Tine Rysman

Protein oxidation in meat products: Effects of apple phenolics during storage and digestion

Thesis submitted in fulfillment of the requirements for the degree of

Doctor (Ph.D.) of Applied Biological Sciences

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Eiwitoxidatie in vleesproducten: Effecten van appelfenolen tijdens bewaring en vertering

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Table of contents

List of abbreviations

4-DPS	4,4′-Dithiodipyridine
4-OH-Phe	4-Hydroxyphenylalanine
4-TP	4-Thiopyridone
AAA	α-Amino adipic acid
AAS	α-Amino adipic semialdehyde
ABA	4-Aminobenzoic acid
BH	Sodium borohydride
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CL-MHC	Cross-linked myosin heavy chain
CRC	Colorectal cancer
CUPRAC	Cupric ion reducing antioxidant capacity
DHA	Dehydroascorbate
DNPH	2,4-Dinitrophenylhydrazine
DOPA	Dihydroxyphenylalanine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
DTPA	Diethylenetriaminepentaacetic acid
DTT	Dithiothreitol
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
ESI	Electrospray ionization
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GGS	γ-Glutamic semialdehyde
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GuHCl	Guanidine hydrochloride

HCAs	Heterocyclic amines
HiOx	High-oxygen
HNE	4-Hydroxy-2-nonenal
HRMS	High resolution mass spectrometry
LC-ESI-MS	Liquid chromatography electrospray ionization mass spectrometry
LDS	Lithium dodecyl sulfate
MAP	Modified atmosphere packaging
MCO	Metal-catalyzed oxidation
MDA	Malondialdehyde
MES	2-(N-morpholino) ethanesulfonic acid
MHC	Myosin heavy chain
MPI	Myofibrillar protein isolation
NOCs	N-nitroso compounds
NTB	2-Nitro-5-thiobenzoate
PAHs	Polycyclic aromatic hydrocarbons
PG	propyl gallate
PIPES	1,4-Piperazinediethanesulfonic acid
ROS	Reactive oxygen species
RSH	Thiol
RSSR	Disulfide
SB	Schiff base
SDS	Sodium dodecyl sulfate
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tert-butylhydroxyquinone
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant activity
TNBS	2,4,6-Trinitrobenzenesulphonic acid
TRIS	Tris(hydroxymethyl)-aminomethane
UHPLC-DAD/ESI-am-MS	Ultra high performance liquid chromatography coupled with diode array and accurate mass spectrometry detection using electrospray ionisation
(U)HPLC-FLD	(Ultra) high performance liquid chromatography with fluorescence detection

Chapter 1

Introduction and research objectives

1.1 GENERAL BACKGROUND

Meat is a good source of high value proteins, vitamins and minerals. Meat proteins contain all the essential amino acids that humans cannot synthesize and therefore must be supplied through the diet. Due to the fresh character of meat and meat products, it is of the utmost importance that the microbiological, sensory, technological and nutritional quality is preserved during processing and storage. Together with microbial spoilage, oxidation of both the lipid and protein fraction is one of the major causes of quality deterioration in meat (Xiong, 2000). The mechanisms of lipid oxidation in meat and meat products have been studied extensively, and its main consequences are the typical rancid off-odour and off-flavour (Velasco, Dobarganes, & Márquez-Ruiz, 2010). The study of protein oxidation, on the other hand, has been relatively new in food science, although it has been studied in medical science for many years (Estévez, 2011). Protein oxidation in meat and meat products may result in impaired sensory quality such as lower tenderness and juiciness (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004; Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007b), impaired technological quality such as poor emulsifying, gel-forming and water holding capacity (Decker, Xiong, Calvert, Crum, & Blanchard, 1993; Ooizumi & Xiong, 2004), or impaired nutritional quality because of loss of essential amino acids and altered digestibility (Xiong, 2000). Although many of these consequences have been studied in meat science, the underlying mechanisms of protein oxidation are not yet fully elucidated (Estévez, 2011).

Despite its nutritional value, meat and meat products have obtained a negative image in recent years, because of the epidemiological evidence linking red meat and processed red meat consumption with colorectal cancer (CRC) (Chan et al., 2011; Ferrucci et al., 2012; Magalhães, Peleteiro, & Lunet, 2012; Johnson et al., 2013). Several hypotheses for the underlying carcinogenic mechanisms have been proposed to explain this epidemiological link, the one more likely than the other, as reviewed by Demeyer, Mertens, De Smet, and Ulens (2015). As such, the presence of polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs), both classified as

carcinogenic, together with the presence of heme, enhancing lipid oxidation, and the formation of carcinogenic N-nitroso compounds (NOCs), are generally considered as important underlying mechanisms (Demeyer et al., 2015). Regarding the intake of oxidized food proteins, it has been suggested that structural modifications in oxidized proteins can change their susceptibility to proteolytic enzymes in the gastrointestinal tract, potentially resulting in an altered colonic fermentation with formation of toxic and carcinogenic compounds (Evenepoel et al., 1998; Hughes, Magee, & Bingham, 2000). In medical science, a growing number of studies have reported an increase of oxidation biomarkers in CRC patients, often measured as protein oxidation such as carbonylation, however it remains unclear whether oxidative stress contributes to or is a consequence of CRC development (Perse, 2013).

In 2007, the World Cancer Research Fund recommended to "limit intake of red meat and avoid processed meat" as one of the guidelines for healthy nutrition (World Cancer Research Fund & American Institute for Cancer Research, 2007). Analogue recommendations followed by e.g. the French National Cancer Institute (2009), the British Scientific Advisory Committee on Nutrition (2010) and the Superior Health Council in Belgium (2013). Changing the eating habits of individuals and a population is however a long process, and it is likely that meat and meat products will continue to have a large share in the total energy and protein intake of many people. Meanwhile, the meat industry is challenged to improve the nutritional profile of meat and meat products. This could be done by reducing (the formation of) potentially unhealthy constituents such as PAHs, HCAs, nitrite and oxidizing lipids and proteins, and/or by enriching meat products with health promoting bioactive compounds (Grasso, Brunton, Lyng, Lalor, & Monahan, 2014). By replacing synthetic with natural antioxidants rich in phenolic compounds, both of the above mentioned strategies could potentially be addressed. However, since bioactive compounds are often highly reactive and prone to interactions with other macronutrients such as lipids and proteins (Le Bourvellec & Renard, 2012; Ozdal, Capanoglu, & Altay, 2013), it remains a challenge to predict their bioactivity and bioavailability during processing, storage and digestion, and ultimately, their effect

on human health when consumed as a meat product additive (Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010; Olmedilla-Alonso, Jiménez-Colmenero, & Sánchez-Muniz, 2013).

1.2 PROTEIN OXIDATION IN MEAT

1.2.1 Mechanisms of protein oxidation

Protein oxidation is a complex phenomenon in which amino acid side chains and the peptide backbone are attacked by reactive oxygen species (ROS), leading to free radical chain reactions consisting of initiation, propagation and termination similar to those of lipid oxidation. Numerous ROS, such as superoxide ($O_2^{\bullet-}$), hydroperoxyl ($HO_2^{\bullet}$) and hydroxyl ($HO^{\bullet}$) radicals and nonradical species such as hydrogen peroxide (H_2O_2) and hydroperoxides (ROOH), can be generated through various chemical and biological reactions, such as lipid oxidation, metal-catalyzed oxidation (MCO), irradiation and oxidative enzymes (Xiong, 2000; Estévez, 2011).

When proteins are subjected to ROS, a hydrogen atom is abstracted and a protein carbon-centered radical ($P^{\bullet}$) is formed (reaction [1.1]). This protein radical will be converted to a protein peroxyl radical ($POO^{\bullet}$) in the presence of oxygen (reaction [1.2]), which in turn is able to abstract a hydrogen atom from another susceptible molecule or a protonated superoxide radical, forming a protein hydroperoxide (POOH) (reactions [1.3] and [1.4]). Further reactions with $HO_2^{\bullet}$ lead to the formation of an alcoxyl radical ($PO^{\bullet}$) (reactions [1.5]) and its hydroxyl derivative (POH) (reactions [1.6]). It is noteworthy that the presence of oxygen is required for reaction [1.2], and therefore all ongoing reactions, to take place. In anaerobic conditions, two protein carbon-centered radicals may interact to form a carbon-carbon cross-link (reaction [1.7]) (Stadtman & Levine, 2003).

$$PH + HO^{\bullet} \rightarrow P^{\bullet} + H_2O \qquad [1.1]$$

$$P^{\bullet} + O_2 \rightarrow POO^{\bullet} \qquad [1.2]$$

$$POO^{\bullet} + PH \rightarrow POOH + P^{\bullet} \qquad [1.3]$$

$$POO^{\bullet} + HO_2^{\bullet} \rightarrow POOH + O_2 \qquad [1.4]$$

$$POOH + HO_2^{\bullet} \rightarrow PO^{\bullet} + H_2O + O_2 \quad [1.5]$$

$$PO^{\bullet} + HO_2^{\bullet} \rightarrow POH + O_2 \quad [1.6]$$

$$P^{\bullet} + P^{\bullet} \rightarrow P\text{-}P \quad [1.7]$$

1.2.1.1 Role of transition metals

In these general mechanisms of protein oxidation, transition metals play an important role. Both their reduced and oxidized forms are able to cleave H_2O_2 through Fenton-like reactions (reaction [1.8] and [1.9]), resulting in the formation of radicals. Furthermore, reduced transition metals such as Fe^{2+} or Cu^{+} are able to replace $HO_2^{\bullet}$ in reactions [1.4] to [1.6], as shown in reactions [1.10] to [1.12]. Hence, iron and other transition metals are able to initiate and catalyze propagation of oxidation. When bound to proteins, this can result in site-specific metal-catalyzed oxidation of amino acids near the metal-binding sites (Stadtman et al., 2003).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{-} + HO^{\bullet} \quad [1.8]$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO^{2\bullet} + H^{+} \quad [1.9]$$

$$POO^{\bullet} + Fe^{2+} + H^{+} \rightarrow POOH + Fe^{3+} \quad [1.10]$$

$$POOH + Fe^{2+} \rightarrow PO^{\bullet} + HO^{-} + Fe^{3+} \quad [1.11]$$

$$PO^{\bullet} + H^{+} + Fe^{2+} \rightarrow POH + Fe^{3+} \quad [1.12]$$

Metal-catalyzed oxidation (MCO) is particularly important in meat because of its high levels of heme and non-heme iron. Most iron is bound to the porphyrin ring of heme proteins such as myoglobin, which is abundantly present in red meat. Upon heating, Fe^{2+} is released from the heme-porphyrin moiety, and non-heme iron comes available for the reactions above (Kanner, 1994; Lombardi-Boccia, Martinez-Dominguez, & Aguzzi, 2002).

1.2.1.2 Role of myoglobin

Besides being a source of non-heme iron upon heating, myoglobin as such is also able to promote protein oxidation. In the presence of oxygen, myoglobin is oxygenated into oxymyoglobin, which in turn can be oxidized into metmyoglobin (MbFe(III)). The latter can be activated by hydrogen peroxide to form perferrylmyoglobin radicals ($^{\bullet}$MbFe(IV)=O) (reaction [1.13]), which are highly reactive and decompose to ferrylmyoglobin (MbFe(IV)=O) by abstracting a hydrogen atom from a neighboring molecule or the protein itself (reaction [1.14]) (Kröger-Ohlsen, Carlsen, Andersen, & Skibsted, 2002; Libardi, Skibsted, & Cardoso, 2014).

$$\text{MbFe(III)} + H_2O_2 \longrightarrow {}^{\bullet}\text{MbFe(IV)=O} + H^+ + H_2O \qquad [1.13]$$

$$^{\bullet}\text{MbFe(IV)=O} + \text{PH} \longrightarrow \text{MbFe(IV)=O} + \text{P}^{\bullet} \qquad [1.14]$$

In meat model systems, myoglobin species have been shown to catalyze protein oxidation to a greater extent than iron-catalyzed oxidation (Park, Xiong, & Alderton, 2006a; Park, Xiong, Alderton, & Ooizumi, 2006b; Park & Xiong, 2007; Estévez & Heinonen, 2010). In meat and meat products, it is however still unclear whether heme or non-heme iron acts predominantly as oxidation catalyst, and so far it is generally accepted that both pathways should be taken into account (Estévez, 2011).

1.2.1.3 Role of lipid oxidation

It is reasonable to assume that there is an interaction between lipid and protein oxidation by transfer of radical species between both phenomena (Estévez, 2011). Some studies have found correlations between the progression of lipid and protein oxidation in model systems as well as meat and meat products (Mercier, Gatellier, Viau, Remignon, & Renerre, 1998; Park et al., 2006a; Park et al., 2006b; Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008b), whereas others found protein oxidation to be hardly affected by lipid oxidation (Liu & Xiong, 1996; Haak et al., 2006). It

is often observed that lipid oxidation progresses faster than protein oxidation, and therefore it is suggested that protein oxidation is induced by radicals formed during initiation and propagation of lipid oxidation, more likely than the other way round. Furthermore, amino acid side chains are able to react with lipid oxidation products, leading to indirect protein oxidation (Estévez, 2011). Although the exact interactions between protein and lipid oxidation in a complex matrix such as meat and meat products are still unclear, it is unlikely that both phenomena take place independently (Lund, Heinonen, Baron, & Estévez, 2011).

1.2.2 Manifestation and assessment of protein oxidation in meat

Free radical attack on proteins may result in modifications of the protein backbone or the amino acid side chains. A list of amino acid residues and their oxidation products is represented in Table 1.1. Among them, cysteine, tyrosine, phenylalanine, tryptophan, histidine, proline, arginine, lysine and methionine have been pointed out as most susceptible to free radical and nonradical ROS (Lund et al., 2011).

1.2.2.1 Thiol oxidation

The thiol group on the cysteine residue is highly reactive and will readily oxidize in the presence of ROS. After a series of reactions as shown in Figure 1.1, thiol oxidation results in the formation of disulfides, sulfenic acid, sulfinic acid, sulfonic acid and thiosulfinates, of which disulfides and sulfenic acid are reversible thiol oxidation products (Nagy & Winterbourn, 2010). In meat science, it is generally believed that thiol oxidation results predominantly into disulfide cross-linking (Lund et al., 2007b; Jongberg, Skov, Tørngren, Skibsted, & Lund, 2011b; Li, Kong, Xia, Liu, & Li, 2013).

Rather than measuring the exact oxidation product, thiol oxidation is often determined as loss of thiol groups. Thiol loss in meat is most commonly measured after reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent). DTNB forms a disulfide bond with free thiol groups, releasing a thiolate ion (TNB dianion) which can be measured spectrophotometrically and plotted

against a cysteine standard curve (Estévez, Morcuende, & Ventanas, 2009a). Although the DTNB method is widely applied, it only provides information on the extent, and not on the nature or reversibility of thiol oxidation.

Table 1.1: Oxidative modification of amino acid side chains (adapted from Xiong, 2000; Levine & Stadtman, 2001; Soladoye, Juárez, Aalhus, Shand, & Estévez, 2015). Amino acids highlighted with * are known to be essential amino acids for healthy adults.

Amino acid	**Type of modification**	**Oxidation products**
Arginine	Carbonylation	γ-Glutamic semialdehyde
Cysteine	Cross-linking, sulfoxidation	Disulfides (Cys–S–S–Cys or Cys–S–S–R), sulfenic acid (Cys-SOH), sulfinic acid (Cys-SO_2H), sulfonic acid (Cys-SO_3H), thiosulfinates (Cys-S(O)-S-R)
Glutamic acid	Hydroxylation, adduct formation, deamination	4-Hydroxyglutamic acid, pyruvate adducts, oxalic acid
Histidine*	Imidazole oxidation	Aspartic acid, asparagine, 2-oxo-histidine, 4-hydroxyglutamate
Leucine*	Hydroxylation	3-, 4-, and 5-Hydroxyleucine
Lysine*	Carbonylation	α-Amino adipic semialdehyde
Methionine*	Sulfoxidation	Methionine sulfoxide, methionine sulfone
Phenylalanine*	Hydroxylation	2-, 3-, and 4-Hydroxyphenylalanine, 2,3-dihydroxyphenylalanine
Proline	Carbonylation, hydroxylation	γ-Glutamic semialdehyde, pyroglutamic acid, 2-pyrrolidone, 4- and 5-hydroxyproline
Threonine*	Carbonylation	2-Amino-3-ketobutyric acid
Tryptophan*	Hydroxylation, nitration	Formylkynurenine, kynurenine, 2-, 4-, 5-, 6-, and 7-hydroxytryptophan, 3-hydroxykynurenine, nitrotryptophan
Tyrosine	Hydroxylation, cross-linking, nitration	3,4-dihydroxyphenylalanine (DOPA), Tyr-Tyr cross-links, 3-nitrotyrosine
Valine*	Hydroxylation	3-Hydroxyvaline

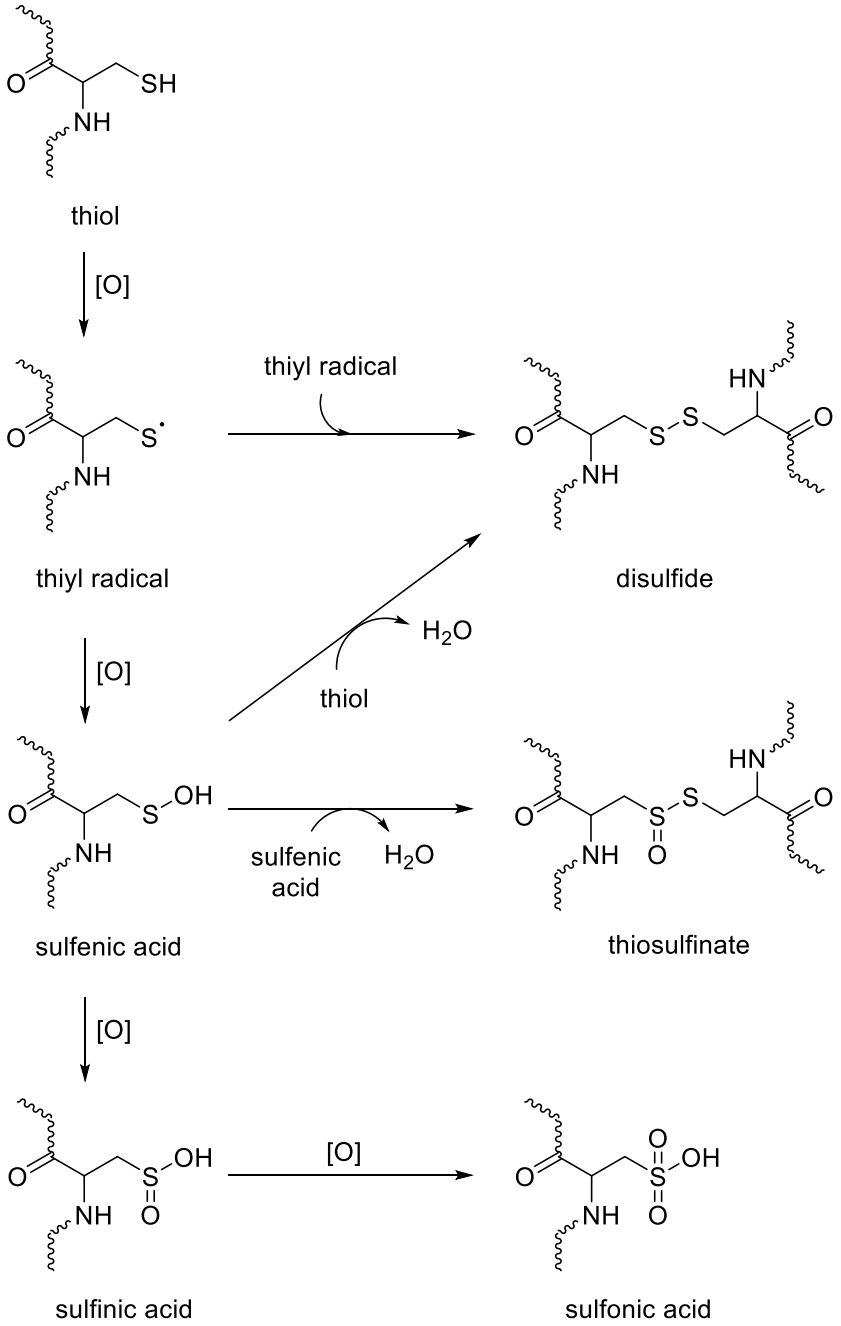

Figure 1.1: Thiol oxidation on the cysteine residue.

Liu, Xiong, and Butterfield (2000a) quantified disulfides in myofibrillar proteins according to the method described by Damodaran (1985), based on disulfide cleavage with sodium sulfite followed by spectrophotometric quantification of the newly formed thiols. The reaction rate of sulfitolysis of disulfide bonds is however rather slow and rarely quantitative in the absence of catalysts (Kella & Kinsella, 1985; Hansen & Winther, 2009b). Furthermore, measures need to be taken to prevent

cross-reaction of sulfite with the thiol detection agent (Damodaran, 1985), making sulfite less appropriate as a reducing agent. Jongberg, Lund, Waterhouse, and Skibsted (2011a) used the strong reducing agent sodium borohydride (BH) to cleave disulfides in myofibrillar proteins, followed by thiol detection with DTNB. The excess of BH could easily be removed by acidification, after which the pH had to be readjusted for the DTNB reaction (pH 8). However, the large pH fluctuations required for this combination of reducing and thiol detection agent are unfavourable for thiol-disulfide redox reactions (Hansen et al., 2009b). Both of these methods for disulfide quantification were performed in isolated myofibrillar proteins (Liu et al., 2000a; Jongberg et al., 2011a). However to the best of our knowledge, no attempts have been made to quantify disulfides in whole meat or meat products by this strategy, that is cleavage of disulfides and subsequent spectrophotometric measurement of newly formed thiols.

Another way to evaluate disulfide formation in meat, in particular disulfide cross-linking of myosin, is by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Myosin makes up approximately 55% of the myofibrillar protein fraction, and in non-oxidized state contains about 42 thiol groups and no disulfide bonds (Hofmann & Hamm, 1978; Liu et al., 2000a). Upon oxidation, intermolecular disulfide cross-links would change the electrophoresis pattern, decreasing the band intensity of myosin heavy chain (MHC), with the simultaneous appearance of a cross-linked myosin heavy chain (CL-MHC) band (Ooizumi et al., 2004; Park et al., 2006b; Lund et al., 2007b; Jongberg et al., 2011b). The major drawbacks of this method are that (1) measurement of the band pixel intensity is merely semi-quantitative, and (2) only CL-MHC dimers are taken into account, since severely oxidized polymers would be too large to enter the gel. Furthermore, this SDS-PAGE procedure is usually applied to muscle tissue samples, whereas hardly any literature can be found on electrophoresis techniques on processed meat. Modifications of the myofibrillar proteins during processing, such as thermal denaturation and/or actions of microorganisms and enzymes, would make SDS-PAGE too nonspecific to be considered relevant (Estévez et al., 2009a).

1.2.2.2 Carbonylation

The generation of carbonyl derivatives (aldehydes and ketones) is believed to be the most abundant manifestation of protein oxidation (Xiong, 2000). Four different pathways for carbonyl formation have been reported: (1) direct oxidation of amino acid side chains (Requena, Chao, Levine, & Stadtman, 2001), (2) covalent binding of non-protein carbonyls, such as secondary lipid oxidation products, through Michael addition (Stadtman et al., 2003). (3) non-enzymatic glycation reactions with reducing sugars (Akagawa, Sasaki, Kurota, & Suyama, 2005), and (4) fragmentation of the protein backbone through α-amidation reactions or oxidation of glutamyl side chains (Berlett & Stadtman, 1997; Hawkins & Davies, 2001), Park et al. (2006a) reported carbonylation through protein backbone scission to be negligible in porcine myofibrillar proteins. The relative contribution of the other three pathways to carbonyl formation in meat proteins remains unknown, although it is generally believed that direct oxidation of amino acid side chains is the major route, especially in MCO systems. In the presence of transition metals, lysine is oxidized into α-amino adipic semialdehyde (AAS) and arginine and proline into γ-glutamic semialdehyde (GGS) as shown in Figure 1.2. Reactive species first abstract a hydrogen atom neighboring the ε-amino group in the side chain, generating a carbon-centered protein radical. Next, oxidized metal ions accept the unpaired electron, forming an imino group which upon hydrolysis yields a carbonyl moiety (Estévez, 2011).

The traditional method to determine protein carbonyl compounds in meat is by means of 2,4-dinitrophenylhydrazine (DNPH). DNPH reacts with carbonyl groups to form 2,4-dinitrophenylhydrazone, which is quantified spectrophotometrically based on its extinction coefficient (Estévez et al., 2009a). The DNPH method is a widely used, relatively rapid and inexpensive procedure rendering information on the total amount of protein carbonyls. However, it does not make a distinction between different carbonyl species, which makes it inappropriate to study the exact mechanisms of protein oxidation in meat.

