FACULTEIT
BIO-INGENIEURSWETENSCHAPPEN

GLYCATION & OXIDATION DURING THE *IN VITRO* DIGESTION OF MEAT

Julie Verbrugge

Student number: 01706032

Promoters: prof. dr. ir. Stefaan De Smet, dr. Thomas Van Hecke

Tutor: Xiaona Tian

Master's Dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of food technology and nutrition

Academic year: 2021 – 2022

UNIVERSITEIT
GENT

Ghent, June 9, 2022

The author,

Julie Verbrugge

The promoters,

prof. dr. ir. Stefaan De Smet

dr. Thomas Van Hecke

The tutor,

Xiaona Tian

ACKNOWLEDGMENT

I would like to thank my supervisors Dr. Thomas Van Hecke and Xiaona Tian for their time and commitment. They provided support where needed at any time. Furthermore, I would also like to specifically thank Xiaona for the extensive cooperation in the lab, the feedback, patience and help during the execution of this thesis. Also Thomas helped me a lot in giving extensive feedback. In addition, I would also like to thank everyone in the lab for the enjoyable working atmosphere and the constant willingness to help me, especially Els, Sabine and Daisy. Without them, this master thesis would not have been possible.

Last but not least, I want to thank my parents, my boyfriend and friends for supporting me through this intense year, with a special thanks to Hannelore for the late night study sessions and help with the coding in LaTeX.

This thesis finalizes a period of five years of studies and I am grateful for everyone I met during these years and who joined me in all the ups and downs of the student life.

ABSTRACT (EN)

High red (processed) meat consumption is associated with the risk to develop several **chronic diseases**, possibly (partly) explained by formation of oxidation, glycation and glycoxidation products during the digestion of meat. **Heating conditions** (temperature and time) and **glucose addition** may stimulate the formation of these potentially harmful products. In this thesis, the effect of heating conditions and glucose addition to pork shoulder on the formation of oxidation, glycation and glycoxidation products during *in vitro* digestion was investigated. Three experiments were executed to eludicate this. Meat samples were prepared and heated in the oven, after which they were subjected to an *in vitro* digestion model. Lipid oxidation products (malondialdehyde), protein carbonyl compounds (PCC) and glycation products (melanoidins) were determined spectrophotometrically, before and after digestion. Glycoxidation products (pentosidine) were determined by high-performance liquid chromatography (HPLC), also before and after digestion. Results showed that the **addition of glucose** and the **increase in heating time** can stimulate **protein oxidation, glycation and glycoxidation** during thermal processing and digestion of pork shoulder. Furthermore, **digestion** of pork shoulder is associated with **more lipid oxidation, glycation and glycoxidation** and therefore potentially harmful substances are formed after red (processed) meat consumption. For protein oxidation, results showed that at gentle and mild heating conditions, whether supplemented with glucose or not, PCC levels increased and were approximately equal after digestion, respectively. However, **extreme heating conditions decreased PCC levels in digests**. This could possibly be explained by further degradation reactions.
Nevertheless, further research is required to determine the extent of association between high red (processed) meat consumption and increased risk on chronic diseases, because our diet contains more than only meat and next to pork, also other meat products (beef, chicken) are consumed, with different heating methods (next to heating in the oven).

Keywords:
Pork shoulder, *in vitro* digestion, oxidation, glycation, glycoxidation

ABSTRACT (NL)

Hoge consumptie van rood (verwerkt) vlees wordt in verband gebracht met het risico om verschillende **chronische ziekten** te ontwikkelen, wat mogelijk (gedeeltelijk) verklaard wordt door de vorming van oxidatie-, glycatie- en glycoxidatieproducten tijdens de vertering van vlees. **Verhittingsfactoren** (temperatuur en tijd) en **glucose toevoeging** kunnen de vorming van deze potentieel schadelijke producten stimuleren. In deze masterproef werd het effect van verhitting en toevoeging van glucose aan varkensschouder op de vorming van oxidatie-, glycatie- en glycoxidatieproducten tijdens de *in vitro* vertering van vlees onderzocht. Er werden drie experimenten uitgevoerd om dit te verduidelijken. Vleesmonsters werden bereid en verhit in de oven, waarna ze werden onderworpen aan een *in vitro* verteringsmodel. Lipide-oxidatieproducten (malondialdehyde), proteïnecarbonylverbindingen (PCC) en glycatieproducten (melanoïden) werden spectrofotometrisch bepaald, voor en na de vertering. Glycoxidatieproducten (pentosidine) werden bepaald met behulp van hoge druk vloeistofchromatografie (HPLC), eveneens vóór en na de vertering. De resultaten toonden aan dat de **toevoeging van glucose** en de **verhoging van de verhittingstijd eiwitoxidatie, glycatie en glycoxidatie** kunnen stimuleren tijdens de thermische verwerking en vertering van varkensschouder. Bovendien gaat de **vertering** van varkensschouder gepaard met **meer lipide-oxidatie, glycatie en glycoxidatie** en daardoor worden potentieel schadelijke stoffen gevormd na de consumptie van rood (verwerkt) vlees. Voor eiwitoxidatie toonden de resultaten aan dat bij lage en gemiddelde verhittingsomstandigheden, al dan niet met toevoeging van glucose, PCC levels toenamen en ongeveer gelijk waren na vertering, respectievelijk. Bij **extreme verhitting reduceerde het PCC-gehalte in de digesten** echter. Dit kan mogelijks worden verklaard door verdere afbraakreacties.

Niettemin is verder onderzoek nodig om de mate van associatie tussen hoge consumptie van rood (verwerkt) vlees en een verhoogd risico op chronische ziekten vast te stellen, omdat onze voeding meer dan alleen vlees bevat en naast varkensvlees ook andere vleesproducten (rundvlees, kip) worden geconsumeerd, met verschillende verhittingsmethoden (naast verhitting in de oven).

Kernwoorden:

Varkensschouder, *in vitro* digestie, oxidatie, glycatie, glycoxidatie

CONTENTS

Acknowledgment i

Abstract (EN) iii

Abstract (NL) v

List of abbreviations ix

1 Introduction 1

2 Literature study 3

2.1 Oxidation . . . 3

2.1.1 Lipid oxidation products . . . 4

2.1.2 Protein oxidation products . . . 7

2.1.3 Health effects . . . 10

2.2 Glycation & glycoxidation . . . 12

2.2.1 Advanced glycation endproducts . . . 12

2.3 Factors influencing oxidation & glycation during digestion of meat . . . 19

2.3.1 Meat composition . . . 19

2.3.2 Processing and storage methods . . . 21

3 Goal & hypotheses 23

4 Material & methods 25

4.1 Experimental design . . . 25

4.1.1 Experiment 1: extreme conditions (5% vs 0% Glu; aerobic; 110°C) 25

4.1.2 Experiment 2: different heating temperatures (3% vs 0% Glu; aerobic vs anaerobic; 80°C vs 130°C vs 180°C) . . . 26

4.1.3 Experiment 3: different heating times (3% vs 0% Glu; aerobic; 180°C;0 vs 20 vs 40 vs 60 vs 80 vs 100 min) . . . 26

4.2 Chemicals . . . 26

4.3 Sample preparation . . . 27

4.4 *In vitro* digestion 28
4.5 Analysis of oxidation products 29
4.5.1 Malondialdehyde 29
4.5.2 Protein carbonyl compounds 31
4.6 Analysis of glycation products 33
4.6.1 Maillard products 33
4.6.2 Pentosidine 33
4.7 Statistical analysis 36

5 Results **37**

5.1 Experiment 1: extreme conditions (5% vs 0% Glu; aerobic; 110°C) 37
5.2 Experiment 2: different heating temperatures (3% vs 0% Glu; aerobic vs anaerobic; 80°C vs 130°C vs 180°C) 40
5.3 Experiment 3: different heating times (3% vs 0% Glu; aerobic; 180°C;0 vs 20 vs 40 vs 60 vs 80 vs 100 min) 44

6 Discussion **47**

6.1 Oxidation 48
6.2 Glycation & glycoxidation 49
6.3 General discussion 51
6.4 Future prospects 52

7 Conclusion **53**

Bibliography **54**

LIST OF ABBREVIATIONS

α-DCs α-dicarbonyl compounds
AAS α-amino adipic semialdehyde
A Absorbance
ABA p-aminobenzoic acid
AD Alzheimer's disease
AGEs Advanced glycation end products
BSA Bovine serum albumin
CHD 1,3-cyclohexanedione
CML Nϵ-carboxymethyllysine
CVD Cardiovascular diseases
DAD Diode array detector
DNPH 2,4-dinitrophynilhydrazine
ELISA Enzyme-linked immunosorbent assay
FLD Fluorescence detector
GC Gas chromatography
GIT Gastrointestinal tract
GGS γ-glutamic semialdehyde
GO Glyoxal
HEX Hexanal
HPLC High performance liquid chromatography
IARC International Agency for Research on Cancer
LC-ESI-MS Liquid chromatography-electrospray ionization-mass spectrometry
LDL Low density lipoprotein
LOP Lipid oxidation products
MDA Malondialdehyde
MOG Methylglyoxal
MOLD Methylglyoxal lysine dimer
MRP Maillard reaction products
MUFA Monounsaturated fatty acids
PCC Protein carbonyl compounds
PD Parkinson's disease
PROP Propanal

PUFA Poly unsaturated fatty acid

RAGE Receptor for advanced glycation end product

ROOH Hydroperoxides

ROS Reactive oxygen species

RR Relative risk

Sens Photosensitizer

SDS Sodium dodecyl sulphate

T2DM Type 2 diabetes mellitus

TBA 2-thiobarbituric acid

TBARs 2-thiobarbituric reactive substances

TCA Trichloroacetic acid

TFA Trifluoroacetic acid

TMP 1,1,3,3-tetramethoxypropane

WCRF World Cancer Research Fund

3-DG 3-deoxyglucosone

4-HHE 4-hydroxy-2-hexenal

4-HNE 4-hydroxy-2-nonenal

LIST OF FIGURES

2.1 Chemical structure of malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE) (Tullberg et al., 2016). 6

2.2 The most common modifications of protein oxidation (Lund et al., 2011a). 8

2.3 Formation of α-amino adipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) in the presence of non-heme iron and hydrogen peroxide, adapted from (Estévez et al., 2009). 9

2.4 Simplified scheme of glycation, adopted from (Akıllıoğlu and Gökmen, 2019). 13

2.5 Generalized pathway for the formation of pentosidine. In addition to arginine and lysine, this scheme includes several possible carbohydrate precursors of pentosidine. Adapted from (Dyer et al., 1991). 14

2.6 A schematic illustration of the structure of the receptor for advanced glycation end product (RAGE) showing that it is composed of an extracelluar portion, a transmembrane portion and an intracytoplasmic tail. The extracelluar portion comprises three domains, V, C1 and C2 (Hegab et al., 2012). 17

4.1 Composition of the simulated digestive juices (/L) used in the *in vitro* digestion model (Van Hecke et al., 2018). 28

4.2 *In vitro* digestion protocol (Van Hecke et al., 2018). 29

5.1 Changes in core temperature of the meat samples in experiment 1 until the core temperature was stable for 10 minutes. The meat samples were heated in the oven at 110°C. 37

5.2 Concentration of the lipid oxidation product, malondialdehyde (MDA) of pork shoulder in experiment 1, expressed in nmol/g (digested) meat, with (green) or without (blue) 5 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation. 38

5.3 Concentration of protein carbonyl compounds (PCC) of pork shoulder in experiment 1, expressed in nmol carbonyls/mg protein, with (green) or without (blue) 5 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation. 38

5.4 Concentration of Maillard reaction products (MRP) of pork shoulder in experiment 1, expressed in nmol melanoidins/g (digested) meat, with (green) or without (blue) 5 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation. 39

5.5 Pentosidine standards 40

5.6 Concentration of the glycoxidation product, pentosidine, of pork shoulder in experiment 1, expressed in μg/kg protein, with (green) or without (blue) 5 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation. 40

5.7 Changes in core temperature of the meat samples in experiment 2 until the core temperature reached 75°C. The meat samples were heated in the oven at either 80°C, 130°C or 180°C. 41

5.8 Concentration of the lipid oxidation product, malondialdehyde (MDA), of pork shoulder in experiment 2, expressed in nmol MDA/g (digested) meat, with (green) or without (blue) 3 % glucose, with (dark coloured) or without (light coloured) oxygen, before and after gastrointestinal digestion. Errorbars represent standard deviation. 41

5.9 Concentration of protein carbonyl compounds (PCC) of pork shoulder in experiment 2, expressed in nmol carbonyls/mg protein, with (green) or without (blue) 3 % glucose, with (dark coloured) or without (light coloured) oxygen, before and after gastrointestinal digestion. Errorbars represent standard deviation. 42

5.10 Concentration of Maillard reaction products (MRPs) of pork shoulder in experiment 2, expressed in nmol melanoidins/g (digested) meat, with (green) or without (blue) 3 % glucose, with (dark coloured) or without (light coloured) oxygen, before and after gastrointestinal digestion. Errorbars represent standard deviation. 42

5.11 Concentration of the glycoxidation product, pentosidine of pork shoulder in experiment 2, expressed in μg/kg protein, with (green) or without (blue) 3 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation. 43

5.12 Picture of the different meat samples after heating in the oven at 180°C at different heating times (experiment 3). Bag 1 and 2 = samples heated for 20 minutes; 3 and 4 = samples heated for 40 minutes; 5 and 6 = samples heated for 60 minutes; 7 and 8 = samples heated for 80 minutes; 9 and 10 = samples heated for 100 minutes. 44

5.13 Concentration of the lipid oxidation product, malondialdehyde (MDA), of pork shoulder in experiment 3, expressed in nmol MDA/g (digested) meat, with (dark blue) or without (light blue) 3 % glucose, before and after gastrointestinal digestion. Errorbars represent standard deviation. Data was statistically analyzed using a linear ANOVA with fixed factors: glucose content (PGlu), heating time (PTime) and the interaction (PInteraction), where $P < 0.05$ indicates significant differences. Different letters (a-c) indicate significant difference ($P < 0.05$) between different heating times within the same glucose level. Asterix (*) indicates significant difference ($P < 0.05$) between different glucose levels within the same heating time. 45

5.14 Concentration of protein carbonyl compounds (PCC) of pork shoulder in experiment 3, expressed in nmol carbonyls/mg protein, with (dark blue) or without (light blue) 3 % glucose, before and after gastrointestinal digestion. Errorbars represent standard deviation. Data was statistically analyzed using a linear ANOVA with fixed factors: glucose content (PGlu), heating time (PTime) and the interaction (PInteraction), where $P < 0.05$ indicates significant differences. Different letters (a-b) indicate significant difference ($P < 0.05$) between different heating times within the same glucose level. Asterix (*) indicates significant difference ($P < 0.05$) between different glucose levels within the same heating time. 45

5.15 Concentration of Maillard reaction products (MRPs) of pork shoulder in experiment 3, expressed in nmol melanoidins/g (digested) meat, with (dark blue) or without (light blue) 3 % glucose, before and after gastrointestinal digestion. Errorbars represent standard deviation. Data was statistically analyzed using a linear ANOVA with fixed factors: glucose content (PGlu), heating time (PTime) and the interaction (PInteraction), where $P < 0.05$ indicates significant differences. Different letters (a-c) indicate significant difference ($P < 0.05$) between different heating times within the same glucose level. Asterix (*) indicates significant difference ($P < 0.05$) between different glucose levels within the same heating time. 46

5.16 Concentration of the glycoxidation product, pentosidine, of pork shoulder in experiment 3, expressed in μg/kg protein, with (dark blue) or without (light blue) 3 % glucose, before and after gastrointestinal digestion. Errorbars represent standard deviation. Data was statistically analyzed using a linear ANOVA with fixed factors: glucose content (PGlu), heating time (PTime) and the interaction (PInteraction), where $P < 0.05$ indicates significant differences. Different letters (a-d) indicate significant difference ($P < 0.05$) between different heating times within the same glucose level. Asterix (*) indicates significant difference ($P < 0.05$) between different glucose levels within the same heating time. 46

LIST OF TABLES

2.1 Overview of structure and type of important AGEs. 13

2.2 Comparison of different techniques for detecting AGEs based on Sun et al. (2012); Nomi et al. (2016); Milkovska-Stamenova et al. (2015); Fang et al. (2014); Matsui et al. (2015). 15

4.1 Overview of experiment 1 set-up. 25

4.2 Overview of experiment 2 set-up. 26

4.3 Overview of experiment 3 set-up. 27

4.4 Specific composition of the standard solutions used in the detection of MDA. 30

4.5 Specific composition of the standard solutions of pentosidine used in the detection of pentosidine. 34

4.6 Specific composition of the BSA standard solutions used in the detection of pentosidine. 34

CHAPTER 1

INTRODUCTION

The World Cancer Research Fund (WCRF) recommends to consume very little to no processed meat [1] and to limit the consumption of red meat [2] to no more than about three portions per week. This is equivalent to about 350-500 g cooked weight (WCRF, 2018). These recommendations are largely based on epidemiological studies suggesting that a high consumption of red or processed meat is associated with the risk to develop chronic diseases such as colorectal cancer, cardiovascular disease or diabetes. Underlining this, the International Agency for Research on Cancer (IARC) concluded that per 50 gram increase in daily processed meat consumption, the relative risk (RR) on colorectal cancer increased by 18%, which is a relatively small increase (Boada et al., 2016). Mechanisms to explain these observations are not clear yet and many different mechanisms have been proposed (e.g. nitrosamines, oxidation products, heterocyclic amines, etc), but none can fully explain (Demeyer et al., 2016). Although these risks are small, they could be important for public health because many people worldwide eat meat and meat consumption is rising in low- and middle-income countries (Domingo and Nadal, 2017).

In this literature study, glycoxidation, a combination of glycation and oxidation, is investigated as a mechanism in the possible link between several chronic diseases and the consumption of red (processed) meat because glycoxidation is considered as harmful for human health (Poulsen et al., 2013). An overview will be given on what is known so far about the formation of the different glycation and oxidation products during heating of meat and during its subsequent gastrointestinal digestion. In addition, their contribution to chronic diseases will be discussed more in detail, together with their analytical methods. Important to know is that different food ingredients (sugar, protein, fat, water, etc.), storage methods (storage temperature, time, etc.) and processing methods (steaming, boiling, frying, baking, roasting, etc.) affect the formation of glycation and oxidation products.

[1] Processed meat is meat that has been transformed through salting, curing, fermentation, smoking or other processes to enhance flavour or improve preservation. e.g ham, salami, bacon, etc (WCRF, 2018).

[2] Red meat is all types of muscle meat from a mammal, including beef, veal, pork, lamb, mutton, horse and goat (WCRF, 2018).

CHAPTER 2

LITERATURE STUDY

2.1 Oxidation

Oxidation in food is a process that concerns both lipids and proteins by the effect of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$). Oxidation is inherent to metabolism, but an excessive formation of ROS can cause damage to vital components in biological systems. Studies confirm that historically, the focus in food was on lipid oxidation rather than on protein oxidation, not only because it is one of the primary mechanisms of quality deterioration in food, but also because lipid oxidation products (e.g. hydroperoxides, hexanal and malondialdehyde) can be measured more easily than protein oxidation products (Zhang et al., 2013).

Meat and meat products are very complex matrices and depending on their composition, susceptible to the oxidation process. Both the fat, protein, free and bound iron in meat can participate in oxidation processes (Van Hecke et al., 2017b). During the self-maintaining Fenton reaction, highly unstable ROS are formed by the oxidation of Fe^{2+} to Fe^{3+} (Equation 2.1) and its reduction back to Fe^{2+} (Equation 2.2). This reaction is catalyzed by hydrogen peroxide (H_2O_2) and the ROS produced are a hydroxyl radical ($HO\cdot$) and a hydroperoxyl radical ($HOO\cdot$), respectively:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO\cdot + OH^- \tag{2.1}$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HOO\cdot + H^+ \tag{2.2}$$

These radicals can initiate a chain of oxidative reactions like modifications to n-3 and n-6 polyunsaturated fatty acids (PUFAs), which lead to the formation of cyto- and genotoxic lipid oxidation products (LOPs), including malondialdehyde (MDA), 4-hydroxy-2-hexenal (4-HHE), and 4-hydroxy-2-nonenal (4-HNE). The ROS and LOPs produced also contribute to protein oxidation, leading to the formation of protein carbonyl compounds (PCC) (Smet et al., 2008). In the following sections, lipid oxidation

and protein oxidation together with their mechanisms, analytical methods and their health effects are briefly discussed.

2.1.1 Lipid oxidation products

Lipids are responsible for many desirable characteristics of meat and meat products as they influence the flavour and contribute to the improvement of tenderness and juiciness of meat. However, PUFAs are susceptible to oxidation, which not only lead to a reduction of nutritional value and sensory quality of meat, but lipid oxidation also results in the production of multiple toxic compounds. Several studies conclude that one of the most important problems of lipid oxidation is the generation of these harmful compounds that implicate several human pathologies, including colorectal cancer and diabetes, among others (Domínguez et al., 2019).

