2003). Antioxidants are elements or compounds that lose an electron and play an essential role in removing either the free radical chain reaction intermediates or inhibit the oxidation reaction. Vitamin C and E, thiol, beta carotene, and polyphenols are the most common antioxidants in plants or animals.

1.10.1.1 Vitamin C

Vitamin C is a water soluble antioxidant and the antioxidant function is due to its property as an electron donor. Vitamin C is also known as ascorbic acid and is abundant in both plants and animals. Ascorbic acid is very important to health and must be obtained from the diet. The most important function of ascorbic acid is collagen synthesis which can convert procollagen to collagen and act through oxidizing proline residues to hydroxyproline. Ascorbic acid is also required for the metabolism of some amino acids and vitamins, and for the synthesis of hormones and neurotransmitters (Padayatty et al., 2003; Traber & Stevens, 2011). The direct antioxidant effect of ascorbic acid is to reduce or neutralize reactive oxygen species, e.g. hydroperoxides (Padayatty et al., 2003). Ascorbic acid reacts with histamine and participates in

the detoxification of toxic substances in the liver, plus reduces inflammation in blood (Padayatty et al., 2003). In addition to this function, ascorbic acid is also a substrate of the redox enzyme ascorbate peroxidase, which plays an important role in stress resistance in plants (Shigeoka et al., 2002).

1.10.1.2 Vitamin E

Vitamin E is a fat soluble antioxidant and the most effective part of the Vitamin E is α-tocopherol. The antioxidant activity of Vitamin E stops the formation of reactive oxygen species when lipid or fat is oxidized (Packer, Weber, & Rimbach, 2001). Vitamin E normally functions as a peroxyl radical scavenger which acts as a chain breaker that inhibits the propagation of free radical reactions (Burton, Joyce, & Ingold, 1983; Ingold et al., 1987). Free-radical scavenging reactions of α-tocopherol happen normally via the intermediate of α-tocopherol radical and α-tocopherol reacts with free radicals and forms tocopheryl radicals, which can be reduced by a hydrogen donor to its reduced state (Traber & Stevens, 2011). The hydrophobic Vitamin E can be incorporated into membranes and protect themselves from oxidation and cell damage.

1.10.1.3 Polyphenols (clove extract)

Antioxidants containing polyphenolic substrates are called polyphenol antioxidants. Clove extracts which can be obtained by distillation of the dry flowers, stems or leaves of the clove (*Eugenia aromatica* or *Eugenia caryophyllata, Fam. Myrtaceae*) contain polyphenol-rich antioxidants (Lee & Shibamoto, 2001 ; Matan et al., 2006). Cloves are easily extracted and its DPPH radical scavenging activity is as high as vitamin C. Clove extract also has a high FRAP (ferric reducing ability of plasma) activity and is an intermediate inhibitor of TBARS (Jin & Cho, 2011). The antioxidant effect of clove extract is mainly due to eugenol, which is the major phenol in the extract (Lee & Shibamoto, 2001). Eugenol is part of the phenylpropanoids class and represents around 80-90% of the polyphenols in clove bud oil and more than 80% in clove leaf oil (Chatterjee & Bhattacharjee, 2015).

The mechanism of polyphenol antioxidants is to up-regulate certain metal chelation reaction and scavenge free radicals. In order to maintain the healthy and normal metabolic function of cells

and prevent the damage from reactive oxygen species, reactive oxygen species, *e.g.* hydro peroxide, have to be continuously removed. The polyphenolic antioxidants (PhOH) can react with the reactive oxygen species, *e.g.* peroxyl radicals ($R^{\cdot}$), by denoting of hydrogen, which interrupts and inhibits the chain reaction and generate phenoxyl radicals ($PhO^{\cdot}$).

$$\mathbf{R^{\cdot} + PhOH \rightarrow R\text{-}H + PhO^{\cdot}}$$

Phenoxyl radicals ($PhO^{\cdot}$) can be stabilized though resonance, intramolecular hydrogen bonding or combine each other to yield dimerization products and therefore terminate the chain reaction (Bors, Heller, Michel, & Saran, 1990).

$$\mathbf{PhO^{\cdot} + PhO^{\cdot} \rightarrow PhO\text{-}OPh}$$

1.10.2 Minerals

In addition to fat, protein, carbohydrates and vitamins, minerals are also an important part of the diet and nutrition intake. The high amount of sodium in processed meat and meat products is negative to health and may lead to diseases. Therefore, it is necessary to adjust the traditional sodium amount or use sodium replacers. Typical sodium replacers are K, Ca, Mg and NH_4. Milk calcium addition to n-3 PUFA-rich sausages is also positive as calcium absorption is enhanced by a n-3 PUFA-rich diet (Owen, Siobhan, Christopher, & C., 2003). Ca has also been pinpointed as a mineral needed to be maintained at an adequate level in order to reduce the risk of colon cancer (Santarelli et al., 2013).

1.10.2.1 Sodium

Meat and meat products are among the top contributors to sodium in the diet. The average NaCl content is 1.8-2.0 g/100 g sausage, and the total consumption of NaCl is about 10 g/person per

day. Overall, the meat industry is a significant contributor of NaCl to the diet, after the cereal industry (*e.g.* bread). Normally, sodium accumulates in the bloodstream and the body needs water to dilute the sodium, which increases the blood volume. Increased blood volume leads to higher blood vessel pressure and more blood fluid in the heart and this extra pressure and work will lead to high blood pressure. High blood pressure is the main cause of many cardiovascular diseases. Therefore, it is necessary to reduce the salt intake or use salt-substitutes to replace the traditional NaCl intake. Milk minerals are a potential good option. In addition to milk (8-9g/L), milk minerals contain calcium, magnesium, sodium and potassium (Gaucheron, 2005). It is a well-balanced mineral salt and one advantage is the fact that milk Ca is largely insoluble and is therefore expected to be tasteless. Salt replacers are mostly based on KCl, or sometimes a mixture of NaCl and KCl. However, KCl may yield a metallic, bitter flavor and it lacks sodium's capacity for flavor enhancement (Nachay, 2005). Therefore, the ratio of K/Na in food may also be important to health.

1.10.2.2 Calcium

Calcium is an essential dietary mineral for the body, needed to keep stabile blood pressure, ensure muscle and nerve function, and keep strong teeth and bones. Calcium is present in many foods and drinks, but can also be ingested as extra supplements. Calcium is either passively absorbed in the intestines by diffusing through the spaces between the cells, or actively absorbed through inter intestinal membrane cells by binding to calbindin, which is a transport protein and produced independently of vitamin D (Bronner, 2003). Traditional sausages in Norway have used dry skimmed milk which provides calcium, but presently the sausages are more often dairy free for allergic reasons. However, long term high intake of calcium carbonate can lead to calcium accumulation in the kidney and to kidney stones, hypercalcemia and kidney failure (Beall, Henslee, Webb, & Scofield, 2006; Caruso, Patel, Julka, & Parish, 2007; Gordon, McMahon, & Hamblin, 2005).

Also, the addition of nitrite to sausages promotes the formation of nitroso compounds (NOS), which are believed to increase the risk of proximal colon, distal colon, and rectal cancer. Adding high amounts of calcium has been shown to suppress the pro-cancer effect of NOS and counteract the toxicity of NOS (Santarelli et al., 2013). Due to decreased intake, diminished sun

exposure and impaired intestinal absorption, Vitamin D and calcium deficiency are associated with muscle weakness in elderly people (Omdahl, Garry, Hunsaker, Hunt, & Goodwin, 1982). Therefore, producing calcium-rich sausage would be a positive alternative to both avoid calcium deficiency in the elderly and decrease colon cancer risk.

1.10.3 Oils

Lipids and oils play an important role in the human diet. Lipids and oils supply essential fatty acids to the body, act as storage of energy, form the cell membrane phospholipids, work as carriers for absorption of certain vitamins, and increase the palatability of foods.

1.10.3.1 Fish oil

Fish oils are oils derived from the fatty tissue of oily fish. Fish oils are rich in n-3 long-chain polyunsaturated fatty acids (PUFAs), which includes eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA). Different organizations recommend a variable amount of EPA/DHA but the European Food Safety Authority recommends that adults should consume about 250mg of n-3EPA/DHA per day. DPA, EPA and DHA play an important role in keeping the bioactivity of membranes and strengthen anti-inflammatory processes (Swanson, Block, & Mousa, 2012). EPA and DHA have recently been reported to be associated with healthy aging and fetal development (Dunstan et al., 2007), while DPA was associated with a lower risk of cardiovascular diseases (Mozaffarian & Wu, 2012). DPA has strong anti-inflammatory properties and helps with the functioning of other fatty acids (Mozaffarian & Wu, 2012). Fast food contains higher amounts of saturated fatty acids and a lower amount of essential n-3 PUFA fatty acids when compared with most slowly cooked food (Dangardt et al., 2010). Fish and fish oil supplements are normally the primary contributors of EPA, DHA and DPA. By adding fish oil to fast food, such as sausages, one can increase the amount of n-3, therefore increasing the n-3 fatty acids and providing long chain PUFAs.

1.10.3.2 Vegetable oils

Vegetable oils from sunflower, soybean and safflower are rich in n-6 fatty acid linoleic acid (LA), which represents the most abundant PUFA in the daily diet. A shorter chain n-3 fatty acid, α-linolenic acid (ALA), is a prominent component of vegetable oils like linseed, rapeseed and soybeans oils. ALA are converted to EPA and DHA by elongation and desaturation enzymes, but the amount that can by synthesized in the body is small (Neff et al., 2011).

1.10.4 Krebs cycle substrates

The Krebs cycle is an important metabolic pathway that oxidizes 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. It is shown that ETS is active a long time post-mortem in meat while the ability to synthesize ATP is lost quickly post-mortem (Phung et al., 2013).

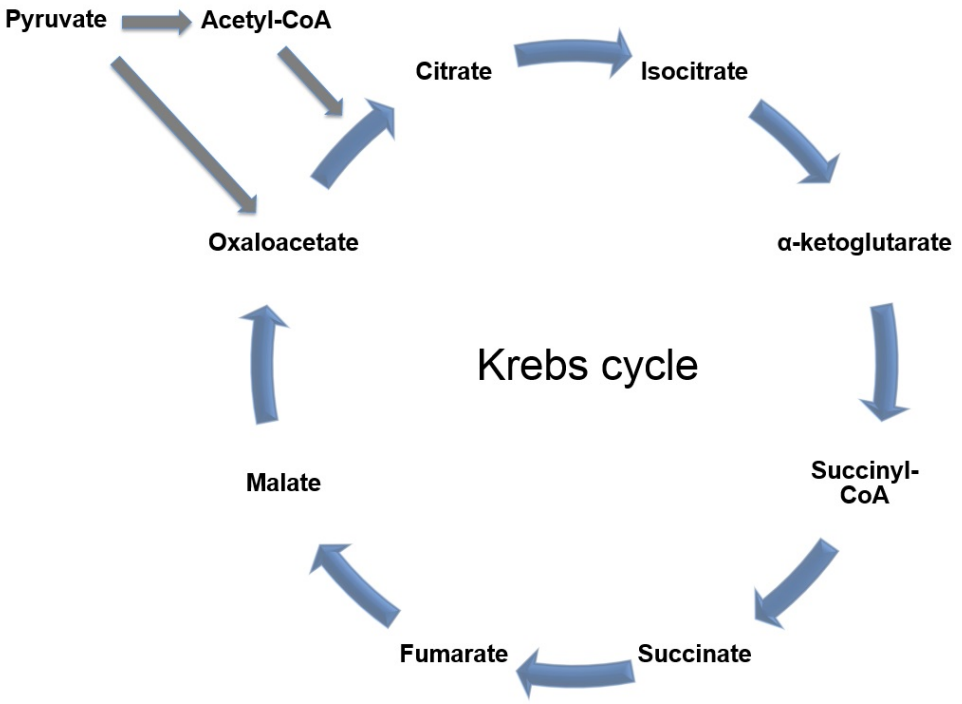


Figure 6. Krebs cycle substrates.

A variety of KCS have been shown to have antioxidant or pro-oxidant properties in different *in vivo* and *in vitro* environments. Succinate can be used either alone or in combination with other KCS *e.g*. glutamate as flavor enhancers. Glutamate is the most often used flavor enhancer by the food industry to provide the umami taste. The antioxidant property of succinate is probably due to it supporting the production of reducing equivalents ($FADH_2$) to ETC (Puntel, Roos, Garcia, Nogueira, & da Rocha, 2011), while glutamate has been reported to induce oxidative stress and lipid peroxidation, thus behaving as a pro-oxidant (Herrera et al., 2001). Glutamate leads enzymatically to α-ketoglutarate, an intermediate in the citric acid cycle (Figure 5). Pyruvate is a dietary supplement that can promote weight loss and have fat burning benefits (Gray et al., 2015). Malate is often used to enhance fruit flavor and is often combined with citrate to make a desirable taste in foods. The antioxidant properties of citrate, malate and pyruvate are due to the formation of an inactive complex with iron and inhibition of iron redox activity (Puntel et al., 2011). A challenge with KCS is that the effect of their combinations on electron transfer (oxidation, reduction) is difficult to predict. This is sometimes the case also for single compounds: malate is a typical example.

1.11 Common nutrient deficiencies in the Nordic diet

Vitamin deficiency can be alleviated through diets such as oil-rich fish or fortified fish oil plus other fortified products like margarine, or food products with added vitamin D (butter and dairy products). Vitamin D deficiency, or inadequate intake, has been reported many times in Nordic countries (Spiro & Buttriss, 2014). Vitamin D can come from intake through diet and from synthesis in the skin by exposing it to sunshine, which has been reported as the main source for vitamin D (Calvo, Whiting, & Barton, 2005). In Norway, not everyone takes cod oil and vitamin D in purified cod oil is mostly lost. Therefore, it needs to be added under the additive regulation as high latitude and weather conditions normally determine whether there is sufficient sunlight to stimulate the synthesis of vitamin D in the body. In the Nordic countries, sunlight is too weak to trigger the synthesis of this vitamin during the winter season. Therefore, in order to maintain a healthy vitamin D status, inhabitants of the Nordic countries have to rely on a vitamin D rich diet, particularly during the winter season (O'Connor & Benelam, 2011).

Calcium is a mineral that is needed to stabilize blood pressure and build stronger bones and teeth. Aging can cause calcium deficiency, *e.g.* loss of memory, depression and muscle dysfunction. Smoking and drinking an excessive amount of alcohol can impair the absorption of calcium. Calcium addition to processed meat can be used to increase the daily intake of calcium, reduce peroxide formation, decrease bone diseases and potentially inhibit colon cancer development (Jorde & Bonaa, 2000; Santarelli et al., 2013).

Previous studies suggest that human beings evolved on a diet with a ratio of n-6 to n-3 polyunsaturated fatty acid of approximately 1:1, whereas in Western diets the ratio is 15:1-16.7:1 (Simopoulos, 2002). The deficiency in n-3 and the excessive amounts of n-3 is partly due to the high intake of processed meat. High amounts of sodium and pork fat with a high n-6:n-3 ratio and a relative high arachidonic acid (ARA) content used in processed meat (especially in sausages) are claimed to increase the risk of many diseases, *e.g.* high blood pressure, heart diseases and cancer (Araujo de Vizcarrondo & Martín, 1997; Sinha et al., 2009; Solakivi et al., 2009). Fish oil can provide EPA and docosahexaenoic acid DHA and adjust the n-6:n-3 ratio to a lower value. A lower ratio of n-6: n-3 is more desirable in reducing the risk of many chronic

diseases and this is favorable for health (Jeun-Horng, Yuan-Hui, & Chun-Chin, 2002). However, fish oil addition may be constrained due to lipid oxidation and therefore the second most relevant target is to add long chain polyunsaturated fatty acids like EPA that are not so easily obtained from meat products.

Fiber is an essential part of a daily diet. It is important to consume enough fiber in order to maintain health and vitality. Meat and meat products are low in fiber. The high consumption of meat products in the diet in Nordic countries may support a fiber poor diet. A low-fiber diet might lead to many diseases, such as high blood pressure, diabetes, cardiovascular disease and cancer (Vanhauwaert, Matthys, Verdonck, & De Preter, 2015). Colon cancer risk has been shown to be associated with a low-fiber diet (Gilsing et al., 2015). Although fiber can be taken as a supplement, the best way to obtain enough fiber is from food such as whole grains, fruits and vegetables.

2 Objectives

The overarching aim of this thesis was to study some meat quality issues that are related to the more common theories behind processed meat as an unhealthy product. Thus, the overall objective of this study was to study the lipid peroxidation in meat and processed meat products and presumed tasteless Ca additives to the sausages.

Partial objectives:

- Use a new model system/ analytical principle for measuring hydroperoxide value in meat products and rank the hydroperoxide formation ability in Norwegian regular diet meats. (Paper I).
- Study how different additives affect lipid peroxidation in meat or processed meat products (Paper II, III and IV).
- Study the use of tasteless Ca additives and how they affect lipid peroxidation in sausages (Paper III and IV).
- Study how the sodium taste varies with the addition of dairy by-products, when the sodium content is constant but other cations vary in sausages (IV).

	Paper I	**Paper II**	**Paper III**	**Paper IV**
Materials	Fresh meat Lean meat: chicken, lamb, pork and beef	Comminuted beef + beef/pork fat*	Processed meat Sausages (Beef+Pork fat)	Sausages (Beef+pork fat)
Objectives	Use a modified FOX method to measuring hydroperoxide value in lean meat; Rank the hydroperoxide formation ability in lean meat	Identify how different KCS affects lipid per-oxidation	Identify how different additives affect lipid per-oxidation and sensory properties	Identify the sodium taste with addition of dairy by-products and how milk calcium effect sensory properties

- may be defined as processed meat using some present definitions since the minced meat has an additive

3 Methods

There are many different analytical methods for measuring oxidation in foods. The methods chosen in this study were used to measure the primary and secondary oxidation products.

3.1 Methods used for the measure of primary oxidation products

Hydroperoxides are reactive oxygen species (ROS) associated with oxidation. The Ferric-xylenol orange (FOX) method was suggested as a simple and sensitive method for monitoring lipidperoxide (Gay & Gebicki, 2003). The FOX method that was used in this study was based on Gay and Gebicki (2003), but adapted to meat instead of serum and with reduced volumes to adapt the technique to Eppendorf tubes. The FOX method is based on oxidation of hydroperoxides under certain acidic condition (pH 1.8) for a maximum response at room temperature (Gay, Collins, & Gebicki, 1999). The quantification of hydroperoxide can be measured using the ferric-xylenol orange (XO-Fe^{3+} complex) product using the absorbance at 590nm.

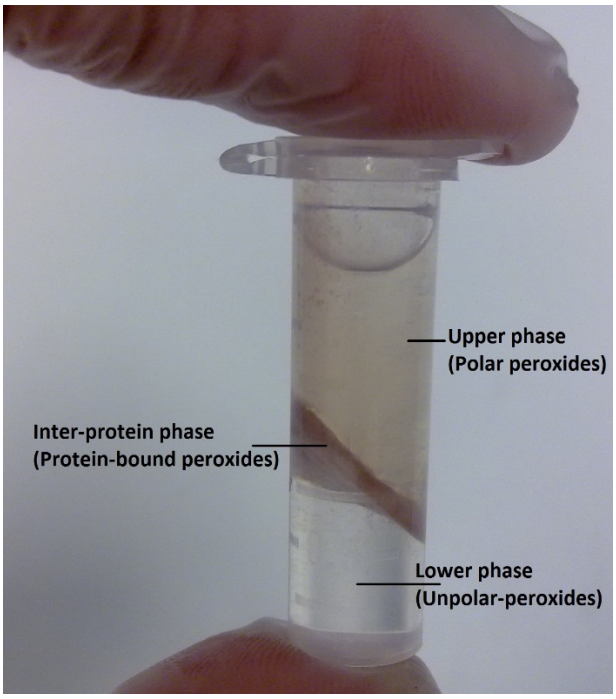


Figure 7. Three extracted phases by using the FOX methods.

The ferrous ion Fe^{2+} can be oxidized to ferric ion Fe^{3+} in the presence of ROS, and XO can measure ROS by spectrophotometrically analyzing the red purple complex which is formed by XO and Fe^{3+} (ferric-xylenol-orange, Fe^{3+} XO).

$$\mathbf{ROOH + Fe^{2+} \rightarrow Fe^{3+} RO + OH^-}$$

$$\mathbf{Fe^{3+} + XO \rightarrow Fe^{3+} XO\ complex}$$

The initially obtained hydroperoxide values were calculated by first subtracting the negative control, then the absorbance was divided by the pigments' molar absorptivities of 14840 (1 cm pathway) and 87583 (1 cm pathway) for the upper phase/inter phase and the lower phase, respectively, before correcting for dilution. In each meaurement, total peroxides were determined by analyzing protein-bound peroxides and hydroperoxide compounds in water-methanol and chloroform extracted phases. More details about this modified FOX method can be found in **Paper I.**

3.2 Methods used for the measure of secondary oxidation products

3.2.1 Thiobarbituric Acid Reactive Substances (TBARS)

Oxidation in meat products results in the formation of ROS and unstable lipid hydroperoxides. Due to the fact that ROS are highly reactive molecules, it is difficult to get a full picture of oxidation by only measuring peroxides. Several byproducts are produced during the oxidative stress damage process, such as Thiobarbituric Acid Reactive Substances (TBARS). TBARS measures malondialdehyde (MDA) generated from the oxidative stress process. MDA is a low molecular weight secondary oxidation product from the decomposition of polyunsaturated fatty acids. MDA can be colorimetrically quantified by following the controlled reaction with thiobarbituric acid. All the meat samples were measured against distilled water at 532 nm.

The value of TBARS was calculated by the following formula:

A_{532}/156*72.1=MDA mg/kg (The annotation ppm is mg/kg, which is often used as MDA equivalents).

More details are given in **Paper II and Paper III.**

3.2.2 Volatile compounds

The primary production of lipid oxidation is hydroperoxides, which are tasteless and have no aroma effect. Upon decomposition, hydroperoxides produce many secondary oxidation products including volatile compounds, which play an important role in the perceived aroma of meat products. The headspace analyses in gas chromatography were performed according to a modified method by Volden et al. (2011) for identification and quantification of the volatile compounds in this study. 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 Bjelanovic et al. (2012). Hexanal and 2-octen-1-ol were added to minced meat that did not contain any of these components in detectable amounts to convert the measured area values of hexanal and 2-octen-1-ol to mg/kg minced meat. Each unknown sample was measured together with one sample with a high concentration of volatile compounds (hexanal and 2-octen-1-ol) and one sample with low concentrations of these two compounds to keep track of the stability of the measuring system. A five-point calibration curve was used to calculate concentrations of hexanal and 2-octen-1-ol in unknown samples. More details can be found in **Paper II.**

3.3 Sensory analysis

Sensory analysis of meat products is a method that includes human elements in order to evaluate meat quality characteristics such as tenderness, juiciness, color, flavor and many other acceptability indicators. Quantitative Descriptive Analysis (QDA) is one of the main descriptive analysis techniques in sensory analysis that uses descriptive panels to measure the sensory characteristics of a product.

Before the QDA analysis, all the panelists were trained and described the differences among the products. Despite the intensive training among the panelistes employed in this method, many studies have assumed that different panelists will use different parts of the scale to make their own determinations (Lawless & Heymann, 1998). Thus, an absolute scale cannot be assessed and it is the relative differences among products that provide the valuable information. Statistics have been used to remove the effect of using different parts of the scale. More details can be found in **Paper III.**

3.4 Fatty acid analysis

Gas chromatography-mass spectometry (GC-MS) is often used to analyze fatty acids in meat fat. Fatty acids of selected fat tissues were transformed into fatty acid methyl esters (FAME), which are derived by transesterification of fats with methanol and then analyzed. The transesterification of lipids to FAME in this study was performed by using a method described previously with minor modifications (Devle, Rukke, Naess-Andresen, & Ekeberg, 2009).

In order to obtain the accuracy and required precision of FAME, all the steps including esterification, injection, separation, identification and quantitation need to be optimized (K. Eder, 1995). More details can be found in **Paper I and Paper II.**

3.5 Hemin analysis

The hemin analysis was carried out on meat samples following the analytical methods from Ginevra et al. (2002) with some optimizations. The main procedure includes meat homogenizing, weighing, and extraction from acetone and HCl. The supernatant was collected and the absorbance was measured at 407 nm against a reagent blank. Myoglobin solutions were used to make a linear standard curve and hemin concentrations were read from the standard curve. More details can be found in **Paper I.**

3.6 Color measurement

The effect of different additives in meat products was measured by Konica Minolta CM-700d portable spectrophotometer with sphere geometry and vertical alignment. The CIE L*, a*and b* chroma meter measures color in three dimensions, where L* for lightness, a* and b* for the color-opponent dimensions. The content of oxymyoglobin, deoxymyolobin, myoglobin and metmyoglobin mainly determines the color of red meat, while in processed meat products, color is also effected by addding different additives. More details can be found in **Paper III** and **Paper IV.**

3.7 High pressure liquid chromatography (HPLC)

HPLC was used to separate, identify and quantify components in the meat samples. Meat samples were analyzed on a coloum, connected to a pump, an autosampler and an oven. Standard solutions for external calibration were prepared similarly to the meat samples, and the compounds were identified according to their retention times compared with the standard solution. HPLC was used for organic acids analysis in **Paper II** and nitrosamine measurements in **Paper III**.

3.8 Statistical analysis

The statistical methods used in this thesis were the analysis of variance (ANOVA) and principal component analysis/regression (PCA/PCR). ANOVA is a standard method to use for designed experiments with different factors while PCA is a useful explorative method to unravel how samples relate to each other. Multivariate analysis of variance (MANOVA) is a method used when more than one response variable is measured on the designed samples.

General linear model (GLM) is an ANOVA procedure in which the calculations are performed using a least squares regression approach to describe the statistical relationship between one or more predictors and a continuous response variable. Predictors can be factors and covariates.

Paper I: Minitab version 16 (Minitab Inc., State College PA, USA) was used for univariate regression analysis, stepwise regression and one way ANOVA. The unscrambler (version X 10.2 CAMO Software AS, Oslo, Norway) was used for PCA and partial least square (PLS) regression. Validation of the PLS regression model was with full cross-validation.