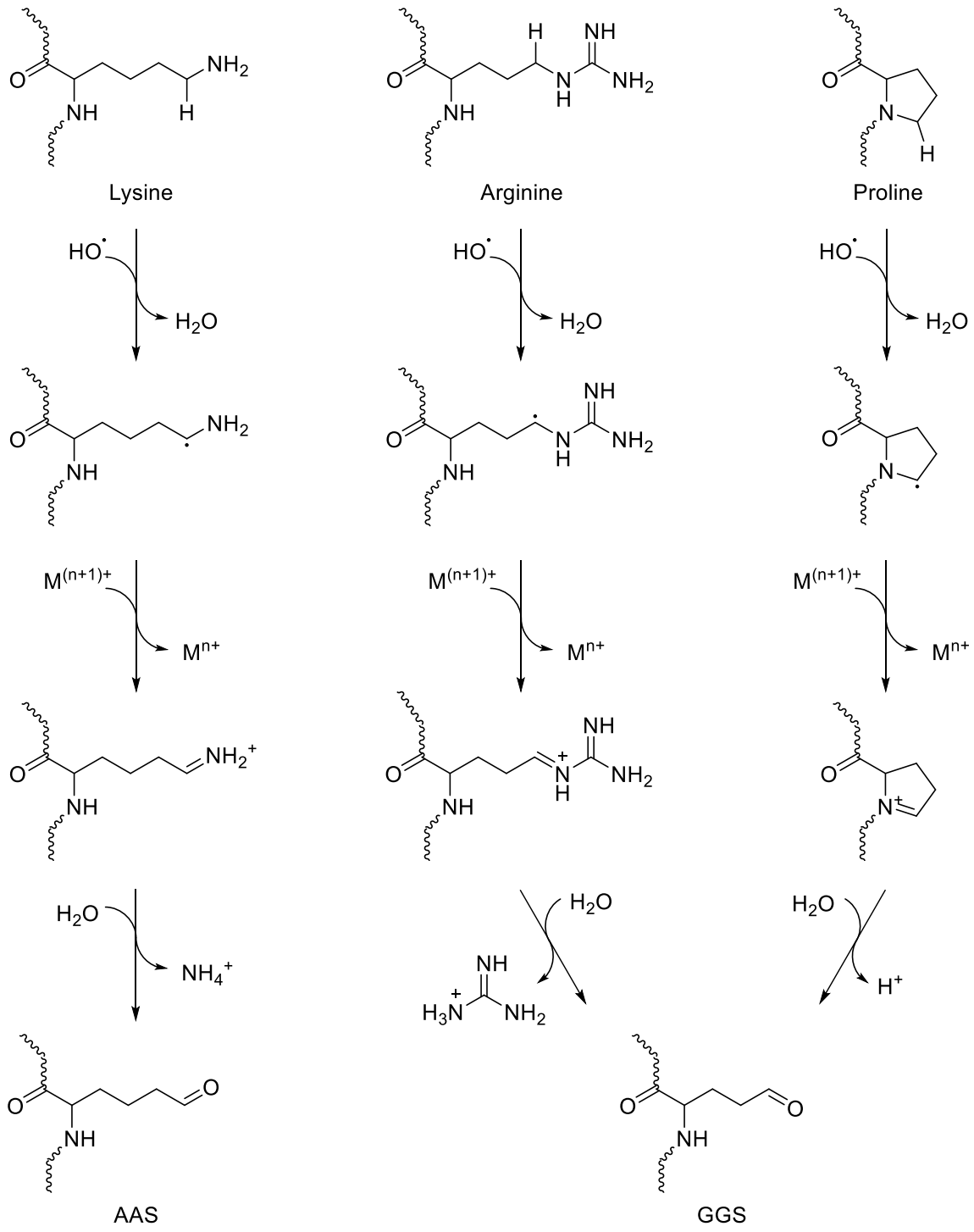

Figure 1.2: Metal-catalyzed oxidation of lysine, arginine and proline into α-amino adipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) (modified from Estévez, 2011).

The specific carbonyl compounds AAS and GGS were initially analyzed as biomarkers of protein oxidation in biological systems (Daneshvar, Frandsen, Autrupand, & Dragsted, 1997). This method was successfully adapted to apply in meat science using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) (Estévez, Ollilainen, & Heinonen, 2009b) or high performance liquid chromatography with fluorescence detection (HPLC-FLD) (Utrera, Morcuende, Rodríguez-Carpena, & Estévez, 2011). The sum of AAS and GGS has been reported to account for 23 to 61% of total (DNPH-derivatized) carbonyls in oxidized plasma, liver and meat proteins (Requena et al., 2001; Akagawa et al., 2006; Utrera et al., 2011), hence AAS and GGS are believed to be good and reliable markers for protein oxidation. However, this value seems to vary greatly depending on the oxidation system and protein matrix. Nonetheless, the specific detection of AAS and GGS allows to better understand the mechanisms and pathways of protein oxidation.

Protein carbonylation is known to be an irreversible process, however carbonyl moieties should not be considered as end-products of protein oxidation. In fact, a biphasic progression of total and/or specific carbonyls in meat proteins has been observed in several studies (Mercier, Gatellier, & Renerre, 2004; Salminen, Estévez, Kivikari, & Heinonen, 2006; Utrera & Estévez, 2013b). The initial increase corresponds to the accumulation of protein carbonyls, whereas the subsequent decrease indicates that carbonyls are subjected to ongoing reactions. Several reactions with the carbonyl groups have been suggested (Figure 1.3), including (1) ongoing oxidation of the carbonyl group into a carboxylic acid (Sell, Strauch, Shen, & Monnier, 2007), (2) reaction with the ε-amino group from another amino acid side chain e.g. lysine, forming a Schiff base cross-link (Dölz & Heidemann, 1989), (3) reaction with another protein-bound carbonyl group, forming an aldol condensation cross-link (Dölz et al., 1989), and (4) reaction with an α-amino group from a free amino acid, forming a Strecker aldehyde (Estévez, Ventanas, & Heinonen, 2011).

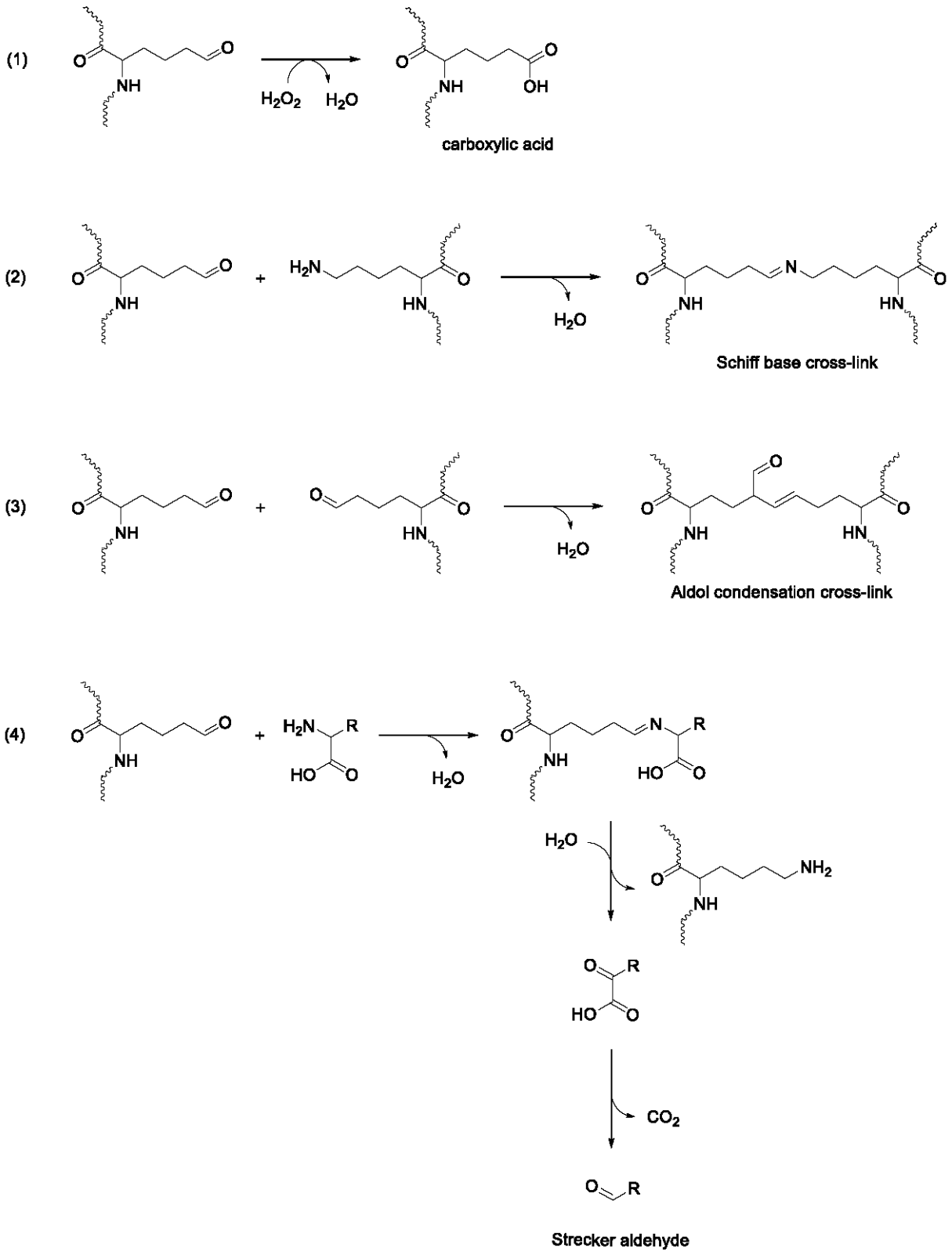

Figure 1.3: Ongoing reactions of protein carbonyls: (1) oxidation into a carboxylic acid, (2) formation of a Schiff base cross-link, (3) formation of an Aldol condensation cross-link, and (4) formation of a Strecker aldehyde (modified from Estévez, 2011).

The natural fluorescence of Schiff base structures can be measured with fluorescence spectroscopy (Utrera & Estévez, 2012b). Although rapid and inexpensive, the specificity towards carbonyl derived Schiff base structures cannot be assured with this type of measurements. The carboxylic acid α-amino adipic acid (AAA) has been identified as an ongoing oxidation product of AAS in cell cultures and model systems (Sell et al., 2007). The research group of Mario Estévez (University of Extremadura, Spain) has attempted to quantify AAA in meat by means of HPLC, helping to understand the pathway of lysine oxidation (Utrera et al., 2012b). Although AAA is said to be a stable end-product and might therefore be a more reliable protein oxidation marker than AAS (Estévez, 2011), the method requires further optimization and has thus far not been incorporated in other meat science laboratories.

1.2.2.3 Hydroxylation

Aromatic amino acids such as tryptophan, tyrosine and phenylalanine are also highly susceptible to oxidation. Rather than hydrogen abstraction, oxidation of these amino acids is mostly expressed as hydroxyl addition to the aromatic ring. As such, oxidation of phenylalanine may generate isomers of tyrosine, which can oxidize further into 3,4-dihydroxyphenylalanine (DOPA) or dityrosine as shown in Figure 1.4 (Hawkins et al., 2001; Lund & Baron, 2010). Hydroxylation of tryptophan can take place on the pyrrole or the benzene ring, generating 2-, 4-, 5-, 6-, and 7-hydroxytryptophan. Attack on the pyrrole ring can eventually lead to kynurenine derivatives (Lund et al., 2010).

In meat science, aromatic amino acid oxidation is mostly analyzed as loss of tryptophan, tyrosin or phenylalanine by means of UV or fluorescence spectroscopy (Gatellier et al., 2009; Utrera & Estévez, 2012a). Similar to measuring thiol loss, these measurements do not provide information on the type of oxidation products formed. Dityrosine formation has been observed in meat model systems (Morzel, Gatellier, Sayd, Renerre, & Laville, 2006; Lund, Luxford, Skibsted, & Davies, 2008), however has thus far not been detected directly in meat (Lund et al., 2010).

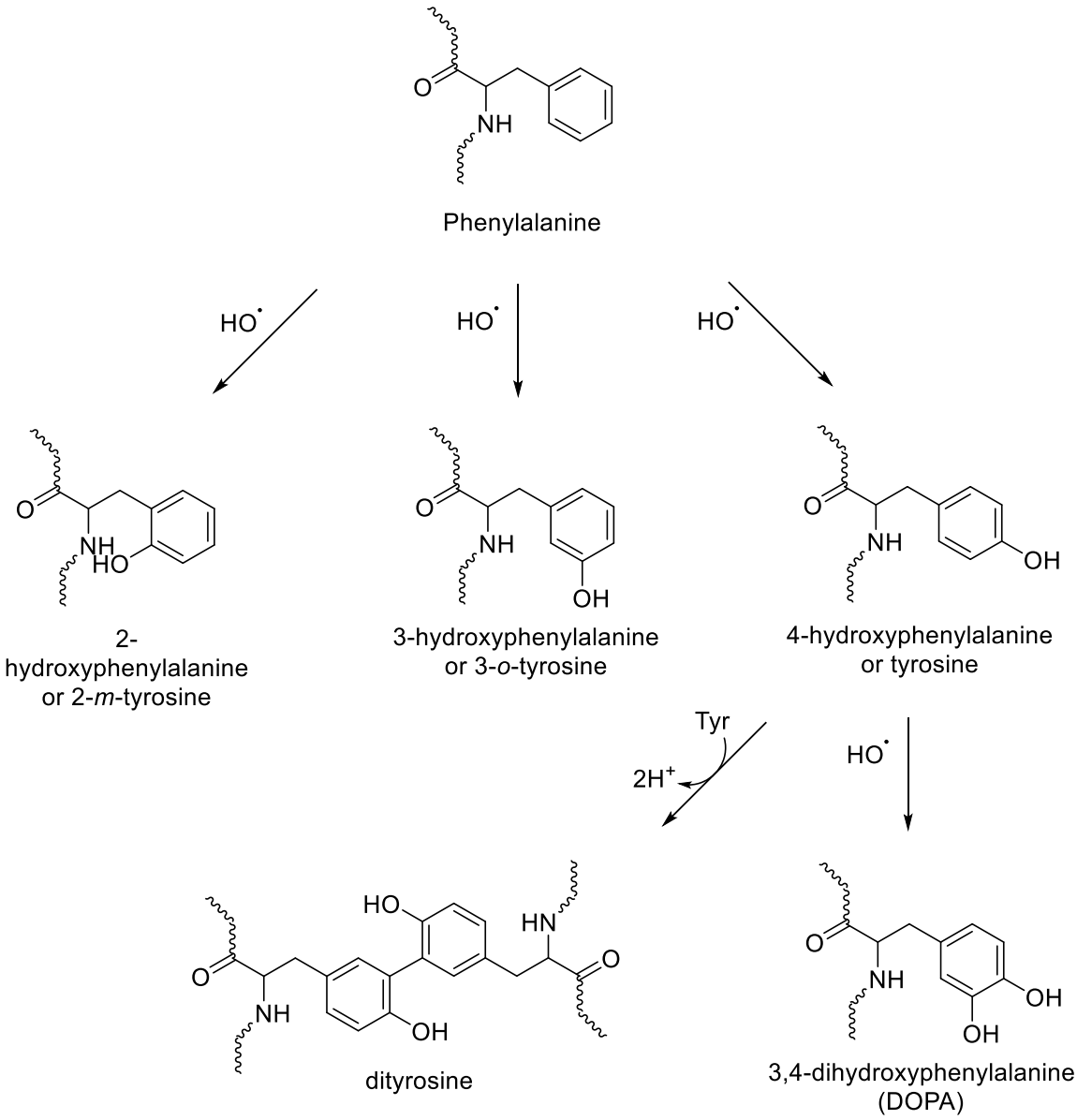

Figure 1.4: Oxidation of phenylalanine and tyrosine (modified from Lund et al., 2010).

1.2.3 Consequences of protein oxidation in meat and meat products

1.2.3.1 Functional and technological properties

Considering the damage that protein oxidation can cause, it is reasonable to believe that the biochemical and structural changes will affect the technological properties of meat proteins (Soladoye et al., 2015). The amino acid composition and sequence (primary structure) of proteins largely influence their native (secondary and tertiary) structure, ultimately determining their functionality. The chemical interactions between amino acids and surrounding water and lipid molecules are highly important for the gelling, emulsifying and water holding capacity (Xiong, 2000; Puolanne & Halonen, 2010). Oxidation of these amino acids would lead to changes in protein-protein, protein-water and protein-lipid interactions. Eventually, the physico-chemical modifications induced by protein oxidation could lead to protein unfolding, increased surface hydrophobicity, aggregate formation, and denaturation. Some of these oxidative changes could be beneficial, however most of them are detrimental for protein functionality, and this appears to depend on the type and extent of oxidation (Xiong, 2000). As such, mild intra- and intermolecular cross-linking could increase gel formation by stabilizing the gel matrix (Srinivasan & Hultin, 1997), however severe cross-linking can impair the gelling capacity by formation of rigid structures (Ooizumi et al., 2004; Bertram et al., 2007). Carbonylation of polar amino acids could lead to altered protein-protein and protein-water interactions, shifting electrical charges and decreasing the isoelectric point (Stadtman, 1990). This, together with cross-link formation, could lead to shrinkage of myofibrillar proteins, resulting in a decreased water holding capacity and increased cooking loss caused by protein oxidation (Bertram et al., 2007; Liu, Xiong, & Chen, 2010).

1.2.3.2 Sensory properties

Oxidative modifications could not only affect the technological properties of meat proteins, but have also been suggested to influence sensory aspects. The decrease in sensory-assessed juiciness and tenderness in meat stored in oxygen-rich packaging, compared to oxygen-free storage, was suggested to be caused by disulfide cross-linking, decreasing the water holding capacity and strengthening the myofibrillar structure (Lund et al., 2007b). Rowe et al. (2004) suggested that the decreased meat tenderness in irradiated beef, measured instrumentally as Warner-Bratzler shear force, was caused by oxidative inactivation of the meat tenderizing enzyme μ-calpain, which has an oxidizable cysteine residue at its active site. Other studies found a significant correlation between protein carbonylation and instrumental hardness of meat products (Estévez & Cava, 2004; Estévez, Ventanas, & Cava, 2005; Ganhão, Morcuende, & Estévez, 2010a), suggesting the contribution of carbonyls to severe cross-linking. Next to textural changes, Estévez et al. (2011) also introduced a possible role of two specific carbonylation products, γ-glutamic and α-amino adipic semialdehyde (GGS and AAS, respectively), in the formation of Strecker aldehydes from (iso)leucine, which could contribute to an off-flavour in meat and meat products.

1.2.3.3 Nutritional properties

Besides a technological and sensory impact, protein oxidation could also have nutritional consequences. It is worth noting that eight out of the nine essential amino acids for healthy adults are known to be susceptible to oxidation (Table 1.1). Since part of the oxidative modifications are irreversible, such as carbonylation, protein oxidation could have a detrimental impact on the quantity and quality of essential amino acids, and thus, the nutritional value of meat (Estévez, 2011; Soladoye et al., 2015). Additionally, protein oxidation has been suggested to affect proteolysis during digestion. Controversial effects have been reported on this matter, depending on the oxidative and proteolytic conditions applied (Xiong, 2000). It is generally believed that relatively

mild oxidative conditions would partially unfold proteins, thereby enhancing the accessibility of digestive enzymes to their recognition sites and thus increasing proteolysis. Severe oxidation on the other hand will induce protein polymerization and aggregation through cross-link formation, structurally hindering proteolytic attack (Davies, 2001; Grune, Jung, Merker, & Davies, 2004; Bax et al., 2012). Furthermore, it is known that gastric pepsin preferentially cleaves at the carboxylic side of phenylalanine, methionine, leucine and tryptophan, while duodenal trypsin and α-chymotrypsin hydrolyze proteins at the carboxylic sides of tyrosine, phenylalanine, tryptophan, leucine, methionine, alanine, aspartic acid, glutamic acid, arginine and lysine (Santé-Lhoutellier, Engel, Aubry, & Gatellier, 2008b). Oxidation of these amino acids, whether through carbonylation, hydroxylation, thiol oxidation or cross-link formation, could be detrimental for protease recognition. Subsequently, severely oxidized proteins that are chemically and physically hindered to be hydrolyzed and absorbed in the stomach and small intestine, would be intensely fermented by the microbial flora in the colon (Evenepoel et al., 1998). During this bacterial fermentation, metabolites can be formed that might have mutagenic or carcinogenic properties. Phenol and *p*-cresol, formed out of tyrosine fermentation, are probably the most widely discussed metabolites in this regard, however other potentially harmful metabolites such as ammonia, amines, NOCs and sulfides might contribute to an increased genotoxicity in the colon (Evenepoel et al., 1998; Hughes et al., 2000). However, all of these potential health effects (loss of essential amino acids, altered digestibility and metabolite formation) are still largely unexplored for meat proteins and require additional research (Soladoye et al., 2015).

1.3 ANTIOXIDANT STRATEGIES AGAINST PROTEIN OXIDATION IN MEAT

Considering all of the above mentioned implications for meat quality, it is obvious that oxidation should be delayed as much as possible. Actions taken to control protein oxidation in meat and meat products are mostly adopted from lipid antioxidant approaches, including dietary strategies by supplementing antioxidants to the animal feed, and technological strategies by adding antioxidants during food processing (Lund et al., 2010; Estévez, 2011).

1.3.1 Mechanisms of antioxidants

Antioxidants can work at different levels in the oxidation cycle: they can scavenge initiating radicals, decompose peroxides, decrease oxygen concentrations or bind oxidation catalysts, such as metal ions (Shah, Bosco, & Mir, 2014; Shahidi & Ambigaipalan, 2015). As such, antioxidants can to a certain extend prevent initiation of the free radical chain reactions, or they can stabilize the radicals formed during propagation, thereby terminating the chain reactions (Shah et al., 2014).

The most effective antioxidants are those interrupting the free radical chain reaction. Radical scavenging is a process in which free radicals are neutralized, by donating a hydrogen atom, or by reaction of antioxidant compounds with radicals to form resonance stabilized carbon centered radical adducts (Nimse & Pal, 2015). Often, chain-breaking antioxidant compounds have at least one aromatic (often phenolic) ring structure containing at least one hydroxyl group. This hydroxyl group is capable of donating a hydrogen atom to radicals generated during various stages of the oxidation cycle (reaction [1.15]) (Brewer, 2011; Shahidi et al., 2015). The resulting antioxidant radical is more stable than protein or lipid radicals because of resonance delocation throughout the ring structure, and therefore is not able to initiate or propagate oxidation itself in normal circumstances (Choe & Min, 2009). Antioxidant radicals are readily neutralized by forming a stable peroxy-antioxidant compound (reaction [1.16]) (Brewer, 2011), or can be regenerated by another hydrogen donating antioxidant.

$$AH + P^{\bullet}/PO^{\bullet}/POO^{\bullet} \rightarrow A^{\bullet} + PH/POH/POOH \quad [1.15]$$

$$A^{\bullet} + A^{\bullet} \rightarrow A\text{-}A \quad [1.16]$$

Besides radical scavenging activity, some antioxidants are able to chelate metal ions. Vicinal hydroxyl groups on the aromatic ring structure of antioxidants stabilize transition metals in an inactive or insoluble form as suggested in Figure 1.5 (Falowo, Fayemi, & Muchenje, 2014), preventing the metal ions to catalyze oxidation.

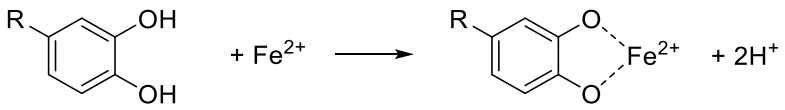

Figure 1.5: Metal chelating activity of antioxidants (modified from Falowo et al., 2014).

Radical scavenging and metal chelating activity are generally considered as the primarily modes of action of antioxidants. Other antioxidative mechanisms include quenching singlet oxygen, absorbing UV radiation, decomposing hydroperoxides, and inhibiting enzymes (Choe et al., 2009; Kumar, Yadav, Ahmad, & Narsaiah, 2015).

Finally, it should be noted that antioxidant compounds are able to exert pro-oxidant activity at certain concentrations or physiological conditions. As such, they are capable of reducing Fe^{3+} to Fe^{2+}, and thus enable formation of initiating radicals through Fenton-like reactions (reaction [1.8] and [1.9]). This pro-oxidant activity is often observed at high concentrations (Moran, Klucas, Grayer, Abian, & Becana, 1997; Procházková, Boušová, & Wilhelmová, 2011).

1.3.2 Synthetic and natural antioxidants

1.3.2.1 Synthetic antioxidants

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroxyquinone (TBHQ), and propyl gallate (PG) have been widely used in meat products (Shah et al., 2014). These antioxidants contain one phenolic ring with one (BHA and BHT), two (TBHQ) or three (PG) hydroxyl groups (Figure 1.6). Hence, their antioxidant activity relies mainly on radical scavenging, and for PG also metal chelating (Kumar et al., 2015). They are lipid soluble, but (practically) insoluble in water.

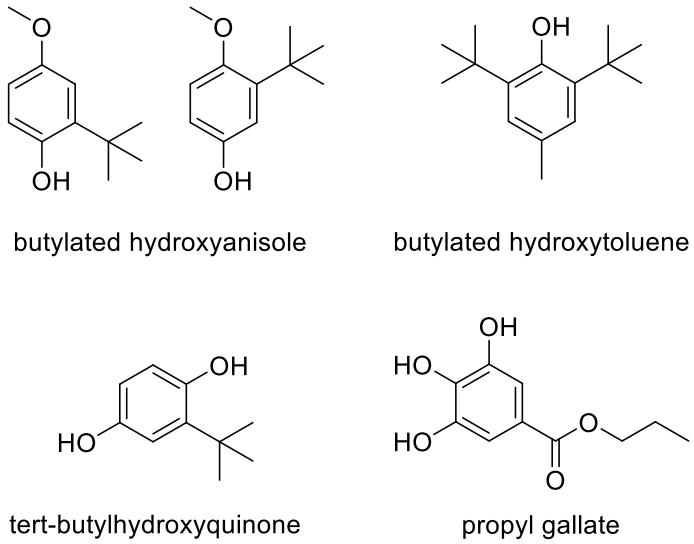

Figure 1.6: Synthetic antioxidants often used in meat products.

Reports on the effects of synthetic antioxidants on consumer health are contradictory. As reviewed by Falowo et al. (2014), some of these compounds have been found cytotoxic or carcinogenic at high concentrations in laboratory animals, while other reports suggest they are not harmful for humans, and might even be anticarcinogenic. The use of synthetic antioxidants in food is strictly regulated by law (Falowo et al., 2014). In Europe, PG, TBHQ and BHA are allowed to be used in meat

products, alone or combined, in a maximum level of 200 ppm based on the fat content (European Parliament & Council of the European Union, 2008).

1.3.2.2 Natural antioxidants

Adverse toxicological reports on synthetic antioxidants, together with consumer's growing interest in natural food additives, have prompted the food industry to search for natural alternatives with antioxidative potential in food matrices (Karre, Lopez, & Getty, 2013; Ahmad, Gokulakrishnan, Giriprasad, & Yatoo, 2015; Kumar et al., 2015). Plant materials of different kinds contain a variety of bioactive substances with antioxidant properties. These natural antioxidants, which can be found in the cell membranes of any plant parts (grains, fruits, nuts, seeds, leaves, roots, etc.), have similar radical scavenger and metal chelating properties as synthetic antioxidants, and have even been shown to have better antioxidant capacities than BHA or BHT*in vitro*, although this strongly depends on dosage and matrix (Kumar et al., 2015).