Chemistry

Lipid oxidation in muscle (meat) is a very complex process and starts from the moment the animal is slaughtered and continues during processing, storage, culinary preparation and gastrointestinal digestion of the meat. Due to biochemical changes in the muscle tissue after slaughtering, the cell does not have a defense mechanism against ROS anymore, so oxidation is promoted. Unsaturated fatty acids can be oxidized in several pathways that interact with each other. Autoxidation, enzymatically catalysed oxidation and photosensitized oxidation are three major mechanisms in lipid oxidation (Domínguez et al., 2019).

Autoxidation is the most important process of lipid oxidation in meat. This process happens in three phases, the initiation, propagation and termination (Domínguez et al., 2019). The initiation occurs by light, heat or the presence of trace metals (Hematyar et al., 2019). In this phase hydrogen is abstracted from an unsaturated fatty acid (RH) due to the reaction with a ROS like $OH\cdot$ (Equation 2.3) (Domínguez et al., 2019).

$$RH + OH\cdot \longrightarrow R\cdot + H_2O \tag{2.3}$$

Polyunsaturated fatty acids (PUFAs) are more susceptible to oxidation than monounsaturated fatty acids (MUFAs), since the removal of a hydrogen atom becomes easier as the number of double bonds in the fatty acid increases (Domínguez et al., 2019). The initiation leads to alkyl radicals ($R\cdot$) which react with molecular oxygen (O_2) in the propagation phase, resulting in unstable peroxyl radicals ($ROO\cdot$) (Equation 2.4).

These radicals are highly reactive and can, in turn, abstract hydrogen from an adjacent fatty acid to form primary oxidation products, hydroperoxides (ROOH) (Equation 2.5) (Domínguez et al., 2019).

$$R\cdot + O_2 \longrightarrow ROO\cdot \tag{2.4}$$

$$ROO\cdot + RH \longrightarrow ROOH + R\cdot \tag{2.5}$$

In contrast to other lipid-derived products, hydroperoxides are odourless and do not contribute to any aroma. However, these compounds are highly unstable, so they decompose rapidly resulting in a large number of secondary products such as hydrocarbons, aldehydes, ketones, alcohols, esters and acids, which cause the appearance of off-flavours and off-odours in meat (Domínguez et al., 2019). The termination of this chain reaction takes place when radicals react with each other (Equation 2.6 or 2.7) or with other non-radical compounds (antioxidants) to give rise to stable low-reactive products (Domínguez et al., 2019).

$$ROO\cdot + ROO\cdot \longrightarrow [LOOOOL] \longrightarrow non{-}radical products + O_2 \tag{2.6}$$

$$R\cdot + R\cdot \longrightarrow RR \tag{2.7}$$

A second important mechanism of lipid oxidation is the photosensitized oxidation. The fact that meat and meat products are directly exposed to light in the supermarket promotes this photo-oxidation process. During this process, an unsaturated fatty acid is exposed to light and a photosensitizer (Sens) such as myoglobin or hemoglobin. By this non free radical process, oxygen in the triplet ground state ($^3O_2$) becomes activated to the singlet state ($^1O_2$) by a transfer of energy from the excited photosensitizer (Sens*) (Equation 2.9). The resulting singlet oxygen is extremely reactive and oxidizes the fatty acid (RH) to a hydroperoxide (ROOH) (Equation 2.10) (Frankel, 1984). According to Frankel (1984) linoleate is reported to react at least 1500 times faster with singlet O_2 than with normal oxygen in the triplet ground state.

$$Sens \longrightarrow Sens\cdot(excited) \tag{2.8}$$

$$Sens\cdot + {}^3O_2 \longrightarrow {}^1O_2 + Sens \tag{2.9}$$

$$^1O_2 + RH \longrightarrow ROOH \tag{2.10}$$

The third mechanism for lipid oxidation is an enzymatic catalyzed oxidation in which, among other things, lipoxygenase catalyzes the formation of hydroperoxides in meat.

It should be noted that a high enzyme concentration favours the oxidation process. In order for this process to happen, the iron present on the active site of the enzyme must be in ferrous form. When this active site is activated, hydrogen is abstracted from an unsaturated fatty acid and reacts, in turn, with molecular oxygen, resulting in a peroxyl radical. This radical removes hydrogen from another unsaturated fatty acid to form hydroperoxides (Domínguez et al., 2019).

All three mechanisms of lipid oxidation lead to secondary oxidation products that are potentially toxic. The predominant harmful products derived from lipid oxidation include MDA, 4-HHE and 4-HNE. Figure 2.1 shows their chemical structure.

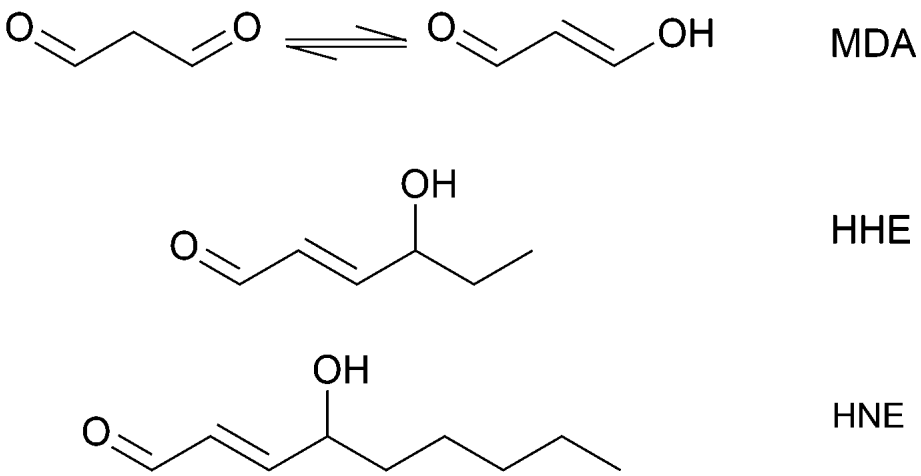

Figure 2.1: Chemical structure of malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE) (Tullberg et al., 2016).

4-HNE, but also hexanal (HEX) are lipid aldehydes originating from n-6 PUFA oxidation such as linoleic acid (18:2n-6) and arachidonic acid (20:4n-6), whereas 4-HHE and propanal (PROP) originate from n-3 PUFA oxidation such as docosahexaenoic acid (22:6n-3). MDA can originate from both n-3 and n-6 PUFAs and results mainly from the oxidative degradation of PUFAs with more than two methylene-interrupted double bonds. In mammalian tissue the precursors for MDA will therefore be mainly the PUFAS 20:4n-6 and 22:6n-3 (Esterbauer et al., 1991).
The toxicity of these lipid aldehydes is mainly due to their potency to covalently modify proteins, but also includes loading of low density lipoprotein (LDL) with 4-HNE, reactions with DNA or reactions with insulin. While 4-HNE is highly toxic, the toxicity of 4-HHE is however less well documented and more typical for fish (Long and Picklo Sr, 2010). Despite that both 4-HNE and 4-HHE have similarities in their chemical structure, 4-HHE is regarded as less damaging and reactive due to its low lipophilicity and reduced chemical reactivity. Depending on their concentration, both toxic and beneficial effects of 4-HNE and 4-HHE have been reported (Van Hecke et al., 2019). MDA also has some toxicity, but this is normally lower than 4-HNE (Michiels and Remacle, 1991). According to Esterbauer et al. (1991) the cytotoxicity increased with increasing

chain length of the aldehydes, which indicates that additional to the general structure the lipophilicity is critical for the cytotoxic properties of an aldehyde.

Analysis

Measurement of the primary oxidation products (hydroperoxides) gives reliable information about the oxidation state during the early phases. In situations where oxidation occurs at an accelerated rate, as in cooked meat and meat products, primary products rapidly decompose to stable secondary products. So measurement of secondary products is usually more appropriate as an index of lipid oxidation in meat and meat products (Domínguez et al., 2019).

The most commonly used procedure for assessing lipid oxidation is the 2-thiobarbituric acid (TBA) test. This test is often criticized due to its lack of specificity and its high inaccuracy, since TBA reacts not only with MDA, but also with multiple other (oxidation) products resulting in considerable overestimation as well as variability of the results (Hoyland and Taylor, 1991). Therefore, TBA is also referred to as TBARs (TBA reactive substances). However, the TBA method is still widely used in evaluating meat oxidative stability, because it is simple and offers considerable sensitivity and versatility for detecting LOPs. In this test, MDA reacts with TBA to form a coloured complex, which can be quantified with a UV-Vis spectrophotometer. Section 4.4.1 gives a detailed description of this procedure.

Next to the TBA-test, a more specific measurement of 4-hydroxy-2-alkenals (e.g. 4-HNE and 4-HHE) and alkanals can be done by high performance liquid chromatography (HPLC) analysis of fluorescent CHD-aldehyde derivatives. In this HPLC method, aldehydes, formed through lipid oxidation, react with 1,3-cyclohexanedione (CHD) to form fluorescent decahydroacridine derivatives. These derivatives are separated by HPLC with gradient elution and the derivatized aldehydes were detected by a fluorescence detector (Esterbauer and Zollern, 1989). Details of this procedure are described in section 4.4.1.

2.1.2 Protein oxidation products

The exposure of food to ROS does not only cause lipid oxidation but also protein oxidation. Protein oxidation is defined as the covalent modification of a protein caused either by the direct reactions with ROS or indirect reactions with LOPs (Hematyar et al., 2019). However, further research is still needed to gain a better and complete understanding in protein oxidation because these reactions are not yet fully understood.

Chemistry

The major mechanisms in protein oxidation are still unclear because there are only a few, mostly non-specific, methods available to evaluate protein oxidation. The second reason is the large variety of protein oxidation products. This is because a protein molecule contains several sites that are susceptible to oxidation (protein backbone, amino acid side chains). In general, protein oxidation proceeds like lipid oxidation via a chain reaction of radicals. In the initiation phase, a hydrogen atom is abstracted from the protein (PH) due to the reaction with a ROS like $OH\cdot$, to generate a carbon-centred radical ($P\cdot$) (Equation 2.11). In the presence of oxygen this radical is converted to a peroxyl radical ($POO\cdot$) (Equation 2.12). Then, another hydrogen atom is, in turn, abstracted from another protein molecule with the formation of a new carbon-centred radical and an alkyl radical (POOH) (Equation 2.13) (Estévez, 2011).

$$PH + HO\cdot \longrightarrow P\cdot + H_2O \tag{2.11}$$

$$P\cdot + O_2 \longrightarrow POO\cdot \tag{2.12}$$

$$POO\cdot + PH \longrightarrow POOH + P\cdot \tag{2.13}$$

Subsequent reactions lead to the formation of the alkoxy radical ($PO\cdot$) and hydroxyl compounds (POH). In addition, two carbon-centred radicals can react with each other in the absence of oxygen to generate carbon–carbon cross-linked derivatives (Hematyar et al., 2019). Protein oxidation leads to multiple modifications of proteins where cleavage of peptide bonds, protein cross-linking and amino acid side chain modification are the most common, as shown in Figure 2.2 Crosslinks are mainly disulfide bonds and dityrosine formation due to the loss of cysteine and tyrosine residues, whereas amino acid modifications give rise to carbonyl groups (aldehydes and ketones) and protein hydroperoxides.

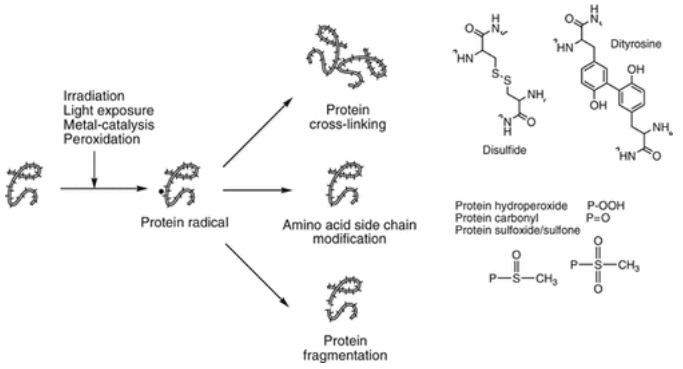

Figure 2.2: The most common modifications of protein oxidation (Lund et al., 2011a).

To conclude, protein oxidation leads to the formation of carbonyls and the loss of thiol groups. The more carbonyls and the fewer thiol groups, the more protein oxidation has occurred (Zhang et al., 2013). The predominant products derived from protein oxidation are the protein carbonyls, among which α-amino adipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS), are oxidation products of lysine, and of arginine and/or proline, respectively. According to Estévez et al. (2009) these amino acids are oxidized in the presence of ferric iron (Fe^{3+}) and H_2O_2 to yield AAS and GGS (Figure 2.3). They account for up to 70 % of the total amount of protein carbonyls in an oxidized meat product (Estévez, 2011). Nevertheless, the mechanisms involved in the formation of these semialdehydes in muscle foods are poorly understood. Both semialdehydes are known to be formed in the presence of glucose, various transition metals, and hydrogen peroxide (Requena et al., 2001). The metal-catalyzed oxidation of proteins as well as the Maillard reaction might be the main formation routes of AAS and GGS in meat samples. It should be noticed that the formation of these semialdehydes does not require a previous cleavage of the peptide bond as protein-bound amino acids can be degraded into their corresponding semialdehydes.

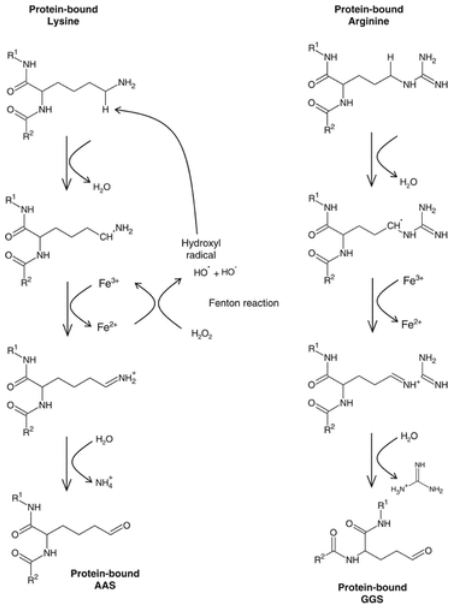

Figure 2.3: Formation of α-amino adipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) in the presence of non-heme iron and hydrogen peroxide, adapted from (Estévez et al., 2009).

Analysis

Methods to measure total carbonyl derivatives as an index of protein oxidation in meat and meat products are still limited as protein carbonyls can be formed by unspecific various pathways including metal-catalyzed oxidation of specific amino acid side chains and the addition of sugars or LOPs such as 4-HNE and MDA (Estévez et al., 2008b). Therefore, more specific and advanced methods are required for the determination of carbonyl derivatives to understand their chemical structure and pathway of formation from specific amino acids. However, the quantification of protein carbonyls through the 2,4-dinitrophynilhydrazine (DNPH) derivatization method has been the most widespread method for assessing protein oxidation in meat products. This method is described as robust, convenient and accurate. In this method, DNPH reacts with the carbonyl groups of proteins to generate orange-colored dinitrophenylhydrazones with characteristic absorbance maxima at 370 nm (Levine et al., 1994). Details of this procedure are described in section 4.4.2. An alternative unspecific technique for evaluating protein oxidation uses fluorescence spectroscopy. In this technique, the compounds formed as a result of the reactions between LOPs (aldehydes) and amino groups from proteins are conjugated fluorophores with spectral properties, which may be detected by recording fluorescence at around 450 nm when excited at 350 nm, and therefore used as a protein oxidation index (Estévez et al., 2008a).
Requena et al. (2001) and Sun et al. (2012) developed several methods based on GC and HPLC, for the determination of specific protein oxidation products, AAS and GGS, in meat samples. In this HPLC method, the aldehydic residues in proteins were derivatized with $NaCNBH_3$ and p-aminobenzoic acid (ABA), a fluorescence reagent, after which ABA-AAS and ABA-GGS were measured by fluorometric HPLC. More recently, Estévez et al. (2008c) detected these semialdehydes by derivatization with ABA using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). According to Estévez et al. (2009) the DNPH measurements were significantly correlated with the amount of AAS and GGS determined by LC-ESI-MS. The strong correlation supports that both AAS and GGS can be representative of the total amount of carbonyl contents and thus can be used as the markers for the protein oxidation in meat systems.

2.1.3 Health effects

Oxidation may contribute to the development and/or progression of colorectal cancer, cardiovascular disease and diabetes in several ways. Furthermore, these diseases can also promote the development and/or progression of other diseases, e.g hyper-

glycemia[1] and hyperinsulinemia[2] are risk factors for colorectal cancer (Giovannucci and Michaud, 2007). The role played by oxidized proteins or lipids in disease pathogenesis is usually linked to the cytotoxicity and mutagenicity potential of these components on the gastrointestinal tract (GIT) and other organs (Van Hecke et al., 2017b). Especially under severe oxidative stress conditions in which the defense mechanisms of the body cannot regain prooxidants/antioxidants balance, the oxidants and free radicals can cause extensive cellular damage and even cause cell death (Davies, 2000).

A first possible mechanism promoting the development of colorectal cancer is DNA damage by adduct formation. ROS and LOPs, coming from high red and processed meat consumption are thought to induce this process, leading to genotoxic effects (Łuczaj and Skrzydlewska, 2003). Although high red and processed meat consumption could lead to a higher risk of developing colorectal cancer, their consumption does not promote cancers of the small intestine. According to Hemeryck and Vanhaecke (2016) and Nair et al. (2007), the formed LOPs are at least partly absorbed before they reach the colon and they may also be degraded or metabolized in the colon itself. Furthermore, the colon mucosa may be exposed to these toxic compounds for a longer time than the small intestine mucosa, as their retention time is longer in the large intestine.

As already discussed in the previous sections, oxidation of proteins is stimulated by the oxidation of lipids during processing or digestion of meat products. Soladoye et al. (2015) indicated that relatively mild protein oxidation leads to a higher susceptibility to protease, whereas intense protein oxidation reduces their in vitro digestibility. Therefore, protein oxidation may result in a higher amount of protein reaching the colon, subsequently leading to increased formation of potentially harmful protein fermentation products. Next to reduced digestibility, protein oxidation also leads to reduced bioavailability and to the loss of essential amino acids. The underlying mechanisms include physicochemical modifications of proteins mediated by ROS or LOPs, leading to impaired functionality and resistance of oxidized proteins to proteolysis (Stadtman and Levine, 2000).

The big challenge in the field of pathological processes is that it is often difficult to determine whether these oxidation products are actually involved in causing the disease or are a consequence of it.

[1] Hyperglycemia is the technical term for high blood glucose (blood sugar). High blood sugar happens when the body has too little insulin or when the body can not use insulin properly (hyp, nd).

[2] Hyperinsulinemia is a condition in which there are excess levels of insulin circulating in the blood in relationship to its usual level relative to blood glucose (Thomas et al., 2019).

2.2 Glycation & glycoxidation

Glycation is often used in food processing to improve many food quality parameters such as color, sensorial properties, textural properties and protein functionality. However, this process is often accompanied by the formation of some potential chemical hazards, like advanced glycation end products (AGEs). Recent studies found that the accumulation of AGEs in the body can cause oxidative stress or nerve cell damage (Gupta et al., 2018). Therefore, glycation is closely linked with oxidation and the combination is often referred to as “glycoxidation”. Glycoxidation is believed to be involved in the development of several chronic diseases, among which especially diabetes, but also cardiovascular and cerebrovascular diseases, and even cancer (Singh et al., 2001).

2.2.1 Advanced glycation endproducts

Although not all AGEs have been identified and the underlying mechanism of glycation is still not fully known, AGEs exist in the body as well as in food. They are called endogenous and exogenous AGEs respectively (Huang et al., 2021). In addition, AGEs can also be classified into free and bound states. Free AGEs are combinations between carbonyls and free amino acids, whereas bound AGEs are combinations between carbonyls and proteins or polypeptides (Zhao et al., 2019). Due to the availability of a wide range of amino acids and a variety of carbonyl compounds, the analysis, structures and definition of AGEs are not completely understood. In the following sections, advanced glycation end products together with their mechanisms, analytical methods and their health effects are briefly discussed.

Chemistry

Glycation refers to a spontaneous non-enzymatic reaction of free reducing sugars such as glucose or fructose with free amino groups in proteins, lipids and nucleic acids. This process (Figure 2.4), also known as the Maillard reaction, takes place in three stages, the early, intermediate and advanced stage.