Paper II: A quarter fraction of the full design (2^9) was prepared and 128 samples were selected by using the 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. Response surfaces were drawn in Minitab Statistical Software version 16 (State College PA 16801-3008, USA) due to the fact that surfaces are the most appropriate way to illustrate the mixture effect.

Paper III: In Exp I, PV and TBARS were analyzed using a general linear model with the five main factors ($CaCO_3$, myoglobin, nitrite, fish oil, clove extract) plus first order interactions between variables. The production day was defined as a covariate and its interactions with design variables were added. In Exp II, PV and TBARS were analyzed using a general linear model with the four main factors (myoglobin, nitrite, fish oil, clove extract) plus first order interactions between variables. Storage groups were used as a random factor, significant factors ($P < 0.05$) were identified, and least squares means were calculated using Minitab version 16 in both systems.

Paper IV: Minitab version 16 was used for one way ANOVA, univariate regression analysis, and stepwise regression. One-way ANOVA was used for sensory, texture, color and TBARS measurements; regression analysis was used to relate mineral analysis to sensory analysis and stepwise regression was used with sodium as a fixed variable in each regression model.

4 Results and Discussion

Paper I

The hydroperoxide formation ability of five groups of lean meat (beef, lamb, pork and two types of chicken meat) was measured. The FOX method used for hydroperoxide determination was adapted from Gay and Gebicki (2002) with some modifications. The assay was adapted to 2 ml Eppendorf tubes and designed to make it possible to measure the total peroxide in meat in one system (Miyazawa, Yasuda, Fujimoto, & Kaneda, 1988; Schmedes & Hølmer, 1989). Total peroxides include three extracted type of peroxides; polar peroxides (polar phase), protein-bound peroxides (protein-interphase in assay tube) and unpolar peroxides (unpolar phase). The polar phase contained lipid degradation compounds as well as most of the water soluble low weight molecules, while the unpolar phase contained highly unpolar lipid components and some of their compounds derived through oxidation. Protein-internphase included most of the proteins and components that are not soluble in any of the other two phases.

Our values of lipid peroxides were higher than the normally given values, which are between 20 to 40 meqv peroxide /kg oil. This might be due to the fact that different types of peroxides are produced during the oxidation procedure and the results are highly sensitive to different laboratory details and analytical methods (Bou, Codony, Tres, Decker, & Guardiola, 2008). The polar peroxides values were lowest, because the dry matter content of water-methanol phase was low. The amount of protein-peroxides/kg protein depended on the type of protein, but the amount of protein-bound peroxides measured in this study was in a comparable range to the previously reported value of 0.44 mmol peroxides/kg ovalbumin when rose bengal was used to generate reactive oxygen species.

Liposomes were added to the meat system to mimic a cell membrane. Meat incubated with liposomes showed higher PV in all three extracted phases when compared to incubation without liposomes. The increase in PV with liposome addition was significant ($P < 0.05$) and independent of the extracted phase. For the protein-bound peroxides, the average PV increase over time was 40% ($P < 0.001$), whereas for lipid hydroperoxides the average increase in PV over time was only 3% ($P < 0.001$) with liposome addition. The protein-bound peroxides

increased the most upon liposomes addition due the interactions between phospholipids and the liposomes (Gay & Gebicki, 2003).

The reduction in protein-bound peroxides with multi-washings was 8% for systems with liposomes and 3.5 % for systems without liposomes, respectively. The effect of multiple washings might be due to the presence of peroxides in the liposomes that were removed along with other components.

All three types of peroxides were transient, having a maximum value at 2-4 h from being subjected to oxygen. The PV in beef and chicken meat increased rapidly during the first 2 h of incubation at 37°C. Thereafter, the PV declined more than 90% in all the three extracted phases. Beef showed 1 to 1.5 folds higher PV than chicken for all three extracted phases, with or without liposomes. It should also be noticed that with extended incubation time, the protein became more difficult to resolubilize in agreement with the fact that protein crosslinking becomes likely when the peroxides decline (Gay & Gebicki, 2002).

The decrease in peroxides with pH was almost linear for both beef and chicken. In all extracted phases, incubated with or without liposomes, beef gave 1 to 2 folds higher PV than chicken. All the meat samples incubated with liposomes showed 1.25 to 2 folds higher PV than the samples without liposomes. The protein-bound peroxides depended most on pH, while the polar peroxides were the least pH dependent. Many factors are expected to provide more polar peroxides, *e.g.* kinetics of formation, stability and hemin catalyed peroxidation (Gay & Gebicki, 2002; Reeder & Wilson, 2001). This was not observed and may be due to the fact that PV had started to decline before 2 h had passed at the lower pH values.

The hemin content is a significant predictor of peroxide formation. The hemin content of the meat groups was ranked as: beef > lamb > pork > chicken-SO group = chicken-LO group. The PUFA content (g/100g meat) was ranked as: chicken-LO > pork > chicken-SO = lamb > beef. For long chain PUFAs: chicken-LO group > chicken-SO group > lamb > beef = pork. Hemin level was correlated to the amount of many unsaturated fatty acids, but it was difficult to identify the importance of specific fatty acids for hydroperoxide formation. In this study, hemin level

alone would explain about 60% of the variation in PV in these five meat groups. By including information about the variation in fatty acid composition, around 70% of the variation could be explained. The total peroxides in these five groups were ranked as: beef > pork > lamb > chicken-LO group = chicken-SO group. The hydroperoxide distribution varied from 13.9% to 22.3% in the polar peroxide, from 38.5% to 41.5% in the protein-bound peroxide, and from 39.2% to 45.6% in the unpolar peroxides among these five groups. Among these three peroxides, polar peroxides were the lowest, while unpolar peroxide were the highest ($P < 0.001$).

This FOX method as used here is different from the other used methods for peroxides meaurement, as this method can measure total peroxides as opposed to only one type of peroxide. The method was also used in **Paper II and III** to elucidate the effect of different additives on the peroxide formation potential of different meat products.

Paper II

Krebs cycle substrates (KCS) can stabilize the color of packaged meat products by reducing oxygen. Whether this oxygen reduction also releases reactive oxygen species (ROC) and leads to lipid oxidation, was investigated for two different storage conditions: a high-oxygen atmosphere for 13 days (75% oxygen and 25% CO_2) and a low-oxygen modified atmosphere packaging (MAP) for 8 days (60% CO_2 and 40% N_2).

Two optimal KCS combinations for color stabilization were studied: 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 storage condition (Slinde et al., 2012). In MAP, deoxymyoglobin (DMb) quickly became stable and the dominant state ~1.0 for the complete experimental period of 13 days. KCS (succinate-glutamate) also led to lower 2-octen-1-ol and slightly increased PV and TBARS. KCS (glutamate, citrate and malate) kept oxymyoglobin (OMb) higher and it remained high for a longer period in high oxygen packaging, and water gave OMb equal to 0.5 after 8 days of chill storage (Slinde et al., 2012).

However, maintaining OMb was generally in conflict with maintaining low TBARS after 8 days. Citrate, glutamate and malate combination (molar ratio 1:3) gave maximum hexanal formation but not for 2-octen-1-ol. Maintenance of OMb cannot take place without the production of ROS and thereby lipid degradation. Despite the fact that the KCS addition increased total peroxide formation, the TBARS increment during storage was small and may not have any contribution to sensory profile.

The type of fat tissue explained the largest (20−40%) cause of variation in the lipid derived volatile compounds on the final storage day (13 days in MAP; 8 days in high oxygen). Pork fat tissue with beef meat from either younger or older cattle gave the highest amounts of hexanal and 2-octen-1-ol. Hexanal is a marker of n-6 fatty acids degradation and 2-octen-1-ol is often as a marker for degradation of arachidonic acid (Volden et al., 2011). A higher percentage of PUFA (16.2% in pork fat; 1.2% in beef fat) therefore increases the prevalence of volatile lipid components. To preserve DMb in MAP, a 50:50 mixture (molar ratio) of succinate: glutamate/malate may be the most efficient (Slinde et al., 2012). This KCS combination provided the highest hexanal value, but below 1 mg/kg. Regarding 2-octen-1-ol, it also seemed that adding a 50:50 mixture of succinate: glutamate/malate would be acceptable with respect to lipid oxidation. In high oxygen, the effect of KCS addition was significant on day 8. Most samples containing KCS had lower values for the volatile 2-octen-1-ol than the samples containing only water. However, hexanal was higher with KCS addition. There was also a significant O_2 reduction in headspace on the third day of chill storage in response to KCS addition ($P = 0.03$).

With KCS addition, meat in MAP seemed to obtain increased peroxide formation potential (PFP). This is due to the fact that succinate and glutamate, possibly true α-ketoglutarate, were oxidized and produced more peroxides. The small increase in PFP upon addition of KCS appears irrelevant to health issues. The addition of KCS also increased ROS formation in high oxygen packaging as assessed from hexanal production. Some of the ROS formation is actually exhausted during the storage in high oxygen packaging. This is in agreement with the formation of volatiles and lipid breakdown products were more abundant in high oxygen packaging.

Succinate and glutamate were metabolized in MAP until the oxygen was removed. This happens 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 mol/kg 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 color. Malate seemed to be used in MAP but there is no indication of it affecting color stability or lipid degradation.

In high oxygen, citrate did not become metabolized and it minimized lipid degradation. However, if the OMb state is desired, glutamate is clearly metabolized. It cannot be concluded how much glutamate is needed as OMb was only measured on the surface. The need for glutamate may be localized; high need (> 0.1 mol/kg mince) in the surface, but not needed in inner layers. When citrate and glutamate were added, malate was not metabolized but produced. It is possible that the oxidation of succinate with the glutamate created more malate.

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). In principle, the combination of glutamate and succinate may be detected since these components have characteristic flavors, and food regulations also specify upper limits for use. The amount of citrate, a *quantum satis* additive, appears limited only by taste, therefore an upper addition taste limit needs to be defined. Concerned consumers challenge glutamate addition and it should be verified if simply adding high glutamate concentrations to the surface could be a better alternative. Thus, KCS would be possible to use to stabilize color without excessive lipid oxidation.

Paper III

In order to identify how different additives influenced lipid peroxidation formation, two sausage systems were studied. One system used beef juice as a pigment source (beef-juice pork sausage) and the other was a standard beef-pork meat sausage where most of the pigment came from beef

meat. Three storage groups were used 1) frozen immediately at -80°C; 2) chilled stored for 2.5 weeks followed by fluorescent light illumination at 4°C for another two weeks; 3) frozen at -20°C for 5 months. The effects of different additives, including fish oil, myoglobin, nitrite, clove extract and calcium source (as $CaCO_3$) on oxidation and sensory properties were examined.

The beef-juice pork sausages (Exp I) had an average total peroxide value of 1.5 mmol/kg for the three storage groups, while the beef-pork meat sausages (Exp II) showed a higher average total peroxide level of 1.9 mmol/kg. The frozen group showed the highest TBARS among the three storage groups in both Exp I and Exp II. The frozen group had three-fold and two-fold higher TBARS than the fresh group in Exp I and Exp II, respectively. Compared to PV, TBARS were less affected by storage than by the additives.

In Exp I, polar and protein-bound peroxides increased significantly with myoglobin addition, while a reduction was observed with clove extract addition. Fish oil addition increased unpolar peroxides while $CaCO_3$ addition decreased unpolar peroxides ($P = 0.002$). Fish oil was the most important source for increasing PV among the design variables in these sausages. Since unpolar peroxides were the dominant peroxides (51% of total), the total peroxides showed a response to additives that resembled the response of unpolar peroxides. In Exp II, polar and unpolar peroxides were significantly increased in response to myoglobin addition. The clove extract significantly reduced all types of peroxides. Calcium was not a design variable in Exp II, but the addition of the dried skimmed milk gave higher amounts of unpolar peroxides than the use of fresh skimmed milk. In Exp I, the addition of fish oil increased TBARS significantly, while the addition of $CaCO_3$ was observed to significantly decrease TBARS ($P=0.005$).

In Exp I, the addition of clove extract resulted in a perceived increase in clove flavor ($P < 0.001$), decrement of rancidity (not significant) and increment of color intensity ($P = 0.002$). Fish oil showed a nominal increment in rancidity (not significant), decrement in meaty flavor ($P = 0.02$) and color intensity ($P = 0.002$). The addition of meat juice nominally decreased the clove flavor (not significant), nominally increased rancidity (not significant), meaty taste ($P = 0.03$) and color intensity ($P < 0.01$). $CaCO_3$ addition did not show any significant effect on sensory properties.

In Exp II, clove extract significantly increased clove flavor ($P < 0.001$), nominally decreased rancidity and meaty flavor (not siginificant). Fish oil was found to nominally decrease the clove flavor (not significant), increase rancidity ($P = 0.04$), decrease meaty flavor and color intensity ($P = 0.03$). Myoglobin resulted in an increment in clove flavor ($P = 0.02$), rancidity (not significant), and color intensity ($P < 0.001$). Fresh skimmed milk was found to decrease clove flavor (not significant). Dry skimmed milk seemed to increase rancidity (not significant), meaty flavor ($P = 0.001$), and color intensity ($P = 0.001$).

Fish oil addition made sausages richer in EPA and DHA and led to peroxides and TBARS in the sausages that are presumed stable, at least for peroxides, in the digestive system of humans. Exp I and Exp II did not reveal the same peroxide pattern but both experiments identified beef meat juice/beef meat as pro-oxidants that increased the amount of peroxides. This is in agreement with previous investigations (G. Yi, Haug, Nyquist, & Egelandsdal, 2013). However, the diversity in TBARS and polar/unpolar peroxides was much larger for the Exp I sausages than for Exp II sausages. The high background PV and TBARS values may actually indicate that the pork fat itself was also a significant source of peroxides.

Significant relationships between the reduction of TBARS and different peroxides, and the presence of the clove extract, were observed in the present study. Clove extract can significantly inhibit and reduce TBARS and PV induced by myoglobin and fish oil addition. In Exp II, the increase in rancidity with these pro-oxidants was the same as the reduction in rancidity provided by adding the clove extract in Exp II. This indicated a strong anti-rancidity effect of clove extract. The clove extract decreased rancidity scores by 20% and 13% in Exp I and Exp II, respectively. It is also generally assumed that the polyphenols in the clove extract are poorly absorbed in the digestive system and enter the colon where they may act as antioxidants against reactive oxygen species (Opara & Chohan, 2014). As the clove extract used was perceived in the sensory test, it will depend on the type of sausage and the desired flavor of the product, combined with consumer preferences to finally determine whether clove extract can be used as an antioxidant in sausages.

Calcium addition to sausages has been implicated in inhibiting colon cancer (Santarelli et al., 2013). The mechanism is suggested to be the precipitation of hemin in the upper part of the

digestive tract (Mackenzie, Iwasaki, & Y., 2008). Another explanation could be the peroxide-reducing effect of calcium since calcium has been found to reduce the amount of unpolar lipid peroxides. This is also in agreement with previous studies (Brookes, Yoon, Robotham, Anders, & Sheu, 2004). There may also be other mechanisms that relate to calcium's effect on the absorbance of other components in the gut. Fresh milk addition would be a good alternative regarding peroxides, when prioritizing the health aspect. Milk calcium addition to sausage is also positive as calcium absorption is enhanced by a n-3 PUFA-rich diet, which is also good for health (Owen et al., 2003). Regarding calcium, this paper studied most clearly the effect of $CaCO_3$. The last manuscript explores more details regarding calcium added as insoluble milk calciumphosphate, and how different milk minerals affect salt flavor at the same time. Also, the effect of different milk minerals on salt flavor was studied.

Paper IV

In this study, commercial milk minerals were investigated, mainly regarding saltiness, but also regarding lipid oxidation in sausages. These minerals were different regarding potassium and calcium content, but also regarding lactose. Skim milk powder (SKM) (52.5% lactose, 4.6% minerals and 35.5% protein), whey permeate (WP) powder (85% lactose, 7% minerals and 3% non-protein nitrogen), milk mineral A powder (MM-A) (47% lactose, 37 % minerals and 5 % non-protein nitrogen); milk mineral B powder (MM-B) (< 10% lactose, 77.5% total minerals, and < 7% protein) and milk mineral C powder (MM-C) (78% minerals, 10% lactose, 30% potassium, 10% sodium, 3% calcium and 0.3% magnesium) were used and compared in the study. These three powders were used to identify salt taste when the sodium content was kept constant but other cations varied through the addition of dairy by-products and also to study how calcium addition affected the sensory properties.

Meat flavor, salt taste, after-taste and chewing resistance were the most differentiating sensory attributes in the sensory test of sausages. MM-A (potassium rich) and MM-B (calcium rich) resulted in sausages with a higher chewing resistance than the commercial sausage control. On average, the meat flavor nominally increased with increased Na content; sausages with added

MM-A being an exception. Sausages with 1.7% $NaCl_{equivalent}$[1] and added WP (lactose rich) had a higher meat flavor than the sausages with added 1.3% $NaCl_{equivalent}$ and MM-B. Salt taste increased significantly with $NaCl_{equivalent}$ level only for WP and MM-B. The highest saltiness, at 1.3% $NaCl_{equivalent}$, was obtained for MM-A. This suggested that MM-A had a potential to contribute to a higher perceived salt taste than the other dairy additives. Potassium addition was highest when MM-A was used. MM-B was exceptional in providing much higher calcium concentrations to the sausages than the other milk by-products. After-taste was not affected by $NaCl_{equivalent}$ but MM-A sausages had a higher after-taste than the sausages with MM-B at 1.3% $NaCl_{equivalent}$. Sausages with added MM-A also had the highest nominal after-taste, but the after-taste was not significantly higher than the after-taste of the commercial sausage.

Meat flavor was not significantly affected by minerals. Na was the major contributor of salt taste. The combined effect of magnesium and calcium in MM-B was to reduce the perception of salt taste. This may explain why MM-B had a lower salt taste than MM-A at 1.3% $NaCl_{equivalent}$. After-taste was affected positively by sodium level while magnesium had a negative influence both on after-taste and salt taste. This result seemed due to the fact that salt taste in itself correlated with after-taste for these specific dairy powders.

In sensory tests of pure mineral and milk mineral solutions, the salt taste of the pure salt solution was ranked as NaCl > KCl > $CaCl_2$. Bitterness and after-taste were ranked as: $CaCl_2$ > KCl > NaCl. Only for bitterness were the interaction terms NaCl*$CaCl_2$ and KCl*$CaCl_2$ significant. This suggested that Na was the mineral that dominated salt taste. The salt taste of the two mineral salts and the WP were ranked as follows: MM-C (extra K enriched) > MM-B = WP. Bitterness was low at high concentrations of MM-C. However, since the salt taste was higher for the MM-A sausage than the commercial sausage, the use of pure NaCl could be further reduced and replaced with MM-A or MM-C. A relevant target would be to reduce the Na: K ratio to 1.5-2.0. Bitterness and after-taste showed no increase when milk minerals were used to increase Ca and K. This is due to the fact that milk calcium is sparely soluble and thereby tasteless. Milk potassium is soluble, but its bitterness is lower than soluble calcium and provided an insignificant contribution to bitterness in this study. This is also why a 25% Na substitution with

[1] $NaCl_{equivalent}$ was used to indicate that the anions to Na^+ were not exclusively Cl^-

dairy K may be possible to exceed. Finally, the addition of WP and MM-B and MM-C to a solution containing 1.3% NaCl increased saltiness, but the sensory panelists could not differentiate significantly between the different milk ingredients added. Similar observations were made for bitterness and after-taste. It should be noted that the after-taste increased when milk ingredients were added compared to a pure NaCl solution and this may actually be the upper limitations for their additions. When lactose increases in these dairy powders, K and Ca will decrease and their suitability as salt replacers is reduced.

TBARS increased after chill storage for 6 weeks in all the systems. There were significant differences between the dairy additives: WP resulted in sausages with higher TBARS while sausages with MM-B had nominally the lowest TBARS value. Only the K-rich powder significantly influenced TBARS. Zhang et al (2014) suggested that K, when replaced for Na, increases lipid oxidation, and actually suggested limiting substitution of Na for K to 30% to reduce lipid oxidation.

Sausages produced by adding only NaCl to get 1.7 NaCl /100g sausage will have a Na: K ratio equal to 36. When MM-A was used, the ratio dropped to 1.87, because MM-A is a high potassium product. This ratio is a significant improvement relative to 36. Yang et al (2011) examined the health impact of a Na: K ratio between 0.46-2.98 and their data suggest that a ratio of < 1.5 would be healthier. Further studies should verify how low a Na: K ratio would be acceptable using milk ingredients rich in K. MM-B gave sausages a Ca: Mg ratio of 10.8. A Ca: Mg ratio higher than 5:1 or 4:1 is regarded as an imbalanced ratio with the risk of impaired bone health and increased heart diseases (Hruby et al., 2014). The MM-B addition gave an undesirable Ca: Mg ratio. In order to improve this imbalance, magnesium content could be increased in milk minerals as both magnesium and calcium levels are a necessary part of the diet.

The recommended daily intake of calcium in the EU is 0.8 g Ca /day. An amount of Ca of 0.6 g /100 g sausage allows the sausage to be declared as being a good source of Ca. A particular benefit of adding the MM-B to sausages is that calcium addition is regarded as particular, because it is claimed that calcium addition will reduce colon cancer risk (Santarelli et al., 2013). Ca has also been implicated in reducing the formation of reactive oxygen species (G. Yi et al.,

2015). MM-B was also associated with the highest phosphate level and phosphate is an element in abundance in the human diet. An adult needs 0.7 g phosphorous/ day (Adatorwovor, Roggenkamp, & Anderson, 2015) and a sausage with MM-B would be a good source of phosphorous. However, more important than the amount of P, is the ratio of Ca: P that should be 1:1 to 2:1 in the total diet.

5 Conclusions and implications of investigations performed

Lipid peroxidation is an important source of deterioration in fresh meat and processed meat products. Iron can directly and/or indirectly catalyze the initiation of lipid peroxidations. However, which type of iron is involved in lipid peroxidation in meat products and how they initiate the lipid peroxidation are still discussed, although hemin is regarded as most powerful pro-oxidant. There are many factors involved in both pre-and post-slaughtering that lipid peroxidation of meat.

In the five goups of fresh lean meat, the modified FOX used in this study ranked the total peroxide contents in lean meat as follows: beef > pork > lamb > chicken groups. The lipid peroxide variations could largely be explained by the hemin level and the variation in fatty acid composition, while the protein-bound peroxides were less explained by the hemin concentration.

In processed minced meat with specific KCS combinations, the optimal KCS combinations (succinate and glutamate in MAP and glutamate, citrate and malate in high oxygen) used for color stabilization can increase lipid degradation when oxygen was removed in the package. Yet, the increase was presumed too small to affect sensory properties and health issue.

In sausages with different additives, PUFA and hemin were major causes of hydroperoxide formation, but the peroxide formation could be counteracted by the addition of a suitable antioxidant, like clove extract. However, the clove extract addition affected both flavor and sausage color. Calcium addition (milk Ca as in skimmed milk/milk powder) have both a nutraceutical and color and flavor impacting potential, and is an alternative to $CaCO_3$. Calcium has been assumed to reduce the formation of reactive oxygen species and is regarded as particularly relevant to reducing the risk for developing colon cancer.

A significant advantage of using milk minerals in sausages is that the Na:K ratio can be reduced from an unhealthy (in this study 36) to a far healthier ratio (~2) with limited or no effects on taste. High additions of milk calcium (6g/kg) added as milk mineral had no influence on detected bitterness or after-taste as typically observed for $CaCl_2$ additions. Ca additions to sausages are presumed to have an advantage with respect to gut health.

6 Present and future perspectives regarding healthier, processed meat products

Healthier processed meat products may be a promising approach to promoting health conditions and preventing the risk of many diseases. More antioxidants, n-3 fatty acids, and minerals can be added in animal diets to improve animal production and fresh meat quality. In addition, some functional ingredients, *e.g.* dietary fibers, herbs, spices, vegetable proteins, can also be added to meat products during processing and modify their functional and nutritional values.

Oxidative degradation of lipids and proteins leads to quality deterioration of meats, and may have harmful effects on health. A very promising way to overcome this is to use antioxidants for nutrition and food quality preservation purposes. The demand for natural antioxidants has been increased due to the adverse effects of synthetic antioxidants. The most difficult challenge is to assess antioxidant capacity and efficacy by using a rapid and accurate method.
On the other hand, calcium addition to processed meat products is recommended. How calcium affects the inhibition of lipid peroxidation needs further investigation.

However, production of healthier processed meat products may be challenging since they should taste good, be convenient, have a reasonable price and be accepted by consumers. Meat and meat products have great potential for supplying more nutrients, such as more dietary fibers, antioxidants, calcium, lower salt and n-3 fatty acids into the diet. Although successful meat products with all these ingredients require technologies to increase stability and decrease the impact on flavor, the production of healthier meat products will become a reality and these products will be a competitive advantage over traditional meat products. The present negative focus on processed meat products is essentially directed towards the use of nitrite, high amounts of total fat and saturated fat and lack of fiber. The debate has been long-lasting when it comes to nitrite and total amount / high amount of saturated fat. In Norway, the nitrite content in sausages was reduced to 30 mg/ kg in the late 60s and low-fat sausages entered the market in the 80s, but low-fat sausages still make up only 10% of the sausage segment. This points to the challenges of making changes when there is an effect on sensory properties, as is the case for fat reduction.

Fiber addition has so far largely been a tool to reduce fat level and not been a target in itself. However, this is presently changing and so is targeting the high n6:n3 ratio in sausages.

There is at present limited debate about targeting other imbalances in sausages and in particular imbalances that are linked to the present hypotheses dealing with colon cancer. This has been the focus of the present work, namely to control/reduce the peroxidation potential of processed meat and to improve on the mineral level and ratio of important minerals. The future will reveal to what extent these variables need to be targeted alongside with fat and nitrite. Also, there is a need for experimental evidence for health improvements when the compositional changes are made. Such evidence could come from efficient animal models.