The most important natural antioxidants in plant tissue include vitamins, carotenoids and phenolic compounds. The water soluble vitamin C (ascorbic acid, Figure 1.7) contains four hydroxyl groups (two pairs on adjacent carbon atoms), and can therefore act as a radical scavenger, metal chelator and oxygen quencher. Vitamin E (α-tocopherol, Figure 1.7) is lipid soluble and is able to scavenge radicals and quench oxygen (Brewer, 2011; Shahidi et al., 2015). Carotenoids such as ß-carotene and lycopene (Figure 1.7) are good oxygen quenchers and radical scavengers, especially for peroxyl radicals. Their long unsaturated alkyl chains make them highly lipophilic (Nimse et al., 2015).

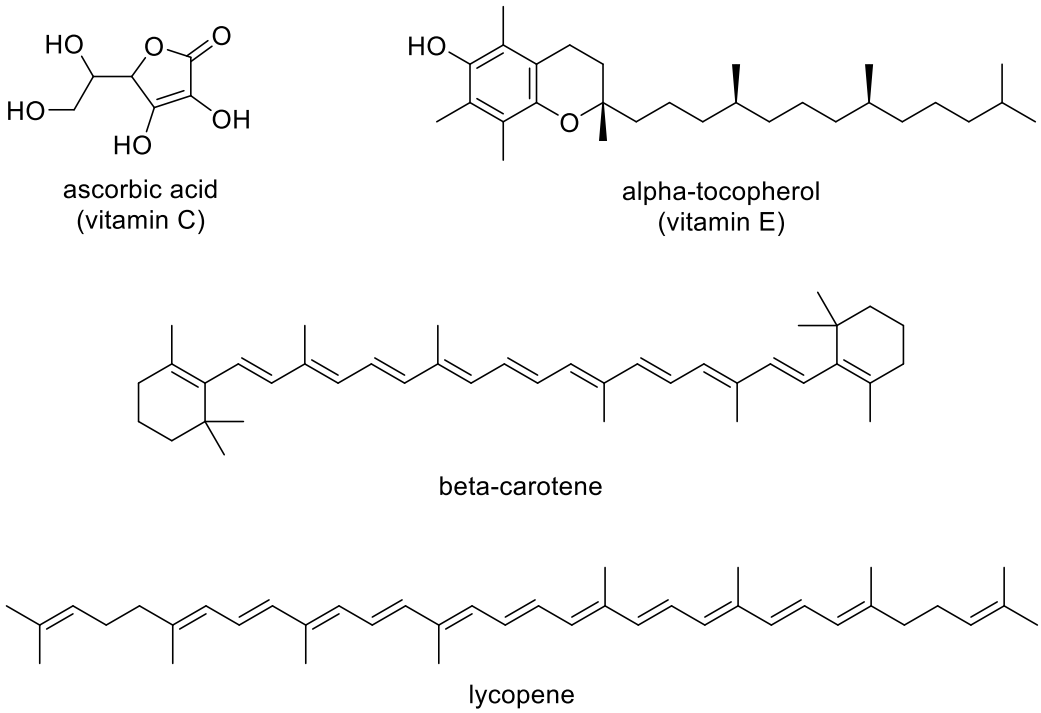

Figure 1.7: Vitamins and carotenoids in plant material with antioxidant activity.

The majority of natural antioxidants in plant material are however phenolic compounds, of which the most important ones are flavonoids and phenolic acids (Gülçin, 2012; Kumar et al., 2015). Flavonoids have a characteristic 15 carbon flavan structure arranged in three rings (C_6-C_3-C_6), labelled A, B and C. Subclasses of flavonoids differ in the level of saturation of the C ring (Figure 1.8A), and compounds within a subclass differ in the substitution pattern (hydroxylation and methoxylation) of the A and B rings (Brewer, 2011; Gülçin, 2012; Shahidi et al., 2015). Phenolic acids (hydroxybenzoic and hydroxycinnamic acid, Figure 1.8B) are hydroxy derivatives of aromatic carboxylic acids (Gülçin, 2012). The predominant mode of antioxidant activity of these phenolic compounds is believed to be radical scavenging through hydrogen donation from hydroxyl groups. Phenolic acids have also been shown to quench oxygen, while flavonoids have metal chelating potential, depending on the arrangements of hydroxyl and carbonyl groups around the molecule Hence, the antioxidant capacity of flavonoids and phenolic acids depend mainly on the pattern (number and location) of hydroxyl groups (Shahidi et al., 2015). Glycosylation of hydroxyl groups usually decreases the antioxidant effectiveness of phenolics (Shahidi, Janitha, & Wanasundara, 1992;

Kumar et al., 2015). Flavonoids and phenolic acids are hydrophilic compounds, and their solubility in fats and oils is very low (Choe et al., 2009).

When using plant material as a source of natural antioxidants, often an extract is made in order to concentrate the target compounds and maximize the antioxidant power. Usually, the plant material is cleaned, dried, and ground into a fine powder followed by solvent extraction. The most suitable type of solvent depends on the chemical characteristics and polarities of the antioxidant compounds of interest. Mixtures of water with ethanol, methanol, acetone, or ethyl acetate are often found to be good extraction solvents. Next to the choice of solvent, the sample preparation, the solvent-sample ratio, and the extraction time, temperature and pressure are also important parameters in determining the extraction yield. Obviously, all traces of organic solvents must be removed before applying the extracts in food stuff, since they can affect human health (Shah et al., 2014).

Finally, it should be noted that even when a food grade extract was produced out of plant material, the safety should not be taken for granted. Natural antioxidants are often perceived as harmless and even beneficial for health, and therefore unlimited to use. However, like synthetic phenolic antioxidants, natural antioxidants might potentially have mutagenic, carcinogenic or other pathogenic activities at high doses or after processing (Shahidi & Zhong, 2005). Hence, not only the efficacy but also the safety of natural antioxidants requires further research.

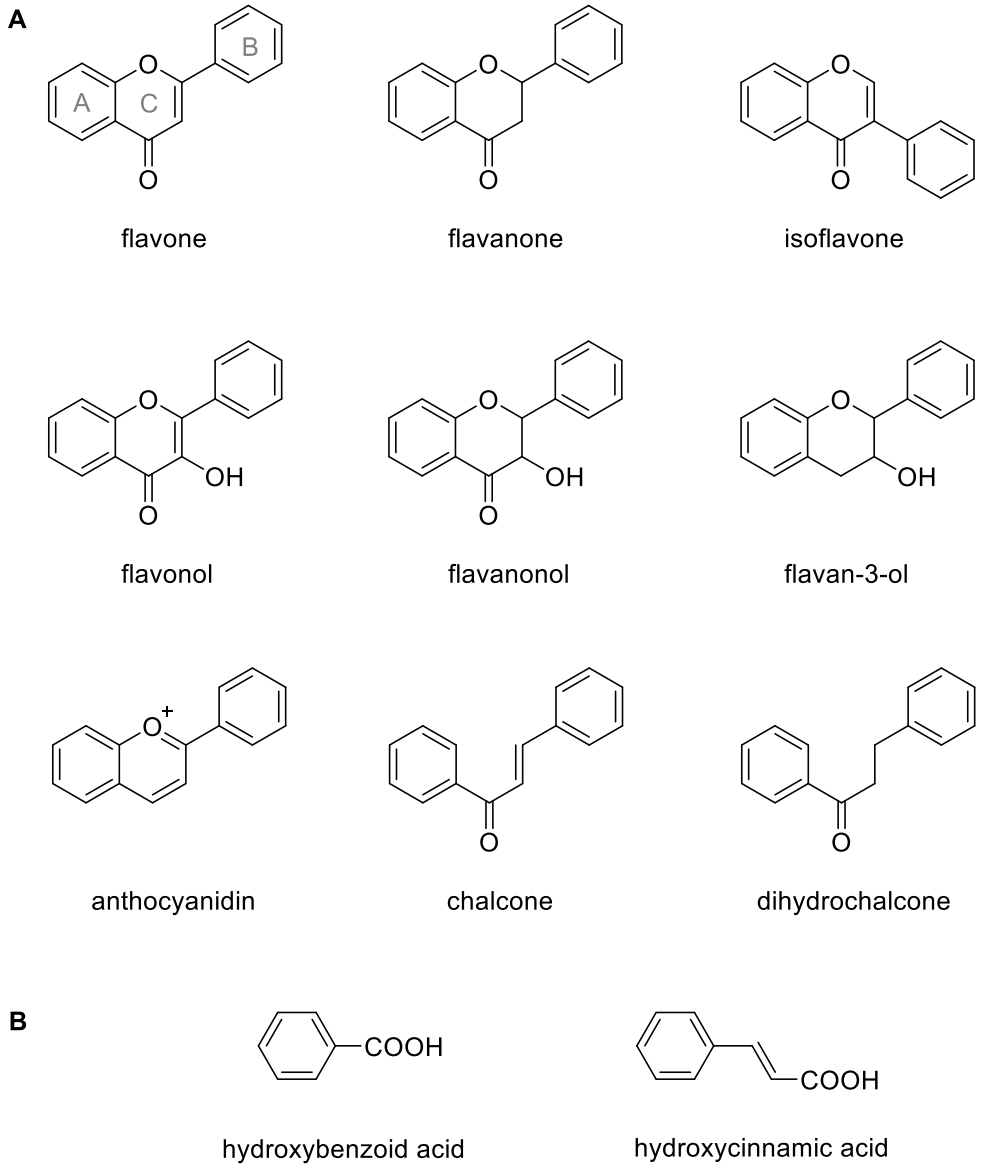

Figure 1.8: Backbone structures of natural phenolic compounds: (A) major subclasses of flavonoids, (B) major subclasses of phenolic acids. Compounds within a subclass differ in the substitution pattern of the phenolic ring(s) (modified from Shahidi et al., 2015).

1.3.2.3 Apple phenolics as a source of natural antioxidants

Apples are an important part of the Belgian diet as well as the Belgian fruit production. Apples take up the highest amount of fresh fruit purchased for home use in Flanders, followed by oranges and bananas (19.5%, 15.8% and 15.7%, respectively, in 2015). The most popular apple cultivar in Flanders is Jonagold, although its market share is decreasing because of the growing popularity of the imported Cripps Pink cultivar (commercial name "Pink Lady"). In 2014, the Belgian apple production accounted for 42% of the total national fruit production volume, only preceded by pears (51%) (Platteau, Van Gijseghem, Van Bogaert, & Vuylsteke, 2016). However, only apples that meet strict quality criteria reach the fresh market, and those with deviating size, shape or color distribution are often categorized as 'low quality fruit', even though there is no quality loss from a nutritional point of view (De Paepe, 2014).

In fact, apples, either of high or so-called low quality, are a good source of phenolic compounds, and it is well documented that apple peel contains higher concentrations of phenolics than apple flesh (Burda, Oleszek, & Lee, 1990; Eberhardt, Lee, & Liu, 2000; Wolfe & Liu, 2003). Recently, De Paepe et al. (2015b) investigated the phenolic profiles of 47 apple cultivars occurring in Belgium, enabling the selection of apple cultivars for breeding programs, and/or for studying processing characteristics and health promoting effects. The same authors studied the preservation of phenolic compounds during the production of cloudy apple juice by spiral-filter pressing and belt pressing (De Paepe et al., 2015a). With both systems, the majority of phenolic compounds ended up in the apple pomace (press residue), since large, phenolic-rich apple skin particles cannot be transferred through the filter element or belt. Because of the fast processing and the low oxygen levels in the extraction chamber of the spiral-filter press, phenolic compounds in both the apple juice and pomace were better preserved with the spiral-filter press technology as compared to conventional belt pressing (De Paepe et al., 2015a). As such, spiral-filter pressing of ('low quality') apples does not only produce phenolic-rich juice, but also a valuable by-product.

The phenolic profile and content in apples varies depending on several factors, such as the variety, cultivar, geographic origin, growing year, weather conditions, maturity stage, crop load, and fruit position within the canopy (De Paepe, 2014). Generally, the main representative phenolics in (whole) apple and apple pomace are the phenolic acid chlorogenic acid (subclass hydroxycinnamic acids), and the flavonoids (epi)cathechin and procyanidin B2 (subclass flavanols), phloridzin (subclass dihydrochalcones), and quercetin and derivatives (subclass flavonols) (Lu & Yeap Foo, 2000; Lee, Kim, Kim, Lee, & Lee, 2003; Diñeiro García, Valles, & Picinelli Lobo, 2009; Neveu et al., 2010; Rothwell et al., 2012; Rothwell et al., 2013; De Paepe et al., 2015a). *In vitro* antioxidant assays showed that these individual apple phenolics exert higher antioxidant activity than vitamin C or E, and that the contribution of vitamin C in apple (on average 12.8 mg/100 g fresh weight) to the total antioxidant activity is only minor compared to phenolics (Wang, Cao, & Prior, 1996; Lu et al., 2000; Lee et al., 2003). Hence, all of these findings suggest that apple pomace could potentially be used as a source of natural, phenolic antioxidants, thereby valorizing the by-product of apple juice production, and answering consumers' demand for natural ingredients.

1.3.3 Antioxidants against protein oxidation in meat and meat products

Although *in vitro* tests may show high radical scavenging and metal chelating activity of antioxidants, it is important to investigate their efficacy in food products. Synthetic antioxidants as well as phenolic-rich fruit and plant materials have been added to meat products to investigate their protection towards the lipid and protein fraction. Theoretically, it could be expected that lipophilic antioxidants prevent lipid oxidation whereas hydrophilic antioxidants are effective inhibitors of protein oxidation. However, even in simplified model systems it has been shown that some hydrophilic antioxidants had a protective effect on lipids (Baron, Berner, Skibsted, & Refsgaard, 2005), and the lipophilic α-tocopherol was able to inhibit the formation of protein carbonyls GGS and AAS in myofibrillar protein (Estévez et al., 2010). In raw pork patties, Haak, Raes, and De Smet (2009) found lipid soluble α-tocopherol and ascorbyl palmitate to inhibit protein

thiol oxidation better than their water soluble variants trolox and ascorbic acid. This emphasizes that it remains a challenge to predict the kinetics and mechanisms of antioxidants against lipid and protein oxidation in complex (emulsion-type) meat systems. Furthermore, the possible interaction of antioxidants with macromolecules in the meat matrix such as lipids, proteins and carbohydrates might modify their radical scavenging and metal chelating properties (Estévez et al., 2008b).

Thus far, most studies on antioxidants in meat have been applied on fresh and freshly prepared meat such as ground meat and patties, whereas their effect on extensively processed meats has been much less studied, especially with regard to protein oxidation. It is however likely that severe muscle structure modification, thermal treatment, and functional ingredients such as salt and phosphate can lead to even more matrix effects (Jiang & Xiong, 2016). Some of the works on antioxidants against protein and lipid oxidation in meat and meat products are presented in Table 1.2. While most of the applied antioxidants exert an inhibitory effect on lipid oxidation, the effects towards protein oxidation are often less pronounced or even pro-oxidative. Unfortunately, often only one (non-specific) protein oxidation marker, or one category (thiol oxidation or carbonylation), is considered (Table 1.2).

Among synthetic antioxidants, BHT at a concentration of 100 ppm has been shown to protect proteins against thiol oxidation and carbonylation during refrigerated storage of raw pork patties (Choe, Kim, & Kim, 2017), while 200 ppm significantly inhibited protein carbonylation in pork liver pâté after processing (90 days storage at 4 °C) (Estévez, Ventanas, & Cava, 2006). During illuminated chilled storage of raw pork patties, 200 ppm of BHA was able to inhibit thiol oxidation, however the decrease in carbonyl content compared to the control (without BHA) was not significant (Jia, Kong, Liu, Diao, & Xia, 2012). TBHQ (200 ppm) significantly decreased carbonylation during cold storage of non-radiated and radiated raw chicken breast (Rababah et al., 2004).

Table 1.2: Effects of antioxidant towards protein and lipid oxidation in meat and meat products.

		Protein oxidation					Lipid oxidation				
Meat type	**Antioxidant**	**thiol oxidation (DTNB)**	**cross-linking (SDS-PAGE)**	**total carbonylation (DNPH)**	**specific carbonylation (GGS + AAS)**	**tryptophan oxidation (spectroscopy)**	**malondialdehyde (TBARS)**	**volatile compounds**	**conjugated dienes**	**peroxide value**	**Reference**
Raw pork patties	Persimmon peel extract (0.05, 0.1 and 0.2%)	A	-	A	-	-	A	-	A	A	Choe et al., 2017
	BHT (0.01%)	A		A			A		A	A	
	Ascorbate (0.05%)	A		A			A		A	A	
Pork cooked ham	Garlic, cinnamon, cloves and rosemary essential oil (1 g/kg)	-	-	A	-	-	A	-	-	-	Armenteros et al., 2016
	Artinox®[1] (3 g/kg)			A			A				
	Rose hip extract (300 mL/kg)			A			A				
Pork emulsions	Green tea extract (100 ppm)	NS	A	-	-	-	A	-	-	-	Jongberg et al., 2015b
	Green tea extract (500 ppm)	P	NS				A				
	Green tea extract (1500 ppm)	P	P				A				
Bologna type sausage	Green tea extract (500 ppm)	P	P	A	-	-	A	-	-	-	Jongberg et al., 2013
	Rosemary extract (400 ppm)	NS	NS	A			NS[2]				
Raw pork patties	Rosemary essential oil (0.05 and 0.4%)	A	A	-	-	-	-	-	-	-	Nieto et al., 2013
	Oregano essential oil (0.05 and 0.4%)	A	A								
	Garlic essential oil (0.05 and 0.4%)	P	P								
Frankfurters	Dog rose extract (5 and 30 g/kg)	-	-	-	A	-	-	A	-	-	Vossen et al., 2012
Raw pork patties	Black current extract (5, 10 and 20 g/kg)	A[3]	-	A[4]	-	-	A	-	-	-	Jia et al., 2012
	BHA (0.2 g/kg)	A		NS			A				

Table 1.2: Effects of antioxidant towards protein and lipid oxidation in meat and meat products.

		Protein oxidation					Lipid oxidation				
Meat type	**Antioxidant**	**thiol oxidation (DTNB)**	**cross-linking (SDS-PAGE)**	**total carbonylation (DNPH)**	**specific carbonylation (GGS + AAS)**	**tryptophan oxidation (spectroscopy)**	**malondialdehyde (TBARS)**	**volatile compounds**	**conjugated dienes**	**peroxide value**	**Reference**
Raw beef patties	White grape extract (500 ppm)	NS	NS	NS	-	-	A	-	-	-	Jongberg et al., 2011b
Raw pork patties	Avocado var. 'Hass' peel or seed extracts (5%) Avocado var. 'Fuerte' peel or seed extracts (5%)	-	-	A NS	-	-	A A	-	-	-	Rodríguez-Carpena et al., 2011
Cooked pork patties	Arbutus berry extract (3%) Common hawthorn extract (3%) Dog rose extract (3%) Elm-leaf blackberry extracts (3%)	-	-	NS NS NS NS	A A A A[5]	A A A A	-	-	-	-	Ganhão et al., 2010b
Beef and pork cooked ham	Apple polyphenol extract (0.3, 0.5 and 1 g/kg) Ascorbic acid (0.4 g/kg)	-	-	NS[6] NS	-	-	A A	-	-	-	Sun et al., 2010
Raw pork patties	Ascorbic acid (100 and 200 ppm) Tocopherol (100 and 200 ppm) Rosemary extract (100 ppm) Green tea extract (100 ppm)	NS NS[7] NS[7] NS[7]	-	-	-	-	P A A A	-	-	-	Haak et al., 2009
Raw beef patties	Rosemary extract (0.05%) Ascorbic acid/sodium citrate (0.05%)	-	-	NS P	-	-	A A	-	-	-	Lund et al., 2007a
Pork liver pâté	Rosemary essential oil (0.1%) Sage essential oil (0.1%) BHT (0.02%)	-	-	A A A	-	-	-	-	-	-	Estévez et al., 2006b

Table 1.2: Effects of antioxidant towards protein and lipid oxidation in meat and meat products.

		Protein oxidation					Lipid oxidation				
Meat type	**Antioxidant**	**thiol oxidation (DTNB)**	**cross-linking (SDS-PAGE)**	**total carbonylation (DNPH)**	**specific carbonylation (GGS + AAS)**	**tryptophan oxidation (spectroscopy)**	**malondialdehyde (TBARS)**	**volatile compounds**	**conjugated dienes**	**peroxide value**	**Reference**
Cooked pork patties	Rapeseed meal extract (0.3, 0.5 and 0.7%) Camelina meal extract (0.3, 0.5 and 0.7%) Soy meal or flour extract (0.3, 0.5 and 0.7%) Rosemary extract (0.04 and 0.08%) Rapeseed, camelina or soy (0.5%) + rosemary (0.04%) extract	-	-	A A NS A[8] A	-	-	-	A A NS A[8] A	-	-	Salminen et al., 2006
Frankfurters	Rosemary essential oil (150, 300 and 600 ppm)	-	-	A[9]	-	-	-	-	-	-	Estévez et al., 2005
Cooked pork patties	Rapeseed meal (141, 282, 353 and 424 mg/100 g) Rapeseed meal extract (15 and 29 mL/100 g) Rapeseed oil extract (2 and 5 mL/100 g) Pine bark extract (7 and 11 mL/100 g)	-	-	A A A A	-	-	-	A A A A	-	-	Vuorela et al., 2005
Raw chicken breast	Green tea extract (3000 and 6000 ppm) Grape seed extract (3000 and 6000 ppm) TBHQ (200 ppm)	-	-	A A A	-	-	A A A	-	-	-	Rababah et al., 2004

TBARS, thiobarbituric acid reactive substances; A, significant antioxidative effect compared to control; P, significant pro-oxidative effect compared to control; NS, non-significantly different from control. [1]Commercial antioxidant consisting of a combination of additives (sodium citrate : sodium erythorbate 1:1). [2]Antioxidative effect close to significance ($P = 0.062$). [3]NS at 5 g/kg. [4]NS at 20 g/kg. [5]Only significant for GGS formation. [6]Antioxidative effect at end of storage in beef. [7]Antioxidative effect after frozen storage. [8]NS at 0.04%. [9]NS at 150 ppm.

Ascorbic acid (vitamin C) and its salt sodium ascorbate have been applied as antioxidants with varying results. During cold storage of raw patties, ascorbate (500 ppm) significantly reduced thiol oxidation and carbonylation, however, inhibition percentages were higher for BHT than for ascorbate (Choe et al., 2017). Ascorbic acid brine injection of cooked hams significantly decreased lipid oxidation during cold illuminated storage, but had no significant effect on protein carbonylation (Sun et al., 2010). Lund et al. (2007a) found the combination of ascorbic acid and sodium citrate (both 0.05%) to be an effective antioxidant towards lipid oxidation in beef patties during cold storage (80% oxygen or absence of oxygen), however it had a pro-oxidative effect on protein carbonyl formation in both packaging atmospheres. Somewhat opposite effects were reported by Haak et al. (2009) on ascorbic acid addition (100 and 200 ppm) to raw pork patties during illuminated cold storage. These authors observed a pro-oxidative effect of ascorbic acid towards lipid oxidation, whereas results for thiol oxidation revealed no significant differences. The same authors also tested α-tocopherol as antioxidant in raw pork patties, which had an antioxidant character towards lipid oxidation, but was only able to significantly inhibit thiol oxidation after frozen storage (Haak et al., 2009).

Several botanical phenolics have been investigated for their antioxidant capacity towards protein oxidation in meat. Often, (commercial) essential oils or solvent extracts of phenolic-rich plant material are used. Rosemary has shown a significant inhibition of protein carbonylation in cooked pork patties (Salminen et al., 2006), frankfurters (Estévez et al., 2005), liver pâté (Estévez et al., 2006), and Bologna type sausages (Jongberg et al., 2013), but not in raw beef patties (Lund et al., 2007a). The effect of rosemary on thiol oxidation was not significant in Bologna type sausages (Jongberg et al., 2013), but in raw pork patties, rosemary showed significant thiol protection (Haak et al., 2009 after frozen storage; Nieto et al., 2013). Green tea phenolics protected meat proteins against carbonylation in raw chicken breast (Rababah et al., 2004) and Bologna type sausages (Jongberg et al., 2013), and against thiol oxidation in raw pork patties, however only after frozen storage (Haak et al., 2009). In Bologna type sausages, a pro-oxidative effect of green tea extract on thiol oxidation was observed (Jongberg et al., 2013). The same research group found green tea extract

to affect thiol oxidation in pork emulsions in a dose-dependent way, and suggested the occurrence of protein-phenolic interactions at high phenolic concentrations (Jongberg et al., 2015b). Dog rose extract was an effective antioxidant against GGS and AAS formation in frankfurters (Vossen et al., 2012), and against GGS formation in cooked pork patties (Ganhão et al., 2010b), although in the latter study no significant effect on total carbonylation was observed. From these results, it is clear that variations in selected plant material, phenolic composition, extraction procedure, applied concentration, meat matrix, meat processing steps and storage conditions may result in a different antioxidant or pro-oxidant outcome. Furthermore, the choice of protein oxidation markers should be made carefully, since the effects of phenolic antioxidants are not always similar for different protein oxidation manifestations.