In the early stage, glycation is initiated by the nucleophilic addition between the amino group from a protein and a carbonyl group from a reducing sugar. This leads to the production of a relatively unstable compound, known as a “Schiff base”, which is formed relatively quickly. This step is reversible but the Schiff base can turn into an reversible and more stable compound called an “Amadori product”. After this, the

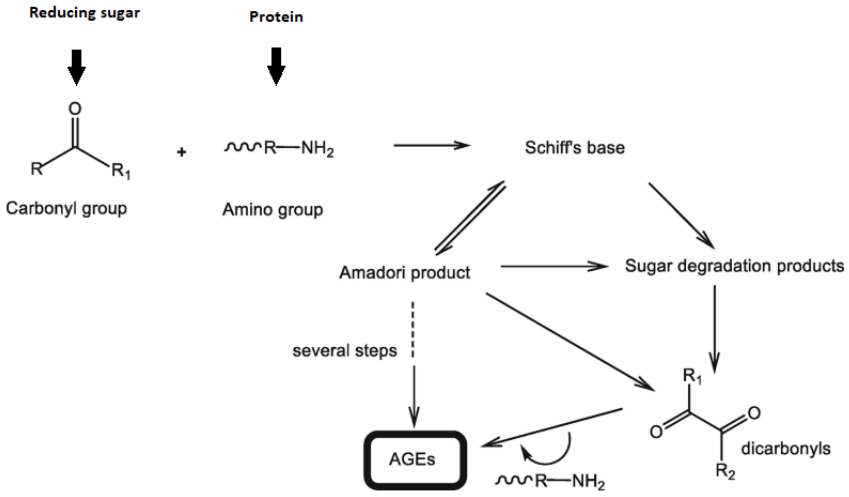

Figure 2.4: Simplified scheme of glycation, adopted from (Akıllıoğlu and Gökmen, 2019).

Table 2.1: Overview of structure and type of important AGEs.

Compound	**Fluorescence**	**Cross-linking**	**Glycation or Glycoxidation**
Nϵ-carboxymethyl lysine (CML)	no	no	Glycoxidation
Crossline	yes	yes	Glycation
Methylglyoxal lysine dimer (MOLD)	no	yes	Glycation
Pentosidine	yes	yes	Glycoxidation
Pyrraline	no	no	Glycation

intermediate stage takes place where dicarbonyl compounds are formed by the degradation of Amadori products[3]. Dicarbonyl compounds are very reactive so they react immediately with the side chains of peptides and proteins in the advanced stage. This leads to the formation of highly stable AGEs (Akıllıoğlu and Gökmen, 2019). Table 2.1 gives an overview of the most important AGEs available.

The oxidative degradation of Amadori products leads, *in vitro*, to the formation of glycoxidation products pentosidine and CML (Grandhee and Monnier, 1991). These products are not specific to glucose, so also other carbohydrates, like fructose or pentose, are able to act as possible precursors of glycoxidation products (Dyer et al., 1991). Furthermore, ascorbate, which oxidizes in the presence of free metals into dehydroascorbate, undergoes further decomposition to form pentoses and tetroses, which can also serve as pentosidine precursors (Nagaraj et al., 1991). Pentosidine and CML are specifically called “glycoxidation” products as oxidation is required at

[3]According to Jakas and Horvat (2003), the major factors that influence the rate and extent of the Amadori product degradation are temperature and water activity as well as the proportion of the acyclic (active form) available under the reaction conditions. This study also showed that the shorter the peptide chain, the more degradation products are formed.

some stage in the formation of these products, so they can not be formed without oxygen (Dyer et al., 1991). Figure 2.5 gives a generalized pathway for the formation of pentosidine.

Pentosidine was discovered in 1991 as a fluorescent protein cross-link between lysine and arginine, mainly through a ribose, in an imidazo [4,5-b] pyridinium ring (Grandhee and Monnier, 1991). Because pentosidine is stable under the conditions used for acid protein hydrolysis and can be detected at very low concentrations based upon its fluorescence properties, it can be regarded as a biomarker for AGEs (Grandhee and Monnier, 1991). AAnother cross-linker formed by the reaction between two lysine side chains and two molecules of methylglyoxal is methylglyoxal-derived lysine dimer (MOLD) (Wei et al., 2018).

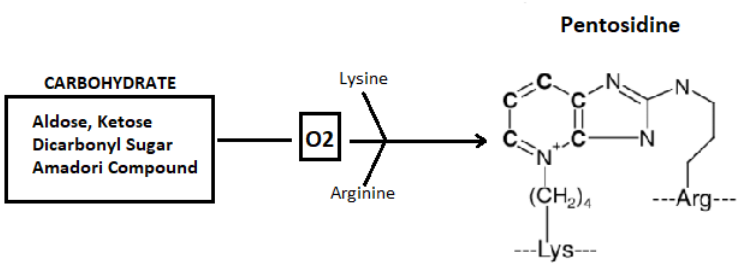

Figure 2.5: Generalized pathway for the formation of pentosidine. In addition to arginine and lysine, this scheme includes several possible carbohydrate precursors of pentosidine. Adapted from (Dyer et al., 1991).

Pyrraline, also called pyrrole-lysine, is a major product formed from 3-deoxyglucosone (3-DG), a dicarbonyl compound, in the nonenzymatic reaction between glucose and the ϵ-amino group of lysine residues on proteins (Monnier et al., 1996). Compared to pentosidine, pyrraline does not require oxidation to be formed and is neither a fluorescent nor cross-linking compound.

Analysis

Due to the complex food matrices and different food compositions, there is still no standard or universal method for detecting different kinds of AGEs, so further research and optimization are needed. However, there are some analytical methods for AGEs based on traditional techniques. For accurate quantification of the total AGE content, it is necessary to separate and purify AGEs in food before measurement, although it is difficult to have an extraction method suitable for all kinds of AGEs (Perrone et al., 2020). In general, free-state AGEs are usually extracted by solid-phase extraction. For the extraction of bound AGEs enzymatic or acid hydrolysis is done, depending upon

Table 2.2: Comparison of different techniques for detecting AGEs based on Sun et al. (2012); Nomi et al. (2016); Milkovska-Stamenova et al. (2015); Fang et al. (2014); Matsui et al. (2015).

Technique	**Advantages**	**Limitations**
GC-MS	Fast & convenient Data acquisition is quite fast	Time-consuming sample preparation Derivatization for samples Not widely used for AGEs
HPLC-DAD	Fast & convenient High sensitivity	Suited for UV-sensitive AGEs Lower specificity
HPLC-FID	High sensitivity High selectivity Ease of operation	Solvents should be highly pure Fluorescent pollution –> environmental issue Lack of specificity
LC-MS/MS	High sensitivity Ease of operation No derivatization	Sample losses during pre-processing and testing
ELISA	Fast Wide application range	Time-consuming sample preparation Specific antibodies Affected by food matrix Low specificity & sensitivity

the acidic stability of AGEs. Enzymatic hydrolysis is mainly used for AGEs that are easily destroyed under acidic conditions, such as pyrraline (Zhao et al., 2019).

According to Wei et al. (2018) and Perrone et al. (2020), there are two main traditional methods to analyze dietary AGEs, namely, instrumental analyses and immunoassay. Instrumental analyses include gas chromatography - mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) coupled with diverse detectors including diode array detector (DAD) or fluorescence detector (FLD) and liquid chromatography (LC) with mass spectrometry (MS) or tandem mass spectrometry (MS/MS). HPLC and GC are important chromatographical techniques where a liquid and a gas are used as the mobile phase, respectively. Samples with different components transfer in this mobile phase to be separated. After this, a detector or mass spectrometer is used to analyse the separated samples (Wei et al., 2018).

The second main traditional method for detecting AGEs is immunoassay analysis. Enzyme-linked immunosorbent assay (ELISA) is the most used method for this. ELISA uses an antigen-antibody reaction and color change to identify an object (Nagai et al., 2016). Although the ELISA method is easy and rapid, it is not regarded as reliable since the precision and accuracy are not high. Table 2.2 provides a summary of pros and contras of instrumental analyses and immunoassay.

The results from immunoassay and instrumental analyses can not be compared in a direct way, because in the former method, the results are expressed in arbitrary units (e.g. AGE kilo units/100 g food) rather than in actual concentrations (e.g mg/kg protein or mg/kg food) (Wei et al., 2018).

Health effects

Several studies have shown a positive association between AGE accumulation in the body and complications of chronic diseases. However, the role of the different types of AGEs in the pathogenesis of these complications is still unclear (Poulsen et al., 2013). The mechanisms of how AGEs can affect human health together with their role in chronic diseases are discussed below.

1. **Mechanisms**
   AGEs can induce a series of undesired effects on human health in various ways. It is for example described that through cross-linking with proteins, the mechanical properties of extracellular proteins (e.g collagen, elastin or laminin), and physiological functions of intracellular proteins (e.g DNA polymerase or RNA polymerase) are affected (Zieman and Kass, 2004). Another possible mechanism is the interaction with cell surface receptors of AGEs (RAGE) (Neeper et al., 1992). These receptors have been detected in a number of cells including endothelial cells, smooth muscle cells, monocytes/macrophages, T lymphocytes, cardiomyocytes, glomerular podocytes, dendritic cells, neurons of the central and peripheral nervous systems and transformed cells (Brett et al., 1993). Figure 2.6 gives a schematic illustration of the RAGE-receptor, showing that it is composed of an extracellular portion, a transmembrane portion and a highly charged cytosolic tail. The extracellular portion comprises three domains, V, C1 and C2. The first two are believed to work together as a single functional complex (VC1) whereas the C2 domain remains attached to the VC1 complex but works independently (Koch et al., 2010).

   Independent of their chemical structure, the negatively charged AGEs bind to the positively charged V domain of the RAGE receptor. Due to fact that RAGE recognizes a class of biochemically heterogeneous ligands rather than a specific ligand, RAGE is considered as one of the pattern recognition receptors.
   In this AGE-RAGE interaction, the cytosolic tail of the receptor is vital to activate multiple signaling cascades, starting with the accumulation of exogenous AGEs in tissues and organs, which leads to an increased expression and activation of RAGE. This induces the expression of transcription factors such as nuclear factor-kappa β (NF-Kβ), signal transducer activator of transcription 3 (STAT3)

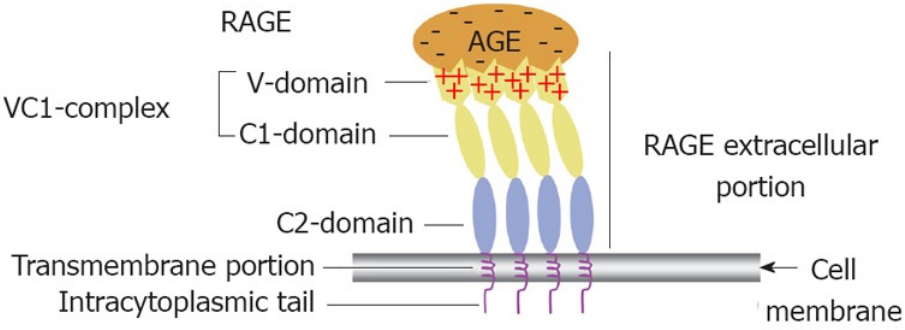

Figure 2.6: A schematic illustration of the structure of the receptor for advanced glycation end product (RAGE) showing that it is composed of an extracelluar portion, a transmembrane portion and an intracytoplasmic tail. The extracelluar portion comprises three domains, V, C1 and C2 (Hegab et al., 2012).

and hypoxia inducible factor 1-α (HIF1α). These transcription factors accelerate cytokine secretion, resulting in the elevation of reactive oxygen species (ROS) production and inflammatory response. The presence of ROS can in turn promote the generation of AGEs to create a cyclic inflammatory response (Turner, 2015).

2. **Role of AGEs in chronic diseases**

   Although AGEs have been implicated in the pathology of diabetes, cardiovascular disease and some neurodegenerative diseases, it is important to emphasize that their role may contribute only in part, or may even be subsidiary to other factors (Delgado-Andrade and Fogliano, 2018).

   - Diabetes

     During long standing hyperglycaemic state in diabetes, glucose forms covalent adducts with the plasma proteins through glycation, so hyperglycemia can promote the glycation process (Tessier, 2010). In the body of diabetic patients, enzymes involved in preventing the aging and injury of proteins are also easily glycosylated, leading to a slower protein metabolism and a reduction of protein renewal, resulting in reversely promoting the accumulation of AGEs (Brings et al., 2017). Protein glycation and formation of AGEs play an important role in the pathogenesis of diabetic complications like retinopathy, nephropathy, neuropathy, cardiomyopathy along with some other diseases such as rheumatoid arthritis, osteoporosis and aging (Singh et al., 2014).

     Diabetic retinopathy is the leading cause of blindness and is characterised by abnormal vessel development leading to haemorrhages and infarction affecting the retina of the eye. Important morphological and functional changes include thickening of the basement membrane and destruction of the blood retinal barrier (Poulsen et al., 2013). Diabetic nephropathy is characterised by expansion of the extracellular collagen matrix, leading

to basement membrane thickening, reduced filtration and ultimately loss of glomerular function (Singh et al., 2001). Diabetic neuropathy is characterised by a number of complex interactions including increased glycation and oxidative stress, functional abnormalities such as reduced nerve conduction and blood flow and structural abnormalities such as axonal degeneration and fibre demyelination. It can cause pain, numbness of limbs, impotence in men together with urinary incontinence, diarrhea, and constipation, impairing the quality of life of diabetic patients (Poulsen et al., 2013).

- Cardiovascular diseases

  The development or progression of cardiovascular disease (CVD) is mainly through the induction of oxidative stress and inflammation (Zieman and Kass, 2004). Like mentioned before, glycation can cause the increase in the degree of crosslinking of collagen in the blood vessel wall, which is a possible reason for AGEs being closely linked to CVD (Luévano-Contreras et al., 2017). The modification by AGEs causes the chemical structure of LDL to be abnormal and finally induces cardiovascular damage. Stirban et al. (2014) found that LDL after glycation reaction was more easily crosslinked with collagen in the vascular wall. This cross-linking could make it difficult for LDL to be taken up by cells and deposited on the walls of blood vessels.

- Neurodegenarative diseases

  Considerable evidence indicates that AGEs accumulate in an age-related way in the brain and other organs of the central nervous system of individuals. So the level of AGEs is a crucial factor for neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Perrone and Grant, 2015). AGEs accumulate in the target tissue of the marked pathology (e.g., the hippocampal regions in AD and the substantia nigra in PD). Interestingly, in most neurodegenerative processes evaluated, changes in AGE concentrations are found not only in the target locations, but also in anatomically distant regions. In other words, AGE formation takes place in several neuronal regions of the affected individuals in comparison with healthy individuals. This suggests that biochemical abnormalities leading to AGE build-up are present along with many different tissues and locations in the central nervous system (Ilieva et al., 2010).

2.3 Factors influencing oxidation & glycation during digestion of meat

Meat is exposed to different parts of the gastrointestinal tract as soon as it enters our body. During digestion, the environment in each part of the digestive system influences oxidative processes. First, meat is exposed to saliva in the mouth, moisturizing the meat so it can pass smoothly to the stomach. Saliva can both exert a pro-oxidative and antioxidant effect, depending on its composition (Gorelik et al., 2007). In the stomach the acidic gastric juice further breaks down the meat into smaller particles and proteins are pre-digested. (Kanner and Lapidot, 2001) showed that the stomach provides an excellent environment for enhanced lipid oxidation in heated muscle tissue due to its low pH and dissolved oxygen content. Next, the mash enters the small intestine, where pancreatic enzymes like protease, amylase, and lipase take care of the digestion and absorption of meat particles and water. According to McClements and Decker (2000) oxidation mechanisms in the small intestine differ between aqueous solutions, fat and emulsions. The final stage is the large intestine, where anaerobic fermentation by microbiota occurs. According to Pierre et al. (2008) a relatively high proportion of ingested heme iron reaches the colon, and, therefore, could be a stimulator of oxidation reactions.

Oxidation and glycation products formed during digestion are at least partly absorbed into the bloodstream (Sirota et al., 2013; van der Lugt et al., 2020) and may exert various (patho)physiological effects due to their cyto- and/or genotoxic properties. Whereas PROP and HEX have only limited biological effects, MDA and especially the hydroxy-alkenals (4-HHE and 4-HNE) are considered more reactive towards macromolecules such as protein and DNA. The formation of LOPs, POPs and AGEs is affected by many factors, including different meat compositions (protein, fat, carbohydrates, moisture levels, haem-Fe, additives, etc.), storage methods (storage temperature, time, moisture) and processing methods (reaction time, processing temperature, etc.) (Wei et al., 2018; Van Hecke et al., 2017b).

2.3.1 Meat composition

The presence of metals, either in the form of heme-proteins (myoglobin or hemoglobin) or in free form, is an important factor that determines the oxidative stability of meat, as they are potent catalysts of lipid oxidation (Guyon et al., 2016). From heme-proteins, free iron could be released by the destruction of the heme group. Free iron and other metals are capable of catalysing the production of ROS by the Fenton

reaction, discussed in section 2.1. In that way, the produced ROS can abstract hydrogen from PUFAs to initiate lipid oxidation. Levels of myoglobin/heme-Fe are generally considered to be a major catalyst for oxidation during digestion. However, Steppeler et al. (2016) showed that the fatty acid profile of the digested meat is the most important factor for oxidation during digestion, since PUFAs provide the substrate for the formation of LOPs. Section 2.1.1 already showed the specific formation of 4-HHE and PROP from oxidized n-3 PUFAs, and the formation of 4-HNE and HEX from oxidized n-6 PUFAs. MDA could originate from oxidation of both n-3 and n-6 PUFAs.

In general, oxidation susceptibility is correlated exponentially with the number of unsaturation of fatty acids (the number of double bonds), due to more reaction sites (Barden and Decker, 2016). In addition, the fatty acid conformation also affects the oxidative stability of lipids. According to Ghnimi et al. (2017), trans isomers are significantly more stable than cis isomers and the oxidation rate is higher for n-3 PUFAs than for n-6 PUFAs. Furthermore this paper indicates that lipid oxidation also increases with fatty acid chain length. Next to metal content and the fatty acid profile, antioxidants also determine the oxidation process.

Antioxidants

Important antioxidants that can protect fresh meat from lipid or protein oxidation are vitamin E and histidine-containing dipeptides, carnosine and anserine. For processed meat, nitrite salt, ascorbate and herbs or spices can prevent oxidation. The mechanism of these antioxidants is mainly due to their ability to neutralize radicals or scavenger metals that catalyse the oxidation process (Karakaya et al., 2011).

Vitamin E or α-tocopherol is a common lipid-soluble vitamin in animal tissue. The main antioxidant activity of α-tocopherol is the competition with PUFAs to donate a hydrogen. Lipid radicals attack α-tocopherol much faster than PUFAs. Therefore, unsaturated fatty acids are protected from the action of these radicals (Traber and Kayden, 1989).

According to Boldyrev et al. (2013), carnosine has been shown to have antioxidant activity through the chelation of metal ions, protection against the formation of AGEs and binding to toxic LOPs. Guiotto et al. (2005) showed interesting activities of anserine and carnosine related to the detoxification of the body from ROS, the protection of membranes, proton buffering capacity, the formation of complexes with transition metals, and the regulation of macrophage functioning.

In processed meat products, nitrite salt is widely used as a curing agent to maintain the desired red meat color and to inhibit the outgrowth of *Clostridium botulinum*,

which causes spoilage and oxidative rancidity. Kanner (1994) demonstrated the antioxidant mechanism of nitrite such as the inhibition of the Fenton reaction, stabilizing unsaturated lipids by reducing their susceptibility to oxidation. Furthermore, Van Hecke et al. (2014a) showed that nitrite inhibits the formation of protein carbonyls during cooking and digestion of meat products. However, in dry-cured meat products, nitrite both has pro- and antioxidant effects on protein carbonyls (e.g. AAS and GGS) (Villaverde et al., 2014).

Herbs or spices also have an antioxidant effect on processed meat, already at concentrations of 0.5% (Van Hecke et al., 2017a). This study showed that the inhibitory effect on LOP formation was more pronounced when the herbs or spices were added before heating of the meat, compared to addition following heating. The extent to which herbs and spices limit oxidation, showed a strong correlation with their phenolic content.

2.3.2 Processing and storage methods

Generally, processes such as slicing, grinding and cooking meat can lead to destruction of cell membranes, accelerating lipid oxidation and leading to a more rapid degradation of primary oxidation products into secondary products. This is due to the fact that destruction of the well organised structure of animal cells increases the contact of lipid components with pro-oxidant components (SATO and HEGARTY, 1971).