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Paper I

Food Chemistry 141 (2013) 2656–2665

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Hydroperoxide formation in different lean meats

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ARTICLE INFO

Article history:
Received 28 November 2012
Received in revised form 4 April 2013
Accepted 13 May 2013
Available online 20 May 2013

Keywords:
Lean meat
Hydroperoxide
Protein-bound peroxide
Hemin
Fatty acid

ABSTRACT

Peroxide is one of the compounds that are indicated to be toxic in the human digestion system. Lean fresh meat samples were collected from beef, lamb, pork and chicken to investigate their hydroperoxide formation potential. Total peroxides of fresh comminuted raw meat were determined by analysing protein-bound peroxides and hydroperoxide compounds in water–methanol and chloroform extracted phases. The amount of total peroxides was ranked as: beef > pork > lamb > chicken. Hydroperoxide formation was examined at different pH values and at different incubation times, using beef and chicken samples. All peroxides were transient, with a maximum value after 2–4 h of incubation at 37 °C. When pH fell from 7 to 1.5, the different peroxides fell by 10–20%. Non-polar peroxide formation could largely (70%) be described by variation in fatty acid composition and hemin content of the meat, while protein-bound peroxide variation was less explained by these variables. Liposome addition increased (40%) the amount of protein-bound peroxides.

1. Introduction

Meat consumption from some land-based animals has come under attack due to unclear status regarding many diseases. Colon cancer is among these diseases, and it is one of the major causes of death in western countries (Sesink, Termont, Kleibeuker, & Van der Meer, 1999). It has been recognised that many genetic factors are involved as determinants of colorectal cancer (Fearon & Jones, 1992), but environmental factors have appeared to contribute to the incidences of colon cancer (MacLennan, 1997). The World Cancer Research Fund panel has judged that the evidence of red meat and processed meat being a cause of colon cancer is convincing (WCRF, 2007), and a western style diet with a high red meat consumption is suggested as a risk factor for colon cancer (Sesink et al., 1999). Increased consumption of meat can be due to improved efficiency in agriculture, which has then created sufficient amounts of relatively cheap meat products. Animal breeding has so far given most priority to rapid animal growth and cost-effective feeds. But meat should also have a good oxidative and microbial shelf life. Sufficient oxidative stabilization is paramount for meat flavour. A present understatement is that oxidised food can be consumed as long as the microbiology and sensory quality are acceptable to consumers. Compounds that could increase the genetic instability of colon cells and the appearance of cancer have received much attention (Ferguson, 2010). Lipid or lipid-derived peroxides are a major source of dietary pro-oxidants speculated to be of toxicological importance (Halliwell & Chirico, 1993).

An *in vitro* study on intake of fat and derived peroxides has identified this as one of many important factors in colon cancer (Angeli et al. 2011). Lipid peroxides are set with an acceptable upper level of 5–10 mmol/kg in oil or fat (Sattar & Deman, 1976). Peroxide limits are normally not defined for products other than oil/fats. However, it is more common to eat larger amounts of lean meat than of pure oil/fats in a meal. Heated turkey meat has been reported to have 1 mmol of lipid hydroperoxide/kg wet weight (Kuffa, Priesbe, Krueger, Reed, & Richards, 2009). This suggests a high peroxide value in the endogenous lipids (~100 mmol/kg lipid). In addition, proteins may also carry peroxides equal to 3–22 mmol/kg of protein (Salminen and Heinonen, 2008). Proteins damaged by free radicals in the presence of oxygen can yield relatively long-lived protein peroxides (Davies, Fu, & Dean, 1995; Gebicki & Gebicki, 1993), which have been shown to readily degrade to free radicals upon reaction with iron (II) complex. It is therefore necessary to include them in an assay for hydroperoxide measurements, in particularly in lean meat where the lipid content is low relative to the protein content.

With sufficient amounts of efficient antioxidants, meat should be a homoeostatic system which remains reduced or without oxidised compounds and reactive components. The aim of this study was: (1) to set up a new model system for measuring total hydroperoxide values of lean meat and the reactivity of lean meat towards liposomes, (2) to discover if the lipid peroxides were always dominant over the protein-bound peroxides, (3) to investigate whether the peroxides were stable when incubated

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http://dx.doi.org/10.1016/j.foodchem.2013.05.041

over time and at different pH values, (4) to establish the hydroperoxide formation ability in some Norwegian regular diet meats.

2. Material and methods

2.1. Meat samples

Chicken muscles (*Musculus pectoralis major*) were collected on the day of slaughter from a hot boning line, vacuum-packed and frozen at −80 °C. The chicken-SO group was chicken fed with a wheat-based diet containing 4% soybean oil and 0.003% selenium-enriched yeast (Ultra Bio-logics., Inc., O.S.Y. 2000× containing 2.15 g Se/kg), whereas the chicken-LO group was fed with a wheat-based diet with 2.4% linseed oil, 1.6% rapeseed oil, and 0.04% selenium yeast. Beef muscles (*Musculus semimembranosus*) were obtained on the day of slaughter from a hot boning line, vacuum-packed and frozen at −40 °C until they could be brought to −80 °C (after 5 days). Pork muscles (*Musculus gluteus medius*) were collected 1 day after slaughter from the cold boning line, vacuum-packed, and frozen at −80 °C. The pig group was homogeneous, as all pigs were of the crossbreed Noroc that was produced to give higher intramuscular fat content than the regular Norwegian Landrace/Yorkshire crossbreed. All the pig samples were from the same farm. Lamb muscles (*Musculus psoas major*) were obtained 1 day after slaughter from a cold boning line, vacuum-packed, frozen at −40 °C until they could be brought to −80 °C (after 5 days). Each group contained 10 animals. These beef (*M. semimembranosus)*, pork (*M. gluteus medius*) and lamb (*M. psoas major*) muscles were randomly chosen from different Norwegian feeding farms from a local meat supplier (Nortura SA, Lillehammer, Norway).

2.2. Chemicals

L-α-Phosphatidylcholine 95% (egg, chicken) powder was purchased from Avanti Polar Lipids, Inc., (Alabaster, USA). Water was purified by a purification system (Millipore, Sydney, Australia). Chloroform (AR grad), sulphuric acid, methanol, acetone, iron (II) sulphate, hexane and Ringer's solution tablets were from Merck (Darmstadt, Germany). Guanidine hydrochloride, hydrochloric acid (37%), streptomycin and C13:0 internal standard were supplied by Sigma–Aldrich Chemical (Sydney, Australia). Butylated hydroxytoluene, xylenol orange sodium salt and triphenylphosphine (99% in purify) were purchased from Alfa Aesar (Lancashire, UK). Sorbitol and hemin were bought from Sigma–Aldrich (St. Louis, USA). Sodium dithionite and KOH were purchased from VWR Inc., (Oslo, Norway). All the other chemicals were of analytical grade as supplied.

2.3. Generation of liposomes

L-α-Phosphatidylcholine 95% (egg, chicken) powder (1 g) was first dissolved and mixed in 50 ml of chloroform to assure a homogeneous mixture of lipids. The organic solvent was evaporated to 1 ml by using a rotary evaporator (R215, Buchi Rotavapor, Switzerland). The solution was dried thoroughly by nitrogen gas to a lipid residue at room temperature. Hydration of the dry lipid cake was accomplished by adding 50 ml of Ringer's solution in a 60 °C water bath for 60 min. Liposomes were produced by using an extrusion technique, which yielded a polydisperse suspension of multilamellar liposomes. The mini-extruder was assembled by inserting two internal membrane filters and one polycarbonate membrane filter (0.1 μm pore size, Avanti polar lipids, Inc. Alabama, USA), and then the system was heated to 60 °C before use. One gas-tight syringe (Hamilton, Bonaduz, Switzerland) was loaded with 1 ml of solution and applied to one end of the mini-extruder while the other end of the mini-extruder was supported with an empty gas-tight syringe so that the fluid could be circulated through filters from both sides. This resulted in large, unilamellar liposome vesicles defined by the pore size of the membrane.

The lipid solution was completely transferred between the original and alternative syringes by gently pushing the plunger (1 min each time) 10 times (20 passes through the membranes). A successfully prepared liposome solution had no sediment after storage at 4 °C overnight. Liposome solutions were stored at −80 °C after preparation for later use.

2.4. Hydroperoxide value (PV) measurements by using the ferric–xylenol orange (FOX) method

Meat cuts were trimmed of all visible fat, frozen in liquid nitrogen and homogenised by blender (800 W Home blender, Invite) to meat powder. Hydroperoxide measurements were made on meat, with or without added liposomes. Triplicates of meat samples (0.1 g) were incubated in 1 ml of Ringer's solution and quadruplicate meat samples were incubated in 200 μl of liposomes (4 mg/ml) and 800 μl of Ringer's solution. To all systems, 10 μl of 20 g/l streptomycin was added and the systems were incubated for 2 h in a 37 °C water bath.

The measurements without added liposomes served to identify endogenous ability to produce peroxides, while the other measurement served to verify the potential of the meat samples to induce peroxides in liposomes (as an *in vitro* model for cell membranes). The samples were mixed with 1 ml of chloroform and methanol (2:1, volume-ratio), vortexed and centrifuged at 24,462*g* for 10 min at 4 °C. After centrifugation the system separated into three phases which were 1.33 ml of polar upper phase (25% methanol + 75% Ringer's solution, pH 7), an interphase (the meat protein aggregate) and 0.67 ml of non-polar lower phase (chloroform) containing soluble lipids. Each of the three phases was removed for separate hydroperoxide measurements. Upper phase (700 μl) was removed and the following chemicals were added immediately in this order: 5 μl of 4 mM BHT, 4 μl of 2 M H_2SO_4, 40 μl of H_2SO_4 at pH 1.8, 30 μl of 5 mM XO + 5 M sorbitol mixture at pH 1.8 and 40 μl of 1.67 mM $FeSO_4$ at pH 1.8. A blank containing the upper phase reduced with 10 μl of 1 M sodium dithionite and subjected to an identical protocol was used as a negative control. The protein aggregate at the interphase was washed three times with 2:1 chloroform:methanol before 1.7 ml of 6 M GuHCl were added to resolubilise the protein for optimal hydroperoxide exposure. The protein aggregate did not always solubilise to a transparent solution, but it swelled to an open system that allowed for low molecular weight diffusion (*i.e.* diffusion of the chemicals added). After 30 min of solubilisation, all chemicals were added immediately in this order: 12 μl of 4 mM BHT, 97 μl of H_2SO_4 at pH 1.8, 73 μl of 5 mM XO + 5 M sorbitol mixture at pH 1.8 and 73 μl of 1.67 mM $FeSO_4$ at pH 1.8. A blank containing suspended protein phase reduced with 10 μl of 1 M sodium dithionite and subjected to identical protocol was used as a negative control. Lower phase (50 μl chloroform) was removed and chemicals were added immediately in this order: 200 μl of chloroform, 460 μl of methanol, 5 μl of 4 mM BHT, 12 μl of 2 M H_2SO_4, 26 μl of 10 mM XO at pH 1.8 and 54 μl of 1.67 mM $FeSO_4$ at pH 1.8. A blank containing the lower phase reduced with 10 μl of 1 M triphenylphosphine and subjected to identical protocol was used as a negative control. All the samples were incubated for 60 min in enclosed Eppendorf tubes at room temperature to ensure colour development. The upper phases and the suspended protein interphases were centrifuged at 24,462*g* for a further 10 min at 4 °C to secure transparency before the measurements by the spectrophotometer, while the lower phases were measured spectrophotometrically at 590 nm immediately after the incubation. The initially obtained hydroperoxide

values were calculated by first subtracting the negative control, then the absorbance was divided by the pigments' molar absorptivities of 14,840 (1 cm pathway) and 87,583 (1 cm pathway) for the upper phase/inter phase and the lower phase, respectively, before correcting for dilution. Our procedure is a modification of Gay and Gebicki (2002a), but adapted to meat instead of serum and with reduced volumes to adapt the technique to Eppendorf tubes. The peroxides of the upper, inter and lower phase are hereafter called polar, protein-bound and non-polar peroxides, respectively.

In order to check the effect of pH on hydroperoxide formation in meat, pH values from 1.5 to 7.0 were examined. Ringer's solution was adjusted to the required pH with 2 M H_2SO_4 before incubation. The FOX method is based on oxidation by hydroperoxide under certain acidic conditions (pH 1.8) for a maximum response at room temperature (Bou, Codony, Tres, Decker, & Guardiola, 2008; Gay, Collin, & Gebicki, 1999). Normally when the samples were incubated at pH 7, a final pH 1.8 (pH of maximum absorbance) was obtained when absorbances were read. But when the samples were incubated at pH 5.5, 3.5 and 1.5, the final pH was <pH 1.8, so the absorbances were lower. So we used the absorbance ratios at pH 7 to pH 5.5 (1.0134), pH 7 to pH 3.5 (1.0321) and pH 7 to pH 1.5 (1.124) to correct absorbances below pH 1.8 back to absorbance at pH 1.8.

The ratio of endogeneous meat fatty acids to the liposome fatty acids varied with the amount of fat in the lean meat, but was always less than 1:2 (weight ratio). The initial peroxide value of the liposomes added was less than 0.037 mmol/kg of phospholipids.

2.5. Conjugated compounds (CC)

The amounts of CC in water–methanol and chlorofrom produced during PV measurements were measured. Both the polar and non-polar phases were removed for CC measurements. Polar phase (100 μl) was removed and diluted 10 times by adding 900 μl of 75% methanol and 25% water solution and the non-polar phase was removed (50 μl) and diluted 20 times by adding 950 μl of chloroform. Both phases were measured spectrophotometrically in the UV range (240–340 nm). The obtained absorbances were multiplied by the dilution factor (×10 in polar phases and ×20 in non-polar phases) then divided by the molar absorptivity of conjugated trienes of 36,300 (1 cm pathway) at 268 nm.

2.6. Hemin distributions among extracted phases

In order to check which phase hemin remained in during hydroperoxide analysis, 1 ml of hemin solution (0.31 mg/ml) was blended with 1 ml of 2:1 chloroform:methanol solution. The same procedure was also carried out for extraction of the three phases for hydroperoxide determination. After centrifugation, undissolved hemin particles were found to appear between polar phase and non-polar phase. The polar phase showed an average absorbance of 0.01 at 407 nm. The non-polar phase had its absorbance tested against chloroform as a blank. By using the molar absorbitivity of 36,000 (1 cm pathway) (Uc, Stokes, & Britigan, 2004), an upper limit of 1.8% of the added hemin was identified as presented in the non-polar phase if the initial solution contained 8 g/l of myoglobin. Therefore hemin, in meat homogenates during the PV assay, was distributed mainly to the interphase with the proteins.

2.7. Hemin analysis

The analyses were carried out on meat samples, following the analytical method described by Ginevra et al. (2002) with some optimizations. Meat cuts were trimmed of all visible fat, frozen in lipid nitrogen and homogenised to meat powder. Meat homogenates (0.155 g) were dissolved in 233 μl of distiled Millipore water, 1.55 ml of acetone and 63 μl of concentrated HCl (37%) in capped Eppendorf tubes. The mixture was vortexed vigorously and then centrifuged at 24,462*g* for 10 min at 4 °C. The supernatant was extracted and the absorbance was measured at 407 nm against a reagent blank. Two replicates were measured, myoglobin solutions were used to make a linear standard curve and hemin concentrations were read from the standard curve.

2.8. Fatty acid analysis by gas chromatography (GC)

Meat samples were placed into 16 × 125 mm screw-cap Pyrex culture tubes and 0.8 ml of the C13:0 internal standard, 0.56 ml of 10 N KOH in water, and 4.24 ml of MeOH were added. All tubes were incubated in a 55 °C water bath for 1.5 h with hand-shaking for 5 s every 20 min to properly permeate, dissolve and hydrolyse the samples. The samples were cooled to below room temperature and 0.464 ml of 24 N H_2SO_4 was added. All the tubes were incubated again in a 55 °C water bath for 1.5 h with hand-shaking for 5 s every 20 min; then the tubes were cooled again in a cold water bath and 2.4 ml of *n*-hexane were added to each tube. All the tubes were vortex-mixed for 5 min and centrifuged for 5 min in a table top centrifuge. The hexane layer, containing the fatty acid methyl esters, was transferred into a GC vial, capped and kept at −20 °C prior to GC analysis (O'Fallon, Busboom, Nelson, & Gaskins, 2007).

The fatty acid composition of the meat samples was determined by gas chromatography on a fused capillary column. The oven temperature was 70 °C at the start, held there for 4 min and then increased to 160 °C at a rate of 20 °C/min. Thereafter the temperature was held for a further 15 min, then the temperature was further increased at 3 °C per minute to 230 °C. Helium was used as the carrier gas at a flow rate of 68.4 ml/min at a temperature of 280 °C and the column head pressure was 309.4 kPa. Both the injector and the detector were set at 260 °C. The split ratio was 30:1. The flame ionisation detector temperature was 290 °C with H_2, air and N_2 make-up gas flow rates of 40, 450 and 45 ml/min, respectively. The run time for a single sample was 92 min. C13:0 was added as an internal standard and used to calculate the amounts of fatty acids in muscle (mg/100 g). The fatty acids were identified by comparing their retention times with the fatty acid methyl standards.

2.9. Statistics

Minitab (version 16; Minitab Inc., State College PA, USA) was used for univariate regression analysis (incl. stepwise regression) and one way ANOVA. The unscrambler (version X 10.2 CAMO Software AS, Oslo, Norway) was used for principal component analysis (PCA), as well as partial least square (PLS) regression. Evaluation of the PLS regression model was with full cross-validation.

3. Results

3.1. Time of maximum content of peroxides at pH 7

Beef and chicken meat samples were incubated for different times, with or without liposomes, to examine when the largest amount of peroxides was formed. The peroxides in raw beef and chicken homogenates increased rapidly during the first 2 h of incubation at 37 °C. Thereafter, the amount of peroxides declined by more than 90% in all three extracted phases. Due to the presence of the largest amount of peroxides after 2 h of incubation, this time point was chosen as a standard incubation time for all meat samples. Beef homogenates showed 1- to 1.5-fold higher amounts of peroxides than did chicken samples for all three extracted phases incubated for 2 h, with or without liposomes (Fig. 1).

3.2. Effect of liposomes addition on hydroperoxides of the three extracted phases at pH 7

Meat homogenates incubated with liposomes showed higher PV in all three extracted phases than did those without liposomes. The increase in PV with liposome addition was significantly ($P < 0.05$) independent of extracted phase. The average increase in polar PV over time, with liposome addition, was 6% ($P < 0.001$, linear regression). For the protein-bound peroxides, the average increase over time was 40% ($P < 0.001$, linear regression) whereas, for lipid hydroperoxides, the average increase in PV over time was only 3% ($P < 0.001$) with liposome addition. Although the PVs of the two systems (with and without liposomes) were correlated, the increased PV with liposome addition of non-polar peroxides

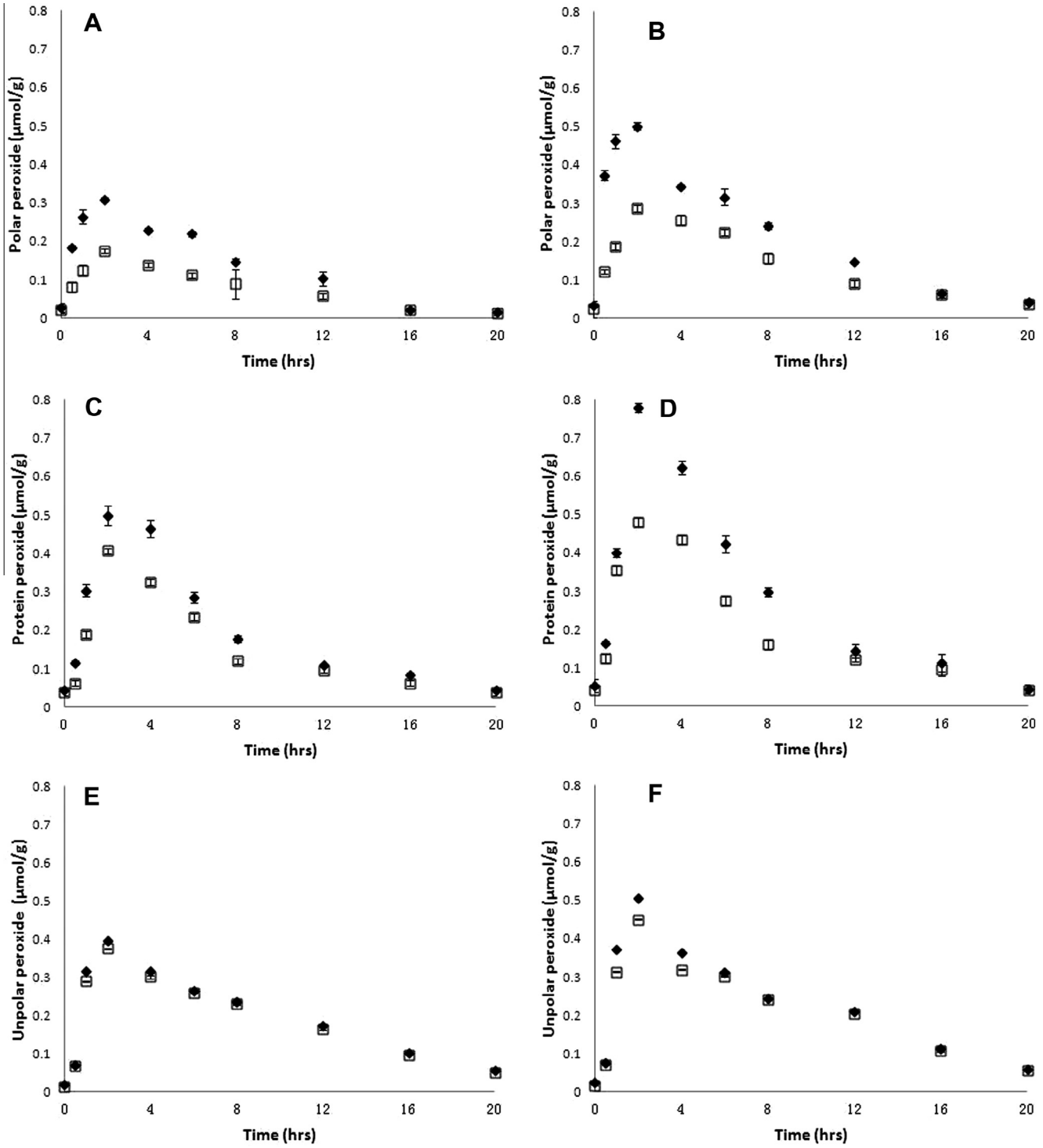

Fig. 1. Peroxides changes of phases over time in raw beef and chicken samples. Closed rhombuses are beef samples and opened squares are chicken samples. (A) Polar peroxide changes over time in polar phase without lipsomes. (B) Polar peroxide changes over time in polar phase with liposomes. (C) Protein-bound peroxide changes over time in interphase without liposomes. (D) Protein-bound peroxide changes over time in interphase with lipsomes. (E) Non-polar peroxide changes over time in non-polar phase without liposomes. (F) Non-polar peroxide changes over time in non-polar phase with lipsomes.

was on average higher (>25%) than at the other incubation time points (Fig. 1). However, the polar peroxides increased the most (~30%, at average) with liposomes addition after 2–4 h. Addition of liposomes gave higher hydroperoxide values when added up to 12 h of incubation.

3.3. Effect of pH on hydroperoxide formation

Both beef and chicken homogenates were incubated for 2 h at pH 1.5, 3.5, 5.5 and 7, with or without liposomes, at 37 °C. Samples that were incubated at lowest pH had the lowest amount of peroxides for

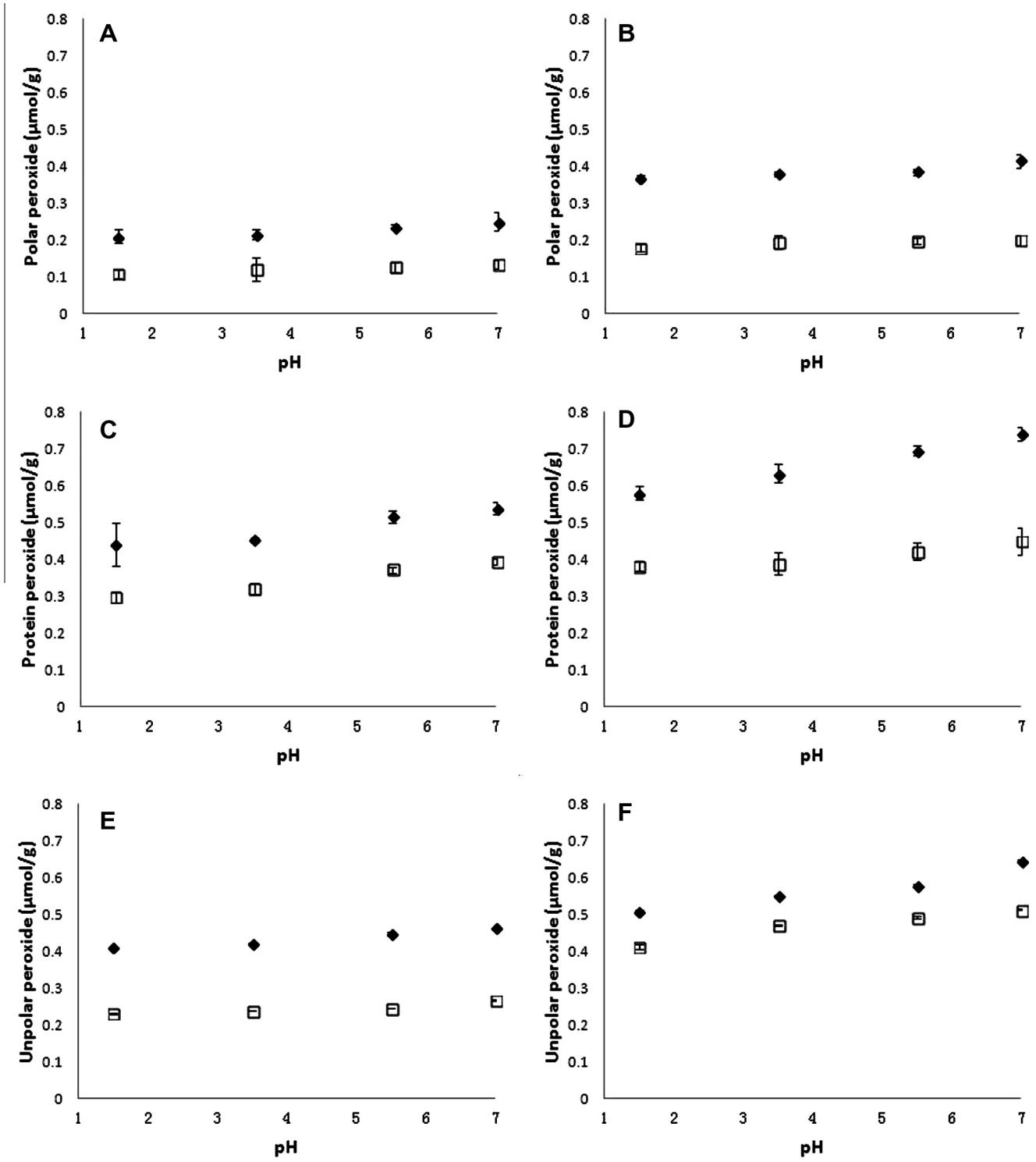

Fig. 2. Peroxide changes among phases at different pH in raw beef and chicken samples. Closed rhombuses are beef samples and open squares are chicken samples. (A) Polar peroxide changes at different pH in polar phase without liposomes. (B) Polar peroxide changes at different pH in polar phase with liposomes. (C) Protein-bound peroxide changes at different pH of interphase without liposomes. (D) Protein-bound peroxide changes at different pH of interphase with liposomes. (E) Non-polar peroxide changes at different pH in non-polar phase without liposomes. (F) Non-polar peroxide changes at different pH in non-polar phase with liposomes.

all phases (Fig. 2). The decrease in peroxides with pH was almost linear for both raw beef and chicken homogenates. In all extracted phases, incubated with or without liposomes, beef homogenates showed 1- to 2-fold higher hydroperoxide value than did chicken homogenates. All the meat homogenates samples incubated with liposomes showed 1.25- to 2-fold higher hydroperoxide values than did the extracted phases without liposomes. As reported previously, the addition of liposomes increased the amount of polar peroxides and protein-bound peroxides more than non-polar peroxides. The protein-bound peroxides depended most on pH, while the polar peroxides were the least pH-dependent.