Few literature can be found on the use of apple phenolics against protein oxidation in meat and meat products. Sun et al. (2010) added a commercial apple polyphenol extract (0.3, 0.5 and 1 g/kg) to the curing brine for beef and pork cooked ham, and observed a significant inhibition of lipid oxidation during cold illuminated storage, similar or better than the ascorbic acid treatment (0.4 g/kg). Protein carbonyls in beef cooked ham were only significantly lower than the control in the 1 g/kg apple phenolic treatment at the end of storage. In pork cooked ham, the level of carbonyl compounds decreased slightly by addition of apple phenolics, though not significantly. Other studies have investigated the effects of apple phenolics towards lipid oxidation in meat, without including protein oxidation measurements. Yu et al. (2015) found a commercial apple phenolic extract to be more effective than BHT in simultaneously inhibiting TBARS and the formation of volatile compounds during storage of Chinese-style (salami-like) sausages. In mutton meat balls, the addition of apple pomace powder (1% ,3% and 5%) significantly decreased TBARS values compared to the control (without antioxidants added) (Rather, Akhter, Masoodi, Gani, & Wani, 2015). Nuñez de Gonzalez, Boleman, Miller, Keeton, and Rhee (2008) evaluated pork sausage treated with 3% and 6% dried plum and apple puree. After cooking and refrigerated storage, TBARS values in the 6% treatment were significantly lower than in the control. Hence, these studies suggest that apple phenolics have the potential to act as an antioxidant in meat and meat products. However,

as shown in Table 1.2, the inhibition of lipid oxidation does not automatically guarantees antioxidant protection of the protein fraction, and a thorough evaluation is needed to elucidate the effect of apple phenolics on meat protein oxidation.

1.3.4 Role of nitrite and ascorbate

In cured meat products, nitrite is mainly used for its antimicrobial effect (especially against *Clostridium botulinum*), and to obtain the desired pink colour of cooked meat products. To obtain this colour, nitrite is first reduced into nitric oxide, which reacts with myoglobin to form nitrosomyoglobin. Upon heating, the latter denatures into globin and the pink nitroso-myochromogen. Ascorbate, as a strong reducing agent, speeds up the process of colour formation, mainly by catalyzing the reduction from nitrite to nitric oxide (Feiner, 2006b; Skibsted, 2011). Besides these functions of nitrite, it has also been shown to have an antioxidant effect towards lipid oxidation. The main mode of action is believed to be an interaction of nitric oxide with lipid radicals, forming non-radical addition products and thereby terminating the lipid oxidation chain reactions (Skibsted, 2011). Other antioxidant activity of nitrite involves NO binding to heme and non-heme iron, inhibiting the catalytic activity of iron towards oxidation (Morrissey & Tichivangana, 1985). Furthermore, in the presence of oxygen, the NO molecule can easily be oxidized to NO_2. In this way, nitrite acts as an antioxidant by oxygen sequestering (Honikel, 2008). On the other hand, the strong oxidizing character of nitrite could make it a pro-oxidant as well, by forming reactive nitrogen species. As such, nitric oxide can react with a superoxide anion to form peroxynitrite, which is able to induce lipid oxidation in food (Brannan, Connolly, & Decker, 2001). The antioxidant or pro-oxidant activity of nitric oxide is highly dependent on the concentration of reactive species (Rubbo et al., 1994).

Although the inhibitory effect of nitrite on lipid oxidation in meat and meat products is well established (Zanardi, Ghidini, Battaglia, & Chizzolini, 2004; Balev, Vulkova, Dragoev, Zlatanov, & Bahtchevanska, 2005), only few studies on nitrite and protein oxidation have been reported in meat science. Vossen and De Smet (2015) found no antioxidant or pro-oxidant effect of nitrite on

protein oxidation (carbonylation and thiol oxidation) in myofibrillar protein isolates or raw pork patties, although TBARS values were significantly lower in patties treated with nitrite. During processing of fermented sausages, Villaverde, Morcuende, and Estévez (2014) observed pro-oxidative activity of nitrite towards carbonyl and Schiff base formation and tryptophan depletion, whereas ascorbate acted as an antioxidant. Their results suggested that ascorbate might be required to compensate the pro-oxidant impact of nitrite on meat proteins. However, in a similar study by Berardo et al. (2016), the combined addition of ascorbate and nitrite to dry fermented sausages resulted in higher carbonyl levels compared to their separate addition. Ascorbate, nitrite and their combination significantly inhibited lipid oxidation, however no significant differences in thiol loss were observed. Hence, it can be concluded that nitrite and ascorbate act differently against lipid and protein oxidation, and the role of these curing agents towards protein oxidation remain to be fully elucidated.

1.4 RESEARCH OBJECTIVES AND THESIS OUTLINE

The aim of this PhD research was to clarify the underlying mechanisms of protein oxidation during storage and digestion of meat and meat products, and the potential inhibition thereof by apple phenolics. In this **Chapter 1**, the mechanisms and consequences of protein oxidation in meat were elucidated, and current methods for measuring protein oxidation in meat were discussed. Furthermore, the mechanisms of synthetic and natural antioxidants were discussed, as well as their use in meat products. In the following chapters, several protein oxidation manifestations will be investigated in meat protein matrices in various oxidation systems, with or without the presence of apple phenolics (Figure 1.9).

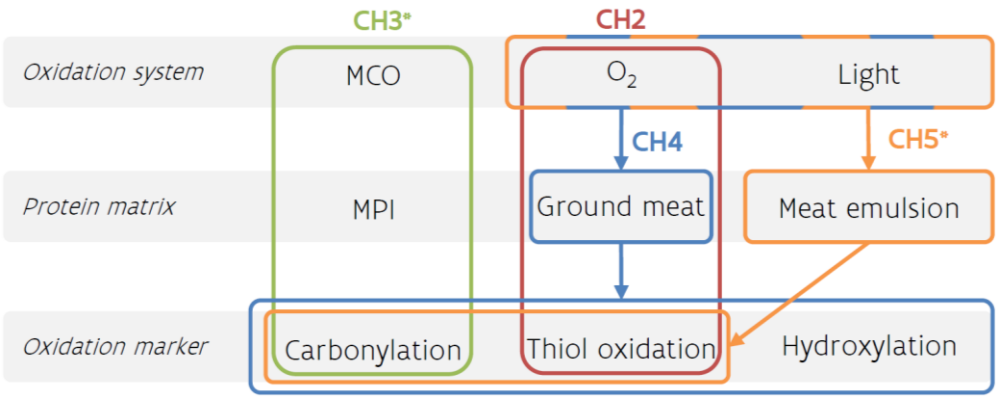

Figure 1.9: Oxidation mechanisms studied in the research and their relation to the chapters. MCO, metal-catalyzed oxidation; O_2, molecular oxygen; MPI, myofibrillar protein isolation. In chapters denoted with *, apple phenolics were used as a source of natural antioxidants.

As discussed in Section 1.2.2.1, thiol oxidation in meat is often measured spectrophotometrically as thiol loss by means of the thiol detection agent DTNB. Spectrophotometric measurement of disulfides in myofibrillar proteins has been performed by using sulfite or sodium borohydride as a reducing agent (Liu et al., 2000a; Jongberg et al., 2011a), however drawbacks to these methods include slow reaction rates, cross-reactivity and unfavourable pH fluctuations (Hansen et al., 2009b). In 2013, Ruan, Chen, Kong, & Hua found 4,4'-dithiodipyridine (4-DPS) to be a more suitable and reliable thiol detection agent in soy proteins, as it is less affected by pH and denaturants. In

Chapter 2, a new method for determination of free and total thiols in meat was introduced, based on reduction with sodium borohydride and thiol detection with 4,4'-dithiodipyridine. This combination of reducing and detection agent has been applied on small proteins in biomolecular science (Hansen, Ostergaard, Norgaard, & Winther, 2007), but has thus far not been tested in meat science. The method was applied on ground beef during storage under high-oxygen atmosphere, since previous work has shown significant thiol oxidation as measured with DTNB in these conditions (Jongberg et al., 2011a; Jongberg et al., 2011b; Zakrys-Waliwander, O'Sullivan, O'Neill, & Kerry, 2012). This method allowed to get more insight on the reversibility of thiol oxidation in meat during high-oxygen storage.

Chapter 3 covers the carbonylation pathway of myofibrillar proteins during *in vitro* metal-catalyzed oxidation in the presence of apple phenolics. A model system was set up to obtain a controlled oxidation of isolated myofibrillar proteins, using Fe^{3+} and H_2O_2 to induce radical formation through Fenton-like reactions. The isolation of myofibrillar proteins allowed to evaluate protein oxidation without the interference of other meat components (Vossen et al., 2015). The suspensions were treated with three pure phenolic compounds and an apple peel extract. Chlorogenic acid, (-)-epicatechin and phloridzin were chosen to represent apple phenolics from different phenolic classes and subclasses (see Section 1.3.2.3). Apple peel (from the apple cultivar Cripps Pink) instead of whole apple or apple pomace was chosen for the extract preparation, because of its superior phenolic content. The experimental conditions (concentrations and temperature) were based on literature (Estévez et al., 2009b; Estévez et al., 2010; Utrera & Estévez, 2013a), specifically chosen to enlarge and accelerate oxidation and the effects of phenolics. HPLC determination of α-amino adipic and γ-glutamic semialdehydes and fluorescence measurement of Schiff base cross-links allowed to evaluate the potential function of apple phenolics as natural antioxidants against meat protein oxidation.

Although *in vitro* model systems are useful for comprehending the mechanisms and pathways of oxidation, the effects of the food matrix are not taken into account. Furthermore, these model systems do not reflect the realistic oxidative conditions that occur during storage or digestion of

meat and meat product. In Chapter 4 and 5, protein oxidation was studied in whole meat and meat products during storage and digestion experiments that mimic realistic conditions. Because of the complexity of protein oxidation, a combination of protein oxidation assays is crucial in order to provide accurate information about oxidative protein damage and its consequences. In **Chapter 4**, a hitherto unique combination of protein oxidation markers was used to shed light on the oxidative degradation of beef and pork patties during illuminated storage and *in vitro* digestion. Meat patties and digests were subjected to thiol measurement as developed in Chapter 2, and carbonyl formation was measured as total and specific carbonylation. For the latter, the HPLC analysis of AAS and GGS from Chapter 3 was adapted to a more rapid and accurate UHPLC analysis. Furthermore, 4-hydroxyphenylalanine was identified and quantified as a new protein oxidation marker in meat. Proteolysis measurement allowed to understand the effect of protein oxidation on digestibility.

To investigate the effects of apple phenolics (as a source of natural antioxidants) on oxidation and digestibility, emulsion-type sausages were enriched with freeze dried apple pomace and subjected to illuminated storage and subsequent *in vitro* digestion in **Chapter 5**. Emulsion-type sausages were chosen for the ease of incorporating new ingredients during processing. Apple pomace (from the apple cultivar Jonagold) instead of apple peel was chosen because the pomace, and not the peel alone, is a by-product of apple juice production that may be valorized, and it is well documented that a large part of the phenolic compounds are retained in the pomace after pressing (Yeap Foo & Lu, 1999; Lu et al., 2000; Diñeiro García et al., 2009; De Paepe et al., 2015a). No extract was made to investigate whether drying of apple pomace alone, without extracting and thus concentrating the phenolics, would be sufficient to inhibit oxidation. Analysis of thiols, total and specific carbonyls, and proteolysis allowed to evaluate the actions and reactions of apple phenolics in meat emulsions.

Table 1.3 provides the hypotheses of the research and their relation to the chapters. To conclude, a general discussion and future prospects are given in **Chapter 6**.

Table 1.3: Hypotheses of the research and their relation to the chapters

	Hypothesis	**Chapter**
H1	Free and total thiols as measured with 4,4'-dithiodipyridine (4-DPS) are good markers for protein oxidation in meat	2, 4, 5
H2	Total carbonyl level as measured with 2,4-dinitrophenylhydrazine (DNPH) is a good marker for protein oxidation in meat	4, 5
H3	γ-Glutamic semialdehyde (GGS) and α-amino adipic semialdehyde (AAS) as measured with (U)HPLC-FLD are good markers for protein oxidation in meat	3, 4, 5
H4	Schiff base structures as measured with fluorescence spectroscopy are good markers for protein oxidation in meat	3
H5	4-hydroxyphenylalanine as measured with UHPLC-FLD is a good marker for protein oxidation in meat	4
H6	Cysteine thiol loss in meat proteins is caused by reversible disulfide formation	2
H7	γ-Glutamic semialdehyde (GGS) and α-amino adipic semialdehyde (AAS) are the most abundant protein carbonyls in meat	4, 5
H8	Apple phenolics can be used as a source of natural antioxidants against protein oxidation in meat	3, 5
H9	Protein oxidation in meat affects proteolysis during *in vitro* digestion	4, 5
H10	The antioxidative role of apple phenolics in meat reduces the effects of protein oxidation on proteolysis during *in vitro* digestion	5

Chapter 2

Reversible and irreversible thiol oxidation in ground beef as detected with 4,4'-dithiodipyridine

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Redrafted after

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ABSTRACT

Quantification of protein thiols (free and total) in ground beef during storage under high-oxygen atmosphere at 4 °C was performed by thiol detection using 4,4'-dithiodipyridine (4-DPS) before and after reduction using sodium borohydride. Two independent storage trials were performed, and in trial 1, only reversible thiol oxidation was observed (thiol loss was 30%). In trial 2, irreversible thiol oxidation occurred during the first days of storage, while further loss of thiols was caused by reversible oxidation (thiol loss was 33% of which ca. half was lost due to irreversible oxidation). The results were compared with SDS-PAGE analysis of cross-linked myosin heavy chain formed by disulfide bonding. Assuming that all reversible thiol loss was caused by disulfide cross-linking, both methods confirmed increasing disulfide formation in meat during storage, but the 4-DPS method showed higher disulfide percentages than the SDS-PAGE method (22.2 ± 0.3% and 8.5 ± 1.2%, respectively). The 4-DPS assay provides an accurate method to evaluate the reversibility of thiol oxidation in meat.

2.1 INTRODUCTION

Protein oxidation in meat and meat products is known to have an impact on protein functionality, sensory aspects and nutritional values (Estévez, 2011; Lund et al., 2011). The oxidation of thiol groups (RSH) of the cysteine residue in myosin (the most abundant myofibrillar protein) results in the formation of disulfide cross-links (RSSR) and has shown to alter texture properties resulting in a decreased tenderness and juiciness of meat (Lund et al., 2007b; Kim, Huff-Lonergan, Sebranek, & Lonergan, 2010; Lund et al., 2011; Zakrys-Waliwander et al., 2012; Jongberg, Wen, Tørngren, & Lund, 2014). Furthermore, the structural changes in proteins due to cross-linking may affect their recognition sites for proteases, making the oxidized proteins less susceptible to proteolysis, resulting in a decreased digestibility (Rowe et al., 2004; Morzel et al., 2006; Santé-Lhoutellier, Aubry, & Gatellier, 2007). Thiol oxidation is complex and may lead to the formation of multiple oxidation products, such as disulfides, sulfenic acid, sulfinic acid, sulfonic acid, and thiosulfinates of which disulfides and sulfenic acid are reversible thiol oxidation products (Nagy et al., 2010). Most often oxidation of thiol groups is evaluated by quantification of the loss of thiol groups and not by quantification of thiol oxidation products, but it is generally believed that thiol loss results primarily in formation of disulfides (e.g. Li et al., 2013) although no direct evidence for this statement can be found in the literature. Inhibition of thiol oxidation in meat during storage has been attempted by use of plant extracts rich in phenolic compounds, which are known to effectively reduce lipid oxidation, but have not been shown to be able to prevent disulfide formation in meat (Jongberg et al., 2011a). It is therefore important to quantify the amount of reversibly and irreversibly oxidized thiols in order to further develop antioxidant strategies to avoid or repair thiol oxidation in meat.

The degree of protein disulfide cross-linking in meat and meat products can be evaluated by means of protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Decker et al., 1993). However, measurement of the band pixel intensity on the SDS gel is

merely semi-quantitative, and irreversible thiol oxidation cannot be evaluated by this method. A sensitive method to quantify thiol loss and its reversibility is thus needed to get clear insight into the thiol oxidation chemistry in meat and meat products. The spectrophotometric measurement of the loss of free thiol groups in muscle proteins has been widely used as an indicator of protein oxidation (Estévez et al., 2009a). The quantification of protein disulfides is somewhat more complex, as disulfide bonds must first be cleaved before detecting the newly formed thiols. In order to cleave all disulfide bonds, a strong and efficient reducing agent must be selected. Furthermore, this reducing agent must not cross-react with the thiol detection agent (Hansen et al., 2009b).

An approach for measuring thiols and disulfides is shown in Figure 2.1. Thiols can be detected by reaction with a thiol-specific detection agent with useful spectrophotometric properties (Figure 2.1A). The most common thiol detection reagent is 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent), which forms a disulfide bond with free thiol groups, releasing a yellow NTB dianion (2-nitro-5-thiobenzoate) with maximum absorbance at 412 nm in the pH range from 6 to 9.5 (Estévez et al., 2009a; Hansen et al., 2009b). An alternative to DTNB is 4,4'-dithiodipyridine (4-DPS), which reacts in a similar way with thiol groups, forming 4-thiopyridone (4-TP) that absorbs at 324 nm. The small size of this detection reagent makes it easier to reach poorly accessible thiols within the protein core. 4-TP also has a higher extinction coefficient than NTB, suggesting that 4-DPS is a more sensitive thiol detection agent than DTNB (Riener, Kada, & Gruber, 2002). Furthermore, 4-TP is stable in lower pH ranges (pH 3 – 7), making thiol detection possible at low pH as in common reversed phase HPLC conditions (Hansen et al., 2007). Ruan et al. (2013) found 4-DPS to be less affected by pH and denaturants compared with DTNB, and thus found 4-DPS more suitable and reliable for thiol detection in soy protein. The measurement of disulfide bonds is based on the reduction of disulfides followed by detection of the newly reduced thiols (Figure 2.1B). Assuming that all thiol loss was caused by reversible disulfide formation, the amount of disulfides can then be calculated by subtracting the amount of free thiols from total thiols. To reduce disulfide bonds during SDS-PAGE, dithiothreitol (DTT) is often used. This reagent is less

suitable for disulfide quantification because of its slow reaction rate and the cross-reactivity with thiol detection agents (Hansen et al., 2007; Hansen et al., 2009b). Liu et al. (2000a) have quantified disulfide bonds in myofibrillar proteins based on a method described by Damodaran (1985). In this procedure, disulfides are cleaved by adding an excess of sodium sulfite. However, oxidative sulfitolysis of disulfide bonds is slow and rarely quantitative in absence of catalysts (Kella et al., 1985; Hansen et al., 2009b). An alternative reducing agent is sodium borohydride (BH), a highly reactive and strong reductant whose main advantage is that its excess can be removed by acidification (Gailit, 1993; Hansen et al., 2009b). Jongberg et al. (2011a) have used this reducing agent in myofibrillar proteins, followed by thiol detection with DTNB. However, the large pH variations (from acidic pH after BH excess removal to pH 8.0 for DTNB reaction) are unfavourable for thiol-disulfide redox reactions.

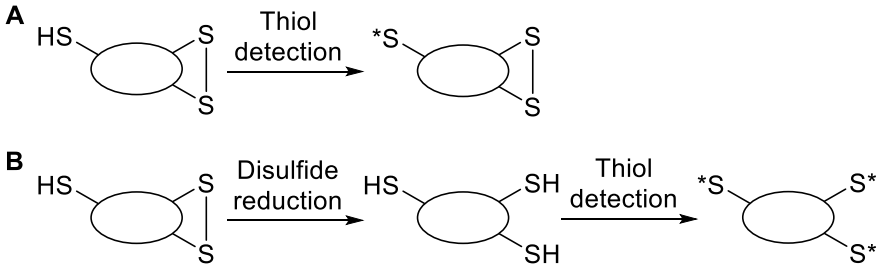

Figure 2.1: Approach for detection of free thiols (A) and total thiols (B). Thiols destined for detection are denoted S* (modified after Hansen et al., 2009b).

The aim of the present Chapter was to investigate the extent of reversible and irreversible thiol oxidation in ground beef during storage. This allowed to evaluate whether (a) thiol loss in meat is caused by (reversible) disulfide formation, which is generally assumed though not proven in literature, or (b) thiol loss is also caused by formation of other (irreversible) oxidation products. A sensitive and reliable method for the quantification of free and total thiols in meat was established based on reduction with sodium borohydride and thiol detection with 4,4'-dithiodipyridine. This combination of BH and 4-DPS has been applied on small proteins in biomolecular science (Hansen et al., 2007), but has thus far not been tested in meat, which is a more complex matrix containing

large proteins with low solubility. The method was subsequently used for quantification of reversible and irreversible thiol oxidation in ground beef during storage in high-oxygen modified atmosphere packaging (HiOx MAP) and compared with SDS-PAGE based methodology.

2.2 MATERIALS AND METHODS

2.2.1 Sampling of HiOx MAP ground meat

Two independent storage trials (Trial 1 and Trial 2) were performed for sampling of HiOx MAP ground beef. For trial 1, ground beef stored in high-oxygen modified atmosphere packaging was obtained from a local Danish supermarket. The meat had been packed under modified atmosphere (high-oxygen) the day before and consisted of nine packages of 400 g each. No information was available on the packaging material or initial oxygen levels. Three packages (replicate A, B, and C) were opened and the meat was vacuum packed in portions of 50 g and stored at -80 °C until analysis (day 0). The remaining six packages were stored in darkness at 4 °C until day 4 or day 9, at which point another three replicates (A, B, and C) were collected, vacuum packed, and frozen to -80 °C.

For trial 2, similar sampling was performed for freshly ground beef obtained from a local Belgian butcher, ground three days after slaughter of the animal. Fifteen polypropylene trays were filled with 450 g of meat each, and flushed with a gas mixture of 70% O_2 and 30% CO_2. Trays were sealed with a PET/CPP NPAF foil (oxygen transmission rate 190 cm^3/m^2/24 h at 25 °C and 50% R.H.) by means of a tray sealer (TS400, VC999 Packaging Systems, Herisau, Switzerland), and stored at 4 °C until sampling day. On day 0, 3, 6, 9 and 12, three packages (replicate A, B, and C) were opened, vacuum packed in portions of 75 g and stored at -80 °C until analysis.

2.2.2 Influence of pH and denaturant on the molar extinction coefficients of 4-TP

A total of 24 buffers were prepared, by varying the buffering capacity, the pH value, and the presence of a denaturant. Six buffers with a low buffering capacity were prepared by mixing 0.1 M

citric acid and 0.2 M Na_2HPO_4 to pH values of 2.5, 3.5, 4.5, 5.5, 6.5 and 7.5. Likewise, six high capacity buffers were prepared by mixing 1 M citric acid and 1 M trisodium citrate dihydrate to the aforementioned pH values. Sodium dodecyl sulfate (SDS) buffers were prepared by adding 5% SDS (w/v) to the low capacity buffers. The same was done for guanidine hydrochloride by adding 6 M GuHCl to the high capacity buffers. Concentrations of L-cysteine ranging from 2.5 to 500 µM were prepared in all buffers. Thiol detection with 4-DPS was performed based on Riener et al. (2002) by diluting 500 µL of L-cysteine solution with 2 mL of the corresponding buffer, and adding 500 µL 4-DPS solution (4 mM 4-DPS in 12 mM HCl). The absorbances were measured at 324 nm against water, before addition of 4-DPS (A_{pre}) and after exactly 30 minutes of reaction with 4-DPS in the dark at room temperature (A_{post}). The absorbance corresponding to the thiol concentration was calculated by subtracting A_{pre} and A_{blank} (2.5 mL buffer + 500 µL 4-DPS solution) from A_{post}. Molar extinction coefficients (ε) were calculated from the slope of the standard curves according to the Lambert-Beer law (equation 2.1: A is absorbance, ℓ is path length).

$$A = \varepsilon \cdot \ell \cdot c \qquad [2.1]$$

2.2.3 Optimization of disulfide reduction

The efficiency of the disulfide reduction was tested by the reduction of known concentrations (25 to 200 µM) of commercially available oxidized glutathione (GSSG). The expectation was to find thiol concentrations in the form of reduced glutathione (GSH) double of the aforementioned molarities (50 to 400 µM). The GSSG was dissolved in 6 M GuHCl in 0.1 M Tris(hydroxymethyl)-aminomethan (TRIS) buffer (pH 8.0), as this was the buffer system that was chosen to solubilize the meat proteins. The method was developed based on disulfide reduction according to Hansen et al. (2007). The reduction was performed by adding 100 µL of 30% (w/v) alkaline borohydride to 3 mL of sample. After incubation at 50 °C, the excess of borohydride was removed with 6 M hydrochloride (final concentration 1.8 M) at room temperature. Different conditions and parameters were tested by varying the solvent for sodium borohydride (1, 2 and 4 M NaOH), the

incubation time for the reduction with borohydride (15, 30, 60 and 150 minutes), and the hydrolysis time of the residual borohydride (2, 10, 20, 30, 40, 45, 60, 90, 150, 165 and 180 minutes).

2.2.4 Quantification of free and total thiols in MAP ground beef using 4-DPS

The vacuum packed meat was thawed in water at room temperature for 30 minutes before homogenizing 1.0 g of meat in 25 mL of 6 M GuHCl in 0.1 M TRIS buffer (pH 8.0) using an Ultra Turrax. The homogenates were centrifuged (20 minutes, 5311 g, 4 °C) and supernatants were filtered (qualitative filter paper, particle retention: 11 μm). Samples were kept on ice at all times. Protein concentration of the filtrates was determined spectrophotometrically at 280 nm using a 5-point standard curve prepared from bovine serum albumin (BSA). Disulfide reduction was performed according to the optimized procedure as described in the previous section. Hence, an aliquot of 3 mL filtrate was subjected to disulfide reduction by adding 50 μL 1-octanol (as anti-foaming agent) and 100 μL freshly prepared 30% sodium borohydride in 1 M NaOH. After incubation at 50 °C for 30 minutes, an aliquot of 1.35 mL of 6 M HCl was added, followed by stirring for 10 minutes.

Free and total thiols were determined with 4-DPS in the non-reduced filtrate and the reduced filtrate, respectively. An aliquot of 500 μL filtrate was mixed with 2 mL of 6 M GuHCl in 1 M citric acid buffer (pH 4.5) and 500 μL 4-DPS solution (4 mM 4-DPS in 12 mM HCl). The absorbance was measured at 324 nm against 6 M GuHCl in 1 M citric acid buffer (pH 4.5), before addition of 4-DPS (A_{pre}) and after exactly 30 minutes of reaction with 4-DPS in the dark at room temperature (A_{post}). A mixture of 2.5 mL 6 M GuHCl in 1 M citric acid buffer (pH 4.5) and 500 μL 4-DPS solution was prepared as a blank sample (A_{blank}). The absorbance corresponding to the thiol concentration was calculated by subtracting A_{pre} and A_{blank} from A_{post}. The thiol concentration was calculated based on a 5-point standard curve ranging from 2.5 to 500 μM $_{L}$-cysteine in 6 M GuHCl in 1 M citric acid buffer (pH 4.5). The thiol content was expressed as nmol thiol per mg protein. Assuming that all reversible thiol oxidation was caused by disulfide formation, and taking into account that

treatment of disulfide bonds with sodium borohydride yields two thiol groups (Brown, 1960), the disulfide content was calculated as half of the difference between total and free thiols.