Time and temperature

Knowing that the oxidative process exists out of multiple chemical reactions, it is clear that oxidation, like any other chemical reaction, oxidation is favored as both the time and temperature increase (Chaijan and Panpipat, 2017).
When meat is heated prior to consumption, the contact between the substrate and the oxidation catalyst is favored. Furthermore, Fe^{2+} is liberated by destruction the heme group, hydroperoxides are formed due to the release of oxygen by oxymyoglobin and antioxidant enzymes become inactivated by heat (Kanner, 1994). These reactions favor stimulation of the Fenton reaction, which leads to more LOP formation. Furthermore, according to Van Boekel (2001) mild heat treatment results mainly in the formation of Amadori products. However, when the processing time or temperature increases, degradation of Amadori products leads to the formation of dicarbonyl compounds and AGEs. They also indicate that glycoxidation proceeds at a higher rate in dry heating conditions than in aqueous conditions due to the dilution effect of the reactants in the aqueous environment.

Next to processing time and temperature, storage time also influences the oxidation process. The possibility that radicals cause damage to lipids increases with time. Long storage periods also promote the release of iron from heme-proteins, which catalyses multiple reactions in the initiation and propagation phase of lipid oxidation (Richards, 2005).

Oxygen content and light

In meat and meat products the most common techniques are vacuum packaging, modified atmosphere packaging and active packaging (Lorenzo et al., 2017). First of all, the gas composition of the atmosphere surrounding the meat product is essential for the development of the oxidative processes. Lower lipid oxidation occurs in meat packaged in low O_2 concentration or vacuum packed, than meat packaged in high O_2 concentration (Cayuela et al., 2004). According to Akıllıoğlu and Gökmen (2019), more glucose attachment is favored in the presence of oxygen and more dicarbonyl compounds are generated, therefore increasing the glycation rate.
Secondly, light is another important parameter to control, in order to increase the oxidative stability of meat. Like mentioned in section 2.1.1.1, light promotes the initiation phase through photo-oxidation and the production of reactive $^1O_2$. Therefore, the use of packaging materials that absorb UV light prevent photo-oxidation and increases the oxidative stability of meat.

CHAPTER 3

GOAL & HYPOTHESES

The aim of this master thesis was to investigate oxidation, glycation and glycoxidation processes during heating and during gastrointestinal digestion of pork shoulder, as influenced by the addition of glucose to the meat, heating conditions (time or temperature) and packaging system (aerobic/anaerobic). Three experiments with three different factors were set up for this purpose. Section 4.1 gives a detailed description of these experiments. Detection of oxidation, glycation and glycoxidation products was done to compare the different experiments and factors.

In this thesis, the following hypotheses were made: a) pork is sensitive to lipid and protein oxidation during heating and digestion, b) glycation accompanies oxidation leading to glycoxidation, which could occur during heating and digestion of pork, giving rise to the formation of the glycoxidation product pentosidine, c) addition of glucose to pork and more extreme heating conditions stimulate glycation and the formation of pentosidine during heating and digestion, d) anaerobic conditions during heating may reduce oxidation and glycoxidation in pork.

CHAPTER 4

MATERIAL & METHODS

4.1 Experimental design

Three experiments were set up to check the effect of glucose, heating conditions (time/temperature) and the packaging system (aerobic/anaerobic) on the formation of oxidation, glycation, and glycoxidation products. After meat preparation, meat samples were subjected to an *in vitro* digestion process, simulating the mouth, stomach and small intestine. Lipid oxidation (malondialdehyde), protein oxidation (protein carbonyl compounds), glycation (melanoidins) and glycoxidation (pentosidine) products were evaluated before and after digestion. For the three different experiments, pork shoulder was used during processing, because pork is mostly used in processed meat products and shoulder has relatively high levels of heme-Fe and fat content.

4.1.1 Experiment 1: extreme conditions (5% vs 0% Glu; aerobic; 110°C)

For the first experiment (Table 4.1), minced pork shoulder was whether or not supplemented with 5% glucose, packed aerobically with aluminum foil (leaving the top open to the air), and subjected to the oven to 110°C for a long heating time (until the core temperature was stable for 10 minutes) (2 x 1 x 1 full factorial design). The core temperature changes during heating can be found in Figure 5.1 in chapter 5. It took approximately 120 minutes to reach a stable core temperature. In this experiment, extreme conditions of the glucose content and heating conditions were chosen to optimize the analysis of pentosidine.

Table 4.1: Overview of experiment 1 set-up.

Meat sample	Glucose content (%)	Packaging system	Heating conditions
M1	0	aerobic	110°C
M2	5	aerobic	110°C

4.1.2 Experiment 2: different heating temperatures (3% vs 0% Glu; aerobic vs anaerobic; 80°C vs 130°C vs 180°C)

For the second experiment (Table 4.2), minced pork shoulder was whether or not supplemented with 3 % glucose, packed either aerobically or anaerobically in bags (vacuum sealer bags, resists up to 250 °C, brand: HUISPARK, producer: China, website: Amazon), and subjected to the oven to either 80°C, 130°C or 180°C until a core temperature of 75°C was reached (2 x 2 x 3 full factorial design). 80°C represents the normal cooking temperature for meat (e.g cooked ham), 130°C represents a roasting temperature and 180°C represents a grilling temperature. These exact temperatures were chosen so the range between the different temperatures is equal (50°C). 75°C is the common core temperature to be reached in the meat industry (Wang et al., 2020).

Table 4.2: Overview of experiment 2 set-up.

Meat sample	Glucose content (%)	Packaging system	Heating conditions
M1	3	aerobic	80°C
M2	3	anaerobic	80°C
M3	0	aerobic	80°C
M4	0	anaerobic	80°C
M5	3	aerobic	130°C
M6	3	anaerobic	130°C
M7	0	aerobic	130°C
M8	0	anaerobic	130°C
M9	3	aerobic	180°C
M10	3	anaerobic	180°C
M11	0	aerobic	180°C
M12	0	anaerobic	180°C

4.1.3 Experiment 3: different heating times (3% vs 0% Glu; aerobic; 180°C;0 vs 20 vs 40 vs 60 vs 80 vs 100 min)

For the third experiment (Table 4.3), minced pork shoulder was whether or not supplemented with 3% glucose, and subjected to the oven to 180°C for either 0, 20, 40, 60, 80 or 100 minutes (2 x 6 full factorial design). In this experiment, the samples were packed aerobically with aluminum foil (leaving the top open to the air), so more and faster water loss occurred, promoting the Maillard reaction.

4.2 Chemicals

All digestive enzymes (α-amylase from hog pancreas (50 U/mg; 10080), mucin from porcine stomach type II (M2378), pepsin from porcine gastric mucosa (>250 U/mg solid; P7000), lipase from porcine pancreas type II (10–400 U/mg protein; L3126),

Table 4.3: Overview of experiment 3 set-up.

Meat sample	Glucose content (%)	Heating time (min)	Heating temperature
M1	0	0	180°C
M2	3	0	180°C
M3	0	20	180°C
M4	3	20	180°C
M5	0	40	180°C
M6	3	40	180°C
M7	0	60	180°C
M8	3	60	180°C
M9	0	80	180°C
M10	3	80	180°C
M11	0	100	180°C
M12	3	100	180°C

pancreatin from porcine pancreas (8 $\times$ USP specifications; P7545), and porcine bile extract (B8631)) were purchased from Sigma-Aldrich (Diegem, Belgium). Pentosidine (10010254) was purchased from Cayman Chemical (Sanbio B.V., Netherlands).

4.3 Sample preparation

For experiment 1, four kilograms of pork shoulder were purchased from a local butcher. For experiment 2 and 3, eight kilograms of pork shoulder per experiment were purchased. For all three experiments, the meat was manually chopped into cubes of approximately 1-2 cm^3 and minced in a meat grinder (Omega T-12) to divide the muscle and the fat equally. Thereafter, 400 grams of homogenized meat was weighted per treatment. For the treatment with 3% or 5% glucose, 388 or 380 grams of meat, respectively, together with 12 or 20 grams of glucose, respectively, were manually mixed. After weighing, the different meat samples were either packaged in transparent plastic bags or aluminum foil, and the bags were either or not vacuum packed, depending on the experiment and treatment (see Table 4.1, 4.2 and 4.3). For all experiments, additionally packed meats were used to monitor the core temperature with temperature probes in the middle of the meat. All meat samples were heated in the oven according to Table 4.1, 4.2 and 4.3. After cooling down, all meat samples were homogenized in three 5 s bursts using a food processer (Moulinex DP700), vacuum packed in transparent bags, and stored in the freezer at -80°C until analysis and *in vitro* digestion.

4.4 *In vitro* digestion

The *in vitro* digestions were performed according to a described protocol, specific for studying oxidation processes during passage in the gastrointestinal system (Van Hecke et al., 2018). The digestions were performed in quadruplicate and per experiment. All samples per experiment were digested on the same day with exactly the same digestive juices to exclude any day effect. The composition of the digestive juices is shown in Figure 4.1.

Composition of digestive juices												
	Saliva			Gastric juice			Duodenal juice			Bile		
	Chemicals	Concentration		Chemicals	Concentration		Chemicals	Concentration		Chemicals	Concentration	
A	KCl	0.9	g/L	KCl	0.82	g/L	KCl	0.56	g/L	KCl	0.38	g/L
	NaCl	0.3	g/L	NaCl	2.75	g/L	NaCl	7.01	g/L	NaCl	5.26	g/L
	Na_2SO_4	0.57	g/L	$CaCl_2$ $2H_2O$	0.4	g/L	$MgCl_2$	0.05	g/L	$NaHCO_3$	5.79	g/L
	NaH_2PO_4	0.9	g/L	NaH_2PO_4	0.27	g/L	KH_2PO_4	0.08	g/L	HCl 37%	0.15	mL/L
	$NaHCO_3$	1.69	g/L	NH_4Cl	0.037	g/L	$NaHCO_3$	3.39	g/L			
	KSCN	0.2	g/L	HCl 37%	7.5	mL/L	HCl 37%	0.18	mL/L			
B	Urea	0.2	g/L	Urea	0.15	g/L	Urea	0.1	g/L	Urea	0.25	g/L
	Uric acid	0.0115	g/L	BSA	1	g/L	BSA	1	g/L	BSA	1.8	g/L
	Mucin	0.025	g/L	Mucin	3	g/L	Pancreatin	9	g/L	Bile	30	g/L
	Amylase	0.29	g/L	Pepsin	2.5	g/L	Lipase	1.5	g/L	$CaCl_2$ $2H_2O$	0.222	g/L
	$NaNO_2$	0.0069	g/L	Glucose	0.65	g/L	$CaCl_2$ $2H_2O$	0	g/L			
				Glucosamine-HCl	0.33	g/L						
				Ascorbic acid	0.0176	g/L						
				Glucuronic acid	0.02	g/L						
				$FeSO_4$ $7H_2O$	0.0112	g/L						
C				H_2O_2 (30%)	20	µL						

Figure 4.1: Composition of the simulated digestive juices (/L) used in the *in vitro* digestion model (Van Hecke et al., 2018).

Meat samples (4.5 g) were stirred by hand at room temperature for 5 min in 6 mL saliva. Then the samples were sequentially incubated at 37 °C for 2 h with 12 mL gastric juice [initial stomach pH of 2.3 to final stomach pH of 3.5, according to Van Hecke et al. (2018)], and 2 h with 2 mL bicarbonate buffer (1 M, pH 8.0), 12 mL duodenal juice and 6 mL bile juice [small intestinal pH of 6.5, according to Van Hecke et al. (2018)]. After completion, samples were homogenized with an Ultra Turrax at 9500 rpm. While stirring on a magnetic field, samples were subdivided into 1.3 mL aliquots and stored at -80 °C until further analysis (Figure 4.2).

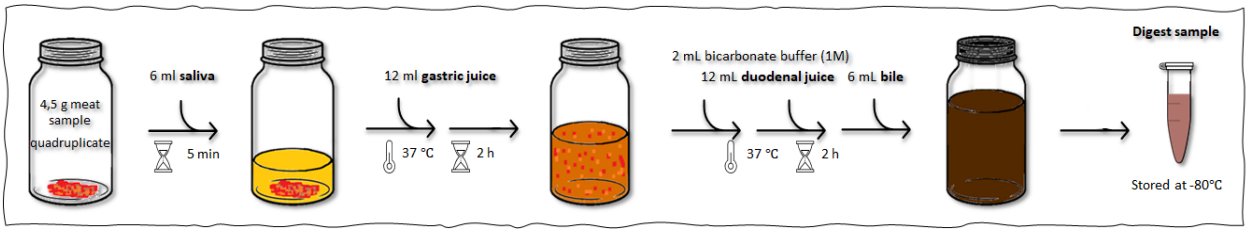

Figure 4.2: *In vitro* digestion protocol (Van Hecke et al., 2018).

4.5 Analysis of oxidation products

4.5.1 Malondialdehyde

Malondialdehyde (MDA) concentrations in meats and digests were measured spectrophotometrically by a modified method in accordance with Grotto et al. (2007). This method is based on the reaction of one mole of MDA with two moles of 2-thiobarbituric acid (TBA), under acidic conditions, forming thiobarbituric acid reactive substances (TBARS). This pink/red colored complex can be measured spectrophotometrically at 532 nm. Since MDA is a highly reactive molecule that is normally bound to other food ingredients (e.g. proteins), complete extraction of the MDA must be performed in order to improve the specificity of MDA. In this regard, this method applies an alkaline hydrolysis step to release the bound MDA and a sample extraction step with 1-butanol.

Preparation of standard solutions

1,1,3,3-tetramethoxypropane (TMP) was used in order to prepare standard solutions. For this purpose, a stock solution was made, by dissolving 82.35 μL of TMP in 50 mL of distilled water. After that, a working solution was prepared, by dilution of 1 mL of the stock solution in 100 mL of distilled water. At this point, a series of standard solutions (10 mL each) were prepared in glass tubes, according to what is indicated in Table 4.4. These standard solutions were then considered in order to build a standard curve as a reference for final measurements of MDA, expressed as nmol MDA/mL solution.

Preparation of reagent solutions

- Sodium hydroxide (NaOH) solution (1.5 M): dissolve 6 g NaOH in 100 mL distilled H_2O (stored at environmental temperature);
- Phosphoric acid (H_3PO_4) solution (6%, v/v): dissolve 7.05 mL H_3PO_4 in 100 mL distilled H_2O (stored at environmental temperature);

Table 4.4: Specific composition of the standard solutions used in the detection of MDA.

MDA concentration (nmol/mL)	**Working solution (mL)**	**Distilled water (mL)**
0	0	10.0
5	0.5	9.5
10	1.0	9.0
15	1.5	8.5
20	2.0	8.0
25	2.5	7.5
30	3.0	7.0
50	5.0	5.0

- 2-Thiobarbituric acid (TBA) solution (0.8%, w/v): dissolve 0.8 g TBA in 100 mL distilled H_2O (prepared fresh, just before the start of analysis);
- Sodium dodecyl sulphate (SDS) solution (10%, w/v): dissolve 10 g SDS in 100 mL distilled H_2O (stored at environmental temperature).

Method

In glass tubes either 450 μL of standard solution (in duplicate), 450 μL of digest (in duplicate), or 100 mg of meat and 350 μL of distilled water (in quadruplicate) were measured, after which 300 μL NaOH (1.5 M) solution was added. Following a brief vortex, glass tubes were subsequently covered with aluminum foil and heated in a water bath at 60 °C for 30 min. After heating, 750 μL of H_3PO_4 solution (6%, v/v) and 750 μL of TBA solution (0.8%, w/v) were added in sequence. Following a brief vortex, glass tubes were covered again with aluminum foil and heated in a water bath at 90 °C for 45 min. After that, the mixtures were cooled down under cold tap water, followed by the addition of 300 μL of SDS solution (10%, w/v) and 3 mL of 1-butanol. Subsequently, samples were mixed well on the vortex for several seconds, and centrifuged at 2700 rpm for 15 minutes. After centrifugation, the upper butanol layer was collected into a plastic macro cuvette using a Pasteur pipette. After extraction in 1-butanol, the absorbance (A) of each colored complex was quickly measured spectrophotometrically at 532 nm with the UV-Vis spectrophotometer. Results were expressed as absorbance and converted to nmol MDA/g meat or g digest. To compare meat with digest samples, a dilution factor of 9.4 was used for the digests to convert the units from nmol MDA/g digests to nmol MDA/g digested meat (dilution of 4.5g of meat with 38 mL digestive juices).

4.5.2 Protein carbonyl compounds

Protein carbonyl compounds (PCC) were measured in meats and digests spectrophotometrically, by an estimation of carbonyl groups following their covalent reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH), which leads to the formation of a stable 2,4-dinitrophenylhydrazone product, according to the method of Oliver et al. (1987), with some modifications of Ganhão et al. (2010).

Preparation of reagent solutions

- Sodium phosphate buffer (20 mM): dissolve 2.3637 g di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) and 1.8236 g Potassium dihydrogen phosphate (KH2PO4) in 1 L distilled H_2O, adjusted to pH 6.5 (stored at environmental temperature);
- Trichloroacetic acid (TCA) solution (10%, w/v): dissolve 10 g TCA in 100 mL distilled H_2O (stored in the fridge at 4 °C and put in the freezer at -20 °C just before the start of analysis);
- Trichloroacetic acid (TCA) solution (20%, w/v): dissolve 20 g TCA in 100 mL distilled H_2O (stored in the fridge at 4 °C and put in the freezer at -20 °C just before the start of analysis);
- Hydrochloric acid (HCl) solution (2 M): dilute 100 mL Hydrochloric acid 37% (HCl 37%) + 500 mL distilled H_2O (stored at environmental temperature);
- 2,4-dinitrophenylhydrazine (2,4-DNPH) solution (0.2%, w/v): dissolve 0.2 g 2.4-DNPH (weighed using a glass stick since metal reacts with the compound itself) in 100 mL HCl solution (2 M) (prepared fresh, just before the start of analysis);
- Ethanol/Ethyl acetate solution (1:1, v/v): ethanol and ethyl acetate in 50/50 ratio (stored at environmental temperature);
- Guanidine-Hydrochloride solution (6 M): dissolve 57.32 g guanidine-HCl in 100 mL sodium phosphate buffer (20 mM).

Method

20 mL of sodium phosphate buffer was added to 2 g of defrozen meat and homogenized by the Ultra Turrax T25 at 16000 rpm. In microtubes, either 700 μL of digest (in triplicate), or 200 μL of the homogenized meat solution (in quadruplicate) were measured, after which 0.5 mL of ice-cold TCA solution (20%, w/v) or 1 mL of ice-cold TCA

solution (10%, w/v) was added to microtubes containing digest or meat respectively. Following a brief vortex, microtubes were left in an ice bath for 10 minutes to ensure the precipitation of proteins. After completion of the previous step, microtubes were centrifuged at 3000 rpm for 15 or 20 minutes, for digest and meat, respectively. After centrifugation, the supernatant was carefully removed. At this point, 1 mL of ice-cold TCA solution (10%, w/v) was firstly added to microtubes, which were secondly vortexed, and again placed in the ice bath for another 10 minutes. Next, samples were centrifuged at 3000 rpm for 15 minutes, and, after that, as was previously done, the supernatant was carefully removed in the same manner. Subsequently, on one hand, 0.5 mL of 2.4-DNPH solution (0.2% w/v) were added only to 3 of the 4 aliquots, on the other hand, 0.5 mL of HCl solution (2 M) were added to the other remaining aliquots. All microtubes were then vortexed and incubated for 1 hour, at room temperature, on a shaker at 350 rpm, fully covered from light with aluminum foil. After this procedure, 0.5 mL of ice-cold TCA solution (20%, w/v) was added to the microtubes, which were then vortexed, just before centrifuging at 3000 rpm for 15 minutes, after which the supernatant was removed from each microtube. 1 mL of ethanol/ethyl acetate solution (1:1, v/v) was added to all samples, with the aim of removing traces of 2.4-DNPH and solubilizing all residual lipids, followed by vortex, centrifugation at 3000 rpm for 15 minutes and removal of the supernatant. At this point, all the previous procedures, precisely from the addition of ethanol/ethyl acetate solution (1:1, v/v) to microtubes until the removal of supernatants, were repeated twice, except the last centrifugation was done at 13000 rpm for 10 minutes. Hence, after removal of supernatants, microtubes were left, one by one, under nitrogen for several seconds, in order to dry the moist pellets. After that step, 1 mL of guanidine-hydrochloride solution (6 M) was added to all samples, in order to dissolve proteins, and also to two new empty microtubes, which were used as blank samples during the absorbance measurements. All microtubes were then vortexed, incubated for 30 minutes, at room temperature, on the shaker at 350 rpm, fully covered from light with aluminum foil. In conclusion, after centrifuging for the last time all microtubes at 13000 rpm for 10 minutes in order to remove insoluble fragments, the content of each microtube was collected and transferred into a plastic semi-micro cuvette.