3.4. Effect of washing times on the amount of protein-bound hydroperoxide

Washing of the protein interphase reduced the peroxide values. The reduction of peroxides by increasing washings in the system without liposomes was larger than the system with addition of liposomes. It should be noted that the reduction in protein-bound peroxides with 6 washings was 8% for systems with liposomes and 3.5% for systems without liposomes (Fig. 3).

3.5. Total peroxides in different species

The total amount of peroxides in meat was ranked as follows: beef > pork > lamb > chicken-LO group = chicken-SO group (Fig. 4). The peroxide values of the three extracted phases were correlated. This relationship (data from all species included) was stronger for the polar and protein-bound peroxides than for the non-polar peroxides. The hydroperoxide distribution varied from 13.9% to 22.3% in the polar phase, from 38.5% to 41.5% in the protein interphase and from 39.2% to 45.6% in the non-polar phase, using data from all five animal groups without liposomes. The hydroperoxide distribution varied between 17.3% and 22.6% in the polar phase, between 36.4% and 44.4% in the protein interphase and between 35.4% and 45.5% in the non-polar phase in all five animal groups with liposomes. Polar peroxides were the lowest while the non-polar peroxides were the highest ($P < 0.001$).

The total hydroperoxide contents in the pork, lamb and beef muscles were 1.4- to 1.8-fold and 1.2- to 1.9-fold higher (with liposomes) than the average total amount of hydroperoxide in chicken muscles. Since the weight-ratio of protein to lipid was approximately 1.5:20, this suggested that the amount of peroxides

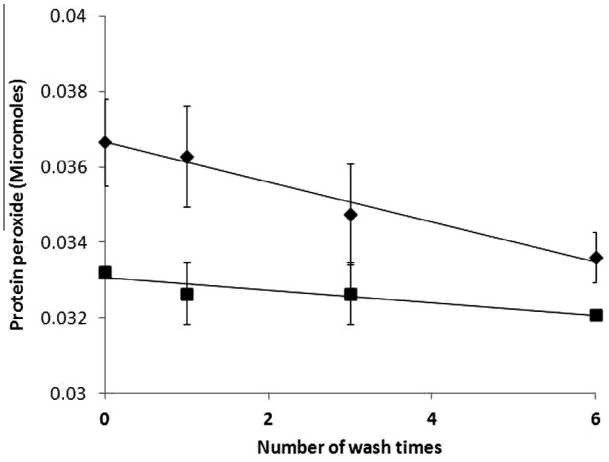

Fig. 3. Effect of multiple washings (x) on the protein-bound hydroperoxide values (y) without liposomes; rhombuses are beef samples ($y = -0.005x + 0.036$, $R^2 = 0.9768$) and squares are chicken samples ($y = -0.0002x + 0.0331$, $R^2 = 0.8571$). Note that the y-axis is not starting at 0.

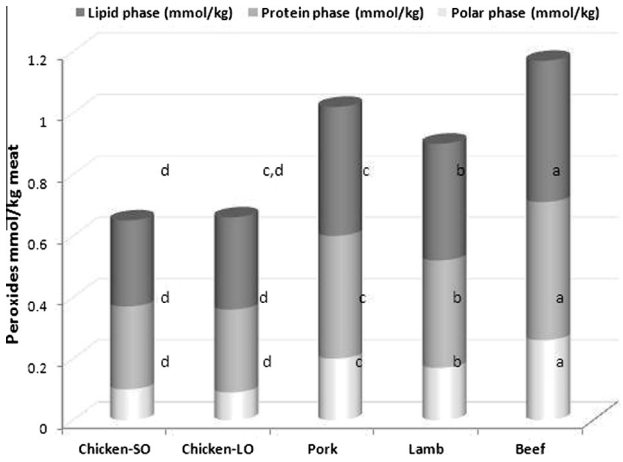

Fig. 4. Total amount of peroxides (mmol/kg meat) without liposomes in different animal species and their distributions among extracted phases. Peroxides from different extracted phases with different letters, across species, were significantly ($P < 0.05$) different. The statistics were only between species.

would be 10- to 15-fold higher per kg of lipid than per kg of protein. As the fat content, on average, was 1 mmol/kg (10 g/kg), Fig. 4 suggests that the lipid peroxides could be induced to contain 20–40 mmol peroxides/kg of meat lipid.

3.6. Conjugated dienes

Conjugated compound measurements of the polar phase at 268 nm were the only measurements that differed between the two chicken groups (Table 1). There were more conjugated compounds in the chicken-LO group that was fed on the diet that included 2.6% linseed oil, which is a rich source to generate more LC-PUFAs (Cleveland, Francis, & Turchini, 2012; Haug, Nyquist, Mosti, Andersen, & Hoestmark, 2012). There was also a tendency for the same chicken-LO group to give more lipid peroxides ($P = 0.067$).

3.7. Hemin and fat composition of the selected meat

The hemin contents of the muscles were in the following order: beef > lamb > pork > chicken-SO group = chicken-LO group (Table 1). The PUFA contents (g/100 g meat) of the muscles were as follows: chicken-LO > pork > chicken-SO = lamb > beef (Table 1). For long chain PUFAs the order was: chicken-LO group > chicken-SO group > lamb > beef = pork. There were some differences in fat content: pork had the highest amount and chicken-SO group had the lowest amount of fat (Table 1). When liposomes were added before incubation for PV measurements, the endogenous fat varied from 38% (pork samples) to 18% (chicken-SO group samples).

3.8. Relationships between peroxides, hemin and fatty acid compositions

The PCA plot (Fig. 5) was calculated with the amounts of unsaturated fatty acids, the more frequent monounsaturated fatty acids, total amount of fat, conjugated compounds, hemin concentrations and the determined peroxide values. The outlier was a pork sample which had a high content of intramuscular fat and belonged to the heaviest pig of the group.

Total amount of fat was, however, not a robust predictor of peroxides; *i.e.* Fig. 5 would not be different, whether the pork sample with the highest fat content was included in the regression or not. Hemin, conjugated compounds, peroxides and C20:5 *n*-3 plus

Table 1
Fat content, selected fatty acid variables, hemin concentration of lean meat and phospholipids used, plus conjugated trienes of polar (water/methanol) phase of meat.

Species	Fat (g/100 g)	PUFAs (g/100 g fat)	PUFAs (g/100 g meat)*	C18:2*n*-6 (g/100 g meat)	C18:3*n*-3 (g/100 g meat)	LC-PUFAs** (g/100 g meat)	CC 268 nm*** (μmol/g)	Hemin (g/kg meat)
Chicken-SO	0.90 ± 0.15[b]	34.6	0.312 ± 0.047[a,b]	0.189 ± 0.039[a,b]	0.012 ± 0.004[b]	0.110 ± 0.007[a,b]	0.188 ± 0.002[a]	0.04 ± 0.004[d]
Chicken-LO	1.19 ± 0.54[b]	32.4	0.387 ± 0.156[a]	0.176 ± 0.084[b]	0.089 ± 0.070[a]	0.121 ± 0.010[a]	0.191 ± 0.002[b]	0.04 ± 0.004[d]
Pork	2.43 ± 1.10[a]	15.3	0.372 ± 0.116[a]	0.266 ± 0.095[a]	0.014 ± 0.008[a]	0.090 ± 0.014[c]	0.209 ± 0.002[c]	0.07 ± 0.004[c]
Lamb	1.50 ± 0.43[b]	20.8	0.312 ± 0.060[a,b]	0.151 ± 0.038[b,c]	0.043 ± 0.013[a,b]	0.116 ± 0.016[a]	0.211 ± 0.002[d]	0.17 ± 0.004[b]
Beef	1.33 ± 0.66[b]	17.0	0.227 ± 0.032[b]	0.099 ± 0.022[c]	0.029 ± 0.013[b]	0.097 ± 0.015[b,c]	0.237 ± 0.002[e]	0.24 ± 0.004[a]
Egg	95	20.7	19.7	16.3	0	3.4	N.m.*****	N.m.*****
PC****								

* Data in each column with different superscripts are significantly different ($P < 0.05$).
** LC-PUFA is fatty acid with chain length >20 *cis* with two or more double bonds).
*** Conjugated trienes of polar (water/methanol) after 2 h of incubation.
**** Used in liposomes (Avanti polar lipids, Inc., Alabama, USA).
***** N.m. = not measured.

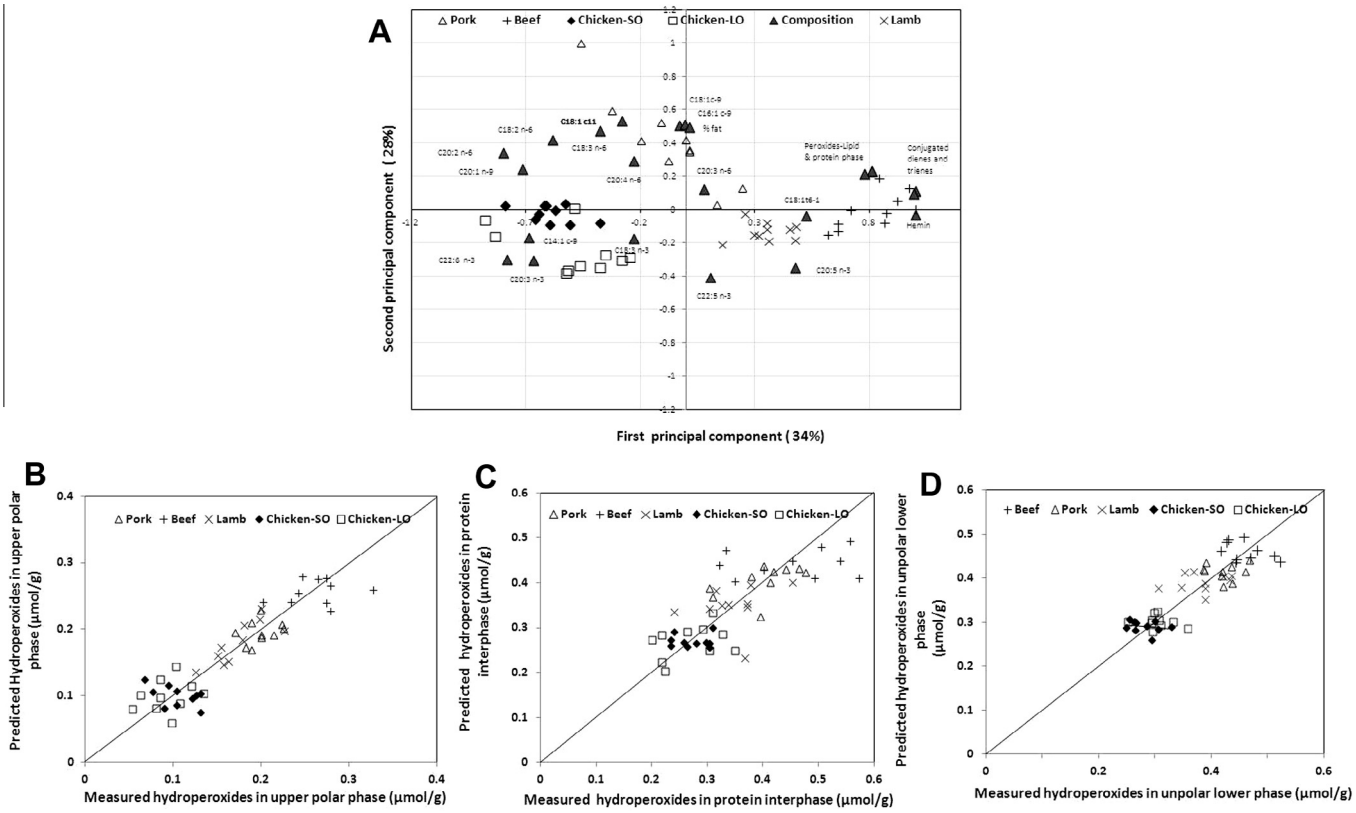

Fig. 5. (A) Principal component (PC) analysis biplot of fatty acids, total fat, hemin, peroxides and conjugated compounds, as well as samples (rhombuses are chicken-SO, opened triangles are pork, plus signs are beef, opened squares are chicken-LO and crosses are lamb species). (B–D) Measured versus predicted values (from the partial least square regression model with six factors) for the peroxides of the different extracted phases: polar peroxides, protein-bound peroxides and non-polar peroxides, respectively. The variables were weighted by 1/standard deviation before carrying out the regression.

C18:1 t6–t11 were the most characteristic components clustering closest to beef meat when the first principal component was plotted against the second principal component (Fig. 5A).

The amount of peroxides was significantly related to hemin level for all extracted phases. For the polar peroxides, 60.7% of the variation in peroxides could be attributed to variation in hemin content. The variation in the protein-bound and lipid peroxides (as opposed to the polar peroxides) depended relatively more on the presence of specific (amounts of) fatty acids.

There were only significant ($P < 0.05$) univariate relationships between induced peroxides (all extracted phases) for a few fatty acids. For example, between the level of C22:6 *n*-3 and the amount of polar peroxides a significant and negative relationship was found. But the level of C22:6 *n*-3 correlated negatively ($P < 0.001$) with hemin level (Fig. 5A, hemin concentration is located opposite to C22:6 *n*-3 concentration) as the species (beef) highest in hemin was also lowest in C22:6 *n*-3. It is possible that C22:6 *n*-3 oxidation is hemin-catalysed, but in order to identify these meat samples with more C22:6, *n*-3 in combination with high hemin levels might be necessary, *i.e.* designed samples, to reduce/eliminate confounding patterns. This was somewhat different for C20:5 *n*-3 due to its higher (up to 0.029 g/100 g of meat) concentration in beef meat (Fig. 5A), as opposed to chicken meat (1/10 of beef value). Thus, the level of C20:5 *n*-3 related significantly and positively ($P > 0.001$) to the hemin level. C20:5 *n*-3 also related significantly to polar peroxides and protein-bound peroxides ($P = 0.013$ and

$P = 0.002$, respectively) while its relation to lipid peroxides in the non-polar phase was on the border of being significant ($P = 0.052$).

Many fatty acids were interrelated, as shown in Fig. 5A, and these made it difficult to identify specific fatty acids as important for peroxide formation in meat using univariate regression methods.

Multivariate regression (partial least square regression) was thus attempted between peroxides and fatty acid composition and hemin (Fig. 5B–D). Polar peroxides correlated with fatty acids and hemin, as indicated by the plotting predicted and measured values of polar peroxides (Fig. 5B; correlation $r = 0.91$). Hemin, C22:6 *n*-3 and C20:3 *n*-6 levels were important predictors of polar hydroperoxide formation. The non-polar peroxides gave similar results but included the fatty acid C20:5 *n*-3 (and C20:1n9) as a predictor of higher hydroperoxide levels (Fig. 5C, $r = 0.87$). The protein-bound peroxides were less well explained ($r = 0.76$) by measured variables but still with hemin as a dominant explanatory variable of peroxide formation. The pork sample had an indicated outlier sample (high in intramuscular fat) that was not removed. Despite the pork meat's limited variation in hemin, this variable (as content) still gave the largest influence on hydroperoxide formation, when studied in a separate pork model. The lamb samples were different from the others and their hemin level was no longer the largest predictor of hydroperoxide levels, and this system alone (10 samples) would not give any significant model to hemin level.

4. Discussion

4.1. FOX hydroperoxide assay adaptations

The FOX method, as set up in this study, can provide a convenient way to measure both lipid and protein-bound peroxides. The method used for hydroperoxide determination was adapted from that of Gay and Gebicki (2002a), with some modifications. The drying (concentration) step for non-polar phase was omitted, as there was no need for it. Also, perchloric acid was replaced with H_2SO_4, due to safety requirements in the laboratory. The assay was adapted to use a 2 ml Eppendorf tube due to the efficiency and convenience during the assay. Effendorf tubes were stable without chemical reactions and did not affect the optical readings in this assay (Ewald, 2010). The assay was designed to make it possible to calculate the total amount of peroxides in meat, as opposed to only the peroxides extracted in one specific solvent (Miyazawa, Yasuda, Fujimoto, & Kaneda, 1988; Schmedes & Hølmer, 1989). Thus, polar peroxides and protein-bound peroxides were included. The assay used in this study relates to the approach described by Volden et al. (2011), where the protein is left as an interphase between extracting solvents.

4.2. Hydroperoxide content of meat

Peroxides can be formed on several amino acid side chains but also on the protein backbone following exposure to reactive oxygen species. Detection of peroxides in a pure protein model system, using the FOX method, has been demonstrated (Gay & Gebicki, 2002a). These authors reported the presence of 0.44 mmol of peroxides/kg of ovalbumin when Rose Bengal was used to generate reactive oxygen species. They also reported that the amount of peroxides/kg of protein depended on the type of protein. There is, to our knowledge, no comparison between the method used by Morgan, Li, Jang, el Sayed, and Chan (1989) and ours regarding the amount of peroxides to be formed on proteins, but the amount of protein-bound peroxides measured here is in a range comparable to their values.

With regard to lipid peroxides, our values were on the high side if compared to the values normally given as 20–40 meqv peroxide/kg of oil (we only had, on average, about 1.5% w/w fat in the samples). But the determination of hydroperoxide is challenging because different types of hydroperoxide can be produced during the oxidation procedure (Bou et al., 2008). Many methods have been carried out to investigate lipid hydroperoxide in biological materials and foods (Dobarganes & Velasco, 2002; Gray & Monahan, 1992; Moore & Roberts, 1998) but the analysis is sensitive to different laboratory details (Bou et al., 2008). Thus our higher non-polar peroxide values could relate to the choice of analytical method. It has been claimed that the more traditional peroxide measurement loses peroxides during the assay (Meisner & Gebicki, 2009). This may explain why our values are relatively high. Regarding polar peroxides, it makes sense that these are the lowest, since the dry matter content of the water–methanol phase will be low.

4.3. The composition of the three extracted phases used for hydroperoxide determination

The polar phase contains degradation products from lipids (Volden et al., 2011) but it should also contain most of the water-soluble low molecular weight compounds (amino acids and peptides) in meat. For example, phenolic antioxidants would be present here. Most proteins will be present in the so-called protein-interphase, but also components that fail to dissolve in any of the other two (polar and non-polar) phases, or have a density that would be intermediate between the densities of the polar and non-polar phases (*i.e.* hemin). Highly non-polar components (lipids), plus components derived through oxidation that are still not soluble in the polar phase, will remain in the non-polar phase.

4.4. Hydroperoxide stability

Transient stability of lipid peroxides has been reported numerous times (Reeder & Wilson, 2001; Takahashia, Shibata, & Niki, 2001). Here we also report that protein-bound peroxides are transient, having a maximum value at 2–4 h from being subjected to oxygen. Since these samples were fresh meat kept at −80 °C under vacuum, we have to consider the sample as being kept anaerobic until incubated with access to oxygen at 37 °C. Addition of extra lipids as liposomes did not affect the transient nature of the peroxides. It should be pointed out that, with extended incubation time, the protein became more difficult to resolubilise, in agreement with the fact that protein crosslinking becomes likely when the peroxides decline (Gay & Gebicki, 2002b). When proteins crosslink, meat becomes tougher and the activities of proteases are reduced. Both processes will be negative for meat quality.

Incubations at lower pH gave consistently lower hydroperoxide values. The effect was largest on the protein-bound peroxides. Both the kinetics of formation and the stability of peroxides may change with lower pH (Gay & Gebicki, 2002b; Reeder & Wilson, 2001). Hemin-catalysed peroxidation is expected to provide more peroxides with lower pH (Gao, Song, Li, & Gao, 2009). The fact that this was not observed here may be due to the fact that the peroxide value had started to decline before 2 h had passed at the lower pH values. The pH effect was also smaller when compared with the effect of incubation time from 1 to 24 h.

4.5. Liposome addition

Addition of liposomes to meat systems is interesting because the liposomes can mimic cell membranes. The fact that protein-bound peroxides increased the most upon liposome addition, may suggest that the added phospholipids interacted with the liposomes. Similar interactions have been reported and ascribed to

electrostatic and hydrophobic interactions (Alipour, Suntres, Halwani, Azghani, & Omri, 2009). It is possible that the effect of multiple washings was partly due to peroxides in the liposomes that were removed, along with other components. However, the effect of washing was nevertheless small and even 10 washes with their removal of peroxides would only explain 1/3 of the increase in protein-bound peroxides upon incubation with liposomes.

4.6. Hydroperoxide formation in different meat species

Five groups of meat homogenates were incubated for 2 h, with or without liposomes. It became apparent that, for the lean meat used here, measuring only the lipid peroxides will give about 40% of the absolute value of peroxides. If there is a need to know the total amount of peroxides, at least protein-bound peroxides should be included.

This experiment was set up to examine fatty acids and hemin levels and to use these variables as predictors of oxidation. This is done in several model systems for accelerating oxidation (Bishov, Henick, & Koch, 1961; Oszmianski & Lee, 1991; Van Dyck, Verleyen, Dooghe, Teunckens, & Adams, 2005). The hemin content emerged as a significant predictor of peroxide formation. However, due to the fact that hemin level was correlated with the amount of many unsaturated fatty acids, it was difficult to identify the importance of specific fatty acids for hydroperoxide formation. This can be exemplified by the fact that C22:6 *n*-3 was a reducer of peroxides in some models due to its correlation with low hemin levels of the biological samples. Nevertheless, our data suggested that the hemin level alone would account for about 60% of the variation in peroxides in commercial meat. By including information about the variation in fatty acid composition, close to 70% of the variation was accounted for. This can explain why beef meat produced more peroxides than did chicken meat, despite the fact that the latter meat had a higher amount of polyunsaturated lipids.

The difference between lamb and pork seemed due to either more efficient fat-soluble antioxidants in lamb meat or a lamb myoglobin that is less pro-oxidative than is pork myoglobin. In addition, the pork samples contained more fat than did lamb samples and that tended to be important for peroxidation of the pork samples.

4.7. Relevance for meat quality and health issues

The peroxide formation ability is relevant to meat quality as it precedes off-flavour formation and protein crosslinking to give tougher meat. In addition, peroxide formation could exhaust the presence of antioxidants in the reduced state.

Angeli et al. (2011) indicated that peroxides originating from lipids and the heme group could be factors that could contribute to cell cytotoxicity. These authors suggested that concentrations higher than 0.1 mM of lipoperoxides would exert toxic effects on cells. According to our data, this concentration is exceeded in all our meat systems, even if only lipid peroxides are accounted for. However, when meat is consumed, it is normally diluted and, in addition, it is heat-treated, except for dried meat products. Other factors, such as processing, storage, added ingredients, pro-oxidants, and antioxidants, are also very important for lipid oxidation (Ladikos & Lougovois, 1990). On the other hand, the results suggest that, in particular for Norwegian pork meat quality, it should be possible to improve it with respect to peroxide levels compared to lamb meat that had a higher hemin level.

5. Conclusion

The fraction of non-lipid hydroperoxide was 40–50% in lean meat. The FOX method ranked the total peroxide as follows: beef > pork > lamb > chicken groups. The lipid peroxide variations could largely (70%) be explained by the hemin level and the variation in fatty acid composition, while the protein-bound peroxides were less well explained by the hemin concentration.

Acknowledgements

We thank Nortura SA for their support when obtaining lamb, pork and beef samples. Professor Jan Gebicki is acknowledged for sharing his experience with the FOX-assay with us. This work was supported by project 224798 granted by FFL/JA.