2.2.5 SDS-PAGE analysis

The meat from trial 1 was subjected to SDS-PAGE analysis. The meat was thawed at room temperature for 30 minutes before an aliquot of 1.0 g meat was homogenized in 25 mL of 5% SDS (w/v) dissolved in 0.1 M TRIS buffer at pH 8.0 using an Ultra Turrax. The homogenates were placed in a water bath at 80 °C for 30 minutes, followed by centrifugation for 20 minutes at 950 g. The supernatant was filtered (particle retention: 5-13 μm), and the protein concentration was determined spectrophotometrically at 280 nm using a standard curve prepared from BSA. The homogenates were analyzed by gel electrophoresis using NuPAGE Novex 3-8% TRIS-acetate gels according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reduced samples were prepared by mixing 1.6 μL diluted sample (2 mg protein/mL) and 14.4 μL loading solution, which was prepared from 60 μL LDS (lithium dodecyl sulfate) sample buffer, 24 μL 1.0 M DTT and 132 μL MilliQ water. The non-reduced samples were prepared by mixing 1.6 μL diluted sample (2 mg protein/mL) and 14.4 μL loading solution, which was prepared from 60 μL LDS sample buffer and 156 μL MilliQ water. Aliquots of 10 μL reduced or non-reduced samples, as well as 3 μL of Precision Plus Protein Standard All Blue marker were loaded to the wells of the gels. Electrophoresis was run for 90 minutes at 150 V in cassettes containing ice cold SDS TRIS-acetate running buffer. After electrophoresis, the gels were fixed overnight in fixation solution (50% ethanol, 7% acetic acid) at room temperature on a laboratory shaker. Following staining overnight by the fluorescence SYPRO Ruby Protein Gel Stain, the gels were photographed by a charge-coupled device (CCD) camera (Raytest, Camilla II, Straubenhardt, Germany). The protein concentration of the bands were estimated by the pixel intensity as determined by the freeware GelAnalyzer2010a© by Dr. Istvan Lazar, www.gelanalyzer.com. Band intensities were quantified from the volume of the pixel intensity when analyzing the gels with GelAnalyzer. In order to correct for total protein content of

each sample, the pixel intensities of cross-linked myosin heavy chain (CL-MHC) and myosin heavy chain (MHC) were divided by the total pixel volume of all bands in each lane of the gel to obtain the relative intensity of CL-MHC and MHC for each sample. The degree of cross-linking was calculated based on gels containing both reduced and non-reduced samples, and was considered equal to the ratio of the relative intensity of CL-MHC in the non-reduced samples to the relative intensity of MHC in the reduced samples at day 0 ($CL\text{-}MHC_{non\text{-}red}$ / $MHC_{red,\ day\ 0}$; %).

2.2.6 Statistical analysis

Statistical analysis was performed using SAS® 9.3 package (SAS Institute Inc., Cary, NC, USA). Recovery percentages were analyzed using a linear model with concentration as fixed effect. Thiol content was analyzed using a linear model with storage time (days), treatment (with reduction = free thiols and without reduction = total thiols) and their interaction as fixed effects. Disulfides and degrees of cross-linking were analyzed using a linear model with storage time as fixed effect. The correlation between the 4-DPS assay and SDS-PAGE was assessed using Pearson correlation. Effects were considered significant at $P < 0.05$.

2.3 RESULTS AND DISCUSSION

2.3.1 The influence of pH and denaturant on the molar extinction coefficients of 4-TP

In order to determine the optimal pH for thiol detection with 4-DPS, two types of buffer solutions with a low and high buffering capacity were prepared that ranged from pH 2.5 to pH 7.5. It is strictly necessary to include protein denaturants in the buffer during analysis of thiols in meat to prevent precipitation of some of the cross-linked protein. In the absence of protein denaturants, such precipitants would not be included in the assay resulting in erroneous quantifications. The effect of denaturants was therefore tested by adding 5% SDS and 6 M GuHCl to the low and high capacity buffers, respectively. Molar extinction coefficients were calculated from the slope of an $_L$-

cysteine standard curve, which was subjected to the 4-DPS assay at all pH and denaturant conditions.

According to Riener et al. (2002), the molar extinction coefficient of 4-TP is 21400 M^{-1} cm^{-1} at pH 7.0. As shown in Figure 2.2, the molar extinction coefficients in all low and high capacity buffers without denaturants stay close to this value. However, adding denaturants to the buffers clearly narrows the pH range in which the molar extinction coefficients are stable. This is in agreement with the findings of Hansen et al. (2009b), who found that the presence of denaturants decreased the alkylation rate of NTB by N-ethylmaleimide. The lowest 4-TP absorptions were measured at pH 2.5 with buffers added 5% SDS and 6 M GuHCl (ε = 8500 M^{-1} cm^{-1} and ε = 13800 M^{-1} cm^{-1}, respectively). The amount of GuHCl was not soluble at pH 6.5 and 7.5, thus Figure 2.2 shows no results for these conditions.

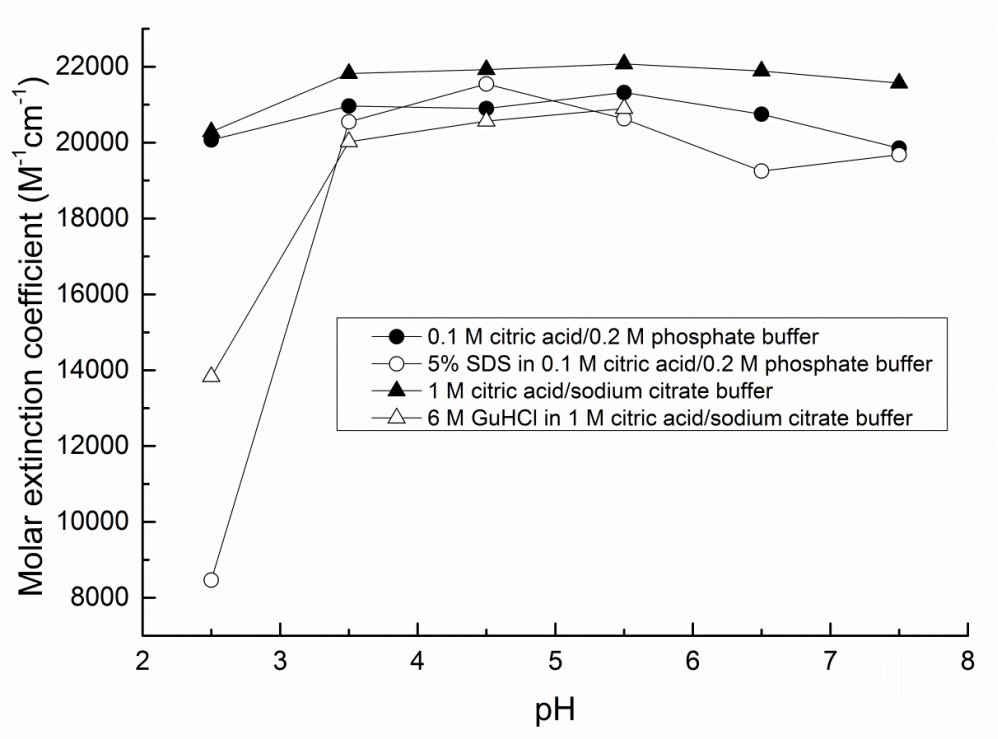

Figure 2.2: Molar extinction coefficients of 4-DPS as a function of pH.

Overall, optimal molar extinction coefficients were found in buffers containing denaturants ranging from pH 3.5 to 5.5. Two factors were considered to select the optimal working buffer. First,

the pH value in the samples is very low after acidification in the reduction step. Therefore, a high capacity buffer was needed to ensure correct pH adjustment. Second, a strong denaturant is crucial to ensure good solubility of meat proteins. We therefore chose to use 6 M GuHCl in 1 M citric acid buffer pH 4.5 as a working buffer for determining the free and total thiols in meat using 4-DPS.

2.3.2 Optimization of disulfide reduction

It was necessary to optimize the disulfide reduction in the buffer systems chosen for the meat samples, and this was established by determination of recovery percentages for the reduction of GSSG based on incubation at 50 °C with alkaline borohydride followed by removal of residual borohydride by acidification. The reduction procedure was tested by combining different sodium hydroxide concentrations with varying times of incubation and hydrolysis as described in Section 2.2.3. For each combination, a known concentration of GSSG in 6 M GuHCl in 0.1 M TRIS buffer (pH 8.0), as well as a blank (6 M GuHCl in 0.1 M TRIS buffer pH 8.0), were subjected to the reduction assay. Twice as much GSH as GSSG in molar concentrations was expected, thus the recovery percentages were calculated according to equation 2.2:

$$\text{recovery\%} = \frac{\text{thiols quantified}}{\text{thiols expected}} \times 100 \qquad [2.2]$$

It is well known that sodium borohydride rapidly decomposes at neutral or acidic pH (Banfi, Narisano, Riva, Stiasni, & Hiersemann, 2001). Therefore, solutions of 30% (w/v) borohydride in 1, 2, and 4 M NaOH were freshly prepared for the reduction of GSSG. After mixing 3 mL of sample with 100 µL of BH dissolved in 1, 2, and 4 M NaOH, the pH was approximately 8.6, 11.0, and 12.0, respectively. Although Patsoukis and Georgiou (2005) highlighted that a high alkaline environment (pH 12.0 rather than 8.0) is needed for optimal disulfide reduction by borohydride, we found similar recovery percentages with the 1 M NaOH treatment as with 2 and 4 M NaOH (data not shown). The lack of correlation between our results and those reported by Patsoukis et al. (2005) may be due to the use of different buffers and derivatization agents.

After addition of BH, the mixtures were incubated at 50 °C as recommended by Patsoukis et al. (2005). The optimal incubation time for effective reduction depends on the concentration and reactivity of BH and on the accessibility of the protein disulfides. Incubation must take long enough in order to let the BH reach and reduce all disulfides. Prolonged incubation, however, might promote re-oxidation of the newly formed thiols (Gailit, 1993). Furthermore, $NaBH_4$ can decompose over time, even at alkaline pH (Gailit, 1993; Minkina, Shabunya, Kalinin, Martynenko, & Smirnova, 2012). For the current method development, oxidized glutathione was incubated with BH during 15, 30, 60, and 150 minutes, and optimal recovery percentages were found with 30 minutes of incubation (data not shown).

Subsequent to incubation, the residual BH was removed by acidification with 6 M HCl, bringing the final concentration of HCl in the mixture to 1.8 M. Acidification of $NaBH_4$ completely discharges the reactive hydride, yielding hydrogen gas (H_2) (Hansen et al., 2009b; Winther & Thorpe, 2014) and causing the protein solution to foam. This reaction was avoided by adding a small amount of 1-octanol (Hansen et al., 2009b). During the method optimization, the acidified solutions were left to stir on a magnetic stirrer at room temperature for various time periods, ranging from 2 minutes to 3 hours. The best recovery percentages were found when stirring for 10 minutes after addition of HCl, and subsequently re-adjusting the pH with 6 M GuHCl in 1 M citric acid buffer (pH 4.5) for the 4-DPS assay (data not shown). Although the low pH of the BH-HCl mixture is likely to protect the reduced thiols from re-oxidation (Hansen et al., 2009b), oxygen inclusion due to prolonged and vigorous stirring may induce further oxidation after the reducing agent has been deactivated (Gailit, 1993).

The method for disulfide reduction was adjusted based on all the above mentioned considerations. Optimal recovery percentages in the range from 25-200 μM GSSG were obtained when carrying out the reduction as follows: 50 μL 1-octanol and 100 μL freshly prepared 30% sodium borohydride in 1 M NaOH were added to 3 mL of sample. The mixture was incubated at 50 °C for 30 minutes with occasional stirring. After incubation, the excess of borohydride was removed by adding 1.35 mL of 6 M HCl followed by stirring for 10 minutes. This procedure was applied for the reduction

of various concentrations of GSSG. The quantified thiols and recovery percentages are presented in Table 2.1.

Table 2.1: Recovery of GSH after reduction of GSSG in 6 M GuHCl and 0.1 M TRIS buffer (pH 8.0), with 30% sodium borohydride in 1 M NaOH at 50 °C for 30 minutes, followed by acidification with 6 M HCl (final concentration 1.8 M) and stirring for 10 minutes. Recovery percentages are expressed as the ratio quantified to expected thiols (described in equation 2.2).

GSSG (μM)	**Thiols expected (μM)**	**Thiols quantified (μM)**	**Recovery (%)**
0 (blank)	0	3.8 ± 1.6	
25	50	42.9 ± 3.6	85.8 ± 7.1
50	100	82.7 ± 4.7	82.7 ± 4.7
75	150	129.1 ± 2.6	86.1 ± 1.7
100	200	175.3 ± 1.2	87.7 ± 0.6
125	250	211.5 ± 8.6	84.6 ± 3.4
150	300	262.7 ± 5.6	87.6 ± 1.9
175	350	304.7 ± 6.5	87.1 ± 1.9
200	400	350.1 ± 8.1	87.5 ± 2.0

No significant differences between recovery percentages were found among the GSSG concentrations (P = 0.642), indicating that the reduction procedure is precise for concentrations ranging from 25 to 200 μM of disulfides and resulted in an approximate recovery of 86%. According to AOAC International the expected recovery is from 80-90% to 107-110% in the concentration range of the quantified thiols used in the present study, so the observed accuracy is acceptable (AOAC International, 2012).

2.3.3 Quantification of disulfides in MAP ground beef with 4-DPS

MAP ground beef obtained from trial 1 and trial 2 were subjected to free and total thiol quantification by the 4-DPS assay at various storage times. Caution should be made when comparing the meat from trial 1 and 2 for several reasons: 1) the batches of meat were not obtained from the same animal, 2) ground beef often comes from different muscles so any observed

difference in thiol or disulfide concentrations between the two trials could be ascribed to differences in the types of muscles used in each batch of meat, and 3) difference in time between slaughter and packaging and the exact gas composition (unknown for trial 1).

Table 2.2: Thiol and disulfide quantification with 4-DPS in ground beef from trial 1, stored under high-oxygen atmosphere at 4 °C for up to 9 days. Results are shown as mean ± SD of three independent replicates. Different superscripts (a-c) within a column denote statistical differences among storage times ($P < 0.05$). Total thiols marked with * are significantly different from free thiols on the same day.

Storage time	**Free thiols (nmol/mg protein)**	**Total thiols (nmol/mg protein)**	**Disulfides (nmol/mg protein)**
Day 0	$37.6^{a} \pm 0.5$	$51.7^{a} \pm 0.8$ *	$7.0^{a} \pm 0.6$
Day 4	$30.0^{b} \pm 1.1$	$49.2^{a} \pm 1.5$ *	$9.6^{b} \pm 1.1$
Day 9	$26.5^{c} \pm 1.7$	$49.4^{a} \pm 1.4$ *	$11.5^{c} \pm 1.4$
	$P_{\text{storage time}} < 0.001$		$P_{\text{storage time}} < 0.001$
	$P_{\text{treatment}} < 0.001$		
	$P_{\text{storage time * treatment}} < 0.001$		

The significant decrease in free thiols in the meat from trial 1 as well as trial 2 (Table 2.2 and Table 2.3, respectively) shows that thiol oxidation (ca. 30% and 33% loss of free thiols, respectively) took place during storage, which is in agreement with previous studies (Lund et al., 2007b; Jongberg et al., 2011b). After reducing the proteins from the beef samples from trial 1 with borohydride, the amount of total thiols was significantly higher than the amount of free thiols at all sampling days indicating the formation of disulfides. No significant changes in total thiol levels were found during the nine days of storage (Table 2.2). This suggests that all thiols lost during nine days storage could be fully reduced by borohydride, and were, hence, lost due to reversible thiol oxidation (most likely disulfide formation).

Table 2.3: Thiol and disulfide quantification with 4-DPS in ground beef from trial 2, stored under high-oxygen atmosphere at 4 °C during 12 days. Results are shown as mean ± SD of three independent replicates. Different superscripts (a-c) within a column denote statistical differences among storage times ($P < 0.05$). Total thiols marked with * are significantly different from free thiols on the same day.

Storage time	**Free thiols (nmol/mg protein)**	**Total thiols (nmol/mg protein)**	**Disulfides (nmol/mg protein)**
Day 0	$58.3^{a} \pm 1.6$	$61.8^{a} \pm 0.2$	$1.8^{a} \pm 0.9$
Day 3	$44.4^{b} \pm 1.2$	$55.1^{b} \pm 1.2$ *	$5.3^{b} \pm 1.1$
Day 6	$42.4^{bc} \pm 0.8$	$54.4^{b} \pm 1.6$ *	$6.0^{b} \pm 1.1$
Day 9	$40.4^{bc} \pm 2.4$	$53.1^{b} \pm 2.2$ *	$6.4^{b} \pm 2.1$
Day 12	$39.2^{c} \pm 0.8$	$52.1^{b} \pm 1.6$ *	$6.4^{b} \pm 1.0$
	$P_{\text{storage time}} < 0.001$		$P_{\text{storage time}} < 0.001$
	$P_{\text{treatment}} < 0.001$		
	$P_{\text{storage time * treatment}} < 0.001$		

When subjecting the beef from trial 2 to borohydride reduction, no significant difference was found between the free and total thiol level on day 0 ($P = 0.191$), indicating that the initial level of oxidation in this meat was minimal. After three days of storage, the concentration of free thiols was significantly lower than at day 0 (Table 2.3) ($P < 0.001$) confirming thiol oxidation during storage. Reduction of the beef proteins resulted in a significantly higher concentration of total thiols than free thiols at day 3 ($P < 0.001$) suggesting the formation of disulfides or other reducible thiol oxidation products. However, the concentration of total thiols significantly decreased from day 0 to day 3 ($P < 0.001$) showing that irreversible thiol oxidation had taken place concomitantly to the suggested disulfide formation. Although the amount of total thiols stayed constant between day 3 and 12, there was a significant decrease of free thiols between day 3 and 12, indicating that predominantly protein disulfides were formed in this storage period. By subtracting the free thiol concentration on day 0 with the free thiol concentration on day 12, a loss in free thiols of 19 nmol/mg protein was obtained. Similarly, the loss in total thiols over 12 days of storage was only 10 nmol/mg protein, which means that over the full duration of storage about half of the thiols were lost due to irreversible oxidation and the other half due to reversible oxidation. The consequence of thiol oxidation in meat has previously been ascribed mainly to disulfide formation

(Lund et al., 2007b; Jongberg et al., 2011b) but thiol oxidation is very complex and numerous oxidation products may be formed such as sulfenic acid, sulfinic acid, sulfonic acid and thiosulfinates (Nagy et al., 2010). The present study shows that other thiol oxidation products than disulfides may be formed in meat during storage. While the formation of sulfonic acid is irreversible, sulfenic acid is extremely reactive and may react further to create compounds such as disulfides or sulfonic acid. Due to the efficient reducing capacity of BH it is likely that sulfenic acids are also reduced to thiols by this reducing agent, so it is unknown whether a part of the quantified total thiols is in fact derived from the reduction of sulfenic acid, thus overestimating the amount of disulfides. However, the approach based on BH and 4-DPS has previously been used for the quantification of the thiol-disulfide redox status in cells without considering a potential sulfenic acid formation (Hansen, Roth, & Winther, 2009a). The formation of sulfenic acids in living cells has been shown to be rather widespread in some cases (Saurin, Neubert, Brennan, & Eaton, 2004) but it has never been determined in meat samples during storage.

2.3.4 Comparison between SDS-PAGE and 4-DPS for the quantification of disulfides in MAP ground beef

The quantification of free and total thiols in the ground beef from trial 1 with 4-DPS was compared with the conventional cross-linking analysis by SDS-PAGE. Myosin heavy chain has previously been found to be the meat protein that is most susceptible to oxidation and SDS-PAGE analysis has been widely used to study disulfide cross-link formation in meat and meat products (Decker et al., 1993; Morzel et al., 2006; Lund et al., 2007b; Jongberg et al., 2011b). The non-reduced samples showed an increase of CL-MHC band intensity on the SDS gel as the storage time increased (Figure 2.3), whereas no CL-MHC was seen in the reduced samples (data not shown).

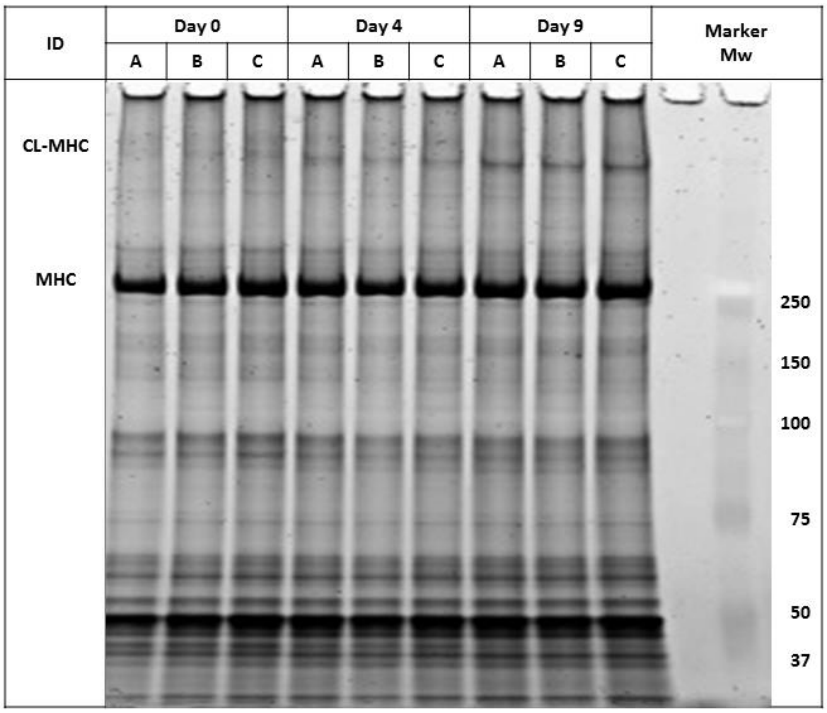

Figure 2.3: SDS-PAGE gel of non-reduced ground beef from trial 1 stored under high-oxygen atmosphere at 4 °C during 9 days. The gel shows all samples in triplicate (A-C). CL-MHC, cross-linked myosin heavy chain; MHC, myosin heavy chain.

The degrees of disulfide formation in the ground beef according to the 4-DPS assay and SDS-PAGE are compared in Table 2.4. With both methods, a significant increase of disulfides was found in the ground beef during nine days of storage under high-oxygen atmosphere ($P < 0.001$ and $P = 0.005$ for 4-DPS and SDS-PAGE, respectively). A Pearson correlation of 0.913 was found between the two methods, but the concentration of disulfides found by the 4-DPS assay was considerably higher than by SDS-PAGE. This can be explained by the fact that two proteins may be cross-linked by several disulfide bridges, consequently, cleaving these disulfide bridges leads to more than two newly formed thiol groups. From the three-dimensional protein structure of MHC it can be seen that several cysteine residues are transversely positioned in the tail of MHC, thus it is likely that several intermolecular disulfides are formed between two MHC molecules. Furthermore, thiols may be lost to disulfide formation intramolecularly in MHC, without causing a cross-link between two proteins; that is without generating the CL-MHC band that appears in the SDS-PAGE gel. Additionally, it is worth noting that severely cross-linked MHC polymers are probably too large to

enter the SDS gel (Jongberg et al., 2011a), leading to an underestimation of CL-MHC. This is a problem which is actually one of the many drawbacks with the gel electrophoresis methods. Upon severe and ongoing oxidation, such polymerization of MHC would eventually lead to a hyperbolic instead of linear correlation between 4-DPS and SDS-PAGE results. Finally, even though myosin heavy chain has been found to be the meat protein that is most prone to thiol oxidation (Stagsted, Bendixen, & Andersen, 2004), disulfide formation in other meat proteins may also contribute to the values obtained with the 4-DPS assay, and as mentioned above, the potential presence of sulfenic acid may contribute to an overestimation of disulfides by the 4-DPS assay.

Table 2.4: Degrees of cross-linking in ground beef obtained from trial 1 during 9 days of storage under high-oxygen atmosphere at 4 °C, measured with 4-DPS and SDS-PAGE. Degrees of cross-linking are expressed as the level of cross-linking (disulfides and CL-MHC for 4-DPS and SDS-PAGE, respectively) divided by the reference level of reduced protein on day 0 (total thiols and MHC_{red} for 4-DPS and SDS-PAGE, respectively). Results are shown as mean ± SD of three independent replicates. CL-MHC, cross-linked myosin heavy chain; MHC, myosin heavy chain. Different superscripts (a-c) within a column denote statistical differences among storage times ($P < 0.05$).

	4-DPS assay	**SDS-PAGE**
Storage time	**Disulfides / Total thiols$_{day\ 0}$ (%)**	**CL-MHC$_{non-red}$ / MHC$_{red,\ day\ 0}$ (%)**
Day 0	13.6^{a} ± 0.4	3.1^{a} ± 1.6
Day 4	18.6^{b} ± 0.9	6.9^{b} ± 1.0
Day 9	22.2^{c} ± 0.3	8.5^{b} ± 1.2
	$P_{storage\ time} < 0.001$	$P_{storage\ time} = 0.005$

2.4 CONCLUSIONS

SDS-PAGE analysis provides information about intermolecular disulfide formation in MHC for moderately oxidized samples, while the 4-DPS assay provides a more accurate and quantitative assessment of protein thiols and intra- and intermolecular disulfides and thus offers a good tool to investigate the thiol-disulfide reactions in meat and potentially also in meat products.