Hence, at the end, the absorbance (A) of each sample was quickly measured, firstly at 280 nm (A_{280}) and secondly at 370 nm (A_{370}) with the spectrophotometer (using guanidine-hydrochloride solution as zero, in order to calibrate the machine). More specifically, carbonyl concentrations were measured considering the amount of 2.4-DNPH incorporated/mg of protein, by using the following formula (Levine et al., 1994):

$$C_{hydrazone}/C_{protein} = A_{370}/(\epsilon_{hydrazone,370}) * (A_{280}\text{-}A_{370} * 0.43)) * 1000000$$

where $\epsilon_{hydrazone,370}$ = 22000 M^{-1} cm^{-1} and 0.43 = $\epsilon_{hydrazone,280}$/ $\epsilon_{hydrazone,370}$. The final results were expressed as nmol 2.4-DNPH fixed/mg protein or as nmol carbonyl/mg protein.

4.6 Analysis of glycation products

4.6.1 Maillard products

Maillard products (MRPs) were measured in meats and digests spectrophotometrically by the extraction of the colored material formed, according to the modified method of Silva et al. (2016). The concentration of MRPs was calculated using a molar extinction coefficient (1.0 $\pm$ 0.03 L $mmol^{-1}$ cm^{-1}) described by Martins and van Boekel (2003).

Method

In hermetically closed tubes either 0.125 mL of digest (in triplicate) or 0.125 g of meat (in triplicate) were measured, after which 1 mL of methanol was added. Following a brief vortex, tubes were placed at an orbital shaker for 1 hour for the extraction to take place. Subsequently, samples were centrifuged at 5000g for 15 minutes, after which the supernatant of each tube was collected and transferred into a plastic semi-micro cuvette. Finally, the absorbance (A) of each sample was quickly measured at 420 nm (A_{420}) with the spectrophotometer. The concentration of MRPs was calculated by using the following formula:

$A = \epsilon * c * l$

where ϵ represents a molar extinction coefficient of melanoidin[1] (= 1.0 $\pm$ 0.03 L $mmol^{-1}$ cm^{-1}), c represents the concentration of melanoidin and l represents the length of the cuvette (= 1 cm). The results were expressed as nmol melanoidin/g meat or g digest. To compare meat with digest samples, a dilution factor of 9.4 was used for the digests to convert the units from nmol melanoidin/g digests to nmol melanoidin/g digested meat (dilution of 4.5g of meat with 38 mL digestive juices).

4.6.2 Pentosidine

Pentosidine was measured in meats and digests based on its fluorescence characteristics using high-performance liquid chromatography with fluorescence detection

[1] Melanoidins are the final products of the Maillard reaction, which is the coloured material Silva et al. (2016) mentioned. Therefore, we use melanoidin to represent maillard reaction products.

(HPLC-FLD, Agilent 1200 series, Diegem, Belgium), according to the modified method of Scheijen et al. (2009).

Preparation of standard solutions

- **Standards of pentosidine**

  Trifluoroacetic acid (TFA) (0.1%, v/v) was used in order to prepare standard solutions. For this purpose, 150 μL TFA was diluted by distilled H_2O to a final volume of 150 mL. Then, a stock solution was made, by dissolving 1 mg pentosidine in 132.135 mL TFA (0.1%, v/v) and stored at -80 °C before use. After that, a working solution was prepared, by dilution of 1 mL of the stock solution in 100 mL citric acid (25 mM). At this point, a series of standard solutions (1 mL each) were prepared in glass tubes, according to what is indicated in Table 4.5. These standard solutions were then considered in order to build a standard curve as a reference for final measurements of pentosidine, expressed as nmol/L solution.

Table 4.5: Specific composition of the standard solutions of pentosidine used in the detection of pentosidine.

Standard	**Pentosidine concentration (nM)**	**Working solution (μL)**	**Citric acid (μL)**
0	0	0	10
1	1.25	62.5	9.9375
2	2.5	125	9.875
3	5	250	9.75
4	10	500	9.5
5	20	1000	9

- **BSA standards**

  Bovine serum albumin (BSA) and citric acid were used in order to prepare standard solutions. A series of standard solutions (3000 μL each) were prepared in glass tubes, according to what is indicated in Table 4.6. These standard solutions were then considered in order to build a standard curve as a reference for final measurements of protein concentration, expressed as μg protein/mL solution.

Table 4.6: Specific composition of the BSA standard solutions used in the detection of pentosidine.

Standard	**1**	**2**	**3**	**4**	**5**	**6**
BSA (μg/mL)	0	500	1000	2000	4000	6000
BSA (μL)	0	500	1000	2000	4000	6000
Citric acid (μL)	6000	5500	5000	4000	2000	0

Preparation of reagent solutions

- Sodium borohydride borate buffer (200 mM, pH 9.2): 1.237 g H_3BO_3 and 0.757 g $NaBH_4$ in 100 mL NaOH (0.2%);
- TCA solution (10%, w/v): dissolve 10 g TCA in 100 mL distilled H_2O (stored in the fridge at 4 °C);
- TCA solution (40%, w/v): dissolve 40 g TCA in 100 mL distilled H_2O (stored in the fridge at 4 °C);
- HCl solution (6 M): dilute 100 mL HCl (37%) in 100 mL distilled H_2O (stored at environmental temperature);
- Citric acid buffer (25mM) named buffer A: dissolve 9.606 g citric acid in 2L HPLC—H_2O (= ultrapure water);
- Acetonitrile/citric acid solution (1:1, v/v) named buffer B: mix acetonitrile and 25mM citric acid in 50/50 ratio.

Method

20 mL of sodium phosphate buffer was added to 0.67 g meat and homogenized by the Ultra Turrax T25 at 16000 rpm. In hermetically closed tubes, either 150 μL of digest, or 150 μL of the homogenized meat mixture were measured, after which 500 μL sodium borohydride borate buffer (200 mM, pH 9.2) was added to prevent a potential artifactual formation of pentosidine from early glycation products during sample preparation. Following a brief vortex, tubes were placed in the dark and stood for 2 h at room temperature. Subsequently, 1 mL of TCA solution (40%, w/v) was added to the tubes. Following a brief vortex to ensure the precipitation of proteins, the tubes were centrifuged (4 °C) at 4500g for 10 minutes, after which the supernatant was carefully removed just by turning each tube. At this point, 1 mL of TCA solution (10%, w/v) was firstly added to wash the pellets. Secondly, the tubes were vortexed, followed by centrifugation (4 °C) at 4500g for 10 minutes and removal of the supernatant as described above. Thereafter, 50 μL of HCl (6 M) was added and then they were incubated for 18 h at 110 °C to hydrolyze the samples. After hydrolysis, samples were placed in a water bath at 80 °C for approximately 40 minutes to be dried, after which 200 μL of citric acid (25 mM) was added to reconstitution. Following a brief vortex, the tubes were centrifuged (4 °C) at 14000 rpm for 15 minutes. In the meantime, either 1 mL of the pentosidine standards (in duplicate), or 1 mL of the BSA standards (in duplicate) were measured in hermetically closed tubes. Finally the content of the tubes (standards and samples) was transferred into vials using a Pasteure

pipette, after which 100 μL of this solution was injected into the HPLC system. The separation was done on an Allsphere ODS-2 column (3 μm, 150 x 4.6 mm, catalog no. 760053.46, MACHEREY-NAGEL) at a flow rate of 1 mL/min. The pentosidine was detected by a FLD at an excitation wavelength of 325 nm and an emission wavelength of 385 nm. Protein was detected by a DAD at an absorption wavelength of 280 nm. Pentosidine and protein content were quantified using standard curves, respectively. The final results were expressed as μg pentosidine/kg protein.

4.7 Statistical analysis

The data obtained during the study was first processed in Microsoft Excel. The results were further processed statistically using the program SAS Enterprise Guide 8. Data on MDA, PCC, MRPs and pentosidine were analyzed for each experiment separately in the meat products and gastroduodenal digests. For experiment 1 and 2, no statistical analysis was done, as conclusions were clear from the results. For experiment 3, a linear model ANOVA procedure (SAS Enterprise Guide 8) was used with the fixed effects of glucose content, heating time and their interaction term. Post hoc comparisons were performed using the Tukey HSD test. P-values < 0.05 were considered statistically significant.

CHAPTER 5

RESULTS

5.1 Experiment 1: extreme conditions (5% vs 0% Glu; aerobic; 110°C)

In the first experiment, more extreme conditions were used to stimulate potential pentosidine formation as much as possible during heating of meat and/or during digestion, in order to optimize and gain experience with the analysis of pentosidine. In Figure 5.1, changes in the core temperature of the meat samples during heating at 110°C (in the oven), are presented for experiment 1. Meat samples were removed from the oven when the core temperature was stable for 10 minutes. A stable core temperature of circa 81°C was reached after 120 minutes.

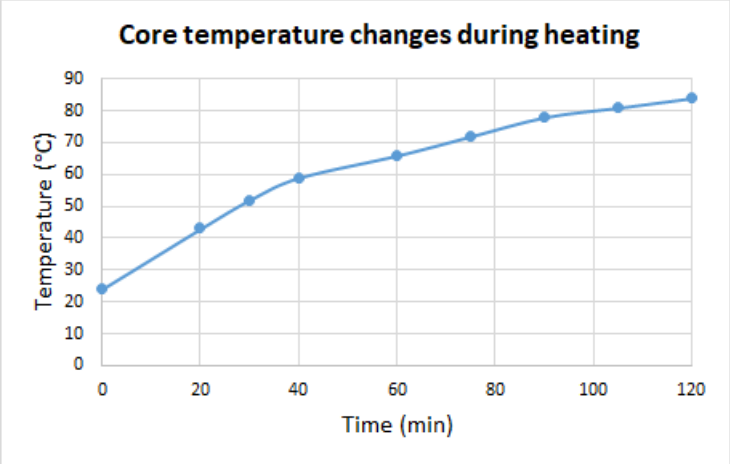

Figure 5.1: Changes in core temperature of the meat samples in experiment 1 until the core temperature was stable for 10 minutes. The meat samples were heated in the oven at 110°C.

Figure 5.2 shows a distinct increase in the concentration of the lipid oxidation product MDA in meat, after supplementation with 5% glucose. Following gastrointestinal digestion, increased MDA concentrations were observed in both the control (0 % glucose) and treated (5 % glucose) samples, however the relative difference between the meats became much smaller following digestion.

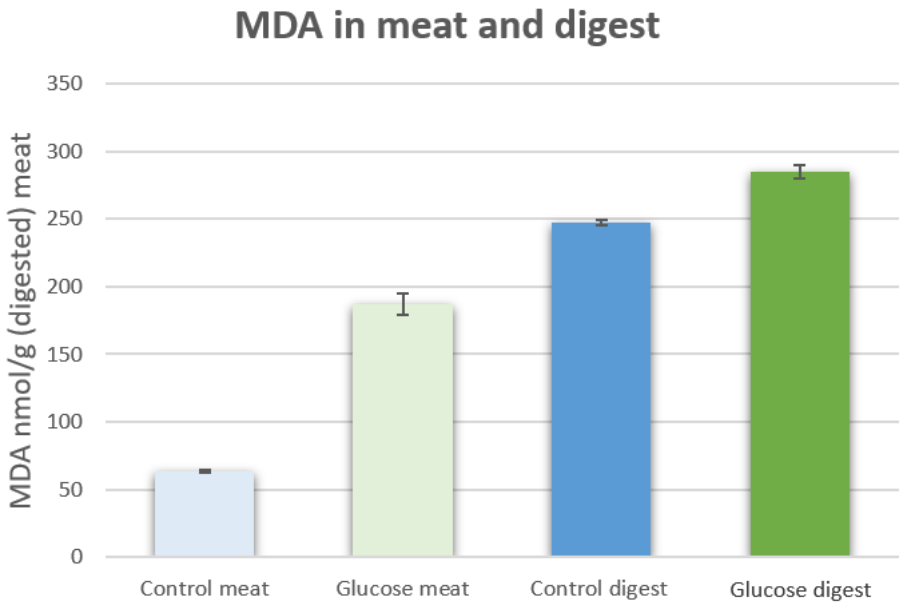

Figure 5.2: Concentration of the lipid oxidation product, malondialdehyde (MDA) of pork shoulder in experiment 1, expressed in nmol/g (digested) meat, with (green) or without (blue) 5 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation.

For protein oxidation, results show that supplementation with 5 % glucose increased PCC levels in meat (4-fold higher) and digested meat (2-fold higher) (Figure 5.3). In contrast with MDA levels, gastrointestinal digestion of these meat samples with 5 % glucose resulted in distinctly lower PCC levels, whereas in the meat without glucose, PCC remained approximately at the same level.

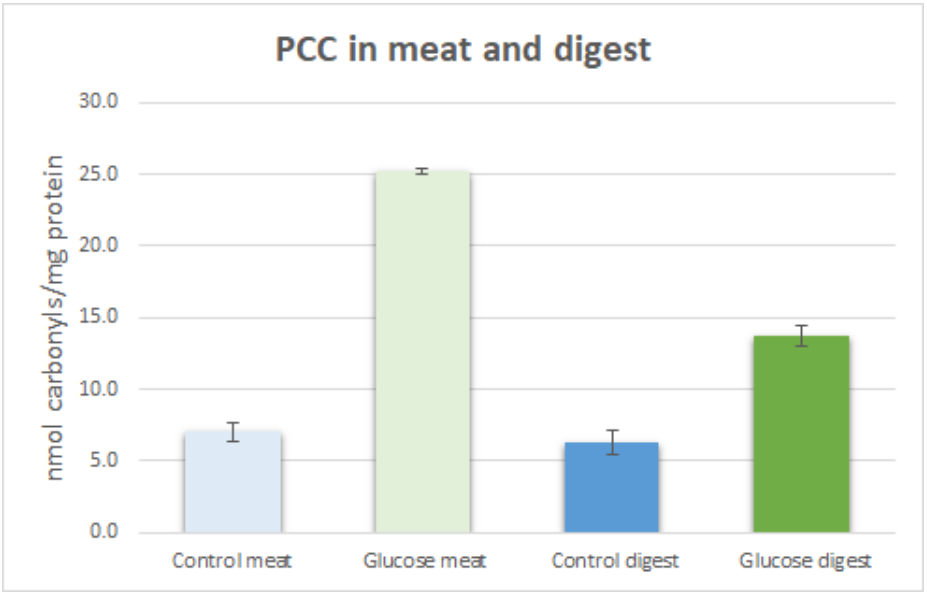

Figure 5.3: Concentration of protein carbonyl compounds (PCC) of pork shoulder in experiment 1, expressed in nmol carbonyls/mg protein, with (green) or without (blue) 5 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation.

Both supplementation with 5 % glucose and gastrointestinal digestion increased glycation in the samples, which is demonstrated by the more than 2.3-fold higher MRP concentrations in digests of pork with glucose compared to control digests without glucose (Figure 5.4).

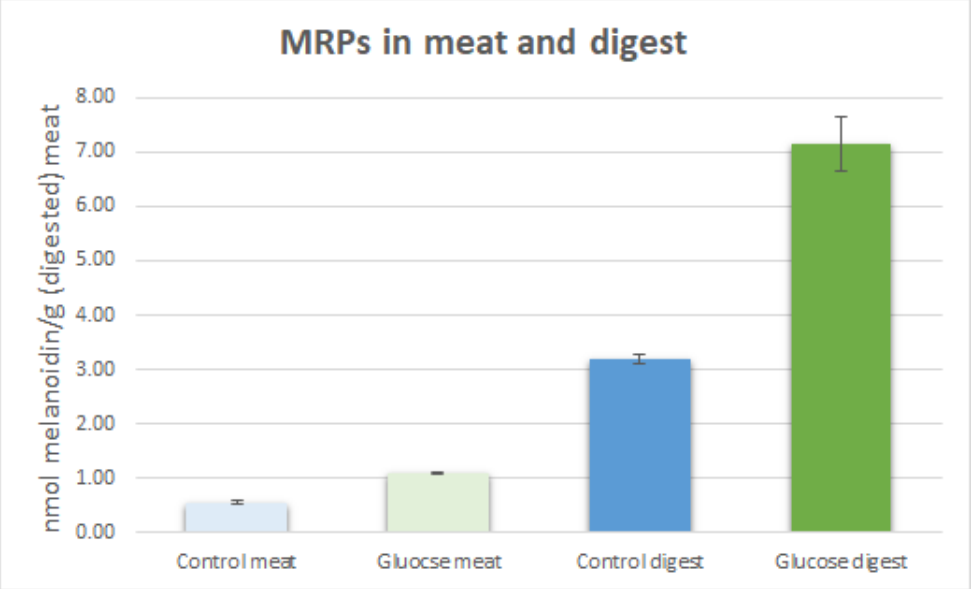

Figure 5.4: Concentration of Maillard reaction products (MRP) of pork shoulder in experiment 1, expressed in nmol melanoidins/g (digested) meat, with (green) or without (blue) 5 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation.

The combination of oxidation and glycation leads to glycoxidation products such as pentosidine. Figure 5.6[1] shows that supplementation with 5 % glucose and gastrointestinal digestion result in higher pentosidine concentrations. Digests of pork with glucose contained approximately 3-fold higher levels of pentosidine compared to the control digests without glucose.

The limit of detection of pentosidine was 0.95 nmol/L. Recoveries of pentosidine were 91.58 % and 85.07 % in meat and digests, respectively. Regarding the linearity of the detection of pentosidine, R^2 of 0.9999 was reached, as shown in the standard curve (Figure 5.5).

[1]These samples were used for optimization of the method for pentosidine by a lab technician. I did not do the analysis, nor the optimization and received the numbers from my tutor.

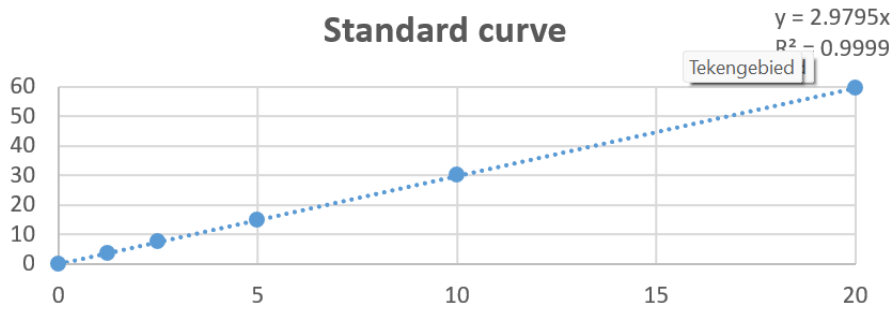

Figure 5.5: Pentosidine standards

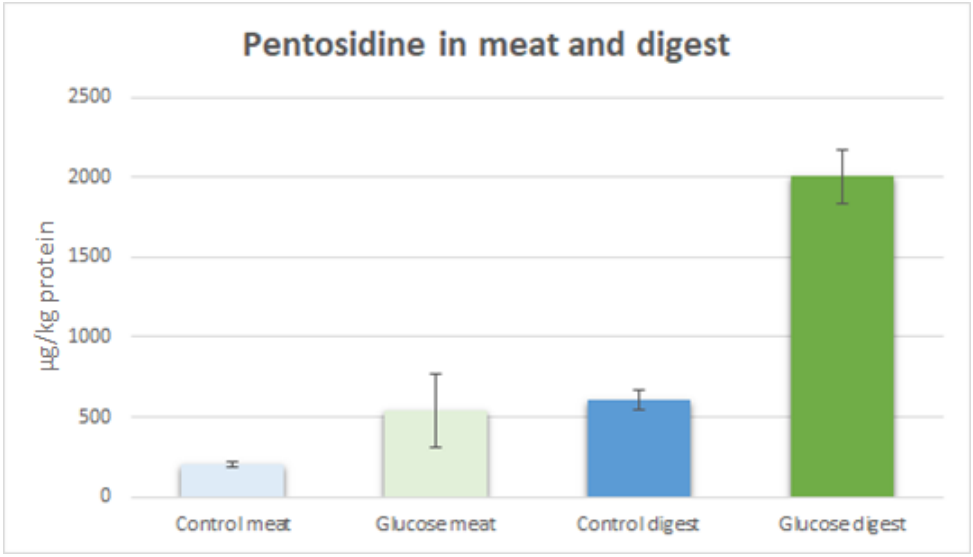

Figure 5.6: Concentration of the glycoxidation product, pentosidine, of pork shoulder in experiment 1, expressed in μg/kg protein, with (green) or without (blue) 5 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation.

5.2 Experiment 2: different heating temperatures (3% vs 0% Glu; aerobic vs anaerobic; 80°C vs 130°C vs 180°C)

In Figure 5.7, changes in the core temperature of the meat samples during heating at different heating temperatures (in the oven), are presented for experiment 2. Meat samples were removed from the oven when the core temperature reached a stable temperature of 75°C. Therefore, a higher heating temperature resulted in a shorter heating time. Meat samples heated in the oven at 80°C needed 100 minutes to reach 75°C, whereas samples heated at 180°C only needed approximately 20 minutes to reach this core temperature. Samples heated at 130°C reached 75°C after approximately 30 minutes.