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Paper II

Food Chemistry 187 (2015) 563–571

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

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ARTICLE INFO

Article history:
Received 5 September 2014
Received in revised form 26 March 2015
Accepted 2 April 2015
Available online 7 April 2015

Keywords:
Krebs cycle substrates
Minced meat
Colour stability
Lipid oxidation

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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http://dx.doi.org/10.1016/j.foodchem.2015.04.002
0308-8146/

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

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

2. Materials and methods

2.1. Chemicals

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

2.2. Animal tissues

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

2.3. Preparation of minced meat

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

2.4. Preparation of solutions

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

2.5. Modified atmosphere packaging (MAP) and storage

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

2.6. Fatty acid composition analysis

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

2.7. Headspace analysis

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

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

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

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

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

2.9. Thiobarbituric acid reactive substances (TBARS) measurements

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

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

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

2.11. Amino acid analysis for glutamate

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

Prior to analysis, 350 μL of borate buffer (0.4 M, pH 10.2, Agilent Technologies) was added to 50 μL of sample. Separation of AAs was performed using an Agilent series 1200 pump and autosampler (Agilent Technologies, Singapore), a Perkin Elmer 200 column oven, and an Agilent 1200 series thermostat and fluorescence detector (Agilent Technologies). The system used software from EZChrom Elite (Agilent Technologies). An XTerra RP 18 column (150 × 4.6 mm; Waters, Milford, MA) was used for separation of AAs at 42 °C. Derivatisation with o-phthalaldehyde/3-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 ± 0.14[*,a]	0.00 ± 0.34[a]	0.01 ± 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 ± 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

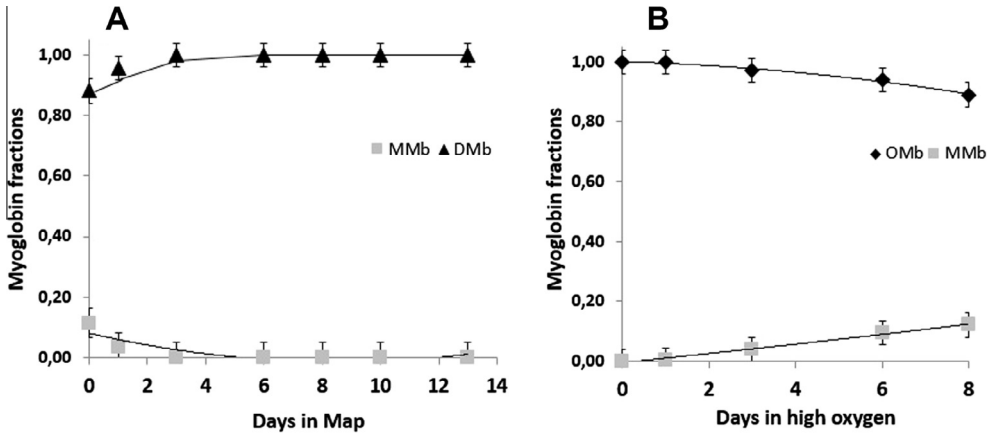

Fig. 1. Myoglobin fractions on surfaces of minces in two different packaging. (A) Myoglobin states with added succinate, glutamate and malate (molar ratio 50:37.5:12.5) in MAP; (B) myoglobin states with added glutamate, citrate and malate (molar ratio 56:25:19) in high oxygen. Total concentration of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

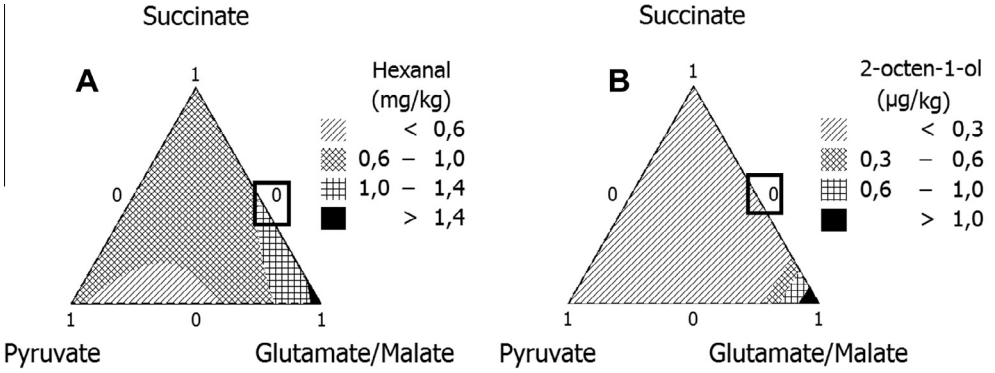

Fig. 2. Hexanal (A) and 2 octen-1-ol (B) concentration as a function of the composition of succinate, pyruvate and glutamate/malate (KCS, mean conc. = 0.075 mol/kg) for minces chill stored for 13 days in MAP. The slopes of the response surfaces were significant ($P < 0.05$). Square indicates the area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

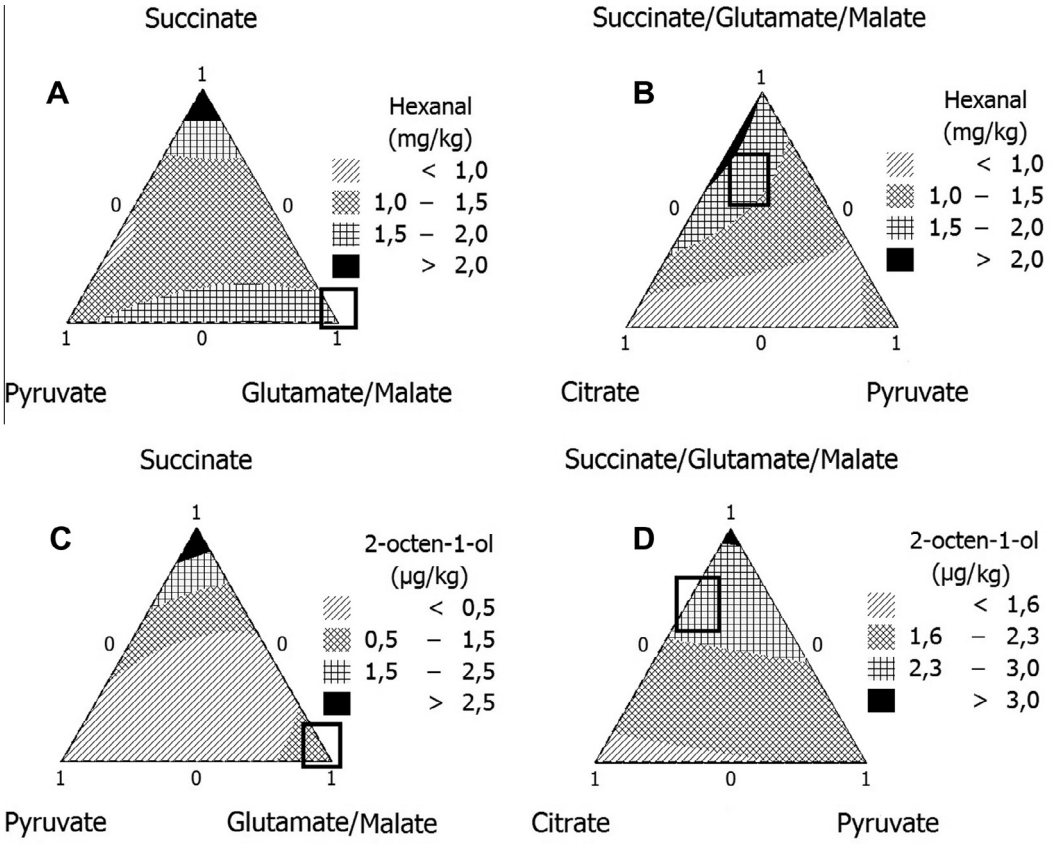

Fig. 3. Hexanal concentration (A) and 2 octen-1-ol (B) as a function of the composition of succinate, pyruvate and glutamate/malate (Krebs cycle substrates, mean conc. = 0.075 mol/kg) for minces chill stored for 8 days in high oxygen. The slopes of the response surfaces were significant ($P < 0.05$).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

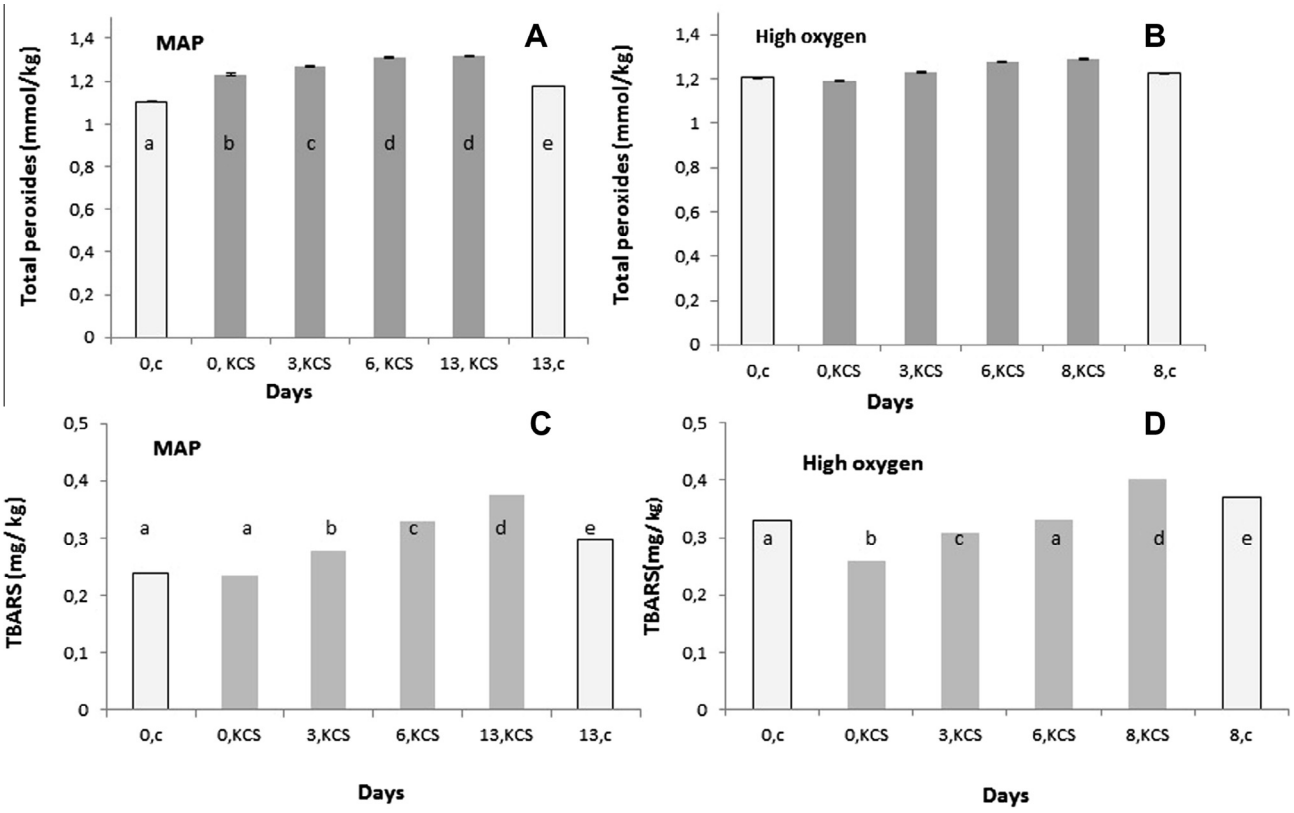

Fig. 4. (A) Total PFP over time for a succinate and glutamate mixture in MAP; (B) total PFP over time for a glutamate, citrate and malate mixture in high oxygen; (C) TBARS formed over time for a succinate and glutamate mixture in MAP; (D) TBARS formed with time for glutamate, citrate and malate mixture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

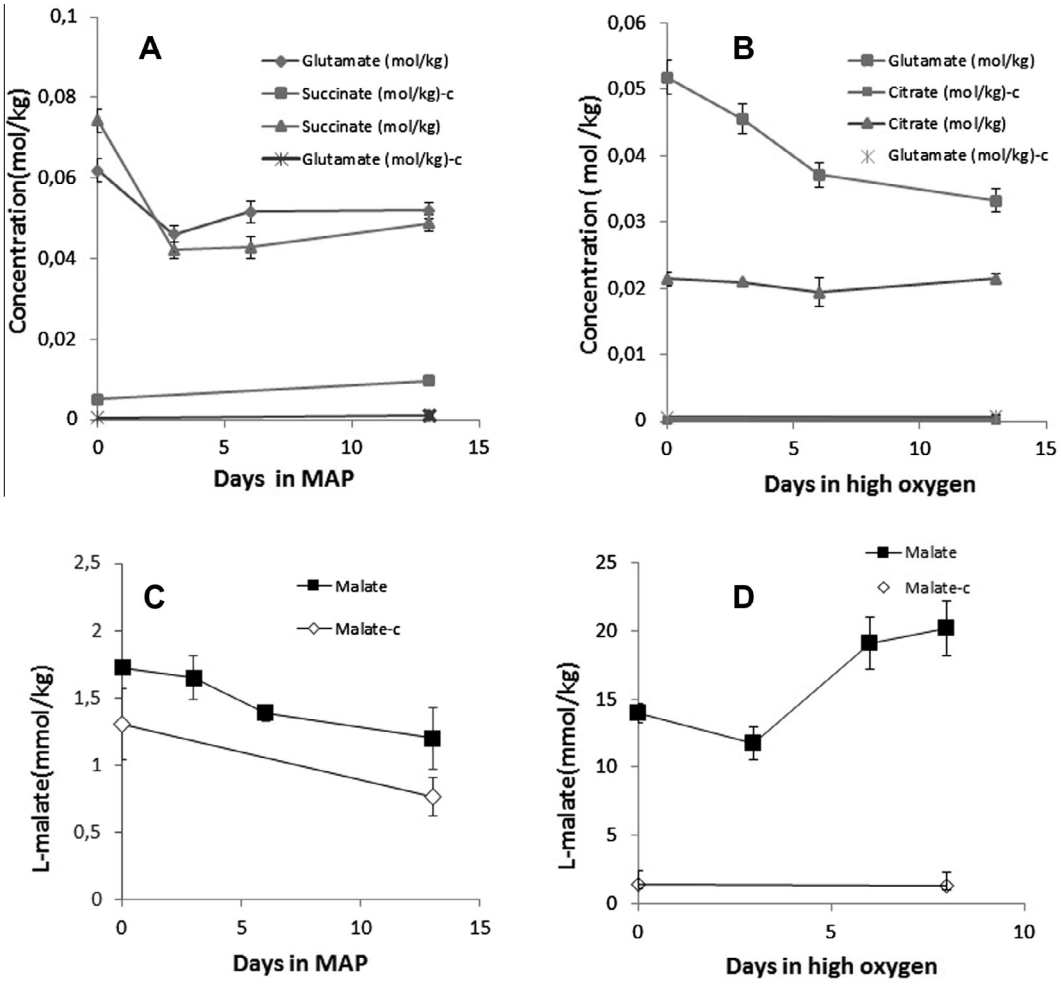

Fig. 5. (A) Succinate and glutamate concentrations (molar ratio 50:50, totally 0.1 mol KCS/kg) in minces stored in MAP; (B) glutamate, citrate and malate in high oxygen (molar ratio 56:25:19, totally 0.1 mol KCS/kg); (C and D) malate concentration in the same minces stored in MAP and in high oxygen,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

Acknowledgements

This work was supported by Grant No. NFR184846/I10 from the Research Council of Norway and from HERD project "Comparison of lamb Carcass and meat quality of Breeds in Western Balkan and Norway achieving improved palatability, sale and sustainability". We also thank TINE SA and Nortura SA for their support. The two companies provided funding, but had no influence on the design, choice of methodology or interpretation of the results.

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Paper III

Compositional Factors that Influence Lipid Peroxidation in Beef Juice and Standard Sausages

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Abstract: In order to identify how different additives influenced lipid peroxidation formation, a sausage only using beef juice as pigment source and a standard beef–pork meat sausage were studied. The effects of different additives, including fish oil, myoglobin, nitrite, clove extract, and calcium sources on oxidation and sensory properties were examined. Both sausage systems were stored in 3 different manners prior to testing: (1) frozen immediately at −80 °C; (2) chilled stored for 2.5 weeks followed by fluorescent light illumination at 4 °C for another 2 wk; (3) frozen at −20 °C for 5 mo. The frozen group 3 showed the highest peroxide formation and thiobarbituric acid reactive substances (TBARS) for both sausage systems. Unpolar peroxides dominated in both systems. The clove extract could offset the peroxide formation from myoglobin/beef juice and/or fish oil, but the addition of clove flavor was recognized by the sensory panelists. Calcium addition reduced lipid peroxide formation. Added nitrite and fish oil seemed to interact to stimulate nitroso-myoglobin formation. Nitrite was identified to interact with clove addition and thereby, relatively speaking, increased TBARS. The 2 sausage systems generally ranked the additives similarly as pro– and antioxidants.

Keywords: antioxidant, calcium, clove extract, hydroperoxide forming potential, sausages

Practical Application: Components such as peroxides are argued to potentially be involved in conditions leading to colon cancer. This study assessed how sausages' peroxide forming potential, along with other measures of oxidation, depended on sausage composition. An added clove extract provided flavor and prevented hydroperoxide formation in sausages caused by increased amounts of polyunsaturated fatty acids and heme in beef meat juice. Added $CaCO_3$ decreased the lipid peroxide forming potential. A calcium source and a good antioxidant are recommendable additions to sausages.

Introduction

Processed meat such as sausages and hams are popular daily and iconic foods due to their convenience, nutrition, and taste. However, substantial amounts of sodium and pork fat with a high n-6:n-3 ratio and a relative high arachidonic acid (ARA) content used in traditional sausages are claimed to increase the risk of many diseases, such as high blood pressure, heart diseases, and cancer (Araujo de Vizcarrondo and Martín 1997; Solakivi and others 2009; Sinha and others 2009). Parr and others (2013) suggested that high consumption of processed meat, mainly driven by the intake of sausages (≥ 30g/d), was associated with increased risk of proximal colon, distal colon, and rectal cancer in the Norwegian population. Some additives used in traditional Norwegian sausage production are of concern. The addition of nitrite gives a slightly pink color but also promotes the formation of nitroso compounds (NOS) during preparation of sausages. Heme-rich raw material used to adjust the color of traditional sausages is also assumed to stimulate the endogenous production of mutagenic nitrosamines and lipid oxidation in colon and therefore increase the risk of colon cancer (Araujo de Vizcarrondo and Martín 1997; Joosen and others 2009; Oostindjer and others 2014). Addition of ascorbate as an antioxidant to sausages is another tradition, which can reduce the nitrosamine formation and increase the formed fraction of nitroso-myoglobin (Beyer 1994). Thus, altering the additives used in sausages may alter the healthiness of sausages. Added fish oil can provide eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and adjust the n-6:n-3 ratio to a lower value. This is assumed favorable for health (Jeun-Horng and others 2002). Calcium addition can be used to increase the daily intake of calcium, reduce peroxide formation, and potentially inhibit colon cancer development (Jorde and Boenaa 2000; Santarelli and others 2013).

Natural antioxidants, such as polyphenol-rich extract of cloves (*Syzygium aromaticum* L.) might be used to slow the rate of oxidation during storage (Valencia and others 2006). The antioxidant effect of clove extract is due to eugenol which is also the major phenol in the extract (Lee and Shibamoto 2001). Clove extract has a positive effect on the color stability and functions as an efficient antioxidant in dry fermented sausages (Rohlík and others 2010). In addition, clove has antimicrobial properties and contains polyphenolic compounds that stabilize free radicals and break the oxidation chain (Lee and Shibamoto 2001; Issac and others 2015).

Phenols are not necessarily absorbed and may therefore act as antioxidants in the colon. This could be relevant as the negative aspects of meat in the colon are speculated to relate, among others, to reactive oxygen species formed there. Calcium has been reported to both enhance and inhibit peroxidation presumably depending on the added level. This can be through a direct (reacting with reactive oxygen species) or an indirect mechanism preserving antioxidants (Babizhayev 1988; Dogterom and Mulder 1993). Calcium and antioxidant plant component additions may counteract peroxide formation (Lee and Shibamoto 2001; Lordan and others

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doi: 10.1111/1750-3841.13123

Journal of Food Science A Publication of the Institute of Food Technologists

2011; Santarelli and others 2013). Although previous publications have reported on secondary oxidation products like thiobarbituric acid reactive substances (TBARS; Berasategi and others 2014; Karwowska and others 2015), much less work has been done on total peroxide formation potentials in staple food such as sausages, despite the fact that peroxides are the more reactive compounds than aldehydes that maybe included in the TBARS assay. In particular, the total amount of peroxides seems neglected. The study below has a creative design as pure beef juice is elucidated as a prooxidant in sausages produced with the "white" (pigment-poor) pork muscle *M. Gluteus medius* as a protein source. Then beef juice in itself was studied as a pro-oxidant Hydroperoxide formation can then be solely be attributed to beef juice. This is relevant information as the prooxidative power of the beef juice can be directly assessed against antioxidants.

The aims of this were to quantify: (1) peroxidation/oxidation in a beef juice-pork meat sausage where the n-6:n-3 fatty acid ratio, beef meat juice (adjusts myoglobin level), clove extract and calcium level varied; (2) the effect of the same variables on sensory properties. Finally, the results of the beef juice-pork meat sausage system were compared to a traditional beef and pork meat sausage.

Materials and Methods

Material and chemicals for sausage samples

Pork fat tissue, pork muscles (*M. Gluteus Medius*), and beef muscles (*M. Semimembranosus*) were purchased fresh (3 and 4 days *post-mortem*, respectively) from a local commercial slaughter house (Flatland, Norway), vacuum packaged and frozen immediately as intact muscles. The pork fat tissue contained 2.2% ARA, with a 10:1 ratio of n-6:n-3 and 38%, 46%, and 15% of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), respectively. These values are presently typical for Norwegian pork fat. Beef meat juice (from *M. semimembranosus*) was collected as thaw drip loss during 2 d from a commercial meat supplier (Nortura SA, Norway). The thaw drip was refrozen at −20 °C in a plastic container and used within 2 wk for sausage production. Dried cloves (Lyckby Culinar AS, Sweden) were transformed into a clove extract (see section 2.3) and frozen at −20 °C before use. Food-grade vacuum salt (NaCl), nitrite salt (600 mg $NaNO_3$/kg), and dipolyphosphate ($P_2O_7^{4-}$) were from Arne B. Corneliussen AS (Norway). Fresh skimmed milk was purchased from a local supermarket (supplier Tine SA, Norway). Dry skimmed milk powder (355 g protein, 525 g carbohydrate, and 10.5 g Ca all *per* kg powder) was from Tine SA. Fish oil (21%, 55%, and 24% SFA, MUFA, and PUFA, respectively, n-6:n-3 ratio was 1:16; 0.5% ARA) was also supplied by Tine SA. Ethanol and $CaCO_3$ were from Merck (Darmstadt). Liquid smoke was purchased from Smokez Enviro (Red Arrow, Manitowoc, U.S.A.). All the raw materials were vacuum packed and kept frozen for 2 d at –20 °C until production. The sausages were prepared during 2 different periods: the beef juice–pork meat sausage in Experiment I (Exp I) was produced 1st during 2 d and the beef–pork meat sausage for Experiment II (Exp II) was produced 10 d later. The 2 productions did, for practical reasons, not follow immediately after each other. This led to a higher amount of peroxides in the fish oil used in Exp II compared to Exp I. The fish oil started to oxidize once the package was opened, despite being contained in a sealed oxygen-free Bag-in-Box.

Chemicals

Purified aqueous water was from Millipore (Millipore, Sydney, Australia). Chloroform (AR grade), sulfuric acid, methanol, acetone, iron (II) sulfate, sodium carbonate, and Ringer's solution tablets were purchased from Merck (Darmstadt, Germany). Guanidine hydrochloride, hydrochloric acid (37%), 3,4,5-trihydroxybenzoic acid, and streptomycin were supplied by Sigma-Aldrich Chemical (Sydney, Australia). Butylated hydroxytoluene, xylenol orange sodium salt, and triphenylphosphine (99% purity) were purchased from Alfa Aesar (Lancashire, U.K.). Folin-Ciocalteu reagent, trichloroacetic acid, thiobarbituric acid, and D-sorbitol were bought from Sigma-Aldrich (St. Louis, U.S.A.). Sodium dithionite was purchased from VWR Inc. (Oslo, Norway). All the other chemicals were of analytical grade.

Preparation of clove extracts

Dry clove buds (2 kg) were frozen in liquid nitrogen and homogenized by a blender (800 W Home blender, Invite). The homogenized clove powder was mixed in 16 L of 96% (v/v) ethanol and extracted for 12 h in closed bottles with constant shaking (SM-30, Edmund Buhler). After filtration through a cheese cloth, the residual clove powder was reextracted with an additional 8 L of 96% ethanol (v/v) for 12 h and then filtered again. The filtrates were concentrated on a rotary evaporator (R215, Buchi Rotavapor, Switzerland) in a water bath to 0.8 L at 50 °C (B-490, Buchi Heating Bath). The residue was refiltered through a filter paper (Φ185 mm, 589^3, Blauband) and evaporated to 200 mL. The extract was placed in a sealed bottle and stored for 14 d at −20 °C until used.

Total phenols measurement

Assessment of the total phenolic content of the clove extract was done by a modified Folin–Ciocalteau (FC) procedure (Slinkard and Singleton 1977; Singleton and others 1999). Gallic acid (500 μg/mL), sodium carbonate (7.5% w/v), and 5 mL FC-reagent were dissolved in 50 mL water and prepared on the day of analysis. FC-reagent (100 μL) and the diluted (1:20) phenol extract solution were pipetted directly into the cuvettes, mixed and incubated at 37 °C for 1 min. Eighty microliters of 7.5% (w/v) sodium carbonate was added to the mixture and incubated at 37 °C for another 15 min. The absorbance of the mixture was measured at 765 nm. The absorbance of a diluted (diluted 2, 3, 5, 10, and 25 times) stock gallic acid solution was used to estimate gallic acid equivalents of the clove extract. The concentration was adjusted according to the exact mass of gallic acid and the total phenolic content of the clove extract was calculated as 6.1 g gallic acid equivalents (GAE)/L extract.