In the present study, we have shown that both reversible and irreversible thiol oxidation takes place in meat during storage. Disulfide formation is often the consequence of thiol oxidation as observed by a thiol loss, but our results clearly show that disulfide formation in meat during storage is not the only consequence of thiol oxidation. This is an important observation in relation to the further development of strategies to avoid or repair thiol oxidation.

Chapter 3

Apple phenolics as inhibitors of the carbonylation pathway during *in vitro* metal-catalyzed oxidation of myofibrillar proteins

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Redrafted after

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ABSTRACT

The effect of apple phenolics on the oxidative damage caused to myofibrillar proteins by an *in vitro* metal-catalyzed oxidation system was investigated. Three pure phenolic compounds (chlorogenic acid, (-)-epicatechin and phloridzin) and an apple peel extract were added to myofibrillar proteins in three concentrations (50, 100 and 200 μM), and a blank treatment was included as a control. All suspensions were subjected to Fe^{3+}/H_2O_2 oxidation at 37 ºC during 10 days, and protein oxidation was evaluated as carbonylation (α-amino adipic and γ-glutamic semialdehydes) and Schiff base cross-links. Significant inhibition by apple phenolics was found as compared to the control treatment, with (-)-epicatechin being the most efficient antioxidant and phloridzin showing the weakest antioxidant effect. The higher concentrations of apple peel extract showed effective antioxidant activity against protein carbonylation in myofibrillar proteins, emphasizing the potential of apple by-products as natural inhibitors of protein oxidation in meat products.

3.1 INTRODUCTION

Among food products, meat and meat products belong to the group that is most prone to oxidative deterioration due to their chemical composition. Oxidation in muscle foods can cause chemical modifications of both lipids and proteins, affecting quality traits (Hygreeva, Pandey, & Radhakrishna, 2014). To cope with consumer's demand for qualitative, shelf-stable meat and meat products, the use of antioxidative strategies is almost inevitable (Monahan, 2000).

Over the last decade, the utilization of vegetable, fruit, herbs and spice derivatives as natural antioxidants has been a topic of great interest. Plant materials provide a good alternative for synthetic antioxidant additives because of their high phenolic content (Hygreeva et al., 2014). These natural phenolics, like synthetic antioxidants, are able to act as free radical scavengers and metal ion chelators, and thus inhibit oxidation and extend the shelf-life (Falowo et al., 2014). However, a concern is growing among consumers about synthetic antioxidants because of adverse, potential toxicological reports (Devatkal, Narsaiah, & Borah, 2010; Karre et al., 2013). Replacing synthetic with natural antioxidants can thus provide a solution to consumers demand for shelf-stable meat products (Hygreeva et al., 2014).

The antioxidative effects of plant derivatives on lipid oxidation in muscle foods have been reported extensively (Maqsood, Benjakul, Abushelaibi, & Alam, 2014; Ahmad et al., 2015). The inhibition of protein oxidation by natural antioxidants has also been a rising research topic while much less studied than lipid oxidation (Estévez, 2011; Falowo et al., 2014). The possible nutritional impact (loss of amino acids), sensory deterioration (tenderness, off-odour, off-flavour) and impaired protein functionality (gelation, emulsification, water holding) and health concerns caused by protein oxidation, emphasize the importance of understanding the effects of antioxidants based on the underlying chemical mechanisms and pathways related to protein oxidation (Estévez, 2011; Soladoye et al., 2015; Estévez & Luna, 2017).

The oxidative conversion of amino acid side chains into carbonyl compounds is believed to be one of the most important consequences of protein oxidation, and the spectrophotometric measurement of carbonylation using 2,4-dinitrophenylhydrazine (DNPH) has been widely used for evaluating protein oxidation (Estévez et al., 2009a). Estévez et al. (2009b) identified two specific carbonylation markers in oxidized myofibrillar proteins, α-amino adipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS), which can be quantified using HPLC with fluorescence detection (Utrera et al., 2011). AAS is a direct carbonylation product of a lysine residue, whereas GGS is oxidatively derived from arginine and proline residues (Requena, Levine, & Stadtman, 2003). As carbonyl groups are known to be highly reactive moieties, they can be involved in several ongoing reactions. Protein carbonyls can interact with the primary amine group of lysine residues, forming intra- or intermolecular Schiff base cross-links (Xiong, 2000; Stadtman et al., 2003).

In this Chapter, porcine myofibrillar proteins were subjected to an *in vitro* metal-catalyzed oxidation (Fe^{3+}/H_2O_2), and the effect of natural antioxidants was studied. Because apples are one of the most important sources of phenolic compounds in the human diet, and because apple peel is known to contain a high concentration of phenolics (Wolfe et al., 2003), a selection of three phenolic compounds that are abundantly present in apple peel (chlorogenic acid, (-)-epicatechin and phloridzin) were separately added to the myofibrillar proteins as natural antioxidants. Furthermore, an apple peel extract was prepared and added as a fourth treatment, as to compare with the pure phenolic treatments. Hence, the objective of this research was to investigate the effects of apple phenolics (chlorogenic acid, (-)-epicatechin, phloridzin, and apple peel extract) on the oxidative stability of myofibrillar proteins in terms of carbonylation (AAS and GGS) and Schiff base formation.

3.2 MATERIALS AND METHODS

3.2.1 Extraction of phenolic compounds from apple

In order to obtain an optimal extraction of phenolic compounds from apple peel, a total of 8 extraction solvents were tested: 20%, 40%, 60% and 80% of acetone or methanol (v/v) in water. The extraction was done following a protocol described by Cando, Morcuende, Utrera, and Estévez (2014) with minor modifications as follows. A volume of 20 mL of extraction solvent was added to 1 g of freeze dried and homogenized peel of the apple cultivar "Cripps Pink". The mixture was homogenized with an Ultra Turrax and centrifuged (8 minutes, 1328 g, 4 °C), and the supernatant was filtered. This procedure was repeated on the pellet with once more 20 mL of extraction solvent. Filtrates were collected and diluted to a total of 50 mL with the corresponding extraction solvent.

3.2.2 Measurement of total phenolic content: Folin-Ciocalteu assay

Total phenolic content of the apple peel extracts were measured following the Folin-Ciocalteu method according to Soong and Barlow (2004) and Rodríguez-Carpena, Morcuende, Andrade, Kylli, and Estévez (2011a) with slight modifications. An aliquot of 200 μL of diluted apple peel extract (1:100 in water) was mixed with 1000 μL of 1:10 diluted Folin-Ciocalteu's phenol reagent, and 800 μL of 7.5% (w/v) sodium carbonate. The mixture was shaken and allowed to stand for 30 minutes at room temperature in the dark, after which the absorbance was measured at 765 nm using a spectrophotometer. Total phenolic content was calculated from a standard curve of gallic acid, and results were expressed as millimolar gallic acid equivalents (GAE).

3.2.3 Measurement of radical scavenging activity: DPPH• assay

The radical scavenging activity of the apple peel extracts was measured by means of the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assay as reported by Rodríguez-Carpena et al. (2011a). Briefly,

30 µL of extract was mixed with 2 mL of DPPH• solution (0.14 mM in methanol). The reaction mixture was stirred and allowed to stand at room temperature in the dark for 11 min, followed by absorbance measurement at 517 nm. The radical scavenging activity was calculated from a Trolox standard curve, and results were expressed as mM Trolox equivalent antioxidant capacity (TEAC).

3.2.4 Measurement of cupric ion reducing antioxidant capacity: CUPRAC assay

The cupric ion reducing antioxidant capacity (CUPRAC) assay was carried out as described by Apak, Güçlü, Özyürek, and Karademir (2004) and Rodríguez-Carpena et al. (2011a) with slight modifications. One milliliter of 10 mM $CuCl_2{\cdot}2H_2O$, 1 mL of neocuproine solution (7.5 mM in ethanol), and 1 mL of 1 M ammonium acetate buffer (pH 7.0) were added to 0.1 mL of diluted extract (1:3 v/v in water). One milliliter of water was added, making a final volume of 4.1 mL. The absorbance of the final solution at 450 nm was read against a reagent blank after 30 min of standing at room temperature in the dark. Results were calculated from a Trolox standard curve, and results were expressed as mM TEAC.

3.2.5 Preparation of apple peel extract for *in vitro* oxidation assay

The apple peel extract for the *in vitro* oxidation assay was made following the extraction procedure as described in Section 3.2.1, with 3 g of freeze dried apple peel, and 60% acetone in water as extraction solvent. Filtrates were collected and acetone was removed using a rotary evaporator. The total phenolic content of the remaining watery solution was quantified according to the Folin-Ciocalteu method as described in Section 3.2.2, and the solution was diluted with water to obtain a stock solution of 20 mM GAE.

3.2.6 LC-MS quantification of phenolic compounds in apple peel extract

The apple peel extract was characterized according to De Paepe et al. (2013) with slight modifications. Briefly, an acetone extract was prepared from the freeze dried apple peel as

described In Section 3.2.5. Subsequently, 1 mL of the acetone extract was dried under a nitrogen flow and re-dissolved in 1 mL of methanol:40 mM ammonium formate buffer (60:40). The obtained solution was diluted 10-fold with methanol:40 mM ammonium formate buffer (60:40), filtrated through a 0.22 μm Millex-HV Syringe filter (Millipore Corporation, Bedford, MA, USA), and stored in a capped vial at 4 °C prior to injection into the UHPLC coupled with diode array and accurate mass spectrometry detection using electrospray ionisation (DAD/ESI-am-MS) as described by De Paepe et al. (2013). The concentration of chlorogenic acid, (-)-epicatechin and phloridzin in the apple peel were calculated by means of external standards, and expressed as mg per kg of dry weight.

3.2.7 Extraction of myofibrillar proteins

The extraction of myofibrillar proteins was adapted from Park et al. (2006b) with slight modifications (Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008a). Minced muscle (lean pork loin) obtained from a local supermarket was homogenized using an Ultra Turrax for 30 seconds at 8000 rpm with 4 volumes (v/w) of isolation buffer (10 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$ and 1 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA); pH 7.0). The homogenate was centrifuged at 850 g for 15 minutes, after which the supernatant was discarded and the pellet was washed twice with 4 volumes (v/w) of the same buffer. The solution was stirred and centrifuged after each washing step. Subsequently, the myofibrillar pellet was washed three times with 4 volumes of 0.1 M NaCl. Before the third centrifugation, the myofibrillar suspension was filtered through cheese cloth and the pH was adjusted to 6.0 with 0.1 M HCl. After the last centrifugation, the myofibrillar protein isolation (MPI) was suspended (ca. 5 mg mL^{-1}) in 15 mM 1,4-piperazinediethanesulfonic acid (PIPES) buffer (pH 6.0) containing 0.6 M NaCl.

3.2.8 Protein concentration determination

The protein concentration of the MPI suspension was measured according to the biuret method (Doumas, Bayse, Carter, Peters, & Schaffer, 1981) with minor modifications. A volume of 2.5 mL of

biuret reagent (6 mM copper (II) sulfate pentahydrate; 20 mM potassium sodium tartrate tetrahydrate; 0.75 M NaOH) was mixed with 0.5 mL of MPI suspension. The absorbance was read after 5 minutes at 550 nm. The calibration curve was constructed using bovine serum albumin (BSA) at various concentrations ranging from 0.2 to 1 mg mL^{-1} under the described conditions. Results were expressed as mg BSA equivalents per mL of MPI suspension.

3.2.9 *In vitro* oxidation of myofibrillar proteins

Thirteen different suspensions (20 mL) were prepared depending on the addition and concentration (50, 100 and 200 μM) of the selected phenolic compound: chlorogenic acid (C50, C100 and C200), (-)-epicatechin (E50, E100 and E200), phloridzin (P50, P100 and P200), apple peel extract (A50, A100 and A200) and a control group (no phenolic compounds). All suspensions were prepared in triplicate in capped flasks and oxidized using 10 μM $FeCl_3$ as oxidation promoter in combination with 1 mM H_2O_2. During the *in vitro* oxidation, suspensions were kept on a magnetic stirrer in an oven at 37 °C for 10 days. Sampling was carried out at days 0, 2, 5, 7 and 10 for analysis.

3.2.10 Synthesis of AAS-ABA and GGS-ABA standards

Standards for AAS-ABA and GGS-ABA were prepared from Nα-acetyl-$_L$-lysine and Nα-acetyl-$_L$-ornithine using lysyl oxidase activity of egg shell membrane according to the procedure of Akagawa et al. (2006) with minor modifications. Briefly, 10 mM of Nα-acetyl-$_L$-lysine and 10 mM of Nα-acetyl-$_L$-ornithine were individually incubated with egg shell membrane (3 g) in 30 mL of 20 mM sodium phosphate buffer (pH 9.0) during 24 hours at 37 °C on a laboratory shaker. After removal of the egg shell membrane by centrifugation, the pH was adjusted to 6.0 with 1 M HCl. Subsequently, the resulting aldehydes, Nα-acetyl-$_L$-AAS and Nα-acetyl-$_L$-GGS, were reductively aminated with 3 mmol of 4-aminobenzoic acid (ABA) and 4.5 mmol of $NaCNBH_3$ for 2 hours at 37 °C on a magnetic stirrer. Then, the resulting derivatives, Nα-acetyl-$_L$-AAS-ABA and Nα-acetyl-$_L$-GGS-ABA, were hydrolyzed in the presence of 12 M HCl (30 mL) during 10 hours at 110 °C. The hydrolysates were

dried *in vacuo* at 40 °C using a SpeedVac (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the resulting AAS-ABA and GGS-ABA were finally reconstituted in HPLC water and filtered through a 0.45 μm Millex-HV Syringe filter (Millipore Corporation, Bedford, MA, USA).

3.2.11 Determination of α-amino adipic and γ-glutamic semialdehyde

The MPI suspensions were prepared for HPLC analysis of α-amino adipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) according to Utrera et al. (2011). At sampling times, 200 μL of MPI suspension was dispensed in 2 mL-Eppendorf tubes. Proteins were precipitated with 1.5 mL of ice cold 10% trichloroacetic acid (TCA) followed by centrifugation at 2000 g for 30 minutes at 4 °C. The resulting pellets were treated again with 1.5 mL of cold 5% TCA and proteins precipitated after centrifugation at 5000 g for 5 minutes at 4 °C. Pellets were then treated with 500 μL of 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer at pH 6.0 containing 1% sodium dodecyl sulfate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA), 500 μL of 50 mM ABA in 250 mM MES buffer (pH 6.0) and 250 μL of 100 mM $NaCNBH_3$ in 250 mM MES buffer (pH 6.0). The derivatization was completed by allowing the mixture to react for 90 minutes, while tubes were incubated at 37 °C and stirred regularly. All solutions for the derivatization procedure were freshly made on the day of analysis. The derivatization reaction was stopped by adding 500 μL of ice cold 50% TCA followed by centrifugation (10000 g, 10 minutes, 4 °C). Pellets were then washed twice with 1 mL of 10% TCA and 1 mL of ethanol:diethyl ether (1:1). Centrifugations at 5000 g for 5 minutes at 4 °C were performed after each washing step. Subsequently, protein hydrolysis was performed at 110 °C for 18 h in the presence of 6 M HCl. After that, hydrolysates were dried *in vacuo* at 40 °C using a SpeedVac (Thermo Fisher Scientific Inc., Waltham, MA, USA). Hydrolysates were finally reconstituted with 200 μL of HPLC water and filtered through a 0.45 μm Millex-HV Syringe filter (Millipore Corporation, Bedford, MA, USA). Samples and standards (Section 3.2.10) were analysed using a PerkinElmer Series 200 HPLC, connected to a LS 45 Fluorescence Spectrometer with LC flow cell (PerkinElmer Life and Analytical Science inc., Massachusetts, USA). The HPLC system was equipped

with a Cosmosil 5C18-AR-II RP-HPLC column (5 μm, 150 × 4.6 mm, Nacalai Tesque inc., Kyoto, Japan) and a SecurityGuard Analytical Guard Cartridge System (Phenomenex inc., Torrance, USA). Eluent A and B were 50 mM sodium acetate buffer (pH 5.4) and acetonitrile, respectively, and a gradient was programmed varying eluent B from 0% to 8% in 20 minutes. The injection volume was 10 μL, the flow rate was kept constant at 1 mL min^{-1}, and the oven temperature was set at 30 °C. Excitation and emission wavelengths were set at 283 and 350 nm, respectively. The AAS-ABA and GGS-ABA peaks were identified by comparing the retention times with those of the standards, and were manually integrated and plotted in an ABA standard curve ranging from 0.5 to 10 μM (R^2 > 0.998). Results are expressed as nmol of carbonyl compound per mg of protein.

3.2.12 Determination of fluorescent Schiff base cross-links

The natural fluorescence of Schiff base structures were measured with a PerkinElmer LS 45 Fluorescence Spectrometer equipped with a single cell thermostatted sample holder according to Utrera et al. (2012b) with slight modification (Iqbal, Kenney, & Klandorf, 1999; Chelh, Gatellier, & Sante-Lhoutellier, 2007). An aliquot of 400 μL of MPI suspension was mixed with 3.6 mL of 20 mM sodium phosphate buffer (pH 6.5) in a 4 mL UV spectrofluorometer cuvette. Emission of Schiff base structures was recorded at 420 nm with the excitation wavelength established at 350 nm. Excitation and emission slit widths were set at 10 nm and integration time 3 s. The contents of Schiff base structures were expressed as fluorescence intensity units emitted at 420 nm.

3.2.13 Statistical analysis

Statistical analysis was performed using SAS® 9.4 package (SAS Institute Inc., Cary, NC, USA). Results for the extraction solvents were analyzed using a linear model with solvent and concentration, as well as their interaction, as fixed effects. The effect of apple phenolics on GGS, AAS and Schiff base structures was tested using a repeated measures linear mixed model. Days of sampling (0, 2, 5, 7 and 10), compound (chlorogenic acid, (-)-epicatechin, phloridzin, and apple peel extract) and concentration (0, 50, 100 and 200 μM), as well as their interactions were tested as categorical fixed

effects. Significant difference was considered for $P < 0.05$. Post-hoc comparison was performed with a Tukey test.

Table 3.1: Total phenolic content (Folin-Ciocalteu assay), radical scavenging activity (DPPH• assay) and cupric ion reducing antioxidant capacity (CUPRAC assay) of apple peel extracts prepared with acetone or methanol (20, 40, 60 or 80% in water).

		Solvent in water mixture				
Assay	**Solvent**	**20%**	**40%**	**60%**	**80%**	**Mean**
Folin-Ciocalteu[1]	Acetone	1.42 ± 0.02	1.77 ± 0.14	1.80 ± 0.06	1.72 ± 0.05	1.68
(mM GAE)	Methanol	1.17 ± 0.10	1.50 ± 0.06	1.62 ± 0.03	1.52 ± 0.04	1.45*
	Mean	1.29[a]	1.64[b]	1.71[b]	1.62[b]	
DPPH•[2]	Acetone	1.05[a] ± 0.04	1.23[b] ± 0.13	1.10[ab] ± 0.07	0.83[c] ± 0.07	1.05
(mM TEAC)	Methanol	0.77[ab]* ± 0.07	0.90[a]* ± 0.07	0.90[a]* ± 0.04	0.71[b] ± 0.10	0.82
	Mean	0.91	1.06	1.00	0.77	
CUPRAC[3]	Acetone	2.60[a] ± 0.09	3.32[b] ± 0.33	3.22[b] ± 0.26	3.00[ab] ± 0.10	3.03
(mM TEAC)	Methanol	1.89[a]* ± 0.05	2.78[b]* ± 0.14	2.99[b] ± 0.15	2.89[b] ± 0.10	2.64
	Mean	2.24	3.05	3.10	2.94	

GAE, gallic acid equivalents; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TEAC, Trolox equivalent antioxidant capacity; CUPRAC, cupric ion reducing antioxidant capacity

[1] $P_{solvent} < 0.001$; $P_{concentration} < 0.001$; $P_{solvent*concentration} = 0.608$

[2] $P_{solvent} < 0.001$; $P_{concentration} < 0.001$; $P_{solvent*concentration} = 0.037$

[3] $P_{solvent} < 0.001$; $P_{concentration} < 0.001$; $P_{solvent*concentration} = 0.009$

[a-c] Significant difference between concentrations within the same solvent (P < 0.05). * Significant difference between solvents within the same concentration (P < 0.05).

3.3 RESULTS AND DISCUSSION

3.3.1 Selection of extraction solvent

The results for the antioxidant assays of the extracts are shown in Table 3.1. All assays suggested acetone to be a better extraction solvent than methanol. Extraction yields were highest with 40% and 60% acetone in water, with no significant differences between these concentrations. Because generally higher phenol concentrations are reported in extracts with a solvent content above 50% (as reviewed by Shah et al., 2014), it was chosen to make the apple peel extract with 60% aqueous acetone.

3.3.2 Characterization of apple peel extract

LC-MS analysis of the apple peel extract revealed phenolic contents of 582 ± 170 mg of chlorogenic acid, 842 ± 41 mg of (-)-epicatechin, and 124 ± 11 mg of phloridzin per kg dry weight of apple peel. These findings are in agreement with those from Huber and Rupasinghe (2009). However, the comparison of the phenolic content and composition of apples from literature should be undertaken carefully, because of the variety of analytical methods employed including different extraction solvents and fruit varieties investigated in the different studies.

3.3.3 Metal-catalyzed oxidation of myofibrillar proteins in the control treatment

A significant increase of α-amino adipic semialdehyde (AAS), γ-glutamic semialdehyde (GGS), and Schiff base structures (SB) was found in MPI control suspensions (without added phenolic compounds), during 10 days of incubation at 37 °C in the presence of Fe^{3+} and H_2O_2 (Figure 3.1). This may be ascribed to site-specific metal-catalyzed oxidation, in which reactive oxygen species (ROS) are formed through Fenton-like reactions at specific iron-binding sites in the protein (reactions [3.1] and [3.2]) (Stadtman, 1990; Estévez, 2011). When ROS attack the side chains of amino acid residues neighboring the iron-binding site, the latter are oxidized into various oxidation

products, mainly carbonyls (Stadtman, 1990). The significant AAS and GGS increase during incubation of myofibrillar protein in the presence of iron and hydrogen peroxide suggests that site-specific metal-catalyzed oxidation as described above has taken place, and lysine, arginine and proline were converted into their corresponding specific carbonyl derivatives.

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\bullet} + H^+ \qquad [3.1]$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet} \qquad [3.2]$$

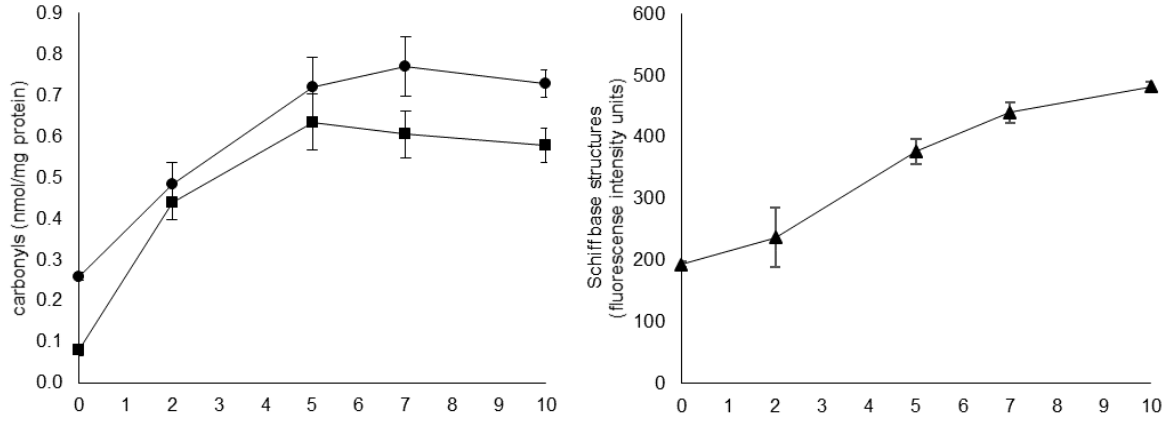

Figure 3.1: Progression of α-amino adipic semialdehyde (AAS, ●) and γ-glutamic semialdehyde (GGS, ■) (left panel), and Schiff base structures (SB, ▲) (right panel) during *in vitro* oxidation (37 °C/10 days) of myofibrillar proteins (5 mg mL^{-1}) without phenolic compounds added (control treatment). Values are shown as mean ± standard error (N=3).

Regarding the progression of AAS and GGS formation in the control treatment (Figure 3.1), a slight but non-significant decrease was observed towards the end of the experiment. This is in agreement with previous studies on myofibrillar proteins and meat products (Estévez et al., 2009b; Estévez et al., 2010; Utrera et al., 2012b; Utrera, Rodríguez-Carpena, Morcuende, & Estévez, 2012c; Utrera et al., 2013a; Utrera, Parra, & Estévez, 2014), and can be ascribed to ongoing reactions of carbonyl groups. In fact, simultaneously with the decrease of carbonyl compounds, a significant increase was found in Schiff base (SB) structures (Figure 3.1). As SB cross-links can be the result of interactions between carbonyls and the amino group of lysine residues in the same or a different protein (Xiong, 2000;

Stadtman et al., 2003), the decrease in carbonyls may be likely ascribed to the formation of SB (Utrera et al., 2012b).

The abovementioned observations in the control treatment are in agreement with previous studies on Fe^{3+}/H_2O_2 catalyzed oxidation of myofibrillar proteins (Estévez et al., 2009b; Estévez et al., 2010; Utrera et al., 2012b, 2013a), and thus provide a reliable basis to compare with the apple phenolic treatments.