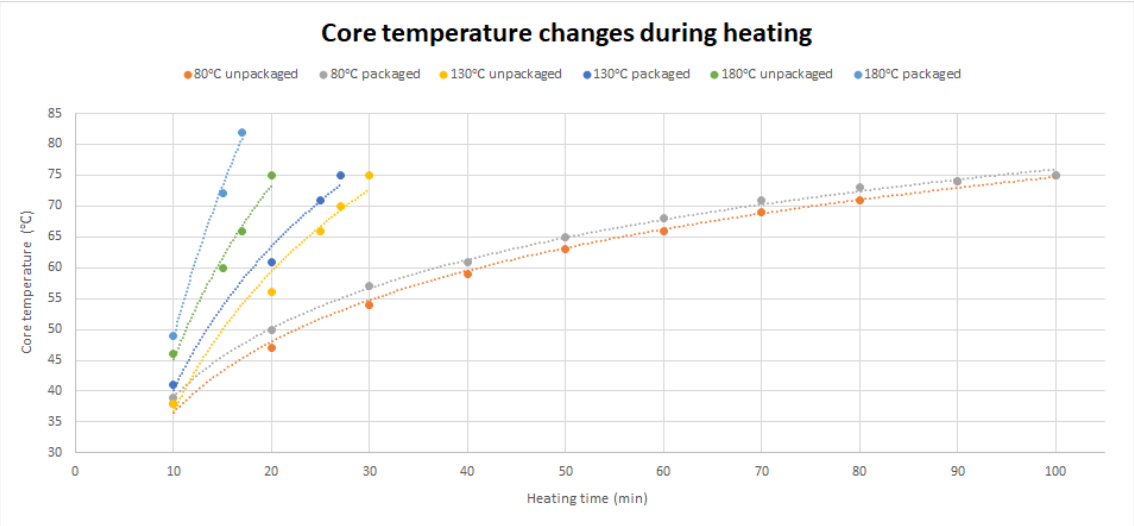

Figure 5.7: Changes in core temperature of the meat samples in experiment 2 until the core temperature reached 75°C. The meat samples were heated in the oven at either 80°C, 130°C or 180°C.

Figure 5.8 shows that gastrointestinal digestion clearly increased lipid oxidation, as the concentrations of MDA increased. For meat samples, supplementation with 3 % glucose had relatively minor effects on MDA concentrations, whereas this supplementation for the digest samples clearly increased MDA levels, especially for the two lowest heating temperatures (80°C and 130°C). Differences in MDA concentrations between heating with oxygen compared to heating without oxygen were relatively minor, if present.

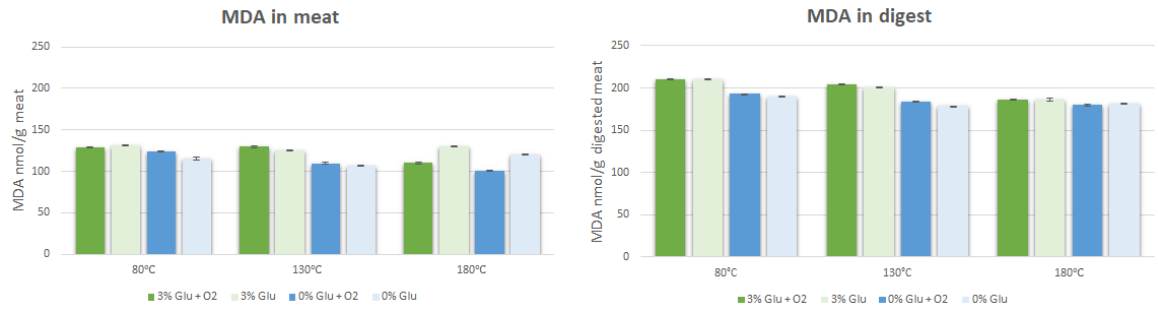

Figure 5.8: Concentration of the lipid oxidation product, malondialdehyde (MDA), of pork shoulder in experiment 2, expressed in nmol MDA/g (digested) meat, with (green) or without (blue) 3 % glucose, with (dark coloured) or without (light coloured) oxygen, before and after gastrointestinal digestion. Errorbars represent standard deviation.

Looking at protein oxidation, gastrointestinal digestion also increased PCC concentrations (Figure 5.9), which is demonstrated by the 2-fold (average) higher PCC concentrations in digest compared to meat samples. Both for meat and digest samples, supplementation with 3 % glucose or oxygen had hardly any influence on PCC concentrations, both for low and high heating temperatures.

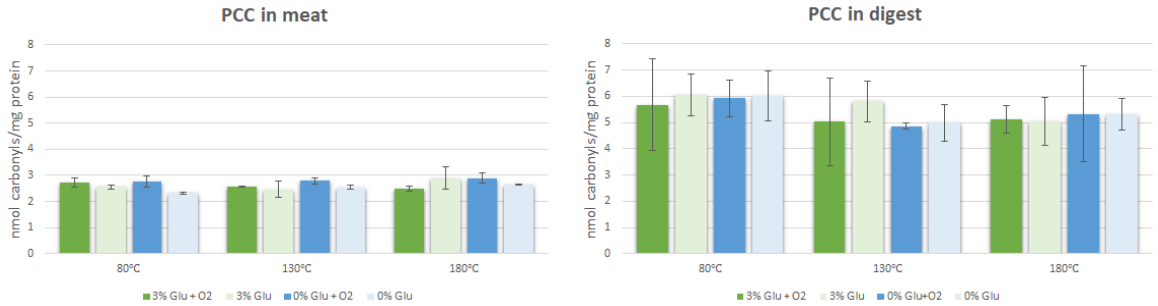

Figure 5.9: Concentration of protein carbonyl compounds (PCC) of pork shoulder in experiment 2, expressed in nmol carbonyls/mg protein, with (green) or without (blue) 3 % glucose, with (dark coloured) or without (light coloured) oxygen, before and after gastrointestinal digestion. Errorbars represent standard deviation.

Following gastrointestinal digestion, increased MRP concentrations were observed (Figure 5.10). For both meat and digest samples, no big differences between heating with oxygen and heating without oxygen were observed. From the data, it was clear that supplementation with 3 % glucose increased MRP concentrations in all meat samples, both for heating with oxygen and without oxygen. The effect of glucose supplementation on digest samples is less clear. In most cases, addition with 3 % glucose reduces MRP levels in the digest samples, with some exceptions.

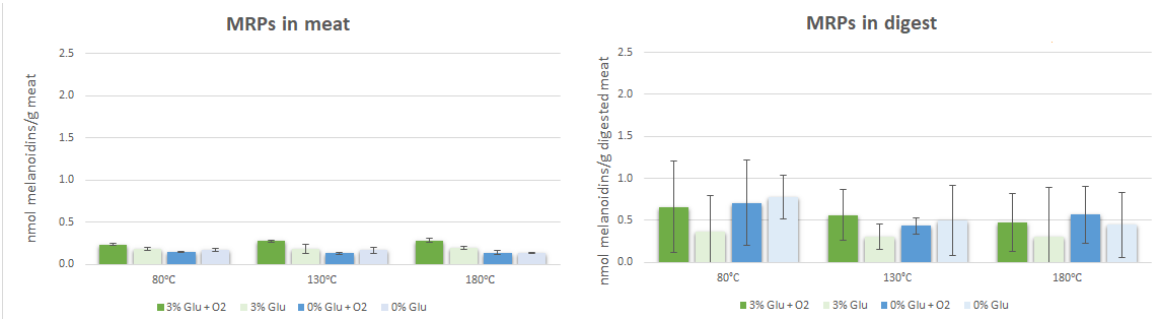

Figure 5.10: Concentration of Maillard reaction products (MRPs) of pork shoulder in experiment 2, expressed in nmol melanoidins/g (digested) meat, with (green) or without (blue) 3 % glucose, with (dark coloured) or without (light coloured) oxygen, before and after gastrointestinal digestion. Errorbars represent standard deviation.

Looking at the glycoxidation product, pentosidine[2], gastrointestinal digestion of the meat samples clearly increased pentosidine concentrations. Results show that both supplementation with 3 % glucose and oxygen have hardly any influence on pentosidine concentrations in both meat and digest samples (Figure 5.11).

[2]For the pentosidine content in meat, the results only show data from one replicate, because of a problem with the HPLC. This is why there are no error bars present. These numbers were again obtained from my tutor who did the analysis in the lab.

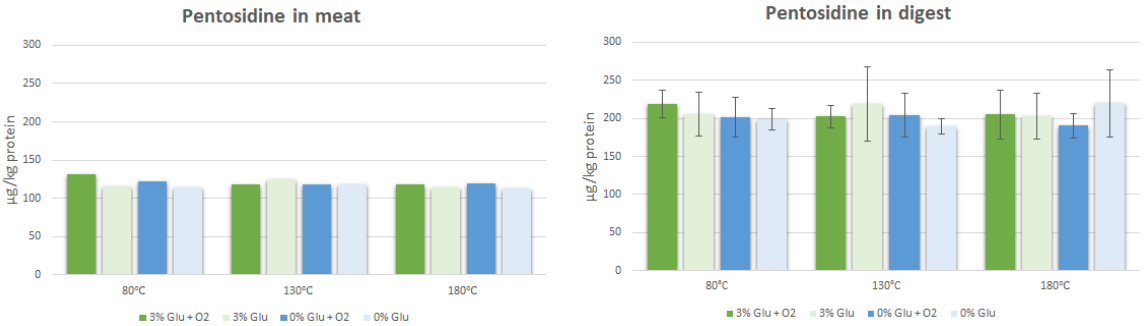

Figure 5.11: Concentration of the glycoxidation product, pentosidine of pork shoulder in experiment 2, expressed in μg/kg protein, with (green) or without (blue) 3 % glucose, before (light coloured) and after (dark coloured) gastrointestinal digestion. Errorbars represent standard deviation.

From experiment 2, it was clear that heating temperature did not have a lot of influence on the formation of oxidation, glycation and glycoxidation products since similar concentrations were found for meats subjected to the three temperatures. Therefore, experiment 3 was set up to check whether heating time is an important factor in their formation.

5.3 Experiment 3: different heating times (3% vs 0% Glu; aerobic; 180°C;0 vs 20 vs 40 vs 60 vs 80 vs 100 min)

Figure 5.12 gives a picture of the different meat samples of experiment 3, heated at 180°C for different heating times. More browning is observed as the heating time increases. In other words, the longer the heating time, the darker the meat samples.

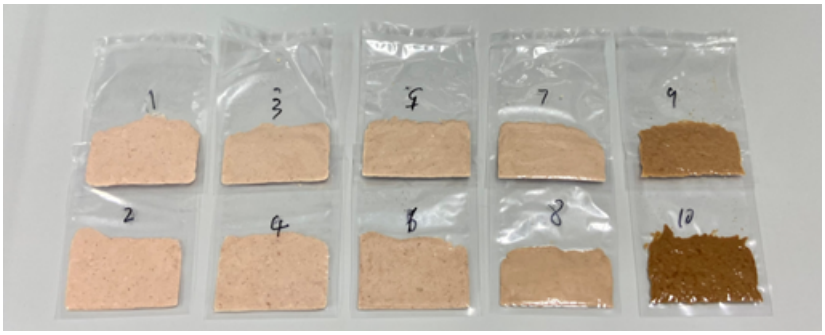

Figure 5.12: Picture of the different meat samples after heating in the oven at 180°C at different heating times (experiment 3). Bag 1 and 2 = samples heated for 20 minutes; 3 and 4 = samples heated for 40 minutes; 5 and 6 = samples heated for 60 minutes; 7 and 8 = samples heated for 80 minutes; 9 and 10 = samples heated for 100 minutes.

Like experiment 2, experiment 3 shows that gastrointestinal digestion increased lipid oxidation, as the MDA concentrations increased (Figure 5.13). In both meat and digest samples, it could be expected that the MDA concentration would increase with heating time. Figure 5.13 contradicts this, showing that the MDA concentration increased until the heating time reached 60 minutes. From that time, a further increase of the heating time resulted in lower MDA concentrations.
Furthermore, Figure 5.13 confirms that differences in MDA between meat digests with or without glucose are relatively minor ($P = 0.4233$). Although, supplementation with 3 % glucose had a contrasting effect on the MDA concentration, depending on heating time.

Figure 5.14 gives the comparison of the PCC formed in meat and in digest samples. Different glucose levels resulted in significant differences in PCC levels after digestion ($P = 0.0075$). For both meat and digest samples, the influence of glucose on PCC levels increased with heating time. Furthermore, longer heating time and supplementation with 3 % glucose clearly increased PCC levels. The effect of digestion on PCC levels was relatively minor for the lower heating times, but reduced PCC levels at the heating time of 100 minutes.

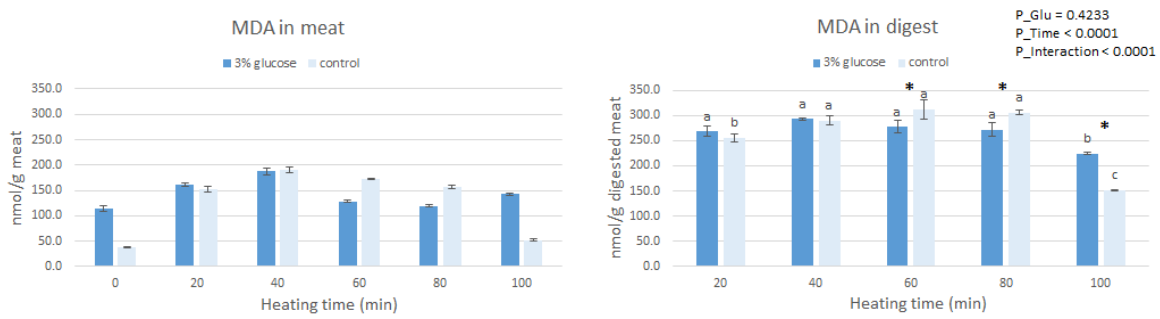

Figure 5.13: Concentration of the lipid oxidation product, malondialdehyde (MDA), of pork shoulder in experiment 3, expressed in nmol MDA/g (digested) meat, with (dark blue) or without (light blue) 3 % glucose, before and after gastrointestinal digestion. Errorbars represent standard deviation. Data was statistically analyzed using a linear ANOVA with fixed factors: glucose content (PGlu), heating time (PTime) and the interaction (PInteraction), where $P < 0.05$ indicates significant differences. Different letters (a-c) indicate significant difference ($P < 0.05$) between different heating times within the same glucose level. Asterix (*) indicates significant difference ($P < 0.05$) between different glucose levels within the same heating time.

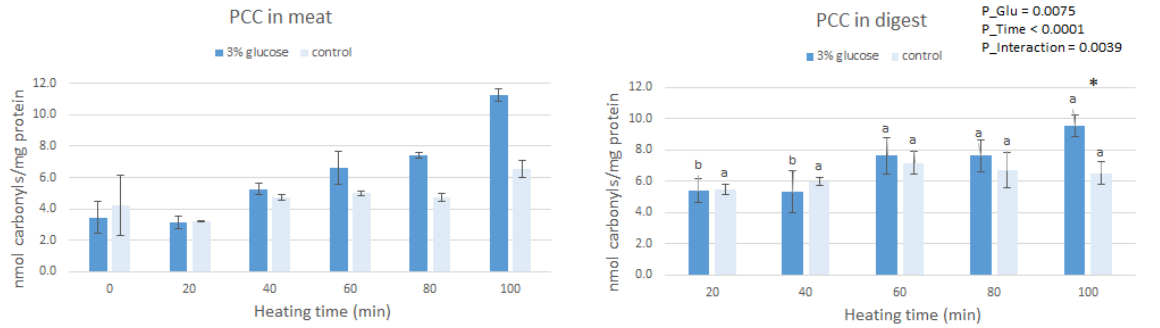

Figure 5.14: Concentration of protein carbonyl compounds (PCC) of pork shoulder in experiment 3, expressed in nmol carbonyls/mg protein, with (dark blue) or without (light blue) 3 % glucose, before and after gastrointestinal digestion. Errorbars represent standard deviation. Data was statistically analyzed using a linear ANOVA with fixed factors: glucose content (PGlu), heating time (PTime) and the interaction (PInteraction), where $P < 0.05$ indicates significant differences. Different letters (a-b) indicate significant difference ($P < 0.05$) between different heating times within the same glucose level. Asterix (*) indicates significant difference ($P < 0.05$) between different glucose levels within the same heating time.

Following gastrointestinal digestion, increased MRP and pentosidine concentrations are observed, with some exceptions (Figure 5.15 and 5.16). In addition, it is clear that longer heating time ($P < 0.0001$) and supplementation with 3 % glucose ($P < 0.0001$) increases their concentrations. Especially the longer heating times (80 and 100 minutes) show a significant difference between different glucose levels.

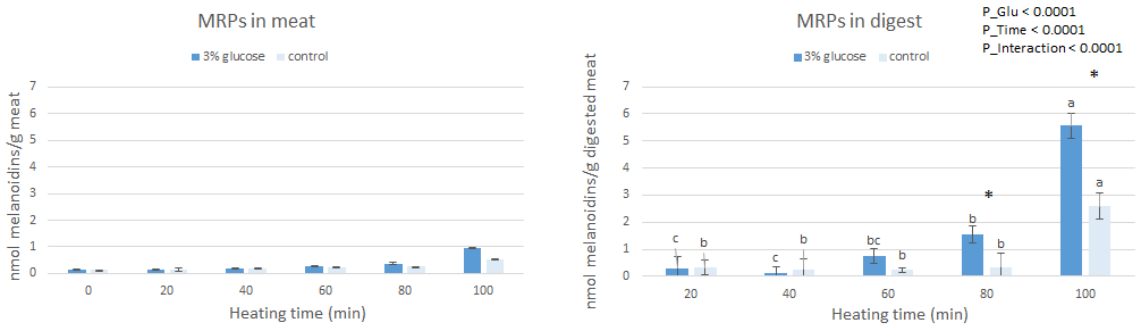

Figure 5.15: Concentration of Maillard reaction products (MRPs) of pork shoulder in experiment 3, expressed in nmol melanoidins/g (digested) meat, with (dark blue) or without (light blue) 3 % glucose, before and after gastrointestinal digestion. Errorbars represent standard deviation. Data was statistically analyzed using a linear ANOVA with fixed factors: glucose content (PGlu), heating time (PTime) and the interaction (PInteraction), where $P < 0.05$ indicates significant differences. Different letters (a-c) indicate significant difference ($P < 0.05$) between different heating times within the same glucose level. Asterix (*) indicates significant difference ($P < 0.05$) between different glucose levels within the same heating time.

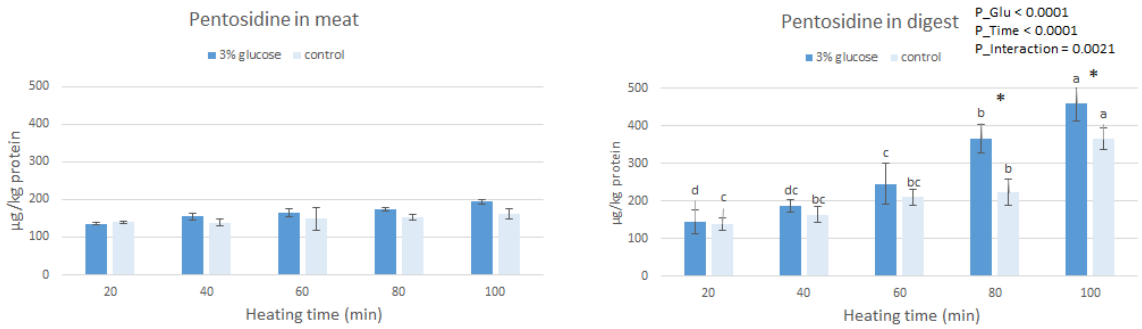

Figure 5.16: Concentration of the glycoxidation product, pentosidine, of pork shoulder in experiment 3, expressed in μg/kg protein, with (dark blue) or without (light blue) 3 % glucose, before and after gastrointestinal digestion. Errorbars represent standard deviation. Data was statistically analyzed using a linear ANOVA with fixed factors: glucose content (PGlu), heating time (PTime) and the interaction (PInteraction), where $P < 0.05$ indicates significant differences. Different letters (a-d) indicate significant difference ($P < 0.05$) between different heating times within the same glucose level. Asterix (*) indicates significant difference ($P < 0.05$) between different glucose levels within the same heating time.

CHAPTER 6

DISCUSSION

This thesis was designed to investigate the effect of heating conditions and glucose addition to pork shoulder, on the formation of oxidation, glycation and glycoxidation products during gastrointestinal digestion. This is of relevance since these compounds may potentially contribute to explain the possible link between high red (processed) meat consumption and the development of several chronic diseases. Three experiments with three different factors (glucose content, heating conditions and packaging system) were executed to elucidate this. The average glucose amount that is commonly added to a wide range of commercial luncheon meat products is 2%. In some products (e.g. pate), the glucose content can go up to 4% (and sometimes even 9% in e.g. marinades)[1]. So the used 3% glucose in experiment 2 & 3 is somewhat higher than the average amount, but is still a realistic concentration.