Preparation of sausage samples

Exp I included 32 samples with the design variables: $CaCO_3$, beef meat juice, nitrite, fish oil, and clove extract according to a full factorial (2^5) design. A block production order was used regarding the fish oil (4 samples with fish oil were produced in a sequence) to avoid transfer of fish oil between samples. Extensive washing was performed thereafter. Exp II included 16 samples with the design variables: beef meat, nitrite, fish oil, and clove extract, according to a full factorial (2^4) design. Exp II samples had a constant calcium level, but used dry skimmed milk powder or fresh skimmed milk as calcium source; that is, calcium level was not a design variable (Table 1). Because heme/hemin is such a significant pro-oxidant, we decided to keep its level constant in both systems. Lean beef muscles (*M. Semimembranosus*) were used in Exp II instead of beef

Table 1–Composition of the sausage samples in both sausage systems.

Exp.	Macronutrients (g/kg)	Myoglobin (g/kg)	Fish oil (g/kg)	$NaNO_3$ (g/kg)	Ca-source (g/kg)	Clove phenol (g GAE/kg)
I[a]	Protein: 130[b] Fat: 100 Water: ~720–740	High: 3.7 Low: 0.6	High:10 Low: 0	High: 0.03 Low: 0	High: 8[c] Low: 0	High: 0.3×10^{-3} Low: 0
II	Protein: 130 Fat: 100 Water: ~720	High: 3.8 Low: 0.7	High: 10 Low: 0	High: 0.03 Low: 0	3.4[c]	High: 0.3×10^{-3} Low: 0

[a]Exp I (32 samples) used beef meat juice and Exp II (16 samples) used beef meat and small amount of meat juice to adjust myoglobin concentration to the same level; NaCl was 18 g/kg and phosphate (calculated as P_2O_5) was 1.8 g/kg for both systems.
[b]Meat was from the pork muscle *M. Gluteus Medius* in the Exp I.
[c]$CaCO_3$.
[d]Skimmed milk source. There was no significant difference between fresh skimmed milk and dry skimmed milk powder ($P > 0.397$) for all 3 storage groups (ANOVA); therefore, we do not differentiate them regarding storage. The Ca content was 3.2 and 3.4 g/kg for the Exp I and Exp II, respectively.

meat juice which was only used to adjust to requested heme level. Beef meat and beef juice are hereafter called myoglobin in the design, as these raw materials were meant to mimic the presumed most important pro-oxidant, that is, myoglobin. A control sausage sample without any of the 5 additives was also made.

Lean pork, lean beef, and pork fat were passed through a mincer (4 mm, Seydelmann Stuttgart, Germany). NaCl and nitrite fortified NaCl (if wanted) were added to minced lean meat and mixed (WMC5, Stephan Food Service Equipment GmbH, Germany) for 1 min. Approximately, half of the water and meat juice or beef were added and mixed for 45 s. The remaining water, meat juice, and polyphenol were then added and mixed for 30 s. Minced pork fat, fish oil, and the different calcium resources were added and mixed for 1 min. The mixtures were filled into casings (E-25-1, Fatosa SA, Spain), the raw sausage was thereafter dipped in liquid smoke for 1 min and air dried for 10 min. The sausages were heated in a cabinet (SC2000, Doleschal Unitronic, Austria) with the following cooking procedure: 10 min heating at 60 °C, 20 min drying at 60 °C, 20 min cooking at 78 °C, 5 min ventilation at 60 °C, and 10 min showering at room temperature. After drying and chilling, all the sausages were vacuum-packed (Vacuum packaging systems, Vacuum webomatic, Germany), light-protected, and stored.

Storage of sausages

The sausages were split in 3 groups. Group 1 (fresh) was frozen immediately at −80 °C, whereas group 2 (chilled) was 1st stored at 4 °C for 2.5 wk and then put under fluorescent lamps (intensity of light 3920 lux, wavelength 350 to 750 nm) for 2 wk to simulate retail display. The sausages were moved around twice per day in order to secure homogeneous illumination, then frozen at −80 °C. Group 3 (frozen) was frozen at −20 °C for 5 mo and then frozen at −80 °C. Freezing at −80 °C was only done to stop the samples for further chemical changes and was not a design variable. The following analysis were carried out after 6 mo, part of the time stored at −80 °C: sensory analysis, color analysis, and nitrosamine analysis. PV and TBARS analysis were measured, again from frozen storage at −80 °C, after finalizing the selected storage periods at −20 °C or chill storage.

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

Sausages samples were frozen in liquid nitrogen and homogenized by a blender (800 W Home blender, Invite) to a sausage powder. Sausage homogenates (0.1 g) were mixed with 1 mL Ringer's solution and 10 μL 20 g/L streptomycin and incubated for 2 h in a 37 °C water bath. The samples were then mixed with 1 mL chloroform and methanol (2:1, volume-ratio), vortexed and centrifuged at 25186 × g for 10 min at 4 °C. After centrifugation, the mixture was separated in 3 phases: a polar phase, an interphase (the meat protein aggregate), and an unpolar phase (chloroform phase) containing soluble lipids. Each of the 3 phases was removed, respectively, for separate hydroperoxide measurements. Chemicals used were as described by Yi and others (2013). All the peroxides were measured at 590 nm. Hydroperoxide values were calculated by 1st subtracting the absorbance of the negative control, then dividing the absorbance was by the pigments' (ferric-xylenol orange) molar absorptivities of $\varepsilon = 14840$ and 87583 M^{-1} cm^{-1} for the upper phase/inter phase and the lower phase, respectively, before correcting for dilution. The peroxides of the upper, inter, and lower phase are hereafter called polar, protein-bound and unpolar peroxides, respectively. The peroxides distributed through the 3 phases represent the total amount of peroxides.

TBARS measurements

TBARS were measured using the reaction between 2-thiobarbaturic acid (TBA) and compounds reacting with it in a meat extract. Sausages were frozen in liquid nitrogen and homogenized with a blender (800W Home blender, Invite) to sausage powder. Two grams of sausage powder were added to 10 mL of TBA-stock solution (0.375% TBA and 15% trichloroacetic acid in 0.25 N HCl). Following homogenization, 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 25186 × g at 4 °C. The resulting supernatant was immediately measured by spectrophotometer at 532 nm using $\varepsilon = 156000$ M^{-1} cm^{-1} (Buege and Aust 1978).

Color measurement

The CIE $L^*a^*b^*$ color variables and spectra were measured using the Konica Minolta CM-700d portable spectrophotometer with sphere geometry and vertical alignment. All the sausage samples were cut 2-cm thick, put on trays (Polystyrene Weigh Boats 85 × 85 × 24 mm; VWR Intl., Darmstadt, Germany) and covered with oxygen-permeable polyvinyl chloride film. Each sausage sample was measured in triplicates after calibration using a white ceramic calibration cap CM-A177. Standard D65 (Daylight, color temperature 6504 K) was used and the angle of the field of view was 10° (CIE Konica-Minolta 1964). The average CIE $L^*a^*b^*$ variables and reflectance spectra (400 to 700 nm, 10 nm resolution) were measured. The reflectance ratio R570/R650 was used to measure the effects of the different additives.

Nitrosamine measurements

Nine samples (8 sausage samples from Exp I and a control sample) were chosen for the following nitrosamine measurements: N-nitrosodimethylamine, N-nitrosomethylethylamine, N-nitro-

soethylamine, N-Nitrosopropylamine (NPDA), N-nitrosobutylamine, N-nitrosopiperidine, N-nitrosopyrrolidine, and N-nitrosomorpholine. The samples made up a 2^3 design including myoglobin fishoil, and nitrite, but contained no $CaCO_3$ and polyphenol extract. This design was chosen for the purpose of studying the nitrosamine formation without additives that functioned as antioxidants (see below).

Sausage samples (10 g) were cut into small pieces. Water and internal standard (14 mL) containing N-nitrosodiisopropylamine (NDiPA) were added. The mixture was homogenized using an IKA blender and treated by steam distillation for 2 h. The water phase was collected from the distillation and then injected into an Extrelut column (Merck KGaA, Darmstadt, Germany). Nitrosamines were eluted from the column together with 60 mL dichloromethane and the solution was evaporated to 1 mL using toluene as a keeper. The final toluene solutions were analyzed by gas chromatography thermal energy analysis, using *N*-nitrosodiisopropylamine as an internal standard. The recovery of the standard was >90%. The detection limit was just below 2 μg/kg.

Quantitative descriptive sensory analysis

Six panelists were chosen after a basic sensory test that asessed their sweet, salty, bitter, umami, and sour perception ability (Lawless and Heymann 1998). Forty-nine sausage samples from Exp I, Exp II from chilled group and 1 commercial, reduced fat sausage (Go' og mager grillpølse, 9% fat, Nortura SA, Norway) were analyzed. All the sausages were randomly portioned into individual coded plates for testing. All panelists were asked to score for clove flavor, bitterness, saltiness, rancidity, meaty flavor, and color intensitiy of each sausage sample in isolated booths. These 7 attributes were graded on a scale from 0 to 7 where 7 indicated high perception of a specific attribute.

Statistical analysis used for PV and TBARS

Exp I: PV and TBARS were analyzed using a general linear model with the following fixed main factors: $CaCO_3$, myoglobin, nitrite, fish oil, clove extract plus 1st-order interactions between variables. Storage groups were used as a random factor. The production day and its interactions with design variables were added. Production day was defined as a covariate. Significant factors ($P < 0.05$) were identified and least squares means were calculated using Minitab version 15.

Exp II: PV and TBARS were analyzed using general linear model with the following main factors: myoglobin, nitrite, fish oil, clove extract, plus 1st-order interactions between variables. Storage groups were used as a random factor. Significant factors ($P < 0.05$) were identified and least squares means were calculated using Minitab version 15.

Results

Effect of type of storage on peroxide formation and TBARS

In beef juice–pork meat sausage (Exp I), the peroxide formation abilities of all 3 (polar, protein-bound, and unpolar) peroxides of sausages from the 3 storage groups were ranked as follows: frozen group > chilled group > fresh group. The differences in all 3 PV values were larger between chilled group and frozen group than between chilled group and fresh group. The peroxides formed were distributed in the frozen group as: unpolar peroxides > protein-bound peroxides > polar peroxides (Figure 1A).

The beef–pork meat sausages (Exp II) showed similar PV results to products from Exp I. The absolute value for peroxide formation in Exp II was highest in the frozen group, in particular for protein-bound peroxides ($P = 0.003$, *t*-test) and unpolar peroxides ($P < 0.001$, *t*-test). However, this difference could not exclusively be attributed to the presence of fresh skimmed milk or dried skimmed milk powder or using beef meat instead of beef juice as myoglobin source, as the pork meat and pork fat added in Exp II were stored frozen and vacuum packed 10 d longer than in Exp I. Exp I samples had an average total peroxide value of 1.5 mmol/kg for the 3 storage groups, whereas the Exp II samples showed a higher average total peroxide level of 1.9 mmol/kg. The frozen group also showed the highest TBARS among the 3 storage groups, both in Exp I and Exp II. The frozen group had 3- and 2-fold higher TBARS than the fresh group in Exp I and Exp II, respectively. However, the TBARS differences between each storage group were not as large as for the peroxides (Figure 1B).

The 2 storage conditions were selected because they were of commercial interest. One commercial approach would be chill-storage 1st in the dark and then later under light characteristic of retail display. The 2nd possible commercial approach would be frozen storage. PV (Exp I) and TBARS (Exp I and Exp II) did not increase more with storage than the increase observed during the actual production of fresh sausages using certain combinations of additives (fish oil and meat juice). As TBARS were less affected by storage than by the additives, we decided to show the average changes in TBARS found with different storage principles. We therefore focused on presenting the average effects of additives on PV and TBARS.

Effect of additives on peroxide formation and TBARS

In Exp I (Table 2), both polar and protein-bound peroxides increased significantly with myoglobin (as meat juice) addition, whereas a reduction was observed with clove extract addition. Fish oil addition increased unpolar peroxides whereas $CaCO_3$ addition decreased unpolar peroxides (not shown, $P = 0.002$). Also, a weak interaction between fish oil and $CaCO_3$ addition was observed ($P = 0.03$). The most important source for inducing peroxides among the design variables in these sausages (with 10% total fat) was the 1% fish oil added. Because unpolar peroxides were the dominant type of peroxides (51% of total), total peroxides showed a response to additives that resembled the response of unpolar peroxides.

In Exp II (Table 3), the added myoglobin significantly increased unpolar peroxides, which was the dominant type of peroxides (55% of total) also in these sausages. The polar peroxide formation was also significantly increased by myoglobin addition. Surprisingly, we only found in Exp II an effect of fish oil addition on polar peroxide formation in contrast to Exp I sausages. This may actually suggest a more extensive degradation of lipids in the sausage system. The clove extract significantly reduced all types of peroxides. Calcium was not a design variable in Exp II, but compared to the fresh skimmed milk addition, the addition of the dried skimmed milk gave higher amounts of unpolar peroxides. Addition of 30 mg/kg $NaNO_3$ did not affect any type of peroxide value in any system.

In Exp I (Table 2), the addition of fish oil increased TBARS significantly, although the addition of $CaCO_3$ was observed to significantly decrease TBARS ($P = 0.005$) in agreement with its effect on unpolar peroxide values. In sausages (Exp II), the fish oil and myoglobin addition gave higher TBARS whereas clove extract reduced TBARS in agreement with the polar peroxide results. The TBARS results also supported that the beef meat–pork

Table 2–Significant factors[a] effects of PV and TBARS for Exp I.

Exp I				
	Significant factors[b]			
Polar peroxides	Myoglobin ($P < 0.001$)	Polyphenol ($P < 0.001$)	LSM-PV[c] (mmol/kg)	Constant (mmol/kg)
	No[d]	No	0.202 ± 0.004bc	0.208
	No	Yes	0.190 ± 0.004c	
	Yes	No	0.235 ± 0.004a	
	Yes	Yes	0.206 ± 0.004b	
Protein-bound peroxides	Significant factors			
	Myoglobin ($P = 0.045$)	Polyphenol ($P = 0.048$)	LSM-PV (mmol/kg)	Constant (mmol/kg)
	No	No	0.486 ± 0.017[ab]	0.491
	No	Yes	0.464 ± 0.017[b]	
	Yes	No	0.530 ± 0.017[a]	
	Yes	Yes	0.486 ± 0.017[ab]	
Unpolar peroxides	Significant factors			
	Fish oil ($P < 0.001$)		LSM-PV (mmol/kg)	Constant (mmol/kg)
	No		0.747 ± 0.002[b]	0.810
	Yes		0.955 ± 0.002[a]	
TBARS	Significant factors			
	Fish oil ($P = 0.002$)		LSM-TBARS (mg/kg)	Constant (mg/kg)
	No		0.052 ± 0.012[b]	0.062
	Yes		0.072 ± 0.012[a]	

[a]Significant factors by using ANOVA model using PV (peroxide values) and TBARS as responses for the Exp I.
[b]Five main factors in Exp I: $CaCO_3$, myoglobin (beef meat juice), nitrite, fish, and polyphenol. Significant factors ($P < 0.05$) were identified and least squares means (LMS) were calculated with standard errors. Only the 3 most commonly significant factors (polyphenol, myoglobin, and fish oil) are listed.
[c]When LMS-PV > constant value, it means the additive is a pro-oxidant whereas when LMS-PV < constant value, the additive is an antioxidant. The number of samples analyzed was 32.
[d]Yes means high level, no means low level.

meat sausage system was a more reactive system as judged from the presence of more degraded lipids (and more TBARS) in the polar phase. Nitrite addition was only found significant for the sausage system, resulting in increased TBARS ($P = 0.021$). It is not likely that this is due to a spectroscopic artifact, because the addition of $NaNO_3$ was low. It could be more likely that nitrites interfered with the polyphenol activity because their interaction factor was significant ($P = 0.02$). For both polar peroxides and protein-bound peroxides, the production day had only weak interactions with all 5 additives. For the unpolar peroxides, production day showed a significant interaction with both fish oil ($P < 0.001$) and polyphenol addition ($P = 0.001$).

It should be noted that the larger part of hydroperoxides and TBARS is the background value (Table 2). This may be due to the fact that most of the fat (9% pork fat added) was a part of the background matrix, that is, our additives contributed less to total TBARS and peroxides than the main background matrix. One exception was TBARS for the Exp II (Table 3).

The effects of additives on reflectance ratio

Color differences between the 3 storage conditions were marginal. Average values showing the most important design factors are therefore shown in Figure 2. Exp I: Nitrosomyoglobin (as indicated by R570/R650) formation was stimulated by beef meat juice and nitrite addition (Figure 2). Fish oil addition also seemed to stimulate nitrosomyoglobin formation (more redness) in the beef juice–pork meat sausage. When fish oil was added

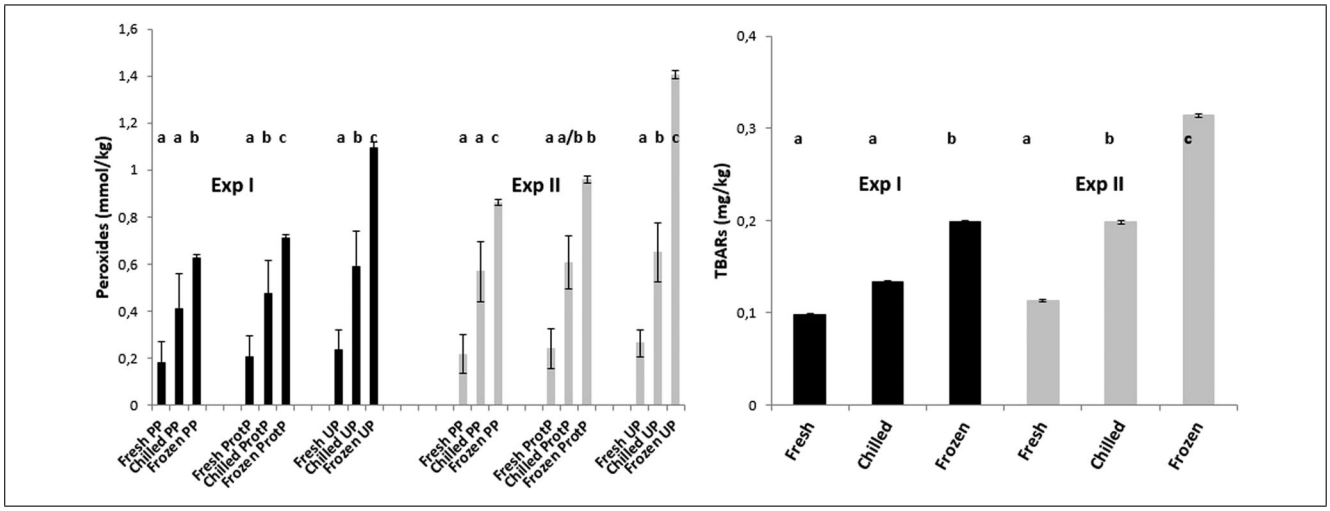

Figure 1–(A) Peroxides among phases of the 3 storage groups in Exp I and Exp II; (B) TBARS of the 3 storage groups in Exp I and Exp II. Error bars are standard deviations. Means with different letters are significantly different ($P < 0.05$). One-way ANOVA with Tukey's *post hoc* test was used. PP, polar peroxides; ProtP, protein bound peroxides; UP, unpolar peroxides. The number of samples analyzed was 32 (Exp I) and 16 samples (Exp II), respectively.

Table 3–Significant factors effects of PV and TBARS for Exp II[a].

	Exp II				
	Significant factors[b]				
Polar peroxides	Myoglobin ($P < 0.001$)	Fish oil ($P < 0.001$)	Polyphenol ($P < 0.001$)	LSM-PV[c] (mmol/kg)	Constant (mmol/kg)
	Yes[d]	Yes	No	0.262 ± 0.005a	
	Yes	No	No	0.253 ± 0.005ab	
	No	Yes	No	0.250 ± 0.005ab	
	No	No	No	0.246 ± 0.005b	0.241
	Yes	Yes	Yes	0.246 ± 0.005b	
	No	Yes	Yes	0.229 ± 0.005c	
	Yes	No	Yes	0.220 ± 0.005c	
	No	No	Yes	0.219 ± 0.005c	
Protein-bound peroxides	Significant factors				
	Polyphenol ($P < 0.001$)			LSM-PV (mmol/kg)	Constant (mmol/kg)
	Yes			0.570 ± 0.029b	0.608
	No			0.646 ± 0.029a	
	Significant factors				
Unpolar peroxides	Myoglobin ($P = 0.03$)	Polyphenol ($P < 0.001$)		LSM-PV (mmol/kg)	Constant (mmol/kg)
	No	No		1.108 ± 0.023a	1.074
	No	Yes		1.004 ± 0.023b	
	Yes	No		1.147 ± 0.023a	
	Yes	Yes		1.036 ± 0.023b	
	Significant factors				
TBARS	Myoglobin ($P < 0.001$)	Fish oil ($P = 0.002$)	Polyphenol ($P = 0.01$)	LSM-TBARS (mg/kg)	Constant (mmol/kg)
	No	No	No	0.038 ± 0.019b	0.090
	No	No	Yes	0.035 ± 0.019b	
	No	Yes	No	0.056 ± 0.019b	
	No	Yes	Yes	0.039 ± 0.019b	
	Yes	No	No	0.068 ± 0.019b	
	Yes	No	Yes	0.097 ± 0.019b	
	Yes	Yes	No	0.297 ± 0.019a	
	Yes	Yes	Yes	0.092 ± 0.019b	

[a]Significant factors by using ANOVA model using PV (peroxide values) and TBARS as responses for the Exp II.
[b]Four main factors in Exp II: myoglobin (beef meat), nitrite, fish oil, and polyphenol. Significant factors ($P < 0.05$) were identified and least square means (LMS) were calculated with standard errors. Only the 3 most common and significant factors (polyphenol, myoglobin, and fish oil) are listed.
[c]When LMS-PV > constant value, it means the additive is a prooxidant whereas when LMS-PV < constant value, the additive is an antioxidant. The number of samples analyzed was 16 samples.
[d]Yes means high level, no means low level.

together with nitrite, R570/R650 nitrosomyoglobin formation was further increased ($P = 0.03$; Figure 2). Exp II: Only the main factors nitrite and myoglobin (beef meat) were significant ($P < 0.05$) effects in agreement with Exp I.

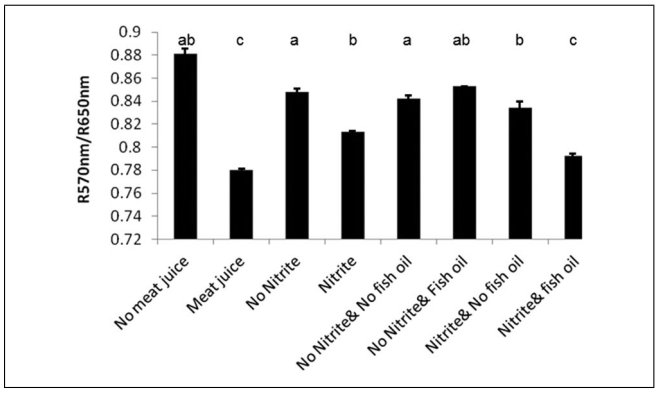

Figure 2–The effects, with standard error, of meat juice, nitrite, and 1 interaction effect (fish oil*nitrite) on the reflectance ratio R570/R650 for Exp I sausages. Samples with different letters are significantly different with $P < 0.05$. The number of samples analyzed was 32 at 3 different storage times.

Table 4–Number of samples (Exp I) with NPDA above detection limit (2 μg/kg).

	Myoglobin		Nitrite		Fish oil	
Amount	**High**	**Low**	**High**	**Low**	**High**	**Low**
NPDA (μg/kg)	1/4[a]	1/4	1/4	1/4	0/4	2/4

[a]1/4 means one of 4 samples. Eight samples were analyzed (2^3 design).

NPDA differences in sausages

Eight samples (from Exp I) made up a 2^3 design differing in myoglobin, fish oil and nitrite. Among all the measured nitrosamines, only NPDA was found above the detection limit, in 2 samples devoid of fish oil (Table 4). No other tendencies were found regarding the other design variables (myoglobin and nitrite).