Table 3.1: AAS formation (nmol/mg protein) during *in vitro* oxidation (37 °C/10 days) of myofibrillar proteins (5 mg mL^{-1}) with added phenolic compounds.

	day 0	**day 2**	**day 5**	**day 7**	**day 10**
control	0.26^{a} ± 0.02	0.48^{ab} ± 0.09	0.72^{bc} ± 0.13	0.77^{c} ± 0.13	0.73^{bc} ± 0.06
C50	0.24^{a} ± 0.01	0.40^{ab} ± 0.04	0.49^{abc} ± 0.02	0.57^{bc} ± 0.02	0.69^{c} ± 0.10
C100	0.28^{a} ± 0.01	0.42^{ab} ± 0.02	0.56^{ab} ± 0.07	0.67^{b} ± 0.19	0.70^{b} ± 0.05
C200	0.24^{a} ± 0.01	0.41^{ab} ± 0.04	0.55^{bc} ± 0.04	0.68^{bc} ± 0.03	0.72^{c} ± 0.06
E50	0.24 ± 0.02	0.35 ± 0.04	**0.41 ± 0.05**	**0.37 ± 0.01**	0.46 ± 0.04
E100	0.25 ± 0.02	0.33 ± 0.01	**0.37 ± 0.01**	**0.34 ± 0.01**	0.45 ± 0.04
E200	0.27 ± 0.00	0.31 ± 0.01	**0.34 ± 0.01**	**0.30 ± 0.01**	**0.31 ± 0.04**
P50	0.25^{a} ± 0.01	0.47^{ab} ± 0.06	0.74^{b} ± 0.13	0.69^{b} ± 0.07	0.61^{b} ± 0.15
P100	0.22^{ab} ± 0.02	0.42^{ac} ± 0.04	0.71^{d} ± 0.07	0.51^{cd} ± 0.16	**0.41^{bc} ± 0.06**
P200	0.22^{a} ± 0.01	0.41^{ab} ± 0.00	0.54^{b} ± 0.08	**0.41^{ab} ± 0.10**	**0.43^{ab} ± 0.14**
A50	0.25^{a} ± 0.03	0.33^{a} ± 0.04	**0.41^{ab} ± 0.11**	0.62^{bc} ± 0.22	0.86^{c} ± 0.31
A100	0.22^{a} ± 0.04	0.32^{a} ± 0.02	**0.36^{a} ± 0.05**	0.50^{a} ± 0.01	0.91^{b} ± 0.12
A200	0.24 ± 0.03	0.33 ± 0.02	**0.32 ± 0.01**	**0.44 ± 0.12**	0.47 ± 0.11

C50, C100, C200: 50, 100 and 200 μM of chlorogenic acid; E50, E100, E200: 50, 100 and 200 μM of (-)-epicatechin; P50, P100, P200: 50, 100 and 200 μM of phloridzin; A50, A100, A200: 50, 100 and 200 μM of apple peel extract. Results are expressed as means ± standard deviations. Values with a different superscript letter (a-c) within a row indicate significant differences ($P < 0.05$). Values in bold denote a significant difference compared with the control treatment within the same column ($P < 0.05$).

3.3.4 Apple phenolics against oxidation of myofibrillar proteins

The results for AAS, GGS and SB formation in MPI samples treated with apple phenolics are shown in Table 3.1, 3.2 and 3.3, respectively. Statistical analyses with 'days of sampling', 'phenolic compound' and 'concentration' as fixed effects revealed significant 3-way interactions for AAS and SB, and 3-way interactions for GGS were close to significance ($P_{day*compound*concentration}$ = 0.058). None of the phenolic treatments led to significantly higher levels of AAS, GGS or SB than in the control treatment. Overall, for all four phenolic treatments, the 200 μM concentration showed the most effective inhibition. However, differences between concentrations within the same phenolic compound were often not significant.

Table 3.2: GGS formation (nmol/mg protein) during *in vitro* oxidation (37 °C/10 days) of myofibrillar proteins (5 mg mL^{-1}) with added phenolic compounds.

	day 0	**day 2**	**day 5**	**day 7**	**day 10**
control	0.08^{a} ± 0.02	0.44^{b} ± 0.08	0.63^{b} ± 0.12	0.60^{b} ± 0.10	0.58^{b} ± 0.07
C50	0.08 ± 0.01	0.30 ± 0.05	**0.32 ± 0.05**	**0.33 ± 0.05**	0.33 ± 0.05
C100	0.07 ± 0.01	0.30 ± 0.05	**0.31 ± 0.06**	**0.32 ± 0.10**	**0.30 ± 0.02**
C200	0.06 ± 0.00	0.30 ± 0.05	**0.29 ± 0.03**	**0.31 ± 0.02**	**0.30 ± 0.03**
E50	0.06^{a} ± 0.01	0.30^{ab} ± 0.06	**0.29^{ab} ± 0.03**	**0.23^{ab} ± 0.03**	0.33^{b} ± 0.06
E100	0.07 ± 0.01	0.23 ± 0.02	**0.21 ± 0.01**	**0.19 ± 0.02**	**0.25 ± 0.04**
E200	0.06 ± 0.01	**0.16 ± 0.03**	**0.13 ± 0.00**	**0.11 ± 0.00**	**0.10 ± 0.02**
P50	0.06^{a} ± 0.01	0.46^{b} ± 0.08	0.71^{b} ± 0.13	0.61^{b} ± 0.05	0.63^{b} ± 0.08
P100	0.08^{a} ± 0.02	0.38^{b} ± 0.08	0.72^{c} ± 0.10	0.58^{bc} ± 0.11	0.59^{bc} ± 0.15
P200	0.05^{a} ± 0.01	0.40^{b} ± 0.04	0.61^{b} ± 0.12	0.45^{b} ± 0.10	0.49^{b} ± 0.15
A50	0.06^{a} ± 0.01	0.19^{a} ± 0.03	**0.20^{a} ± 0.08**	**0.32^{ab} ± 0.14**	0.50^{b} ± 0.27
A100	0.04^{a} ± 0.01	**0.15^{ab} ± 0.03**	**0.12^{ab} ± 0.02**	**0.22^{ab} ± 0.02**	0.36^{b} ± 0.08
A200	0.05 ± 0.01	**0.18 ± 0.00**	**0.11 ± 0.01**	**0.14 ± 0.04**	**0.31 ± 0.20**

C50, C100, C200: 50, 100 and 200 μM of chlorogenic acid; E50, E100, E200: 50, 100 and 200 μM of (-)-epicatechin; P50, P100, P200: 50, 100 and 200 μM of phloridzin; A50, A100, A200: 50, 100 and 200 μM of apple peel extract. Results are expressed as means ± standard deviations. Values with a different superscript letter (a-c) within a row indicate significant differences ($P < 0.05$). Values in bold denote a significant difference compared with the control treatment within the same column ($P < 0.05$).

An explanation for the different outcomes of the phenolic treatments should primarily be found in the chemical structure of the phenolic compounds, as the radical scavenging and metal chelating activity of phenolics mainly depends on the number, position and glycosylation of hydroxyl groups in the molecule (Rice-Evans, Miller, & Paganga, 1996; Michalak, 2006). Generally, there is a positive correlation between antioxidant capacity and the number of hydroxyl groups, whereas the antioxidant capacity decreases with an increase in glycosylation (Shahidi et al., 1992). However, the exact chemical conformation of the phenolic compound, as well as matrix effects should be taken into consideration when evaluating the antioxidant potential of phenolics.

Table 3.3: Schiff base formation (fluorescence intensity units) during *in vitro* oxidation (37 °C/10 days) of myofibrillar proteins (5 mg mL^{-1}) with added phenolic compounds.

	day 0	**day 2**	**day 5**	**day 7**	**day 10**
control	193^{a} ± 8	237^{a} ± 84	377^{b} ± 36	440^{bc} ± 29	481^{c} ± 14
C50	187^{a} ± 18	244^{ab} ± 6	332^{bc} ± 12	348^{c} ± 7	386^{c} ± 12
C100	190^{a} ± 4	238^{a} ± 46	320^{bc} ± 14	360^{c} ± 11	**361^{c} ± 20**
C200	178^{a} ± 1	204^{a} ± 27	307^{b} ± 11	**321^{b} ± 12**	**338^{b} ± 7**
E50	195^{ab} ± 7	185^{a} ± 26	**243^{abc} ± 10**	**284^{bc} ± 12**	**286^{c} ± 14**
E100	205 ± 2	170 ± 16	**248 ± 23**	**261 ± 19**	**255 ± 29**
E200	210^{ab} ± 15	160^{a} ± 28	**218^{ab} ± 12**	**251^{b} ± 38**	**260^{b} ± 17**
P50	163^{a} ± 1	254^{b} ± 46	367^{c} ± 22	410^{c} ± 16	413^{c} ± 36
P100	158^{a} ± 10	217^{a} ± 34	339^{b} ± 23	370^{b} ± 18	**353^{b} ± 19**
P200	158^{a} ± 11	182^{a} ± 49	**237^{ab} ± 43**	**319^{bc} ± 31**	**352^{c} ± 27**
A50	190^{a} ± 7	174^{a} ± 57	281^{b} ± 51	377^{c} ± 40	406^{c} ± 56
A100	196^{ab} ± 2	**122^{a} ± 16**	**206^{ab} ± 31**	**247^{bc} ± 44**	**305^{c} ± 16**
A200	191^{ab} ± 10	140^{a} ± 24	**225^{ab} ± 26**	**264^{bc} ± 27**	**316^{c} ± 26**

C50, C100, C200: 50, 100 and 200 µM of chlorogenic acid; E50, E100, E200: 50, 100 and 200 µM of (-)-epicatechin; P50, P100, P200: 50, 100 and 200 µM of phloridzin; A50, A100, A200: 50, 100 and 200 µM of apple peel extract. Results are expressed as means ± standard deviations. Values with a different superscript letter (a-c) within a row indicate significant differences ($P < 0.05$). Values in bold denote a significant difference compared with the control treatment within the same column ($P < 0.05$).

3.3.4.1 Chlorogenic acid against oxidation of myofibrillar proteins

Although AAS levels in MPI samples with chlorogenic acid treatments were slightly lower than the control, no significant inhibitory effects were observed during 10 days of incubation. For GGS formation however, chlorogenic acid showed significant inhibition as compared to the control treatment on day 5 and 7 (all concentrations) and day 10 (C100 and C200, $P = 0.025$ and $P = 0.028$, respectively). Regarding SB formation, no significant inhibition was found for the 50 μM concentration, however C100 contained significantly less SB than the control on day 10 ($P = 0.011$), and C200 significantly inhibited SB from day 7 to 10. Although slightly less oxidation products were found in higher concentrations of chlorogenic acid, and higher concentrations inhibited for a longer period, these differences between concentrations of chlorogenic acid were not significant. Chlorogenic acid, which is an ester of caffeic and (-)-quinic acid (Figure 3.2), has antioxidant potential because of the two hydroxyl groups on the phenol moiety of caffeic acid (Shahidi et al., 1992; Rice-Evans et al., 1996). The main antioxidant mechanism of these hydroxyl groups is believed to be radical scavenging through hydrogen donation (Shahidi et al., 1992), however their *ortho* position also renders possibility to metal chelating (Kono et al., 1998). Both mechanisms may be applicable to the effects observed in the present study. However, in our study, only limited, often non-significant inhibition against AAS and SB was found in the chlorogenic acid treatments, whereas this phenolic acid worked more efficient as an antioxidant against GGS formation. This suggests that the chlorogenic acid molecule played a more important role in the antioxidative protection of arginine and proline residues, preventing those from oxidizing into GGS, and thus leaving the lysine residues more subjective to metal-catalyzed oxidation into AAS and SB. It is not fully understood how the protein tertiary structure influences oxidation and antioxidant protection of the amino acids. Theoretically, amino acids buried inside the protein (mostly non-polar amino acids) would be less susceptible to ROS attack, while those at the protein surface (polar and charged amino acids) would be more prone to oxidation. A closer look can be taken at

myosin, the most abundant myofibrillar protein. In myosin II (the myosin class responsible for muscle contraction), lysine and arginine (both positively charged amino acids) are distributed quite homogenously in the amino acid sequence of both the myosin head and tail region, and when considering the 3D structure, their side chains are pointed outwards. Proline residues (non-polar) are only present in the myosin head, mainly in loop regions with their side chain pointing inwards (Kopp & Schwede, 2006; Kiefer, Arnold, Künzli, Bordoli, & Schwede, 2009; Bienert et al., 2017). Hence, from a structural point of view, arginine and lysine side chains would be more susceptible to ROS attack, but might also be better accessible for antioxidant protection. The reason why chlorogenic acid seems to protect arginine rather than lysine, might be because of more favourable reduction potentials. Myosin is however a large and complex protein to investigate, and other myofibrillar proteins such as actin might also play a role. In a similar study, Estévez et al. (2010) found pro-oxidative effects of chlorogenic acid, and ascribed this to the auto-oxidation of phenolics into quinones, which in turn could catalyze the oxidative deamination of lysine into AAS (Akagawa & Suyama, 2001). The inconsistencies between our and their results are most likely due to slight differences in model systems (5 vs. 20 mg mL^{-1} MPI suspensions) and dose applied (50 – 200 μM vs. 1 mM).

3.3.4.2 (-)Epicatechin against oxidation of myofibrillar proteins

All three (-)-epicatechin concentrations showed significant AAS inhibition on day 5 and 7, however only E200 was able to retain its antioxidative effect until day 10 ($P < 0.001$). The inhibitory effect of (-)-epicatechin against GGS as compared to the control treatment was significant from day 5 to 7 for E50, from day 5 to 10 for E100, and from day 2 to 10 for E200. As for Schiff base formation, the antioxidant activity of (-)-epicatechin was significant from day 5 to 10 in all concentrations. Like for chlorogenic acid, no significant differences were found between low, medium or high concentrations of (-)-epicatechin, however higher doses trended to inhibit oxidation more and longer.

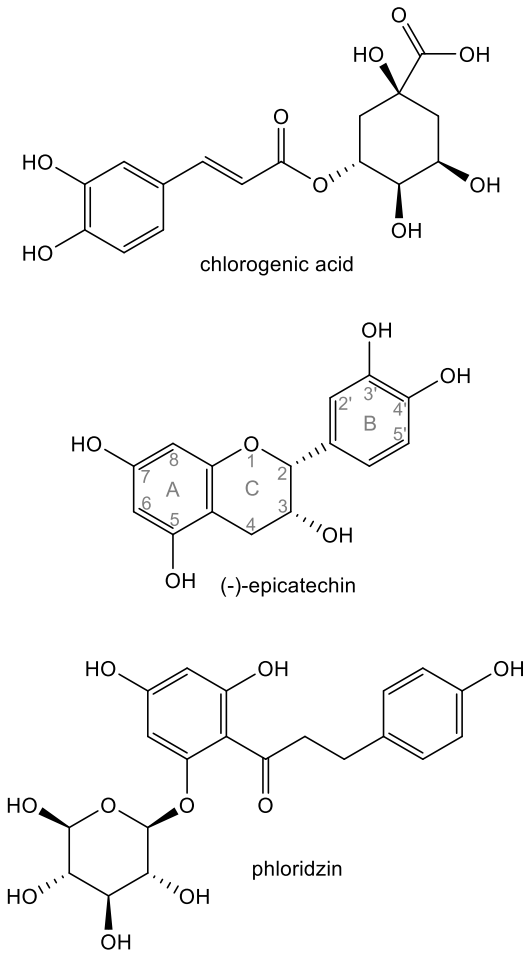

Figure 3.2: Chemical structures of chlorogenic acid, (-)-epicatechin and phloridzin.

In contrast with chlorogenic acid, the (-)-epicatechin molecule (Figure 3.2) contains multiple structural confirmations which contribute to free radical scavenging activity, such as the *ortho* 3',4'-dihydroxy moiety on the B ring, the *meta* 5,7-dihydroxy moiety on the A ring, and the 3-OH group in the C ring (Bors, Heller, Michel, & Saran, 1990; Rice-Evans, Miller, & Paganga, 1997). Furthermore, the 3',4'-dihydroxy position in the B ring forms a metal chelating point within the (-)-epicatechin structure (Rice-Evans et al., 1997). Our results confirm the effective antioxidant capacity of (-)-epicatechin by significant inhibition of AAS, GGS and SB in myofibrillar proteins treated with this phenolic compound. In fact, none of the higher (-)-epicatechin treatments showed a significant increase during the whole experiment, indicating very efficient antioxidative properties. The slight, though non-significant decrease of SB on day 2 is most likely due to formation of aggregates, which interfered with the fluorescence measurement resulting in

underestimation. A study performed by Estévez et al. (2010) revealed AAS and GGS inhibition by catechin, an isomer of (-)-epicatechin, however the antioxidative effect was not as profound as in our study. This can most likely be ascribed to differences in applied concentrations (μM vs. mM range), as well as structural arrangements (isomers) and availability of the phenolic molecule within the protein macromolecule.

3.3.4.3 Phloridzin against oxidation of myofibrillar proteins

The phloridzin treatments only significantly inhibited AAS formation in MPI samples towards the end of the experiment, namely P100 on day 10 ($P = 0.018$) and P200 on day 7 ($P = 0.002$) and, close to significance on day 10 ($P = 0.059$). Similar results were found for phloridzin against SB formation, that is significant inhibition by P100 on day 10 ($P = 0.011$), and by P200 from day 5 to 10. Furthermore, SB levels in the phloridzin treatment significantly differed in 50 and 200 μM concentrations on day 5 ($P < 0.003$). None of the phloridzin concentrations significantly inhibited GGS formation within the duration of the experiment.

Phloridzin, or phloretin 2-β-$_{D}$-glucoside, is a glycosylation product of phloretin (Figure 3.2). Unlike (-)-epicatechin, phloridzin possesses only one hydroxyl group on the B ring, making the molecule more stable, hence less reactive for antioxidative actions (Lavelli & Corti, 2011). Furthermore, it is well documented that glycosylation of phenolic compounds decreases their antioxidant activity (Shahidi et al., 1992; Rice-Evans et al., 1996; Michalak, 2006). This structural chemistry of phloridzin thus explains why little significant inhibition was found in the phloridzin treatments in the current study. The fact that significant AAS and SB inhibition only occurred towards the end of the experiment and in higher concentrations, suggests that the applied phloridzin concentrations were not sufficient, most likely due to the slow reactivity of the mono hydroxyl group on the B ring of phloridzin.

3.3.4.4 Apple peel extract against oxidation of myofibrillar proteins

All concentrations of the apple peel extract treatment inhibited AAS formation on day 5, however, on day 7 inhibition was only significant for A200 ($P = 0.014$). On the last day of observations (day 10), AAS inhibition by the 200 μM apple treatment was no longer significant, and the A50 and A100 even showed higher levels of AAS than the control treatment, although not significant. The amount of GGS was significantly lower than the control from day 2 ($P_{A100} = 0.016$ and $P_{A200} = 0.071$) or day 5 ($P_{A50} < 0.001$) until day 7 ($P_{A50} = 0.018$ and $P_{A100} < 0.001$) or day 10 ($P_{A200} = 0.043$). Only A100 and A200 showed significantly less Schiff base formation than the control from day 2 to 10 or day 5 to 10, respectively. As for differences between concentrations of apple peel extract, the amount of AAS in A200 on day 10 was significantly lower than for A50 or A100 ($P < 0.001$). This dose-dependency of apple peel extract was also clear for SB formation between A50 and A100 ($P = 0.003$), and A50 and A200 ($P = 0.033$) on day 7.

Considering the dilution factors during the preparation of the stock solution of apple peel extract, the weight based concentrations of chlorogenic acid, (-)-epicatechin and phloridzin in freeze dried apple peel (mg/100 g dry weight, Section 3.3.2) can be recalculated to the concentration in the apple treatments. For the 200 μM apple treatment, this comes down to a final concentration of 1.79 ± 0.52 μM, 3.15 ± 0.16 μM and 0.31 ± 0.03 μM of chlorogenic acid, (-)-epicatechin and phloridzin in protein suspensions, respectively. It is worth mentioning that the concentration of the apple peel extract was based on the total phenolic content as determined with the Folin-Ciocalteu method, which measures the reducing capacity of phenolic compounds expressed as gallic acid equivalents (Gülçin, 2012). However, it is most likely that not all phenolics present in the apple peel extract reacted in exactly the same way as gallic acid, therefore leading to an over- or underestimation of total phenolic content. Taken all of this into consideration, caution should be made when comparing the apple extract treatment with the pure phenolic treatments. In fact, since the concentrations of chlorogenic acid, (-)-epicatechin and phloridzin in the apple peel

extract are considerably lower than those of the pure phenolic treatments, it is unlikely that the inhibition patterns of the apple peel extracts can be explained solely by those of the pure phenolic compounds. The inhibition of protein carbonylation by the 200 μM apple peel extract treatment was similar to the (-)-epicatechin treatments and often better than the chlorogenic acid and phloridzin treatments. This suggests that other components contribute to the antioxidant capacity of the apple peel extract, and/or synergistic effects occurred (Bors et al., 1990). As such, a more effective free radical scavenger can be regenerated by a less effective one with a lower reduction potential. A combination of two or more antioxidants with different modes of action can also show synergism, e.g. metal chelating antioxidants inhibiting MCO, thereby producing less radicals to be reduced by radical scavengers. Finally, synergism can be achieved by one antioxidant protecting another one by means of sacrificial oxidation. As such, the less effective antioxidant traps radicals in food, thereby protecting the more effective antioxidant from oxidation. On the other hand, antagonism can arise by regeneration of the less effective antioxidant by the more effective one, by oxidation of the more effective antioxidant by the radicals from the less effective one, by competition between formation of antioxidant radical adducts and regeneration of the antioxidant, and by alteration of the microenvironment of one antioxidant by another one (Choe et al., 2009). The increase of AAS, GGS and SB starting from day 5 of the experiment in the low apple peel extract concentrations, suggests that the extract was no longer sufficiently stable to act as an antioxidant. However, the 200 μM concentration does show some promising inhibition of protein oxidation in myofibrillar proteins.

3.4 CONCLUSIONS

Apple phenolics displayed a potential to protect myofibrillar proteins from oxidation, whereby (-)-epicatechin proved to be more effective against protein carbonylation in the current model system than chlorogenic acid and phloridzin. The characteristics and conformation of the protein is also of importance in terms of accessibility and phenol-protein interactions. Hence, it was observed that chlorogenic acid showed more protection of arginine and proline residues. Low concentrations of apple peel extract seemed not to be as stable as the pure phenolic compounds, however the 200 μM concentration was able to inhibit protein oxidation similar to the (-)-epicatechin treatments.

Although the antioxidative actions of phenolics (the extent as well as the duration of inhibition) tended to be positively correlated with the concentration (50 μM < 100 μM < 200 μM), only few significant differences were found among doses within the same phenolic compound. However, it is worth noting that the applied concentrations (50, 100 and 200 μM) of apple phenolics and apple peel extract showed no pro-oxidant, and often anti-oxidative effects during the time of observation, indicating that these results form a good basis for optimizing the apple phenolic concentration as natural antioxidants in meat products. Obviously, not only the dose-dependency of phenolics, but also the effects of meat matrices should be taken into consideration, and should be investigated more profoundly in ongoing research.

Chapter 4

Protein oxidation and proteolysis during storage and *in vitro* digestion of pork and beef patties

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ABSTRACT

The effect of protein oxidation on meat digestibility was investigated following storage and subsequent *in vitro* digestion of beef and pork patties. Protein oxidation was evaluated as thiol oxidation, total carbonylation, and specific carbonylation (α-amino adipic and γ-glutamic semialdehyde). Furthermore, 4-hydroxyphenylalanine, a hydroxylation product of phenylalanine, was identified and quantified as a new protein oxidation marker. After 7 days of chilled illuminated storage (4 °C under atmospheric air), significant oxidative modifications were quantified and the oxidative degradation was continued during *in vitro* digestion. The observed effects were more abundant in beef patties. Protein oxidation before digestion resulted in impaired digestibility.

4.1 INTRODUCTION

Oxidation is one of the major causes of quality deterioration of meat, together with microbial spoilage. The susceptibility of muscle lipids and proteins to oxidation can be ascribed to both internal and external factors. Meat contains various endogenous initiators or catalysts of oxidation, such as ferric heme pigments, transition metal ions, and oxidative enzymes. Furthermore, processing and storage of meat is likely to intensify oxidative degradation. The mechanical actions during meat grinding leads to cell disruption and oxygen inclusion, thereby increasing the contact with reactive oxygen species (ROS) (Xiong, 2000). Subsequent exposure to oxygen and light during chilled display, such as in retail and butcher shops, enhances further oxidation (Soladoye et al., 2015). Hence, ground meat products are exposed to various pro-oxidative factors and mechanisms by the time they are consumed.

During digestion, partly oxidized meat is further exposed to oxidation catalysts, such as gastric fluid which has a low pH and contains dissolved oxygen, H_2O_2, ascorbate and iron (Nalini, Ramakrishna, Mohanty, & Balasubramanian, 1992; Kanner & Lapidot, 2001). The fate of oxidized meat components during passage through the gastrointestinal tract, and their impact on nutritional value and health, has been a topic of great interest. Lipid oxidation products, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), may be cytotoxic and cause DNA damage through adduct formation (Guéraud et al., 2010). *In vitro* digestion studies of meat have elucidated the effects of heme iron content, nitrite curing, fat content and cooking temperature on the formation of lipid oxidation products (Van Hecke et al., 2014a; Van Hecke et al., 2014b; Vanden Bussche et al., 2014; Van Hecke et al., 2015). Besides the lipid fraction, proteins are also susceptible to ROS attack during storage and digestion. Furthermore, aldehyde moieties from lipid oxidation products such as MDA or HNE can covalently bind to amino acid residues, resulting in indirect protein oxidation. Both direct and indirect protein oxidation can result in modified amino acid side chains and cross-link formation, which in turn may alter recognition sites for proteases in the

gastrointestinal tract. Hence, the nutritional value of oxidized meat proteins may decrease because of loss of essential amino acids and impaired digestibility (Xiong, 2000). Furthermore, undigested proteins may be fermented by bacteria in the colon into toxic or mutagenic metabolites (Evenepoel et al., 1998). However, the biological significance of protein oxidation has yet to be fully clarified (Soladoye et al., 2015), and therefore it is essential to fully understand the chemical nature of oxidative modifications of proteins during digestion (Estévez, 2011). In this respect, the characterization of protein oxidation products after gastric and duodenal digestion can elucidate the oxidation pathways during digestion, and can help to identify the loss of essential amino acids, which could be highly informative from a nutritional point of view.