It has been reported that not only in food production but also during gastrointestinal digestion, oxidation and glycation may be promoted in meat and meat products. This can be explained by the presence of various pro-oxidant components in the GIT, such as peroxidases in the saliva, iron ions and hydroperoxides in the gastric juice and a.o. calcium[2] (which enhances the action of lipase to break down products) in the intestinal juice (Johnson and Decker, 2015). As a result, many harmful compounds like LOPs, POPs, MRPs and pentosidine may occur from oxidation and/or glycation during the digestion of meat.

[1]There are no published papers on the glucose content of meat products. The mentioned data comes from my tutor who executed a survey about the sugar content in meat products.

[2]Calcium plays an important role in lipid oxidation: lipid hydrolysis can be inhibited by the accumulation of long-chain free fatty acids on the surface of fat droplets. Calcium can precipitate these free fatty acids in order to enhance the action of lipase to break down lipids (Zangenberg et al., 2001). Calcium is also an important cofactor for the activity of lipase.

6.1 Oxidation

According to Carvalho et al. (2015), high red (processed) meat[3] consumption by healthy individuals results in high plasma MDA levels because the stomach generates large amounts of ROS, promoting lipid oxidation during digestion. The meat fatty acid profile together with heating conditions play an important role for MDA formation in its passage through the gastrointestinal tract. Also high heme levels seem to contribute to oxidation (Guéraud et al., 2015). This could be explained by the fact that heating above 60°C can trigger oxidative cleavage of the porphyrin ring, resulting in the release of heme iron (Miller et al., 1994), which stimulates the Fenton reaction. This can lead to more formation of hydroxyl radicals, which may then give rise to more lipid and protein oxidation (Bastide et al., 2011). In the experiments of this thesis, pork shoulder was used, which has a relatively high iron content and PUFA content, thus promoting lipid oxidation (Campo et al., 2006). For all three experiments, heating temperatures higher than 60°C were used, resulting in the release of heme iron. Next to digestion of meat, heating is also an important factor in oxidation, as it destroys the cell membranes, in that way that lipid components are exposed to oxygen, so more oxidation should occur.

The results in Figure 5.13 showed that MDA concentrations reached a maximum around a moderate heating time (60 minutes) and then reduced when increasing the heating time. This could be explained by the fact that heating releases free iron in meat, reduces antioxidant enzymes[4] (Hoac et al., 2006) and releases oxygen from oxymyoglobine, which leads to the production of H_2O_2 (Kanner, 1994). These reactions can stimulate the Fenton reaction, leading to more lipid oxidation. The lower concentrations of MDA when reaching a prolonged heating time, could be explained by evaporation of aldehydes caused by intense heating or decreased pro-oxidant effect of oxymyoglobine when heated to core temperatures above 75°C. It could also be possible that, at longer heating times, MDA can further participate in glycation by the reaction with MRPs and contribute to the formation of AGEs, leading to lower observed MDA concentrations. These results are in contrast with the results of Broncano et al. (2009), which showed that raw meat had the lowest MDA values and heating meat gave rise to more lipid oxidation (higher MDA values). Nevertheless, this phenomenon has already been observed previously by Bou et al. (2008).

[3] Fish (which contains a lot of n-3 PUFA) digestion leads to more MDA formation, compared to meat digestion (Larsson et al., 2016). As meat contains especially saturated FA and oxidation is taking place at the level of unsaturated FA, the importance of MDA in the link between red meat and health could be questioned.

[4] MEI et al. (1994) showed that the activity of some antioxidant enzymes (such as catalase and glutathione peroxidase) decreases in cooked meat compared to uncooked meat.

In addition to lipid oxidation, it is currently known that proteins in meat can also be a source and target of ROS (Estévez, 2011). It is known that the oxidation of lipids during the heating and digestion of fresh meat stimulates the oxidation of meat proteins (Van Hecke et al., 2014b), although during heating and digestion of processed meat, other factors like reducing sugars will likely determine the oxidation of proteins. The results of experiment 3 showed that digestion of pork shoulder increased PCC levels for gentle heating conditions (heating time of 20 minutes), which was also observed in experiment 2. For moderate heating conditions, PCC levels remained approximately equal in experiment 3, whereas for the longest heating time (100 minutes) in experiment 3, but also in experiment 1 (extreme heating conditions), a decrease in PCC was observed. The lower amounts of protein carbonyls in the digests during extreme heating conditions can be explained by a possible further degradation of protein carbonyls during digestion into carboxylic acids, Schiff base structures, aldol condensation products, and Strecker degradation aldehydes (Estévez, 2011). Another possible explanation for the lower amounts is the fact that the added glucose can not only react with proteins, but also with LOPs. These products can further interact with glycation and lead to glycoxidation products.
Since lipid oxidation produces free radicals (such as alkyl, alcoxyl, and peroxyl radicals) which have been observed to initiate protein oxidation (Lund et al., 2011b), these will likely contribute to boost protein oxidation during heating. Furthermore, thermal treatments can impair the structure of meat proteins by the breaking of hydrogen or electrostatic bonds. As a consequence, an exposure to the protein surface of hydrophobic amino acids can occur (Santé-Lhoutellier et al., 2008), thereby favouring formation of protein aggregates. The results of this experiment indeed showed that increasing the heating time, resulted in higher PCC concentrations. In general, the literature describes that the carbonyl levels in raw meat are lower than in cooked meat products (expressed in nmol/mg protein). It can be suggested that the actions during the preparation of meat products (such as cutting and heating) could promote the formation of carbonyl groups (Estévez, 2011), which is also confirmed in this experiment.

6.2 Glycation & glycoxidation

During thermal processing of food like meat and meat products, large amounts of dietary AGEs (dAGEs) are formed (Monnier and Cerami, 1981). This formation of dAGEs occurs via the Maillard reaction in which the carbonyl group of a reducing sugar reacts with an amino acid of a protein under the influence of heat. Although in some studies dAGEs are considered to play an important role in the negative health effects

attributed to the Western diet, the health consequences of these compounds are not yet well understood.
Like described in the previous section, during gastrointestinal digestion of food products like meat, several mechanical and enzymatic processes break down the ingested proteins. The natural conditions of the GIT (pH, enzymes, temperature) might be a favorable environment for the Maillard reaction to take place (Nursten, 2005). In the stomach, acid hydrolysis occurs and the proteolytic enzyme, pepsin, cleaves proteins into smaller polypeptides. Further, in the small intestine, the pH increases and different proteolytic enzymes cleave the polypeptides into dipeptides and peptides, which become then available for absorption. Uribarri et al. (2007) and Somoza (2005) have shown that MRPs (like AGEs and melanoidins) are partially absorbed in the GIT. Consequently, the occurrence of the Maillard reaction during the digestion process may reduce the bioavailability of essential amino acids and increase the production of MRPs causing health disorders. Many researchers reported a positive role of dietary lipids and glucose in promoting this Maillard reaction. The reaction mechanism is described in detail in section 2.2.1. At the final stage of this reaction, AGEs are formed, which are largely responsible for the brown colour and palatability of heat-processed meat.

The precursors glyoxal (GO) and methylglyoxal (MGO) of AGEs occur exogenously and endogenously (Sharma et al., 2015; CATAK et al., 2022). These α-dicarbonyl compounds (α-DCs), GO and MGO, can be formed from Maillard reactions during food processing as well as protein oxidation and lipid peroxidation. There are only a limited number of studies on the increase or decrease of GO and MGO in foods *in vitro* and the existing evidence on the effects of digestion on AGEs is conflicting. According to Papetti et al. (2014), digestion could reduce the concentration of α-DCs in foods, because they may convert to harmful AGEs in the presence of digestive enzymes. This decrease in α-DCs during digestion may also result from a reaction of α-DCs with proteins where AGEs are formed as the final product. This could explain the decrease in PCC at longer heating times together along with the increase in pentosidine after digestion, described in chapter 5 and the previous section. However, in the same study, Papetti et al. (2014) have shown that in some products, these AGE precursors were increased after digestion. The reason for these unclear associations is probably related to the food matrix and heating conditions.
Next to digestion, it is clear that heating is also an important factor for the formation of AGEs. Chao et al. (2009) suggested that heating leads to more amino acids release, which allowed reducing sugars to react with the released amino acids and facilitate more formation of MRPs, and further AGEs, which is indeed in accordance with the results showing higher MRP and pentosidine concentrations at longer heating times.

When oxidation accompanies glycation, harmful glycoxidation end products are formed like pentosidine. Pentosidine is an AGE and protein cross-link product formed by the reaction of pentoses or hexoses (like glucose) with lysine and arginine residues of proteins (Sell and Monnier, 1989). Currently, there are still no studies on pentosidine formation during *in vitro* digestion, but there are some studies about its formation during meat processing. The reaction to form pentosidine is favoured by high temperatures (over 100°C) for an extended heating time (more than 15 minutes). For the reaction to take place, both the reagent amount (sugars, proteins) and water are important influencers. A low water content increases viscosity and renders the encounter of reagents difficult, whereas a too high water content hinders dehydration, characterising the Maillard reaction.

6.3 General discussion

To summarize, it was clear that **glucose addition and heating time** had a lot of **influence on the oxidation (especially protein oxidation), glycation and glycoxidation process** during heating and digestion. The **influence of oxygen** was **less clear**, so the food matrix and components in the GIT are probably more important factors determining the formation of oxidation, glycation and glycoxidation products. Furthermore, **gastrointestinal digestion** clearly results in **more lipid oxidation**, so more MDA formation in digested meat, whereas digestion has a debatable effect on protein oxidation. At **gentle heating conditions** (heating conditions in experiment 2 and 20 minutes heating in experiment 3), whether pork shoulder was supplemented with glucose or not, **digestion increased PCC concentrations**. At **mild heating conditions** (like 40/60/80 minutes heating in experiment 3), **PCC was approximately equal** or increased after digestion, whereas **extreme heating conditions** (heating conditions in experiment 1 and 100 minutes heating in experiment 3) **decreased PCC concentrations** in digests. This could possibly (partly) be explained by further degradation reactions. However, something to be noted is the fact that carbonyls in the human body are not only present through oxidation of proteins but also by food itself. This may lead to an overestimation of protein oxidation and oxidative stress. On the contrary, carbonyls are not formed during the oxidation of all amino acids, which may cause an underestimation of protein oxidation (Davies et al., 1999).

Additionally, during the digestive process, the Maillard reaction takes place and glycoxidation products like AGEs are formed with quantities depending on the type of food product and thermal treatment. Overall, **glycation and glycoxidation increase after gastrointestinal digestion** of pork shoulder. This AGE formation

during heating or digestion leads to a noticeable loss of amino acids released from proteins during digestion. This represents an interesting, but usually forgotten issue, which is the loss of nutritional value of meat products resulting from the Maillard reaction. It is already reported that these AGE precursors are known to be the cause of many chronic diseases, such as T2DM, Alzheimer's, several types of cancer, and diabetes complications (Martinez-Saez et al., 2019). This is due to the fact that a small percent of ingested AGEs are absorbed into the circulation system, being incorporated into the body pool of AGEs and therefore contributing to an overall state of chronic oxidative stress (Vlassara and Uribarri, 2014). The largest amount of unabsorbed AGEs will continue its transit into the colon where it can interact with the microbiota, react with AGE receptors (RAGE) within the colonic wall initiating inflammation. In terms of health, it is important to reduce these products.

6.4 Future prospects

It is important to keep in mind that our diet does not consist of only meat. In this study, no other nutritional components were taken into account. For example, fruits and vegetables are an important source of antioxidants (such as ascorbic acid) and could reduce the formation of potentially harmful oxidation and glycation products. Studies combining these components would provide a more accurate picture of the association of red (processed) meat consumption with oxidation, glycation and glycoxidation.

Furthermore, next to the most consumed processed meat (pork), beef and chicken are also highly consumed meats with another fatty acid profile and heme content than pork. Beef contains for example more heme than pork, whereas chicken contains less. In addition, beef is a ruminant and therefore contains more saturated fatty acids than chicken and pork, which are monogastric animals, leading to another degree of oxidation. In addition, also other heating methods like grilling or barbecueing are common, which will probably have faster MRP formation and oxidation. Therefore, to get a better overview on the formation of oxidation, glycation and glycoxidation products, different meats with different heating methods should be compared in further studies.

CHAPTER 7

CONCLUSION

In conclusion, the present study confirmed the first three hypotheses (a, b and c), made in chapter 3, except the effect of digestion on lipid oxidation is not fully clear. Results showed that the **addition of glucose** and the **increase in heating time** can **stimulate protein oxidation, glycation and glycoxidation** during thermal processing and digestion of pork shoulder. Furthermore, it looks like heating time is a more important factor in the formation of these potentially harmful products than heating temperature. The results also showed that **digestion** of thermally processed pork shoulder supplemented with glucose is associated with **more lipid oxidation, glycation and glycoxidation** and therefore potentially harmful substances are formed after red (processed) meat consumption. For protein oxidation, the results were not always similar and for meat samples at **extreme heating conditions, digestion reduced PCC concentrations**. Nevertheless, it is still questionable if higher MDA, PCC, MRP or pentosidine levels are associated with greater risk of colon cancer by weekly consumption of pork shoulder. Because our diet contains more than only meat, it is difficult taking into account this thesis, to give recommendations regarding meat consumption in order to decrease the risk on chronic diseases like cancer. But finding strategies (like adding herbs or spices) to optimize meat processing could certainly help. Furthermore, it is well known that high levels of meat intake are often associated with low levels of intake of fruits and vegetables, less physical activity, a more unhealthy lifestyle etc, which could all play an important role in the link with increased cancer risk.

Considering all the above, it seems obvious that future research is required to help to clarify if oxidation, glycation and glycoxidation products generated from food lipids and proteins during gastrointestinal digestion are present in great enough concentrations to provoke negative health effects. In addition, **other nutritional components** and the **genetic background** of individuals should be taken into account, to fully understand their impact.

BIBLIOGRAPHY

(n.d.). Hyperglycemia (high blood glucose).

Akıllıoğlu, H. G. and Gökmen, V. (2019). Advanced glycation end products (ages). In *Chemical Hazards in Thermally-Processed Foods*, pages 121–151. Springer.

Barden, L. and Decker, E. A. (2016). Lipid oxidation in low-moisture food: a review. *Critical reviews in food science and nutrition*, 56(15):2467–2482.

Bastide, N. M., Pierre, F. H., and Corpet, D. E. (2011). Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved. *Cancer prevention research*, 4(2):177–184.

Boada, L. D., Henríquez-Hernández, L. A., and Luzardo, O. (2016). The impact of red and processed meat consumption on cancer and other health outcomes: Epidemiological evidences. *Food and Chemical Toxicology*, 92:236–244.

Boldyrev, A. A., Aldini, G., and Derave, W. (2013). Physiology and pathophysiology of carnosine. *Physiological reviews*.

Bou, R., Guardiola, F., Codony, R., Faustman, C., Elias, R. J., and Decker, E. A. (2008). Effect of heating oxymyoglobin and metmyoglobin on the oxidation of muscle microsomes. *Journal of agricultural and food chemistry*, 56(20):9612–9620.

Brett, J., Schmidt, A. M., Du Yan, S., Zou, Y. S., Weidman, E., Pinsky, D., Nowygrod, R., Neeper, M., Przysiecki, C., Shaw, A., et al. (1993). Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. *The American journal of pathology*, 143(6):1699.

Brings, S., Fleming, T., Freichel, M., Muckenthaler, M. U., Herzig, S., and Nawroth, P. P. (2017). Dicarbonyls and advanced glycation end-products in the development of diabetic complications and targets for intervention. *International journal of molecular sciences*, 18(5):984.

Broncano, J., Petrón, M., Parra, V., and Timón, M. (2009). Effect of different cooking methods on lipid oxidation and formation of free cholesterol oxidation products (cops) in latissimus dorsi muscle of iberian pigs. *Meat science*, 83(3):431–437.

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

Carvalho, A., Miranda, A., Santos, F., Loureiro, A. P. d. M., Fisberg, R. M., and Marchioni, D. (2015). High intake of heterocyclic amines from meat is associated with oxidative stress. *British Journal of Nutrition*, 113(8):1301–1307.

CATAK, J., Yaman, M., Ugur, H., Servi, E. Y., and FARUK MIZRAK, Ö. (2022). Investigation of the advanced glycation end products precursors in dried fruits and nuts by hplc using pre-column derivatization. *Journal of Food & Nutrition Research*, 61(1).

Cayuela, J. M., Gil, M. D., Bañón, S., and Garrido, M. D. (2004). Effect of vacuum and modified atmosphere packaging on the quality of pork loin. *European Food Research and Technology*, 219(4):316–320.

Chaijan, M. and Panpipat, W. (2017). Mechanism of oxidation in foods of animal origin. In *Natural Antioxidants*, pages 21–58. Apple Academic Press.

Chao, P.-c., Hsu, C.-c., and Yin, M.-c. (2009). Analysis of glycative products in sauces and sauce-treated foods. *Food Chemistry*, 113(1):262–266.

Davies, K. J. (2000). Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB life*, 50(4-5):279–289.

Davies, M. J., Fu, S., Wang, H., and Dean, R. T. (1999). Stable markers of oxidant damage to proteins and their application in the study of human disease. *Free Radical Biology and Medicine*, 27(11-12):1151–1163.

Delgado-Andrade, C. and Fogliano, V. (2018). Dietary advanced glycosylation endproducts (dages) and melanoidins formed through the maillard reaction: physiological consequences of their intake. *Annual review of food science and technology*, 9:271–291.

Demeyer, D., Mertens, B., De Smet, S., and Ulens, M. (2016). Mechanisms linking colorectal cancer to the consumption of (processed) red meat: a review. *Critical reviews in food science and nutrition*, 56(16):2747–2766.

Domingo, J. L. and Nadal, M. (2017). Carcinogenicity of consumption of red meat and processed meat: A review of scientific news since the iarc decision. *Food and chemical toxicology*, 105:256–261.

Domínguez, R., Pateiro, M., Gagaoua, M., Barba, F. J., Zhang, W., and Lorenzo, J. M. (2019). A comprehensive review on lipid oxidation in meat and meat products. *Antioxidants*, 8(10):429.

Dyer, D. G., Blackledge, J. A., Thorpe, S. R., and Baynes, J. W. (1991). Formation of pentosidine during nonenzymatic browning of proteins by glucose. identification of glucose and other carbohydrates as possible precursors of pentosidine in vivo. *Journal of Biological Chemistry*, 266(18):11654–11660.

Esterbauer, H., Schaur, R. J., and Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free radical Biology and medicine*, 11(1):81–128.

Esterbauer, H. and Zollern, H. (1989). Methods for determination of aldehydic lipid peroxidation products. *Free Radical Biology and Medicine*, 7(2):197–203.

Estévez, M. (2011). Protein carbonyls in meat systems: A review. *Meat science*, 89(3):259–279.

Estévez, M., Kylli, P., Puolanne, E., Kivikari, R., and Heinonen, M. (2008a). Fluorescence spectroscopy as a novel approach for the assessment of myofibrillar protein oxidation in oil-in-water emulsions. *Meat Science*, 80(4):1290–1296.

Estévez, M., Morcuende, D., and Ventanas, S. (2008b). Determination of oxidation. handbook of processed meat and poultry analysis. editörler: Lml mollet, f. toldrá.

Estévez, M., Ollilainen, E., and Heinonen, M. (2008c). α-aminoadipic and γ-glutamic semialdehydes as indicators of protein oxidation in myofibrillar proteins. In *Proceedings of the 54th international congress on meat science and technology (ICoMST), Cape Town, South Africa*.

Estévez, M., Ollilainen, V., and Heinonen, M. (2009). Analysis of protein oxidation markers α-aminoadipic and γ-glutamic semialdehydes in food proteins using liquid chromatography (lc)- electrospray ionization (esi)- multistage tandem mass spectrometry (ms). *Journal of Agricultural and Food Chemistry*, 57(9):3901–3910.

Fang, H., Wang, L., Zhang, S., Liu, H., and Li, J. (2014). Advanced glycation end products (ages) formation in high protein foods processing model system. *Journal of Chinese Institute of Food Science and Technology*, 14(2):28–34.

Frankel, E. N. (1984). Lipid oxidation: mechanisms, products and biological significance. *Journal of the American Oil Chemists' Society*, 61(12):1908–1917.

Ganhão, R., Morcuende, D., and Estévez, M. (2010). Protein oxidation in emulsified cooked burger patties with added fruit extracts: Influence on colour and texture deterioration during chill storage. *Meat science*, 85(3):402–409.