Sensory quality of the sausages

Exp I: The addition of clove extract resulted in a perceived increase in clove flavor ($P < 0.001$), whereas the addition of meat juice (myoglobin) and fish oil was found to nominally decrease the clove flavor (not significant; Figure 3A). The rancidity (Figure 3B) shows a nominal increment upon addition of fish oil and meat juice, whereas addition of clove extract resulted in a

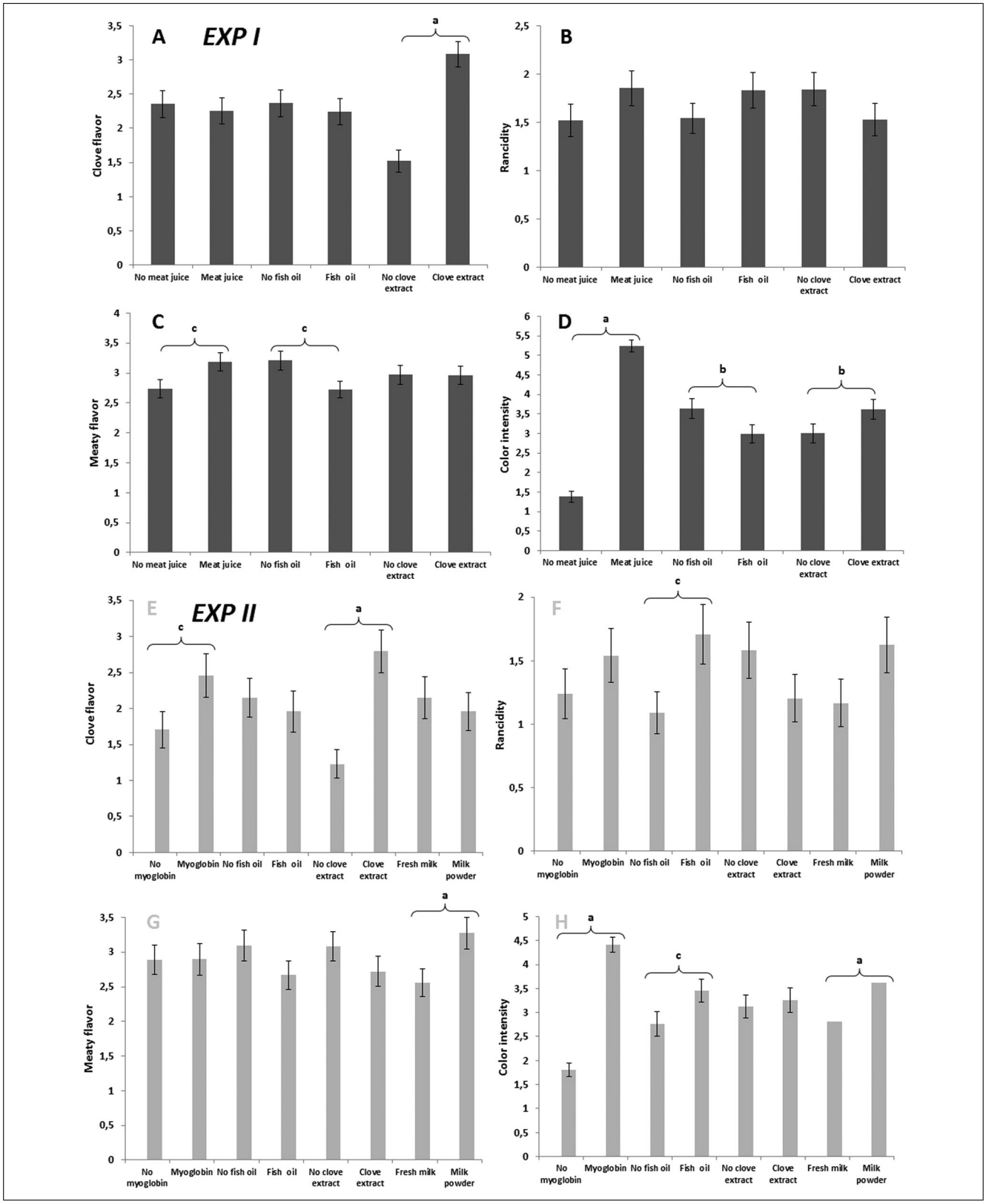

Figure 3–Quantitative decriptive sensory analysis results. A–D are Exp I. (A) Clove flavor; (B) rancidity; (C) meaty flavor; (D) color intensity; E–H are Exp II. (E) Clove flavor; (F) rancidity; (G) meaty flavor; (H) color intensity. Error bars give standard errors of the mean. $^{a}P \leq 0.001$; $^{b}P \leq 0.01$; $^{c}P \leq 0.05$. The number of samples analyzed was 32 (Exp I) and 16 samples (Exp II), respectively; chill-stored only.

nominal decrement; however, none of these additions were significant. The addition of meat juice increased the meat flavor ($P = 0.03$), whereas the addition of fish oil had an opposite effect ($P = 0.02$; Figure 3C). As shown in Figure 3D, the addition of meat juice and clove extract significantly increased color intensity ($P < 0.001$ and $P = 0.002$, respectively), altohugh fish oil was observed to significantly decrease it ($P = 0.002$). $CaCO_3$ addition did not show any significant effect on sensory properties.

Exp II: Both clove extract and myoglobin (beef meat) significantly increased clove flavor ($P < 0.001$ and $P = 0.02$, respectively), whereas sausage samples with fish oil and fresh skimmed milk were found to nominally decrease the clove flavor (not significant; Figure 3E). Numerically, a decrease in rancidity was observed when clove extract was added; on the contrary, meat juice, fish oil, and dry skimmed milk seemed to result in an increased rancid taste. However, only fish oil significantly increased the rancidity ($P = 0.04$). The addition of dried skimmed milk in Exp II significantly increased the meaty flavor ($P = 0.001$), whereas both fish oil and clove extract addition resulted in decreased meaty flavor (Figure 3G). The addition of meat juice and dried skimmed milk increased color intensity ($P < 0.001$ and $P = 0.001$, respectively), although the addition of fish oil decreased the color intensity ($P = 0.03$; Figure 3H).

Discussion

Fish oil as an EPA and DHA additive

Albeit fish oil addition makes sausages richer in EPA and DHA, it leads to peroxides and TBARS in the product that are presumed stable compounds in the digestive system of humans. This is a disadvantage if the presence of peroxides is regarded a health risk (Allard and others 1997). Exp I and Exp II did not reveal the same peroxide pattern but both experiments identified myoglobin/ beef meat juice/ beef meat as responsible for increasing peroxides of 1 type or the other. This is in agreement with previous investigations (Yi and others 2013). However, the diversity in TBARS and polar and unpolar peroxides was much larger for the Exp I sausages than for the beef meat–pork meat sausages, suggesting that the beef meat system acted, relatively speaking, as an antioxidant compared to the beef meat juice–pork meat system, despite the fact that more soluble (polar) lipid degradation products appeared present. The high background PV and TBARS values may actually indicate that the pork fat was also a significant source of peroxides.

Antioxidative property of clove extract

Clove extract has been reported previously as a strong antioxidant; even higher than some often used synthetic antioxidants (Misharina and Samusenko 2008; Wei and Shibamoto 2010). Significant relationships between the reduction of TBARS and the presence of the clove extract, as well as the reductions of different peroxides and the presence of the clove extract, were observed in the present study. Clove extract can significantly inhibit and reduce TBARS and hydroperoxides induced by myoglobin/ beef juice and beef meat addition. Although increased rancidity perception could be discerned by the panelists upon adding fish oil and/or myoglobin, particularly in Exp II, the increase in rancidity detected for sausages with these prooxidants was the same as the reduction in rancidity provided by adding the clove extract. This indicated the strong antirancid effect of clove extract. The clove extract decreased rancidity scores by 20% and 13% in Exp I and Exp II, respectively. Also, it is generally assumed that the polyphenols in the clove extract are poorly absorbed in the digestive system and enter the colon where they may act as antioxidants against reactive oxygen species (Opara and Chohan 2014). As the extract used here was perceived in the sensory test, it will depend on the type of sausage and the desired flavor of the product, combined with consumer preferences, whether clove extract can be used as an antioxidant in sausages.

Addition of different Ca sources

Addtion of calcium to sausages has been implicated in inhibition of colon cancer (Santarelli and others 2013). The mechanism is suggested to be precipitation of hemin in the upper part of the digestive tract (MacKenzie and others 2008). A peroxide-reducing effect could be an additional explanation. Calcium seemed to reduce the amount of both unpolar lipid peroxides. This is also in agreement with previous studies (Brookes and others 2004). There was a tendency that fresh milk addtion would be a better alternative regarding peroxides, when prioritizing the health aspect. Milk calcium addition to sausage is also positive as calcium absorption is enhanced by a n-3 PUFA-rich diet, which is good for health (Kelly and others 2003).

The effect of different additives on reflectance ratio R570/R650

Color differences between the 3 storage conditions were minor. Average values showing the most important design factors are therefore shown in Figure 2. Low values of R570/R650 indicated lower levels of metmyoglobin (MetMb), or higher levels of nitrosomyoglobin (NOMb). Our result may suggest that the presence of fish oil (or unsaturated fatty acids) may actually promote NOMb and reduce nitrosamine formation. Although it is possible to see this as a redox diversification, a possible mechanism should be investigated. The panelists detected the color change with fish oil addition but they could not detect any color change attributed to nitrite and fish oil interactions. Thus, it was only the instrumental method that discovered the interaction between nitrite and fish oil addition.

Conclusion

PUFA and heme as in beef meat juice are causes of hydroperoxide formation in sausages, but their peroxide formation can be counteracted by the addition of clove extract. Beef meat juice/pork meat seemed a stronger prooxidant than beef meat/pork meat. The clove extract added in our sausages had an impact on both flavor and color. Skimmed milk/milk powder with both nutraceutical and flavor impacking potential can be an option to replace $CaCO_3$. However, fresh skimmed milk gave the lowest rancidity and may therefore be the preferred calcium source when making sausages.

Acknowledgments

The authors thank Nortura SA for their support in obtaining pork meat, pork fat, beef meat juice, and beef meat samples. The authors also thank all the panelists for the sensory analysis. Grants NFR184846/I10 from the Research Council of Norway and from the Centre of Advanced Study (2013/2014) supported this work.

Authors' contributions

G. Yi carried out the experiments, wrote the draft of the manuscript, produced, and analyzed data, and revised the manuscript. A. Haug supported preparation of data and writing of the manuscript; B. Nordvi provided input to design, provided raw materials, and participated in writing of the manuscript; K.

Saarem provided raw materials and participated in writing of the manuscript; M. Oostindjer supported presentation of data and writing of the manuscript; Ø. Langsrud designed the experiment and suggested the statistical model to be used; B. Egelandsdal designed and conducted the study, analyzed some data, provided advice and revised the manuscript.

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Paper IV

Commercial Mineral Enhanced Dairy By-products as Sodium Replacers, Antioxidants and Calcium Fortifiers in Sausages

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Total words: 5737

Short version of title: Milk Minerals

Abstract

Presently there are several reasons for targeting the mineral composition of sausages. An important issue is to reduce the sodium content as frankfurter type sausages are regarded as important contributors of sodium in the diet and thereby health risks. Surplus products from the dairy industry are various mineral powders enriched in either potassium or calcium, phosphate and with various amounts of lactose. Sausages were produced at three sodium levels (equivalent to 13, 15 and 17 g NaCl/Kg sausage) using four different milk ingredients (a dried skimmed milk powder, a calcium enriched milk powder, a potassium enriched powder and a lactose enriched powder). The sausages with added calcium and potassium enriched milk powders gave the hardest sausages when compared at the same sodium level. Milk mineral addition also gave whiter and less red sausages. No effect on rancidity after 6 weeks at chill storage was observed by adding milk minerals when compared with adding dried skimmed milk powder. A significant advantage of using these milk minerals in sausages is that the Na:K ratio can be reduced from an unhealthy (in this study 36) to a far more healthier ratio (~2) with limited or no taste changes. High additions of milk calcium (6 g/kg), where Ca-phosphates prevail, added as milk mineral had no influence on sensory bitterness or after taste as typically observed for $CaCl_2$ additions. Ca additions to sausages are presumed an advantage with respect to human colon cancer.

Keywords: milk minerals, sausage, sodium, calcium, potassium

Practical Application: Sausages are presently under critic due to their unhealthy composition. The work presented elucidates how different surplus milk ingredients can be used to improve health status. In particular, potassium and calcium enriched milk minerals can reduce sodium addition and provide healthier Na: K and Ca: P ratio without perceiving the bitter taste of soluble calcium.

Introduction

A high percentage of sodium (approximately 75%) is hidden in processed food. Sodium in NaCl is used for preservation, texturizing and taste enhancer (Kraemer, Oliveira, Gonzalez-Chica, & Proença, 2015). Sausages are often listed as the second most important contributor to sodium, surpassed only by bread. Parr *et al*. (2013) suggested that a high consumption of processed meat, mainly driven by the intake of sausages (≥ 30g/day), was associated with increased risk of diseases like colon cancer in the Norwegian population. Few countries have reached their goal of 5 g NaCl intake per day (or 2 g sodium per day) despite the focus on sodium reduction since the '80s (Sebranek *et al*., 1983).

High sodium intake worldwide is suggested to increase the risk of diseases like hypertension and other cardiovascular diseases (Garza *et al*., 2015; Kim & Lee, 2015). Sodium may increase blood pressure. High blood pressure is the main cause of many cardiovascular diseases. Therefore, it is necessary to use sodium alternatives to improve the sausage receipt and use salt replacers to decrease the total intake of Na (Pietrasik & Gaudette, 2015). NaCl is used to keep the functional and microbial stabilities as well as sensory properties of meat products, and replacement of Na is not always straight forward. A number of commercial salt replacers have been used to create reduced sodium alternatives, mostly based on potassium chloride (KCl) but it is known to have a metallic or bitter aftertaste (Breslin & Beauchamp, 1995). Intake of high potassium and low sodium food such as vegetables and fruits can lower the risk of cardiovascular diseases. High sodium and low potassium were associated with 42 % higher risk of heart disease and a 19 % higher risk of type 2 diabetes (Thorning *et al*., 2015). Therefore, the ratio of Na:K in food is also important to health (Bidlas & Lambert, 2008). A combination of microbial transglutaminase (MTG), KCl, and

$MgCl_2$ can also be used as a suitable sodium replacer in processed meat without compromising sensory properties (Canto *et al*., 2014). Partial replacement of NaCl with $MgCl_2$ and $CaCl_2$ can be done but influences negatively the final flavor if the replacement is too large (Ripollés, Campagnol, Armenteros, Aristoy, & Toldrá, 2011). Barbut *et al*. (1988) produced frankfurters acceptable to a sensory panel using 0.75 % NaCl, 0.75 % KCl and 0.4 % sodium tripolyphosphate, but adding $MgCl_2$ may be restricted for flavour reason (Seman, Olson, & Mandigo, 1980).

Milk minerals could also be a good option as salt replacer. Milk minerals is a by-product of milk and whey processing. Milk and whey are first ultra-filtrated to permeate. Then a combination of lactose crystallization and different membrane filtration technology such as diafiltration, nanofiltration or ion exchange at different pH and temperatures separates molecules to the requested composition (Walstra, Wouters, & Geurts, 2005). Milk minerals consist, among other minerals, of calcium, magnesium, sodium and potassium (Gaucheron, 2005). The milk anions are mostly phosphates, citrate and chloride with phosphates and citrate dominating (Gaucheron, 2005). The lactose level normally determines the mineral level since these are the two major components. Milk minerals have been successfully used in cheese to reduce 80% of the sodium while keeping an acceptable taste (Harju, 2001). Milk Ca can be an alternative to $CaCO_3$ in sausages and alleviate Ca and K deficiency among seniors/elderly in society (Gaucheron, 2005). A dairy ingredient (minimum lactose 47%, 37% minerals; remaining parts being non-protein nitrogen, fat, protein and water) was recently reported to increase the perception of salt flavour in fish pudding (Greiff, Mathiassen, Misimi, Hersleth, & Aursand, 2015). Some commercial powders are marketed as milk minerals since their mineral content dominates (*e.g*. Lactosalt Optitaste – Milk Salt). However, there are still few publications comparing dairy by-

products where milk minerals and lactose are the main constituents with respect to salt perception and nutrition.

Focus on mineral ratio is presently limited in processed meat in particular beyond the Na:K ratio. A Ca:Mg ratio higher than 5:1 or even higher than 4:1 is presently regarded as an imbalanced ratio with risk of impaired bone health and increased susceptibility to heart diseases (Hruby *et al*., 2014). Similarly, the ratio of Ca:P should be between 1:1 to 2:1 in the total diet (NNN, 2012). The aim of this study was: 1) to identify the sodium taste when the sodium content is kept constant but other cations (*e*.*g*. K, Ca and Mg) vary through the addition of dairy by-products that are relatively low in sodium; 2) to identify general sensory properties when Ca is enhanced through the use of the calcium enhanced milk powder to levels suggested protective towards colon health.

Materials and Methods

Raw materials and ingredients

Pork 23% fat, pork 6 % fat, beef 14 % fat and fat tissue were purchased from Kjøttbua A/S (Økern, Oslo). Potato starch, NaCl, $NaNO_2$, nutmeg and white pepper were purchased from Arne B Corneliussen (ABC, Norway). Rape seed oil was from Idun Industri AS (Norway). Five different milk derived powders were used: Skim milk powder (SKM), whey permeate (WP) powder and milk mineral type A (MM-A) were from TINE SA, Norway. Milk mineral type B (MM-B) was from Glanbia Nutritionals, Irland. Milk mineral type C (MM-C) was Lactosalt Optitaste (by Armor Proteines, France) and obtained through Arne B Corneliussen (ABC, Norway).

The SKM powder contained 52.5 % lactose, 4.6 % minerals and 35.5 % protein; the WP powder consisted of 85 % lactose, 7 % minerals and 3 % non-protein nitrogen;

MM-A contained 47 % lactose, 37 % minerals and 5 % non-protein nitrogen; MM-B consisted of <10 % lactose, 77.5 % total minerals, and <7 % protein and MM-C contained 78 % minerals; defined by 30 % potassium, 10 % sodium, 3 % calcium and 0.3 % magnesium and 10 % lactose. MM-C instead of MM-A was used for sensory assessments of solutions. More details are given below.

Design of sausage production

The basic recipe had 10 % total fat and 13 % protein. SKM was added to all productions making the milk protein account for minimum 1.2 % protein in all recipes. Three NaCl levels: 1.3, 1.5 and 1.7 g/100 g were used. The added NaCl was a mixture of real NaCl and Na with other anions that originated from the dairy powders. The concept $NaCl_{equivalents}$ was therefore introduced. At each salt level WP and two milk mineral enhanced powder MM-A and MM-B were used in addition to SKM. Further details can be found in Table 1.

Four samples had only SKM, four had WP, four had MM-A and four had MM-B. For each of the three milk additives, one system was replicated resulting in 16 samples. The primary aim of the recipes was to control Na level since the by-products' capacity for strengthening perceived saltiness should be tested. All by-products were subjected to element analysis (Eurofins, Moss) before the recipes were calculated. Chloride was measured and MM-A was a high Chloride powder, *e.g*. 22 g/100 g. Citrates was not measured since essentially our focus was on cations as replacers for Na.

WP was added up to the amount possible without violating the guidelines for labelling the sausages with the Nordic healthy product code "key hole"; *e.g*. 5 % lactose in the sausage product was a constraint. For MM-A it was decided that 25 % of Na in the products should originate from MM-A. In principle, this was a way of locking the

151 Na:K ratio (see Table 2) to approximately 2. Regarding MM-B, the Ca level was set at
152 0.6 % Ca; *e.g*. content suggested to be relevant for reducing colon cancer incidences
153 in animal models (Santarelli *et al*., 2013).
154 This approach gave us comparable additions of Na as can be seen in Table 1. Pure
155 addition of NaCl was referred to as NaCl addition, but Na originating from added
156 NaCl plus added dairy ingredients were referred to as $NaCl_{equivalents}$. A commercial
157 sausage (Nordfjord) with 10 % fat, 11 % protein, 6 % carbohydrates and 1.7 % NaCl
158 and no dairy ingredients was used as a control for the sensory analysis.
159
160 Processing of sausages
161 The sausages were produced in the pilot plant at TINE R&D (Oslo, Norway). Each
162 recipe was produced in batches of 5 kg. The minces were prepared in a bowl cutter
163 (KILLIA, Neunmünter, Germany). Ingredients were added during chopping in the
164 following order: meat, salt, milk based powders, spices, potato flour and water.
165 Chopping ended at 15 °C. The sausages were produced by heating at 50 °C for 8 min,
166 drying at 55 °C for 15 min, smoking 60 °C for 15 min, cooking at 79-80 °C for 35-40
167 min and chilled in cold water for 10-15 min. The sausages were vacuum-packed
168 (Tecnovac, Confezionatrici Packaging Machines, Grassobio, Italy) and stored at 4 °C
169 until further analysis. On the production day, samples for the oxidation tests were
170 vacuum-packed and frozen at -80 °C as "zero time samples" for later rancidity testing.
171 Fresh samples were chill-stored for 6 weeks before the rancidity testing was carried
172 out.

Table 1- Sausage recipes (kg ingredient/kg sausage batter) containing different dairy additives (1.3 and 1.7 % NaCl equivalents)*.

NaCl Equiv.	WP	MM-A	MM-B	NaCl**	Water
1.3	0	0	0	0.0078	0.304
1.7	0	0	0	0.0118	0.300
1.3	0.038	0	0	0.0072	0.267
1.7	0.038	0	0	0.0112	0.263
1.3	0	0.0102	0	0.0072	0.284
1.7	0	0.0136	0	0.0110	0.277
1.3	0	0	0.0352	0.0078	0.269
1.7	0	0	0.0352	0.0118	0.265

* All the sausage samples also contained equal amount of beef 14 % fat, pork 23 % fat, pork 6 % fat, adipose tissue, rapeseed oil, potato starch, skim milk powder, nitrite salt and spices. **This column indicates what is added as regular NaCl,, the remaining Na comes from the other components including the milk powders.

Preparation of solutions for cation taste maps

Solutions of NaCl (13 g/L), KCl (16.4 g/L) and $CaCl_2$ (12.2 g/ L) were prepared and blended according to a mixture design where the corners were pure salts at a constant molarity of 0.22 mol/L of Cl^-. This was done to erase any taste differences related to anions. The salts were analytical grades dissolved in water of drinking quality. Magnesium was not included as a pure solution in Fig 2, since the magnesium level co-varied with calcium level in MM-B where Ca amounts were the largest, also because bitterness was identified as the most used name both for pure calcium and magnesium chloride solutions. A similar mixture design was made using three milk ingredients where one products corner was high calcium (MM-B), one high potassium (MM-C) and a third was a high lactose powder (WP). The mixture design was a simplex design with the center point triplicated.

Mineral analysis

The data for meat raw materials were taken from Matvaretabellen (a Norwegian food database). The elements of the dairy powders were measured at Eurofins by NMKL No 139 (1991) using atomic absorption spectrophotometry (AAS) after dry ashing. The samples were ashed at 450 °C, under a gradual temperature increase. Hydrochloric acid was added and the solution obtained evaporated to dryness. The residue was dissolved in 0.1 M nitric acid and the metal contents were determined by AAS by flame and graphite procedures. If no milk ingredient was added to sausages, the sausage contained P at 1.1 mg/kg; K at 1.9 mg/kg; Ca at 0.033 mg/kg; Mg at 0.012 mg/kg; Zinc at 0.018 mg/kg and Na at minimum 0.39 mg/kg.

Color measurements

Color measurements were made with a Konica Minolta Chroma Meter CR-400 with illuminant D65. The instrument was calibrated before the measurements according to the instrument's manual. The color measurements (reflection) were done on fresh cross section of the sausages. All color measurements were made with three replications; mean and standard deviations were calculated.

209 Table 2- Composition of selected minerals and lactose of the dairy powders used in sausages at 1.3 % $NaCl_{equivalent}$.

	Meat (g/100 g)			SKM (g/100 g)		WP (g/100 g)		MM- A (g/100 g)		MM-B (g/100 g)	
Element	Beef 14 %	Pork 23 %	Pork 6 %	Powder	Saus-ages	Powder	Saus-ages	Powder	Saus-ages	Powder	Saus-ages
Phosphor (P)	0.160	0.160	0.199	1	0.140	0.65	0.164	0.25	0.142	10	0.492
Potassium (K)	0.290	0.295	0.369	1.8	0.156	1.6	0.217	12	0.278	0.52	0.174
Calcium (Ca)	0.008	0.004	0.005	1.1	0.039	0.48	0.057	0.016	0.039	16	0.602
Magnesium (Mg)	0.019	0.018	0.023	0.12	0.010	0.12	0.015	0.015	0.010	1.3	0.056
Sodium (Na)	0.069	0.056	0.070	0.46	0.52*	0.44	0.52*	2.5	0.52*	0.42	0.52*
Na:K	0.24	0.19	0.19	0.26	3.33	0.28	2.40	0.28	1.87	0.81	2.99
Ca:Mg	0.12	0.22	0.22	9.17	3.90	4.0	3.80	1.07	3.90	12.3	10.8
Ca:P	0.05	0.03	0.03	1.1	0.28	0.07	0.35	0.06	0.03	1.60	1.22
Lactose	0	0	0	52.5	1.77	85	4.9	47	2.4	10	2.1

210 *The sodium level is set at 0.52 % by NaCl (1.3 %) and dairy additives.

211 TBARS measurements

212 The analysis was performed for all control (zero time) samples and samples chill-stored for six
213 weeks after the production. A stock solution of TBA (2-thiobarbituric acid), TCA (trichloroacetic
214 acid) and HCl (hydrochloric acid) was prepared. One gram of minced sausage was weighed and
215 added 10 ml TBA stock solution before it was put in a boiling water bath for 10 min. The
216 samples were then cooled and 1.5 ml of the sample were filled into Eppendorf tubes before being
217 centrifuged for 25 min at 4 °C. The absorbance of the clear solution from the centrifugation were
218 measured at 532 nm. To find the proportion of malondialdehyde equivalents in the sample, the
219 absorbance was divided by the extinction coefficient 1.56*105 M-1 cm-1 and converted into
220 mmol/kg and then to mg/kg (ppm) (Buege & Aust, 1978).

221

222 Texture measurements

223 All samples, and the reference sample were tested one week after production in a Texture
224 Analyzer using compression with a flat probe with diameter 10 cm. Ten samples were measured
225 for each production. Sample cross section was of 2.5 cm. Cross head speed was 1 cm/ sec.

226

227 Sensory analysis

228 A descriptive quantitative sensory analysis (DA) of sausages was carried out 9 days after
229 production of the sausages. The panel consisted of six assessors. The evaluation included 12
230 different attributes: firmness, cohesive, chewing resistance, elasticity, coarseness, solubility, taste
231 intensity, meat taste, saltiness (salt taste), umami taste, after taste and bitter taste. The vocabulary
232 was in accordance with ISO Standard 5492 (ISO, 1992). Each attribute was evaluated on a scale
233 from 1 to 9. Before the session, the panel member participated in a calibration session by

234 evaluating the test extremes in order to agree on the use of attributes and scales. The sausages
235 were served at about 60 °C and in randomized order. The software EyeQuestion (Logic8,
236 Wageningen, Netherlands) was used for analysis setup and data collection.
237 The salt and dairy ingredients solutions were tested by first starting with a pure solutions of
238 $MgCl_2$, $CaCl_2$, KCl and NaCl (all at 17 g/L). The assessors (9) were asked to provide descriptive
239 words for the taste of these solutions. These words were used to organize a second session where
240 $CaCl_2$, KCl and NaCl and their blends were served at constant Cl^- concentration of 0.22 M to 10
241 assessors. Solutions of WP, MM-B and MM-C and their blends as well as these solutions added
242 13 g/L of NaCl was served randomized and assessed for meat flavor, saltiness, bitterness and
243 aftertaste. Meat flavor was included because of the sensory scores on sausages, but it did not
244 differentiate among the dairy solutions and it was therefore ignored.
245
246 Statistics
247 Results from the sensory analysis were evaluated using general linear model (GLM) with
248 assessor and recipe as factors. One-way ANOVA with Tukeys test was used (Minitab version 16)
249 for sensory, texture, color and TBARS measurements.
250 Regression analysis was used to relate mineral content to sensory data. Stepwise regression was
251 used (significance level as defined by α to enter/remove the model was 0.15) with sodium as a
252 fixed variable in each regression model.
253 The mixture designs used for the sensory analysis of solutions, were analyzed by Minitab
254 version16 using either linear or quadratic models.
255
256

Results and Discussion

Sensory properties of sausages fortified with dairy ingredients: flavor and texture

Out of the twelve sensory attributes, four sensory attributes were the most differentiating ones. These were meat flavor, saltiness (salt taste), after taste and chewing resistance (Table 3). Sausages prepared with $NaCl_{equivalent}$ of 1.5 %, were not tested by the sensory panel as these sausages were presumed too close to both the sausages with $NaCl_{equivalent}$ 1.3 % and 1.7 % to be differentiated.