Ghnimi, S., Budilarto, E., and Kamal-Eldin, A. (2017). The new paradigm for lipid oxidation and insights to microencapsulation of omega-3 fatty acids. *Comprehensive Reviews in Food Science and Food Safety*, 16(6):1206–1218.

Giovannucci, E. and Michaud, D. (2007). The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. *Gastroenterology*, 132(6):2208–2225.

Gorelik, S., Kohen, R., Ligumsky, M., and Kanner, J. (2007). Saliva plays a dual role in oxidation process in stomach medium. *Archives of Biochemistry and Biophysics*, 458(2):236–243.

Grandhee, S. and Monnier, V. (1991). Mechanism of formation of the maillard protein cross-link pentosidine. glucose, fructose, and ascorbate as pentosidine precursors. *Journal of Biological Chemistry*, 266(18):11649–11653.

Grotto, D., Santa Maria, L., Boeira, S., Valentini, J., Charão, M., Moro, A., Nascimento, P., Pomblum, V., and Garcia, S. (2007). Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography–visible detection. *Journal of pharmaceutical and biomedical analysis*, 43(2):619–624.

Guéraud, F., Taché, S., Steghens, J.-P., Milkovic, L., Borovic-Sunjic, S., Zarkovic, N., Gaultier, E., Naud, N., Héliès-Toussaint, C., Pierre, F., et al. (2015). Dietary polyunsaturated fatty acids and heme iron induce oxidative stress biomarkers and a cancer promoting environment in the colon of rats. *Free Radical Biology and Medicine*, 83:192–200.

Guiotto, A., Calderan, A., Ruzza, P., and Borin, G. (2005). Carnosine and carnosine-related antioxidants: a review. *Current medicinal chemistry*, 12(20):2293–2315.

Gupta, R. K., Gupta, K., Sharma, A., Das, M., Ansari, I. A., and Dwivedi, P. D. (2018). Maillard reaction in food allergy: Pros and cons. *Critical reviews in food science and nutrition*, 58(2):208–226.

Hegab, Z., Gibbons, S., Neyses, L., and Mamas, M. A. (2012). Role of advanced glycation end products in cardiovascular disease. *World journal of cardiology*, 4(4):90.

Hematyar, N., Rustad, T., Sampels, S., and Kastrup Dalsgaard, T. (2019). Relationship between lipid and protein oxidation in fish. *Aquaculture Research*, 50(5):1393–1403.

Hemeryck, L. Y. and Vanhaecke, L. (2016). Diet-related dna adduct formation in relation to carcinogenesis. *Nutrition reviews*, 74(8):475–489.

Hoac, T., Daun, C., Trafikowska, U., Zackrisson, J., and Åkesson, B. (2006). Influence of heat treatment on lipid oxidation and glutathione peroxidase activity in chicken and duck meat. *Innovative Food Science & Emerging Technologies*, 7(1-2):88–93.

Hoyland, D. and Taylor, A. (1991). A review of the methodology of the 2-thiobarbituric acid test. *Food Chemistry*, 40(3):271–291.

Ilieva, E. V., Naudí, A., Kichev, A., Ferrer, I., Pamplona, R., and Portero-Otín, M. (2010). Depletion of oxidative and endoplasmic reticulum stress regulators in pick disease. *Free Radical Biology and Medicine*, 48(10):1302–1310.

Jakas, A. and Horvat, Š. (2003). Study of degradation pathways of amadori compounds obtained by glycation of opioid pentapeptide and related smaller fragments: Stability, reactions, and spectroscopic properties. *Biopolymers: Original Research on Biomolecules*, 69(4):421–431.

Johnson, D. R. and Decker, E. A. (2015). The role of oxygen in lipid oxidation reactions: a review. *Annual review of food science and technology*, 6:171–190.

Kanner, J. (1994). Oxidative processes in meat and meat products: quality implications. *Meat science*, 36(1-2):169–189.

Kanner, J. and Lapidot, T. (2001). The stomach as a bioreactor: dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biology and Medicine*, 31(11):1388–1395.

Karakaya, M., Bayrak, E., and Ulusoy, K. (2011). Use of natural antioxidants in meat and meat products. *Journal of Food Science and Engineering*, 1(1):1.

Koch, M., Chitayat, S., Dattilo, B. M., Schiefner, A., Diez, J., Chazin, W. J., and Fritz, G. (2010). Structural basis for ligand recognition and activation of rage. *Structure*, 18(10):1342–1352.

Larsson, K., Harrysson, H., Havenaar, R., Alminger, M., and Undeland, I. (2016). Formation of malondialdehyde (mda), 4-hydroxy-2-hexenal (hhe) and 4-hydroxy-2-nonenal (hne) in fish and fish oil during dynamic gastrointestinal in vitro digestion. *Food & function*, 7(2):1176–1187.

Levine, R. L., Williams, J. A., Stadtman, E. P., and Shacter, E. (1994). [37] carbonyl assays for determination of oxidatively modified proteins. *Methods in enzymology*, 233:346–357.

Long, E. K. and Picklo Sr, M. J. (2010). Trans-4-hydroxy-2-hexenal, a product of n-3 fatty acid peroxidation: Make some room hne.... *Free Radical Biology and Medicine*, 49(1):1–8.

Lorenzo, J., Domínguez, R., and Carballo, J. (2017). Control of lipid oxidation in muscle food by active packaging technology. *Natural Antioxidants: Applications in Foods of Animal Origin; Banerjee, R., Verma, AK, Siddiqui, MW, Eds*, pages 343–382.

Łuczaj, W. and Skrzydlewska, E. (2003). Dna damage caused by lipid peroxidation products. *Cell Mol Biol Lett*, 8(2):391–413.

Luévano-Contreras, C., Gómez-Ojeda, A., Macías-Cervantes, M. H., and Garay-Sevilla, M. E. (2017). Dietary advanced glycation end products and cardiometabolic risk. *Current diabetes reports*, 17(8):1–11.

Lund, M. N., Heinonen, M., Baron, C. P., and Estévez, M. (2011a). Protein oxidation in muscle foods: A review. *Molecular nutrition & food research*, 55(1):83–95.

Lund, M. N., Heinonen, M., Baron, C. P., and Estévez, M. (2011b). Protein oxidation in muscle foods: A review. *Molecular nutrition & food research*, 55(1):83–95.

Martinez-Saez, N., Fernandez-Gomez, B., Cai, W., Uribarri, J., and Del Castillo, M. D. (2019). In vitro formation of maillard reaction products during simulated digestion of meal-resembling systems. *Food Research International*, 118:72–80.

Martins, S. I. and van Boekel, M. A. (2003). Melanoidins extinction coefficient in the glucose/glycine maillard reaction. *Food Chemistry*, 83(1):135–142.

Matsui, T., Joo, H. D., Lee, J. M., Ju, S. M., Tao, W. H., Higashimoto, Y., Fukami, K., and Yamagishi, S.-i. (2015). Development of a monoclonal antibody-based elisa system for glyceraldehyde-derived advanced glycation end products. *Immunology letters*, 167(2):141–146.

McClements, D. J. and Decker, E. A. (2000). Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of food science*, 65(8):1270–1282.

MEI, L., CRUM, A. D., and DECKER, E. A. (1994). Development of lipid oxidation and inactivation of antioxidant enzymes in cooked pork and beef. *Journal of Food Lipids*, 1(4):273–283.

Michiels, C. and Remacle, J. (1991). Cytotoxicity of linoleic acid peroxide, malondialdehyde and 4-hydroxynonenal towards human fibroblasts. *Toxicology*, 66(2):225–234.

Milkovska-Stamenova, S., Schmidt, R., Frolov, A., and Birkemeyer, C. (2015). Gc-ms method for the quantitation of carbohydrate intermediates in glycation systems. *Journal of agricultural and food chemistry*, 63(25):5911–5919.

Miller, D., Gomez-Basauri, J., Smith, V., Kanner, J., and Miller, D. (1994). Dietary iron in swine rations affects nonheme iron and tbars in pork skeletal muscles. *Journal of food science*, 59(4):747–750.

Monnier, V., Nagaraj, R., Portero-Otin, M., Glomb, M., Elgawish, A., Sell, D., and Friedlander, M. (1996). Structure of advanced maillard reaction products and their pathological role. *Nephrology Dialysis Transplantation*, 11(supp5):20–26.

Monnier, V. M. and Cerami, A. (1981). Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. *Science*, 211(4481):491–493.

Nagai, R., Shirakawa, J.-i., Ohno, R.-i., Hatano, K., Sugawa, H., Arakawa, S., Ichimaru, K., Kinoshita, S., Sakata, N., and Nagai, M. (2016). Antibody-based detection of

advanced glycation end-products: promises vs. limitations. *Glycoconjugate journal*, 33(4):545–552.

Nagaraj, R. H., Sell, D. R., Prabhakaram, M., Ortwerth, B. J., and Monnier, V. M. (1991). High correlation between pentosidine protein crosslinks and pigmentation implicates ascorbate oxidation in human lens senescence and cataractogenesis. *Proceedings of the National Academy of Sciences*, 88(22):10257–10261.

Nair, U., Bartsch, H., and Nair, J. (2007). Lipid peroxidation-induced dna damage in cancer-prone inflammatory diseases: a review of published adduct types and levels in humans. *Free Radical Biology and Medicine*, 43(8):1109–1120.

Neeper, M., Schmidt, A. M., Brett, J., Yan, S., Wang, F., Pan, Y., Elliston, K., Stern, D., and Shaw, A. (1992). Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *Journal of Biological Chemistry*, 267(21):14998–15004.

Nomi, Y., Annaka, H., Sato, S., Ueta, E., Ohkura, T., Yamamoto, K., Homma, S., Suzuki, E., and Otsuka, Y. (2016). Simultaneous quantitation of advanced glycation end products in soy sauce and beer by liquid chromatography-tandem mass spectrometry without ion-pair reagents and derivatization. *Journal of agricultural and food chemistry*, 64(44):8397–8405.

Nursten, H. E. (2005). *The Maillard reaction: chemistry, biochemistry and implications*. Royal Society of Chemistry.

Oliver, C. N., Ahn, B.-W., Moerman, E. J., Goldstein, S., and Stadtman, E. R. (1987). Age-related changes in oxidized proteins. *Journal of Biological Chemistry*, 262(12):5488–5491.

Papetti, A., Mascherpa, D., and Gazzani, G. (2014). Free α-dicarbonyl compounds in coffee, barley coffee and soy sauce and effects of in vitro digestion. *Food chemistry*, 164:259–265.

Perrone, A., Giovino, A., Benny, J., and Martinelli, F. (2020). Advanced glycation end products (ages): biochemistry, signaling, analytical methods, and epigenetic effects. *Oxidative medicine and cellular longevity*, 2020.

Perrone, L. and Grant, W. B. (2015). Observational and ecological studies of dietary advanced glycation end products in national diets and alzheimer's disease incidence and prevalence. *Journal of Alzheimer's Disease*, 45(3):965–979.

Pierre, F., Santarelli, R., Taché, S., Guéraud, F., and Corpet, D. E. (2008). Beef meat promotion of dimethylhydrazine-induced colorectal carcinogenesis biomarkers is suppressed by dietary calcium. *British Journal of Nutrition*, 99(5):1000–1006.

Poulsen, M. W., Hedegaard, R. V., Andersen, J. M., de Courten, B., Bügel, S., Nielsen, J., Skibsted, L. H., and Dragsted, L. O. (2013). Advanced glycation endproducts in food and their effects on health. *Food and Chemical Toxicology*, 60:10–37.

Requena, J. R., Chao, C.-C., Levine, R. L., and Stadtman, E. R. (2001). Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proceedings of the National Academy of Sciences*, 98(1):69–74.

Richards, M. P. (2005). 8 lipid chemistry and biochemistry. *Handbook of Food Science, Technology, and Engineering-4 Volume Set*.

Santé-Lhoutellier, V., Astruc, T., Marinova, P., Greve, E., and Gatellier, P. (2008). Effect of meat cooking on physicochemical state and in vitro digestibility of myofibrillar proteins. *Journal of Agricultural and Food Chemistry*, 56(4):1488–1494.

SATO, K. and HEGARTY, G. R. (1971). Warmed-over flavor in cooked meats. *Journal of food science*, 36(7):1098–1102.

Scheijen, J. L., van de Waarenburg, M. P., Stehouwer, C. D., and Schalkwijk, C. G. (2009). Measurement of pentosidine in human plasma protein by a single-column high-performance liquid chromatography method with fluorescence detection. *Journal of Chromatography B*, 877(7):610–614.

Sell, D. R. and Monnier, V. M. (1989). Structure elucidation of a senescence cross-link from human extracellular matrix: implication of pentoses in the aging process. *Journal of Biological Chemistry*, 264(36):21597–21602.

Sharma, C., Kaur, A., Thind, S., Singh, B., and Raina, S. (2015). Advanced glycation end-products (ages): an emerging concern for processed food industries. *Journal of food science and technology*, 52(12):7561–7576.

Silva, F. A., Ferreira, V., Madruga, M. S., and Estévez, M. (2016). Effect of the cooking method (grilling, roasting, frying and sous-vide) on the oxidation of thiols, tryptophan, alkaline amino acids and protein cross-linking in jerky chicken. *Journal of food science and technology*, 53(8):3137–3146.

Singh, R., Barden, A., Mori, T., and Beilin, L. (2001). Advanced glycation end-products: a review. *Diabetologia*, 44(2):129–146.

Singh, V. P., Bali, A., Singh, N., and Jaggi, A. S. (2014). Advanced glycation end products and diabetic complications. *The Korean journal of physiology & pharmacology*, 18(1):1–14.

Sirota, R., Gorelik, S., Harris, R., Kohen, R., and Kanner, J. (2013). Coffee polyphenols protect human plasma from postprandial carbonyl modifications. *Molecular nutrition & food research*, 57(5):916–919.

Smet, K., Raes, K., Huyghebaert, G., Haak, L., Arnouts, S., and De Smet, S. (2008). Lipid and protein oxidation of broiler meat as influenced by dietary natural antioxidant supplementation. *Poultry Science*, 87(8):1682–1688.

Soladoye, O., Juárez, M., Aalhus, J., Shand, P., and Estévez, M. (2015). Protein oxidation in processed meat: Mechanisms and potential implications on human health. *Comprehensive Reviews in Food Science and Food Safety*, 14(2):106–122.

Somoza, V. (2005). Five years of research on health risks and benefits of maillard reaction products: an update. *Molecular Nutrition & Food Research*, 49(7):663–672.

Stadtman, E. R. and Levine, R. L. (2000). Protein oxidation. *Annals of the New York Academy of Sciences*, 899(1):191–208.

Steppeler, C., Haugen, J.-E., Rødbotten, R., and Kirkhus, B. (2016). Formation of malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal during in vitro digestion of cooked beef, pork, chicken, and salmon. *Journal of agricultural and food chemistry*, 64(2):487–496.

Stirban, A., Gawlowski, T., and Roden, M. (2014). Vascular effects of advanced glycation endproducts: clinical effects and molecular mechanisms. *Molecular metabolism*, 3(2):94–108.

Sun, C., McIntyre, K., Saleem, A., Haddad, P. S., and Arnason, J. T. (2012). The relationship between antiglycation activity and procyanidin and phenolic content in commercial grape seed products. *Canadian journal of physiology and pharmacology*, 90(2):167–174.

Tessier, F. (2010). The maillard reaction in the human body. the main discoveries and factors that affect glycation. *Pathologie Biologie*, 58(3):214–219.

Thomas, D. D., Corkey, B. E., Istfan, N. W., and Apovian, C. M. (2019). Hyperinsulinemia: an early indicator of metabolic dysfunction. *Journal of the Endocrine Society*, 3(9):1727–1747.

Traber, M. G. and Kayden, H. J. (1989). Preferential incorporation of alpha-tocopherol vs gamma-tocopherol in human lipoproteins. *The American journal of clinical nutrition*, 49(3):517–526.

Tullberg, C., Larsson, K., Carlsson, N.-G., Comi, I., Scheers, N., Vegarud, G., and Undeland, I. (2016). Formation of reactive aldehydes (mda, hhe, hne) during the digestion of cod liver oil: comparison of human and porcine in vitro digestion models. *Food & function*, 7(3):1401–1412.

Turner, D. P. (2015). Advanced glycation end-products: a biological consequence of lifestyle contributing to cancer disparity. *Cancer research*, 75(10):1925–1929.

Uribarri, J., Cai, W., Peppa, M., Goodman, S., Ferrucci, L., Striker, G., and Vlassara, H. (2007). Circulating glycotoxins and dietary advanced glycation endproducts: two links to inflammatory response, oxidative stress, and aging. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 62(4):427–433.

Van Boekel, M. (2001). Kinetic aspects of the maillard reaction: a critical review. *Food/Nahrung*, 45(3):150–159.

van der Lugt, T., Venema, K., van Leeuwen, S., Vrolijk, M. F., Opperhuizen, A., and Bast, A. (2020). Gastrointestinal digestion of dietary advanced glycation endproducts using an in vitro model of the gastrointestinal tract (tim-1). *Food & Function*, 11(7):6297–6307.

Van Hecke, T., Basso, V., and De Smet, S. (2018). Lipid and protein oxidation during in vitro gastrointestinal digestion of pork under helicobacter pylori gastritis conditions. *Journal of Agricultural and Food Chemistry*, 66(49):13000–13010.

Van Hecke, T., Goethals, S., Vossen, E., and De Smet, S. (2019). Long-chain n-3 pufa content and n-6/n-3 pufa ratio in mammal, poultry, and fish muscles largely explain differential protein and lipid oxidation profiles following in vitro gastrointestinal digestion. *Molecular nutrition & food research*, 63(22):1900404.

Van Hecke, T., Ho, P., Goethals, S., and De Smet, S. (2017a). The potential of herbs and spices to reduce lipid oxidation during heating and gastrointestinal digestion of a beef product. *Food research international*, 102:785–792.

Van Hecke, T., Van Camp, J., and De Smet, S. (2017b). Oxidation during digestion of meat: interactions with the diet and helicobacter pylori gastritis, and implications on human health. *Comprehensive Reviews in Food Science and Food Safety*, 16(2):214–233.

Van Hecke, T., Vanden Bussche, J., Vanhaecke, L., Vossen, E., Van Camp, J., and De Smet, S. (2014a). Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect n-nitroso compound (noc)-specific dna adduct formation during in vitro digestion. *Journal of Agricultural and Food Chemistry*, 62(8):1980–1988.

Van Hecke, T., Vossen, E., Vanden Bussche, J., Raes, K., Vanhaecke, L., and De Smet, S. (2014b). Fat content and nitrite-curing influence the formation of oxidation products and noc-specific dna adducts during in vitro digestion of meat. *PLoS One*, 9(6):e101122.

Villaverde, A., Ventanas, J., and Estévez, M. (2014). Nitrite promotes protein carbonylation and strecker aldehyde formation in experimental fermented sausages: Are both events connected? *Meat Science*, 98(4):665–672.

Vlassara, H. and Uribarri, J. (2014). Advanced glycation end products (age) and diabetes: cause, effect, or both? *Current diabetes reports*, 14(1):1–10.

Wang, K., Bao, Y., Yang, H., Wang, Y., Chen, D., Regenstein, J. M., and Zhou, P. (2020). Effect of core temperature on the oxidation of lipids and proteins during steam cooking of large-mouth bass (micropterus salmoides). *Polish Journal of Food and Nutrition Sciences*, 70(3).

WCRF (2018). Recommendations and public health and policy implications. Consulted on 9 October, from https://www.wcrf.org/wp-content/uploads/2021/01/Recommendations.pdf.

Wei, Q., Liu, T., and Sun, D.-W. (2018). Advanced glycation end-products (ages) in foods and their detecting techniques and methods: A review. *Trends in Food Science & Technology*, 82:32–45.

Zangenberg, N. H., Müllertz, A., Kristensen, H. G., and Hovgaard, L. (2001). A dynamic in vitro lipolysis model: I. controlling the rate of lipolysis by continuous addition of calcium. *European Journal of Pharmaceutical Sciences*, 14(2):115–122.

Zhang, W., Xiao, S., and Ahn, D. U. (2013). Protein oxidation: basic principles and implications for meat quality. *Critical reviews in food science and nutrition*, 53(11):1191–1201.

Zhao, D., Sheng, B., Wu, Y., Li, H., Xu, D., Nian, Y., Mao, S., Li, C., Xu, X., and Zhou, G. (2019). Comparison of free and bound advanced glycation end products in food: A review on the possible influence on human health. *Journal of agricultural and food chemistry*, 67(51):14007–14018.

Zieman, S. J. and Kass, D. A. (2004). Advanced glycation end product cross-linking: Pathophysiologic role and therapeutic target in cardiovascular disease. *Congestive Heart Failure*, 10(3):144–151.