Addition of MM-A and MM-B gave sausages with a higher chewing resistance than the commercial control (Table 3). Chewing resistance correlated with hardness ($R^2 = 0.33$, $P < 0.001$).

Meat flavor was not affected by $NaCl_{equivalent}$ level for any additive. Nevertheless, at average, the meat flavor nominally increased with increased Na content; sausages added MM-A being an exception. However, sausages with 1.7 % $NaCl_{equivalent}$ and added WP had a nominally higher meat flavor than the sausage added 1.3 % $NaCl_{equivalent}$ and MM-B.

Saltiness increased significantly with $NaCl_{equivalent}$ level only for whey permeate and MM-B, but a nominal increase in saltiness was observed for all systems when the Na concentration was increased. The nominally highest saltiness, at 1.3 % $NaCl_{equivalent}$, was obtained for MM-A. MM-A was also significantly more salty than MM-B at 1.3 % $NaCl_{equivalent.}$ This suggested that MM-A had a potential to contribute to a higher perceived salt taste than the other dairy additives. Potassium content was at its highest when MM-A was used (Table 2). MM-B is exceptional in providing a much higher Ca concentrations to the sausages than the other milk by-products (Table 2), despite this, no significant variation in sensory bitterness was found.

After taste was not affected by $NaCl_{equivalent}$ but MM-A had a higher after taste than the sausage with MM-B at 1.3 % $NaCl_{equivalent}$ (Table 3). Sausages added MM-A also had the highest nominal after taste, but the after taste was not significantly higher than the aftertaste reported for the commercial sausage.

Table 3- Selected sensory attributes with SEM of sausages with various milk additives.

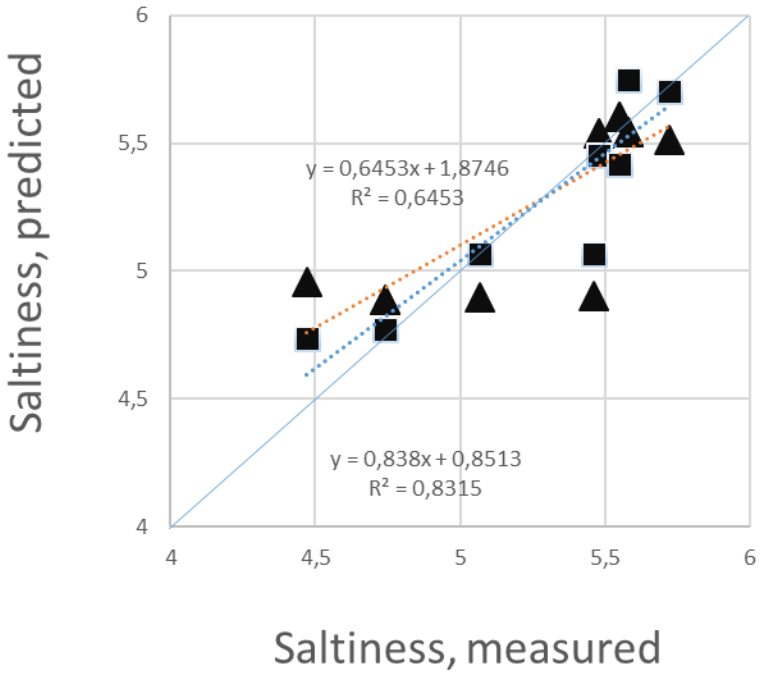
	NaCl* (%)	SKM	WP	MM-A	MM-B	Commercial control**
Meat flavor	1.3	4.10±0.14ab	4.00±0.25ab	4.54±0.12a	3.66±0.27b	4.69±0.14a
	1.7	4.37±0.15ab	4.71±0.22a	4.40±0.16ab	3.98±0.20ab	
Saltiness	1.3	5.07±0.14abc	4.74±0.18bc	5.46±0.14ab	4.47±0.18c	5.35±0.22ab
	1.7	5.58±0.15a	5.48±0.20a	5.72±0.16a	5.55±0.15a	
After taste	1.3	5.80±0.26ab	5.40±0.27ab	6.16±0.21a	5.01±0.19b	6.29±0.29a
	1.7	6.04±0.26ab	5.80±0.26ab	6.40±0.24a	5.63±0.27ab	
Chewing resistance	1.3	5.23±0.10cd	5.15±0.11d	6.02±0.18bc	6.35±0.24ab	5.79±0.44bcd
	1.7	5.80±0.25bcd	5.84±0.28bcd	6.84±0.07a	7.06±0.03a	

*$NaCl_{equivalent}$; Commercial control had 1.7 % NaCl added.

Sensory attributes regressed to element composition

Meat flavor was not significantly affected by the selected cations. Saltiness related to the amount of Na added as NaCl (R^2 = 0.65; P=0.002, general regression, Figure 1). When a stepwise regression was performed (significance level as defined by α to enter/remove the model was 0.15) between saltiness and the minerals listed in Table 2 only sodium (P<0.001), magnesium (P=0.063) and calcium (P=0.089) entered the model. (R^2 = 0.83, P <0.001, general regression,

293 Fig 1). This suggested, as expected, that Na was the major contributor of saltiness. The combined
294 effect of magnesium and calcium in MM-B was to reduce the perception of saltiness. This may
295 explain why MM-B had a lower salt taste than MM-A at 1.3 % $NaCl_{equivalent}$. It may be possible
296 that there is some sequestering of Na to the MM-B compounds and that this reduces saltiness.
297 Sequestering of minerals has been suggested to explain MM's antioxidant properties (Allen &
298 Cornforth, 2007).
299 Potassium did not influence saltiness (P=0.168). This was also observed for phosphate and zinc
300 (not included in Table 3) and lactose. Interestingly, the lower amount of Na from NaCl, the more
301 challenging it was for the panelists to relate saltiness to Na addition from the dairy ingredient
302 only*; e.g*. the triangles in Fig 1 deviated more from the target line than the squares.
303 After taste was affected positively by sodium level (P=0.004), while magnesium had a negative
304 influence (P=0.001) as also observed for saltiness. This result seemed due to the fact that
305 saltiness correlated with after taste (P=0.026, double sided t-test) and was therefore not in itself
306 causal.



307

308 Figure 1- Predicted versus measured sensory saltiness using models including only Na (R^2 =0.65,
309 triangles, dotted red line) and Na, Mg and Ca (R^2 =0.83, cubes, dotted blue line). The target line
310 is drawn as well.

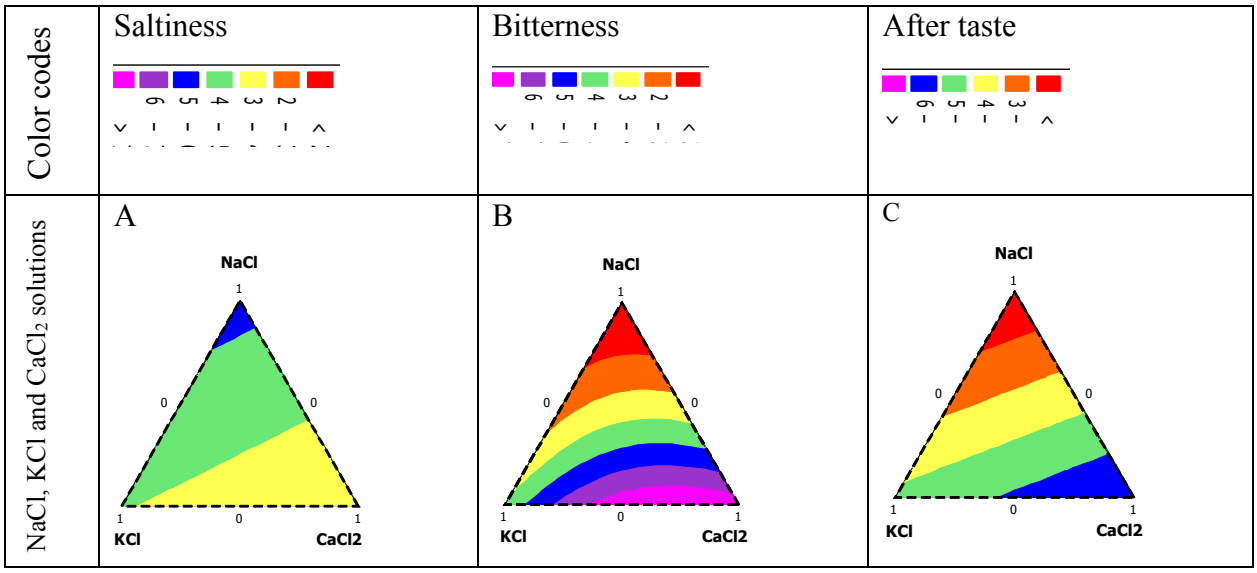
311

312 Sensory perception of pure salt and milk mineral solutions

313 The three pure solutions (MM-A to MM-C, panel 2A-2C) revealed the following saltiness: NaCl
314 > KCl > $CaCl_2$. Bitterness and after taste were ranked as: $CaCl_2$ > KCl > NaCl. Only for
315 bitterness were the interaction terms NaCl*$CaCl_2$ and KCl*$CaCl_2$ significant (*e.g.* *P*=0.023 and
316 *P*=0.001, respectively). These results are largely in agreement with the observation on sausages;
317 *e.g.* Na was the mineral that dominated salt taste.

318 The saltiness of the two mineral salts and the whey permeate (Fig. 2D) was as follows: MM-C >
319 MM-B = WP. MM-C was used instead of MM-A to arrive at a higher milk K level (and lower

lactose level) so that the effect of an enriched milk potassium powder would be clearer. The sensory panelists could not differentiate perceived saltiness of MM-B from perceived saltiness WP (Fig. 2D). The highest concentration of milk K also gave the highest Na concentration in these solutions (Fig. 2D) so both a contribution to saltiness from K and Na in MM-C is likely. As the highest amount of K in the sausages was only 1/3 of the maximum used in Figure 2D, it seems likely that there was a limited effect of adding milk K (or MM-A, Table 3) on saltiness in sausages. Only a nominally higher perceived saltiness was found by increasing MM-A level (Table 1 and 3). However, increasing MM-C more should ultimately increase perceived saltiness. Bitterness was generally low also at high concentration of MM-C (Fig. 2E). However, since the saltiness was nominally higher for the MM-A sausage than the market sausage (Table 3), the use



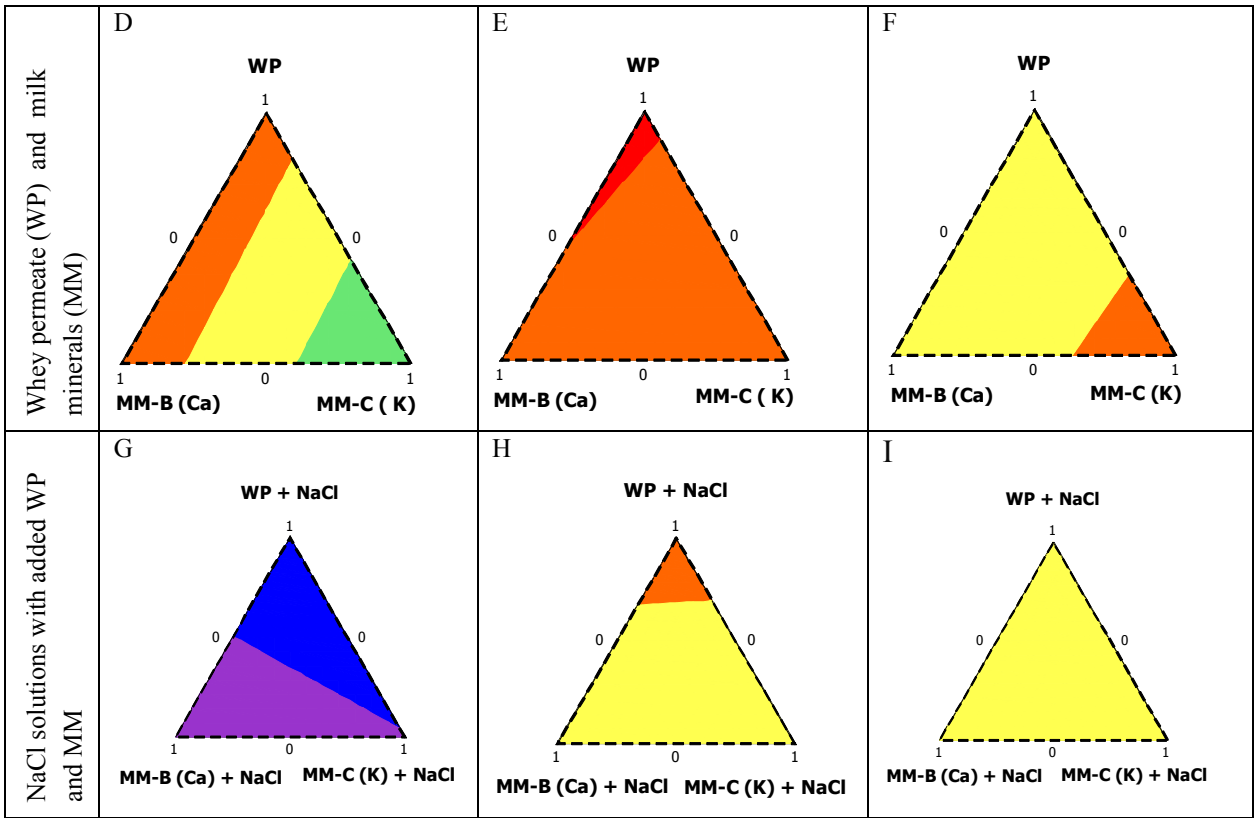


Figure 2- Taste of different blends of NaCl (13 g/L), KCl (16.4 g/L) and $CaCl_2$ (12.2 g/L) solutions (A-C) at a constant molarity of 0.22 M of Cl^-. The following response surfaces had significant slopes: A) P=0.001; B) P= 0.005; C) P < 0.001; D) P < 0.001 while E) - I) had insignificant slopes. WP corner (1): Na=0.5 g/L; K=1.8 g/L; Ca=0.5 g/L; Mg =0.4 g/L; 60 g/L lactose; MM-B corner (1): Na=0.17 g/L; K=0.21 g/L; Ca=6.4 g/L; Mg =0.4 g/L; 4 g/L lactose; MM-C corner (1): Na=2.1 g/L; K=6.4 g/L; Ca=0.6 g/L; Mg =0.4 g/L; 21 g/L lactose. NaCl (13 g/L) was added to the systems in Fig 2D-F to obtain the systems examined in Fig. 2G-2I. MM-B (Ca) and MM-C (K) is used to indicate the dominate anion of the dairy powders in the panels of pure NaCl could be further reduced and replaced with MM-A or MM-C if saltiness is the main targeted attribute. A relevant target would be to reduce the Na:K ratio to 1.5-2.0 (see below).

342 When the lactose content in the powders increases, minerals like K and Ca decrease (Fig. 2D).
343 Sweetness of solutions was commented on by panelist for high lactose milk ingredients. Peters *et*
344 *al*. (2010) has stated that sucrose sweetness tended to be both the least suppressed taste quality
345 and the strongest suppressor of other tastes. Although they did not study lactose, their study
346 favored keeping the lactose content low if saltiness is targeted.
347
348 Fig. 2E and 2F also revealed that the bitterness and after taste showed no increase when milk
349 minerals were used to increase Ca and K. This is due to the fact that milk calcium is not truly
350 soluble and thereby tasteless. Milk potassium is soluble, but its bitterness is lower than that of
351 calcium (Fig. 2A) and therefore it provided an insignificant contribution to bitterness. This is also
352 why a 25 % Na substitution with dairy K may be possible to exceed.
353 Finally, addition of WP and MM-B and MM-C to a solution containing 1.3 % NaCl increased
354 saltiness, but the sensory judges could not differentiate significantly between the different milk
355 ingredients added (Fig. 2G). Similar observations were made for bitterness and after taste. It
356 should be noticed that the aftertaste increased when milk ingredients were added compared to a
357 pure NaCl solution (Fig. 2C and 2I), and this may actual be the upper limitations for their
358 additions.
359
360 Instrumental quality measurements, texture and color
361 The sodium level affected tenderness; the more Na, the higher the peak compression force. This
362 was in agreement with sensory analysis. Texture measurements confirmed that the sausage with
363 MM-B was the firmest (Table 4). The commercial sausage was less hard than our test sausages.

This may in itself not be a problem, as consumers do not easily define an upper constraint for hardness of sausages (Dingstad, Kubberød, Næs, & Egelandsdal, 2005).

Also color was affected by sodium level, but mostly by ingredients. The color coordinates L* and a* were relatively more affected than the color coordinate b*. The sausage with milk minerals was more white (higher L*) and less red (lower a*). However, all sausages with dairy additives were whiter and less red than the commercial sausage. The sausage with WP was most red.

Table 4-Texture, color and TBARS values before and after chill storage of sausages prepared with different dairy ingredients.

Variables		SKM	WP	MM-A	MM-B	Commercial
Peak force (N)	-	33.0±0.7a,*	33.9±0.5a	34.9±0.7ab	36.4±0.6b	24.9±1.7c
Color	L*	66.1±0.6a	66.6±0.6a	67.4±0.3a	71.7±0.3b	63.2±0.8c
	a*	5.7±0.2a	6.4±0.3b	4.9±0.1c	4.0±0.1d	11.4±0.2e
TBARS (mg/kg)	Fresh	0.001±0.005a	0.02±0.01a	0.01±0.01a	0.004±0.01a	Not used
	Stored*	0.012±0.01bc	0.21±0.02d	0.018±0.01c	0.11±0.02b	Not used

WP= whey permeate; MM-A: milk mineral A; MM-B: milk mineral B;* 6 weeks.

Oxidative stability of sausages added dairy ingredients

All systems were low in TBARS before storage. After chill storage for 6 weeks TBARS increased ($P < 0.001$, double sided t-test) but TBARS was not related to added Na neither as pure NaCl (P=0.358) nor as total added Na (P=0.485, regression). Regression to all minerals suggested K, Mg and Ca as influential on TBARS, but stepwise regression only picked out K addition as (positively) linked to TBARS measurements (P=0.044). Zhang *et al*., (2014)

suggested that K, when replaced for Na, increased lipid oxidation, and actually suggested limiting substitution of Na for K to 30 % to reduce lipid oxidation.

Iron was not included in this regression analysis since the dominant form of iron in meat is heme-iron, present at about 2 mg/100 gram meat and this iron is about 20 times more abundant in the meat raw materials compared to dairy additives. The concentration of iron of heme was therefore the same in all sausages.

Milk minerals are reported to act as antioxidants. The mechanism is related to milk phosphates acting as chelators (Allen & Cornforth, 2007). It could also be due to citrate, present at comparable levels to citrate (Gaucheron, 2005) and a known chelator.

There were significant differences between the dairy additives. The whey permeate gave sausages with the higher TBARS values. The sausage with MM-B had nominally the lowest TBARS value. MM-B also had the highest phosphate content (Table 2).

Absolute levels of Na, K, Ca, Mg in sausages

Na: The minimum amount of NaCl to be added to sausages is regarded as 1.4 % (Toldrá, 2008) and then with polyphosphates added. This level (or even 1.3 %, Table 3) can be produced with a saltiness similar to the commercial 1.7 % NaCl without significant changes in sensory properties using MM-A.

K: The average dietary intake of potassium according to European food consumption studies is in the range of 3 to 4 g/day (EFSA, 2006). Since consumption of K above 5 g/day per adult is not recommended, complete replacement of Na with K in the diet is not desirable. The Norwegian

404 public health approach is to reduce Na with 15 % within 2018 and 30 % within 2025
405 (Helsedirektoratet, 2015). Both of these targets may be possible to achieve by potassium
406 enhanced milk minerals as potassium has a sufficient salt flavor.
407 Ca: The recommended daily intakes (RDA) (D-A-CH, 2000; AFSSA, 2001) in EU are between
408 500 - 1300 mg calcium per day depending on target group (NNN, 2012). Main recommendations
409 are 800 mg Ca/day. An amount of Ca of 0.6 g/100 gram sausage can be a declared as being a
410 good source of Ca; an option considered a market advantage. A particular benefit of adding the
411 MM-B to sausages is that calcium addition is regarded as particular relevant to sausages since it
412 is claimed that calcium addition will reduce colon cancer incidences (Santarelli *et al*., 2013). The
413 mechanism is not clear, but investigations point to Ca inducing hemin precipitation in the
414 digestive tract (Santarelli *et a*l., 2013), but Ca has also been suggested to reduce the formation of
415 reactive oxygen species (Yi *et al*., 2015). Only $CaCO_3$ and milk Ca (as phosphate) seem
416 presently to compete for a position as an additive that can be used while at the same time
417 escaping the bitterness of the calcium ion.
418 Mg: Magnesium intake is recommended around 400 mg/day (Hruby *et al*., 2014); somewhat less
419 or more depending on target group. A sausage added MM-B to 0.6 % Ca cannot also be a source
420 of Mg. Magnesium intake has declined in the population since 1900. For the age group > 80
421 years in institutions in Australia, calcium, zinc, potassium and magnesium are all below desirable
422 intake (Iuliano, Olden, & Woods, 2013). There is a general need in the population to increase
423 both magnesium and calcium levels.
424 P: MM-B is, however, associated with the highest phosphate level and phosphate is an element in
425 abundance in human diet. An adult needs 700 mg phosphorous/day (Adatorwovor, Roggenkamp,
426 & Anderson, 2015)

Optimal ratios of Na:K, Ca:Mg and Ca:P in sausages

Na:K: Sausages produced by adding only NaCl to get 1.7 g NaCl /100 g sausage will have a Na: K ratio equal to 36 (0.68/0.019). Using these commercial dairy ingredients, the ratio dropped to 1.87 if MM-A was used since it is a high potassium product (Table 2; 1.3 % $Na_{equivalents}$). This ratio is a significant improvement relative to 36. If the addition of MM-A is increased by adding more MM-A or exchanging more Na, the ratio Na:K could drop even further. Yang *et al*. (2011) examined the health impact of a Na:K ratio between 0.46 - 2.98. The adjusted Hazard ratio (HR, as used in survival analysis) for US respondents was 1.46 (CVD and all-cause mortality, CI 1.27-1.67) when the Na:K ratio was 1.57. With lower Na:K ratio, HR dropped. Further studies should verify how low a Na:K ratio would be accepted using milk ingredients rich in K.

Ca:Mg: The present Ca:Mg ratio in our diet is regarded as too high, albeit the fact that Ca is too low and both these observations are challenging for osteoporosis (Hruby *et al*., 2014). The sausage with added MM-A will not have a good Ca:Mg ratio, but the absolute levels of Ca and Mg are low and thus have less influence on the daily intake of Ca and Mg. MM-B gave sausages high in Ca and Mg and with a Ca:Mg ratio of 10.8 (Table 2). This ratio was not affected by pure NaCl addition. The MM-B addition gave an undesirable Ca:Mg ratio. Actually, the common practice of adding skimmed milk to sausage provides a products with a more suitable Ca:Mg ratio, too (Table 2). However, the imbalance related to the MM-B addition is more relevant since the calcium concentration is much higher. In order to improve this imbalance, magnesium content could be attempted increased in milk minerals or the milk mineral could be combined with the use of magnesium carbonate.

Ca:P: However, more important than the amount of P is the ratio of Ca:P that should be 1:1 to 2:1 in the total diet, *e.g*. MM-B provides a good Ca:P ratio in sausages in particular if sodium phosphates are not used for binding water and to improve texture in sausages, but replaced by fibers (*e.g*. SavorPhos AF200; Formtech Solutions Inc.). Diet among elderly has a too low Ca:P ratio (Adatorwovor *et al*., 2015). A sausage with MM-B would be a good source of phosphorous. Further research is needed to determine an optimal blend of Ca and K enhanced milk mineral powder.

Lactose level and intake.

As shown in Table 2, dairy additives would vary in lactose to mineral ratio. These two compounds being characteristic of dairy by-product components and their ratio should be optimized for specific uses.

Conclusion

Among the three commercial dairy by-products tested (skimmed dry milk being present in all productions), their properties were as follows:

MM-A gave a good Na: K ratio and increased saltiness when added to sausages. Milk K may from a sensory perspective replace more than 25 % of Na added as NaCl. Pro-oxidative properties of potassium enriched milk powders, relative to control, was observed.

MM-B gave sausages with high Ca levels but without bitterness, a too high Ca:Mg ratio (11.4) but a good Ca:P ratio (1.4). MM-B addition reduced meat flavor of sausages when NaCl additions were low. Anti-oxidative properties of MM-B relative to control were observed. Whey permeate also reduced the Na:K ratio of sausages, but contributed little to saltiness. Pro-oxidative

properties, relative to control, were observed for WP. Further optimization of dairy milk minerals should take place to satisfy health needs. All sausages produced with milk minerals were harder, whiter and less red than the commercial low fat sausage variant.

Acknowledgments

Financial support was received from project number 210431/O10 (SALTO). Efforts from Marie Steinslien and Hilde Kraggerud from TINE are appreciated. We also thank the companies Glanbia Nutritionals and Arne B Corneliussen for providing milk minerals.

Author Contributions

Anne Maren Engeloug planned the experiment, took part in the production of samples, did most measurements, preliminary treatment of data and took part in the writing process. Gu Yi defined the experimental hypothesis in a literature context, participated in the experimental part and took part in the writing process. Bjørg Egelandsdal took part in the experimental planning and in the writing process. Anna Haug was responsible for nutrition parts of the work and amended the manuscript. Berit Nordvi planned the experiment and took part in the writing process.

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