In this Chapter, the effects of protein oxidation on the digestibility of beef and pork patties were investigated. Meat samples were stored under light at 4 °C and subsequently subjected to a gastric and duodenal digestion simulation, specifically designed to study oxidation during passage of meat through the gastrointestinal tract (Van Hecke et al., 2014a). A hitherto unique combination of protein oxidation markers was used to acquire an optimal insight in the oxidative damage of meat proteins during storage and digestion. Thiol oxidation was measured with 4,4'-dithiodipyridine (4-DPS), and carbonylation was determined as total carbonyls (2,4-dinitrophenylhydrazine or DNPH method) and as γ-glutamic and α-amino adipic semialdehyde (GGS and AAS, respectively). The HPLC analysis of GGS and AAS as described by Utrera et al. (2011) was adapted to a more rapid and accurate UHPLC analysis. Furthermore, 4-hydroxyphenylalanine, which has previously been highlighted as one of the oxidation products of the aromatic amino acid phenylalanine (Maskos, Rush, & Koppenol, 1992), was identified and quantified as a new protein oxidation marker in meat. Secondary lipid oxidation products were measured as thiobarbituric acid reactive substances (TBARS). Finally, proteolysis was determined by reaction of free amino groups with 2,4,6-trinitrobenzenesulphonic acid (TNBS). All markers were determined after storage and after digestion, in order to understand the effects of oxidation on proteolysis which occurs during digestion of meat proteins. Hence, the chemical changes caused by oxidation were studied extensively to give more insight in the potential effects on nutritional value and health of meat.

4.2 MATERIALS AND METHODS

4.2.1 Preparation and sampling of pork and beef patties

Lean pork shoulder cuttings and beef cuttings as well as subcutaneous pork fat were obtained from a local meat cutter 4 days *post mortem*. No details were available on the animal breeds and the types of beef muscles. All meat cuttings had been kept at 4 °C in a plastic bag covered from light. The pork and beef, both with 5% added pork back fat to level out possible differences in fatty acid composition, were separately grounded in a meat mincer equipped with a 3 mm plate (Seydelmann, Stuttgart, Germany). Portions of 100 g were shaped into patties of 10 cm diameter and stored at 4 °C (atmospheric air) under fluorescent light of 1000 lux, which was turned on for 12 hours per day to simulate retail display. Upon sampling time (day 0, 4 and 7), patties were homogenized using a food processor, vacuum packed and stored at -80 °C until analysis.

4.2.2 Physico-chemical characterization of meat samples

Moisture, crude protein and crude fat content were determined according to ISO 1442:1997, ISO 937:1978 and ISO 1444:1996, respectively.

4.2.3 *In vitro* digestion

The *in vitro* digestion of the meat products was performed according to a protocol described by Versantvoort, Oomen, Van de Kamp, Rompelberg, and Sips (2005) with modifications according to Van Hecke et al. (2014a). Oxidants and antioxidants that are normally present in digestive juices were included: peroxidase and $NaNO_2$ were added to the saliva juice, and ascorbic acid, H_2O_2 and ferrous iron were added to the gastric juice. Hence, although in many aspects comparable to the protocol from the consensus paper by Minekus et al. (2014), this protocol was specifically designed for studying oxidation processes during passage in the gastrointestinal system (Van Hecke et al., 2014a). Digestions consisted of an enzymatic digestion simulating the mouth, stomach and

duodenum gastro-intestinal tract compartments. Briefly, meat samples (4.5 g) were sequentially incubated at 37 °C for 5 minutes with 6 mL saliva, 2 hours with 12 mL gastric juice, and 2 hours with 2 mL bicarbonate buffer (1 M, pH 8.0), 12 mL duodenal juice and 6 mL bile juice. The composition of the synthetic digestive juices are presented in Table 4.1. All of the 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), pancreatin from porcine pancreas (8 × USP specifications; P7545), porcine bile extract (B8631)] were purchased from Sigma-Aldrich (Diegem, Belgium). Lipase and bile acted as emulsifiers. The incubations were performed in triplicate. After completion, samples were homogenized with an Ultra Turrax homogenizer (9500 rpm) and aliquots were stored in Eppendorf tubes at -80 °C pending analysis.

4.2.4 Quantification of lipid oxidation

MDA concentrations in meat products and digests were measured colorimetrically as TBARS by a modified method in accordance with Grotto et al. (2007). Results were expressed as nmol MDA equivalents per mg free fat.

4.2.5 Quantification of free and total thiols

Free and total thiols were quantified according to Rysman et al. (2014) with slight modification. Briefly, the vacuum packed meat was thawed in water at room temperature for 30 minutes before homogenizing 0.5 g of meat in 25 ml of 6 M guanidine hydrochloride (GuHCl) in 0.1 M Tris(hydroxymethyl)-aminomethane (TRIS) buffer (pH 8.0) using an Ultra Turrax. The homogenates were centrifuged (5311 g, 20 minutes, 4 °C) and supernatants were filtered. Digests were thawed, diluted (1:7) with 6 M GuHCl in 0.1 M TRIS buffer (pH 8.0) and filtrated. Samples and filtrates were kept on ice at all times. Protein concentration of the filtrates was determined spectrophotometrically at 280 nm using a 7-point standard curve prepared from bovine serum

albumin (BSA). Reduction with sodium borohydride and measurement of free and total thiols were performed as described by Rysman et al. (2014) (Chapter 2, Section 2.2.4). The thiol content was expressed as nmol thiol per mg protein.

Table 4.1: Composition of the synthetic digestive juices (1 L) used for *in vitro* digestion of meat samples (Van Hecke et al., 2014a).

	Mouth		**Stomach**		**Duodenum**			
	Saliva (pH 6.8)		**Gastric juice (pH 1.3)**		**Duodenal juice (pH 8.1)**		**Bile (pH 8.2)**	
Inorganic solution	0.90 g	KCl	2.75 g	NaCl	7.01 g	NaCl	5.26 g	NaCl
	0.20 g	KSCN	0.27 g	NaH_2PO_4	3.39 g	$NaHCO_3$	5.79 g	$NaHCO_3$
	0.90 g	NaH_2PO_4	0.82 g	KCl	0.08 g	KH_2PO_4	0.38 g	KCl
	0.57 g	NaSO4	0.40 g	$CaCl_2{\cdot}2H_2O$	0.56 g	KCl	0.15 mL	HCl 37%
	0.30 g	NaCl	0.31 g	NH_4Cl	0.05 g	$MgCl_2$		
	1.69 g	$NaHCO_3$	6.50 mL	HCl 37%	0.18 mL	HCl 37%		
Organig solution	0.20 g	Urea	0.09 g	Urea	0.10 g	Urea	0.25 g	Urea
	11.5 mg	Uric acid	0.02 g	Glucuronic acid	1.00 g	BSA	1.80 g	BSA
	25.0 mg	Mucin	0.65 g	Glucose	9.00 g	Pancreatin	30.0 g	Bile
	2.50 IU	peroxidase	0.33 g	Glucosamine-HCl	1.50 g	Lipase		
			17.6 mg	Ascorbic acid				
			1.00 g	BSA				
			2.50 g	Pepsin				
			3.00 g	Mucin				
Add	6.90 mg	$NaNO_2$	10.0 µL	H_2O_2 (30%)	0.200 g	$CaCl_2{\cdot}2H_2O$	0.222 g	$CaCl_2{\cdot}2H_2O$
			11.2 mg	$FeSO_4{\cdot}7H_2O$				

4.2.6 Quantification of total carbonyls

Total carbonyls were determined as described by Vossen et al. (2015) with slight modifications. Briefly, the vacuum packed meat was thawed in water at room temperature for 30 minutes. Subsequently, 3.0 g was homogenized in 30 mL of 0.6 M NaCl in 20 mM phosphate buffer (pH 6.5) using an Ultra Turrax (in triplicate), and four aliquots of 100 µL of the homogenates were dispensed in 1.5 mL-Eppendorf test tubes. All aliquots were treated with 1 mL of ice cold 10% trichloroacetic acid (TCA) and left at 4 °C for 15 minutes. For the digested samples, 200 µL of

thawed digest was pipetted in 1.5 mL-Eppendorf test tubes and treated with 1 mL of ice cold 10% TCA in quadruplicate. Samples were incubated at 40 °C for 15 minutes in order to free the digested proteins from the lipid particles. Subsequently, meat and digest samples were centrifuged (2000 g, 30 minutes) and supernatants were discarded. The protein pellets were treated with another milliliter of 10% TCA, left at 4 °C for 15 minutes, and centrifuged (2000 g, 30 minutes). After discarding the supernatant, two pellets of each sample were treated with 500 µL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl, and the two other pellets were treated with 500 µL of 2 M HCl as a blank. Samples were left to derivatize covered from light on a laboratory shaker (300 rpm) during 1 hour. Subsequently, samples were treated with 500 µL of 20% TCA, and left at 4 °C for 15 minutes before centrifugation (2000 g, 20 minutes). After discarding the supernatant, the pellets were washed three times with 1 mL of ethanol:ethylacetate (1:1). After each washing step, samples were centrifuged (2000 g, 20 minutes) and supernatants were discarded. After the final wash, the test tubes were left open under the fume hood for 15 minutes in order to remove the residual washing solvent. Pellets were dissolved in 1 mL of 6 M GuHCl in 20 mM phosphate buffer (pH 6.5) and placed on a laboratory shaker (300 rpm) covered from light during 30 minutes. The final solution was centrifuged (3800 g, 10 minutes) to remove insoluble parts. Protein concentration of the samples (mg mL^{-1}) was determined spectrophotometrically at 280 nm using a 7-point BSA standard curve. DNP hydrazone absorbance was measured at 370 nm, and protein carbonyl concentration (nmol mL^{-1}) was calculated as [$A_{370}/\varepsilon_{hydrazone}$*10^6], where $\varepsilon_{hydrazone}$ is 22000 M^{-1} cm^{-1}. Protein carbonyl concentration expressed as nmol carbonyl per mg protein was calculated from the above mentioned concentrations, and DNPH-treated samples were corrected by subtracting the protein carbonyl concentration of their blank equivalents.

4.2.7 Quantification of γ-glutamic semialdehyde (GGS) and α-amino adipic semialdehyde (AAS)

Samples were prepared for ultra high performance liquid chromatography with fluorescence detection (UHPLC-FLD) analysis of γ-glutamic semialdehyde (GGS) and α-amino adipic semialdehyde (AAS) according to Utrera et al. (2011) with modifications. The vacuum packed meat was thawed before 3.0 g of meat was homogenized in 30 mL of cold isolation buffer (10 mM sodium phosphate buffer, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA, pH 6.5) using an Ultra Turrax. The homogenates were made in triplicate for each meat sample. Four aliquots of 100 µL of each homogenate were dispensed in 2 mL-Eppendorf test tubes. Proteins were precipitated with 1 mL of ice cold 20% TCA followed by centrifugation at 3000 g for 30 minutes. Digested samples were thawed and four aliquots of 200 µL were treated with 1 mL of ice cold 20% TCA. After incubation at 40 °C during 15 minutes, samples were centrifuged at 3000 g for 30 minutes. The resulting meat and digest pellets were treated again with 1.5 mL of ice cold 5% TCA followed by centrifugation at 5000 g for 5 minutes. Subsequently, pellets were treated with 500 µL of 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer at pH 6.0 containing 1% sodium dodecyl sulfate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA). Two aliquots were treated with 500 µL of 50 mM 4-aminobenzoic acid (ABA) in 250 mM MES buffer (pH 6.0) and two aliquots were treated with 500 µL of 250 mM MES buffer (pH 6.0) as a blank. To create a reductive environment, an aliquot of 250 µL of 100 mM $NaCNBH_3$ in 250 mM MES buffer (pH 6.0) was added to all test tubes. The derivatization was completed by allowing the mixture to react for 90 minutes, while tubes were incubated at 37 °C and stirred regularly. The derivatization reaction was stopped by adding 500 µL of ice cold 50% TCA followed by centrifugation (10000 g, 10 minutes). Pellets were then washed twice with 1 mL of 10% TCA and 1 mL of ethanol:diethyl ether (1:1). Centrifugations at 8000 g for 5 minutes were performed after each washing step. Following the final wash, the blank pellets were dissolved in 1.0 mL of 6 M guanidine hydrochloride in 20 mM phosphate buffer (pH 6.5) and placed on a laboratory shaker (300 rpm) for 30 minutes. After final centrifugation (3800 g,

10 minutes) to remove insoluble material, the protein concentration was determined spectrophotometrically at 280 nm using a 7-point standard curve prepared from BSA. For the ABA-treated samples, protein hydrolysis was performed in 1.5 mL of 6 M HCl at 110 °C for 18 h. After that, hydrolysates were dried *in vacuo* at 45 °C using a SpeedVac (Thermo Fisher Scientific Inc., Waltham, MA, USA). Hydrolysates were finally reconstituted with 1 mL of HPLC water and filtered through a 0.22 μm Millex-HV Syringe filter (Millipore Corporation, Bedford, MA, USA). GGS-ABA and AAS-ABA standard was prepared according to the procedure of Akagawa et al. (2006) with minor modifications as described in Chapter 3 (Section 3.2.10). Samples and standards were analysed using an ACQUITY UPLC H-Class system coupled to a fluorescence detector (Waters Corporation, Massachusetts, USA). The UHPLC system was equipped with an AccQ-Tag Ultra C 18 column (1.7 μm, 2.1 × 100 mm) (Waters Corporation, Massachusetts, USA). Eluent A and B were 5 mM sodium acetate buffer (pH 5.4) and acetonitrile, respectively, and a gradient was programmed varying eluent B linearly from 0% to 8% in 3 min, to 90% B at 4 minutes and held at 90% B to 6 min. Initial conditions of 100% A were re-equilibrated from 6.5 to 8.5 min. The injection volume was 2.5 μL, the flow rate was kept constant at 0.5 mL min^{-1}, and the oven temperature was set at 30 °C. Excitation and emission wavelengths were set at 283 and 350 nm, respectively. The GGS-ABA and AAS-ABA peaks were identified by comparing the retention time with that of the standard, and were automatically integrated and plotted in an ABA standard curve ranging from 0.1 to 4 μM (R^2 > 0.999). Results are expressed as nmol of GGS or AAS per mg of protein.

4.2.8 Identification and quantification of 4-hydroxyphenylalanine

To identify the peak with retention time 0.995 minutes in the GGS/AAS chromatograms, the accurate mass of the eluting compound was determined using an ACQUITY UPLC H-Class system coupled to a Synapt G2 S (Waters Corporation, Massachusetts, USA) high resolution mass spectrometer (HRMS). The used UHPLC column and gradient were the same as described in Section 4.2.7, however eluent A (5 mM sodium acetate buffer pH 5.4) was replaced by 5 mM ammonium

acetate buffer (pH 5.4) and the injection volume was 10 μL. Two sequential analysis were done, one in positive electrospray ionization (ESI) and one in negative ESI. Prior to analysis the HRMS was calibrated (50-1200 Da) using a sodium-formate solution. The HRMS was operated in resolution mode (20.000 FWHM) and a leucine-enkephalin solution (200 pg/μL) was constantly infused during analysis as lockmass. Once identified, the peaks in the GGS/AAS chromatograms were quantified with UHPLC-FLD as described in Section 4.2.7, using a 4-hydroxyphenylalanine standard curve ranging from 1 μM to 1 mM (R^2 > 0.998).

4.2.9 Quantification of proteolysis

Proteolysis was determined as described by Polychroniadou (1988) with modifications. For the meat samples, 1.5 g of thawed meat was homogenized in 25 mL of 0.1 M TRIS (pH 8.0) using an Ultra Turrax. After centrifugation (5311 g, 20 minutes), 2 mL of supernatant was precipitated with 1 mL of TCA (30%). Solutions were left to stand at room temperature for 15 minutes before filtration. Simultaneously, a glycine standard curve ranging from 0.5 to 4 mM was prepared in 0.1 M TRIS (pH 8.0), and diluted (2:1) with TCA (30%). For the digested meat samples, thawed digests were diluted (1:50) with TCA (10%), left to stand for 15 minutes at room temperature, and filtrated. A glycine standard curve ranging from 10 to 100 mM was prepared in water, and diluted (1:50) with TCA (10%). For the colorimetric reaction, 200 μL of sample or standard was added to 2 mL of 1 M borate buffer (pH 9.2). After adding 800 μL of 0.15% (w/v) of 2,4,6-trinitrobenzenesulphonic acid (TNBS), the solution was left to stand for 30 minutes in the dark at room temperature. Subsequently, 800 μL of 2.3 M NaH_2PO_4 containing 18 mM Na_2SO_3 was added to stop the reaction. After 20 minutes of incubation at room temperature in the dark, the absorbance was measured at 420 nm and proteolysis in the samples was calculated from the glycine standard curves. Incubated meat samples were corrected with a blank incubation. Results were expressed as μmol glycine equivalents per mg of dry weight.

4.2.10 Statistical analysis

All analyses were done in triplicate. Results were analyzed using a linear model with storage day, type of meat and digestion, as well as their interactions, as fixed effects (SAS 9.4). Tukey-adjusted *post hoc* tests were performed for pairwise comparisons, and the significance level was selected as $P < 0.05$.

4.3 RESULTS AND DISCUSSION

4.3.1 Composition of meat patties

The composition (moisture, protein and fat content) of the beef and pork patties are represented in Table 4.2. As lipid oxidation occurs at the double bonds of unsaturated fatty acids, the fatty acid profile and the degree of unsaturation is one of the most determining factors in the rate of lipid oxidation (Velasco et al., 2010). Because of the addition of 5% pork back fat to both the beef and pork patties, only minimal differences in fatty acid composition are expected, as was observed by Van Hecke et al. (2014a; 2015) in similar meat formulations.

Table 4.2: Moisture, protein and fat content in beef and pork patties. Results are expressed g/100 g.

	Moisture	**Protein**	**Fat**
Beef	72.3	22.4	4.1
Pork	72.2	20.5	5.9

4.3.2 Oxidation during storage and digestion of beef and pork patties

During storage and digestion, oxidative degradation of the meat patties was observed (Table 4.3, Figure 4.1). It is most likely that the disruption of cells during manufacturing of the patties, followed by the exposure to light and oxygen, has led to the development of ROS. Subsequently, ROS attacked protein and lipid molecules in the meat patties resulting in oxidative deterioration. Since the simulated digestive juices contained pro-oxidant substances such as H_2O_2, ascorbate and iron,

which are components of human digestive juices (Nalini et al., 1992; Van Hecke et al., 2014a), the free radical chain reactions involved in both protein and lipid oxidation were further propagated during *in vitro* digestion.

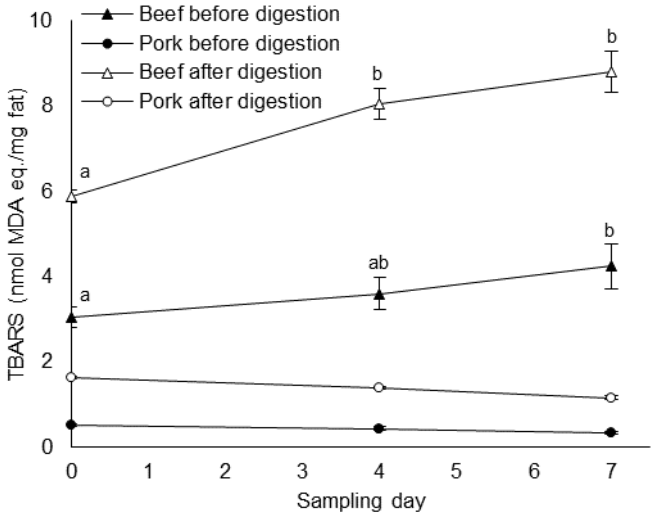

Figure 4.1: Lipid oxidation in beef and pork patties during chilled illuminated storage and in vitro digestion. [a-b] Significant difference between storage days within the same type of meat and digestive state ($P < 0.05$). Results are shown as mean ± standard deviation (N=3).

Our results show that the oxidative effects were more noticeable in beef patties than in pork patties (Table 4.3, Figure 4.1). This implies the role of endogenous pro-oxidants in the development of ROS. As heme pigment, and thus heme bound iron, is more abundant in beef than pork muscle (Lombardi-Boccia et al., 2002), the formation of ROS through metal-catalyzed (Fenton-like) reactions is likely to be more pronounced in beef.

4.3.3 Lipid oxidation

Since oxidizing lipids are believed to be promoters of protein oxidation (Xiong, 2000), a correlation between lipid and protein oxidation has been observed by several authors (Estévez et al., 2004; Ventanas, Estévez, Tejeda, & Ruiz, 2006; Estévez, Ventanas, & Cava, 2007). In meat science, the TBARS assay is the most common method to evaluate lipid oxidation (e.g. see Table 1.2). This assay is based on the reaction of thiobarbituric acid (TBA) with the secondary lipid oxidation product MDA,

forming a red chromophore which is measured spectrophotometrically. Although the method is often questioned regarding its specificity towards MDA, it is still a widely used method for determination of secondary lipid oxidation (Böhm & Müller, 2009). TBARS values in beef and pork patties before and after *in vitro* digestion are represented in Figure 4.1. During 7 days of chilled illuminated storage, a significant increase in TBARS was found for beef patties, whereas no significant change was observed for pork patties (Figure 4.1, dark marks). Similar TBARS patterns were found by Min, Nam, Cordray, and Ahn (2008) after chilled storage of raw beef and pork patties, where a significantly higher heme iron content was measured in the beef patties. Hence, it is likely that the lower heme iron content in the pork patties did not promote ROS formation to induce lipid oxidation during the course of our experiment.

After digestion, all beef and pork patties showed significantly higher TBARS values than their undigested equivalents (Figure 4.1, white marks). This is in agreement with Van Hecke et al. (2014a), who found a 2- to 3-fold increase in lipid oxidation products in beef, pork and chicken after *in vitro* digestion. In beef patties, the relative increase seemed to be slightly higher in patties that had been subjected to illuminated storage (94%, 124% and 108% increase after digestion of patties on day 0, 4 and 7 respectively). This suggests that lipid oxidation during digestion is more intense when the meat products are already oxidized before digestion. Since more lipid oxidation had already taken place in the 4- and 7-day old patties before digestion, it is likely that those beef patties contained more lipid oxidation radicals to further promote lipid oxidation during digestion.

Table 4.3: Protein oxidation in beef and pork patties during chilled illuminated storage and *in vitro* digestion.

		Before digestion			After digestion				*P* values						
	Meat	**Day 0**	**Day 4**	**Day 7**	**Day 0**	**Day 4**	**Day 7**	**RMSE**	**D**	**M**	**S**	**D∗M**	**D∗S**	**M∗S**	**D∗M∗S**
Free thiols (nmol/mg protein)	Beef	44.0^{a}	36.1^{b}	30.8^{c}	**2.8**	**3.0**	**3.4**	1.0	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Pork	46.7	46.8*	45.2*	**2.7**	**3.2**	**4.7**								
Total thiols (nmol/mg protein)	Beef	51.5	49.9	48.4	**40.3**	**34.8**	**36.8**	2.5	<0.001	0.043	0.027	0.774	0.415	0.177	0.704
	Pork	52.4	53.2	50.2	**40.0**	**39.5**	**37.0**								
Total carbonyls (nmol/mg protein)	Beef	1.75^{a}	2.00^{a}	2.89^{b}	1.98^{a}	**2.62^{b}**	**3.51^{c}**	0.12	<0.001	<0.001	<0.001	0.003	0.033	<0.001	0.272
	Pork	1.34^{ab} *	1.11^{a}*	1.67^{b}*	1.48^{a}*	1.38^{a}*	1.90^{b}*								
GGS (nmol/mg protein)	Beef	0.20^{a}	0.33^{b}	0.47^{c}	0.16	**0.19**	**0.19**	0.03	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
	Pork	0.12	0.14*	0.17*	0.16	0.11	0.16								
AAS (nmol/mg protein)	Beef	0.12^{a}	0.43^{b}	0.79^{c}	0.11	**0.14**	**0.16**	0.05	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Pork	0.14^{a}	0.20^{ab}*	0.30^{b}*	0.09	0.11	0.18								
4-OH-Phe (µmol/mg protein)	Beef	0.44^{a}	0.61^{b}	0.68^{b}	**0.14**	**0.18**	**0.22**	0.03	<0.001	<0.001	<0.001	<0.001	0.013	0.015	0.024
	Pork	0.36	0.39*	0.45*	**0.14**	**0.17**	**0.23**								

GGS, γ-glutamic semialdehyde; AAS, α-amino adipic semialdehyde; 4-OH-Phe, 4-hydroxyphenylalanine; RMSE, root mean square error; D, digestion; M, type of meat; S, storage day. $^{a-c}$ Significant difference between storage days within the same type of meat and digestive state ($P < 0.05$). * Significant difference between types of meat within the same storage day and digestive state ($P < 0.05$). Values in bold indicate significant difference from its undigested equivalent ($P < 0.05$).

4.3.4 Protein oxidation

4.3.4.1 Thiol oxidation

The loss of thiol groups in pork and beef during storage in an oxygen-rich environment has been reported before (Lund et al., 2007b; Jongberg et al., 2011b). Similar to these studies, the thiol content in the current paper was determined in a denaturant-treated protein filtrate and expressed as nmol thiol per mg of protein. This was calculated from the thiol concentration (μM), as determined by a colorimetric reaction with a thiol detection agent (in this case 4-DPS) and a cysteine standard curve, and the protein concentration, as determined spectrophotometrically at 280 nm with a BSA standard curve. Hence, all proteins as well as peptides and amino acids present in the filtrate were included in the assay. If severely oxidized proteins would not be able to pass the filter paper, this underestimation of thiols concentration would be levelled out by a lower protein concentration.

In the present study, a significant decrease in free thiols was observed during chilled illuminated storage of beef patties before digestion (Table 4.3), indicating that thiol groups were lost due to oxidative reactions. This thiol loss could be attributed to direct ROS attack at thiol groups on the cysteine residue in meat proteins, converting them to disulfide bonds and other thiol oxidation products such as sulfenic, sulfinic and sulfonic acid, and thiosulfinates (Nagy et al., 2010). Furthermore, free thiols can be lost by reaction with the lipid oxidation product HNE through Michael addition (Stadtman & Berlett, 1997). In contrast with beef patties, only a slight, non-significant decrease in free thiol content was found in pork patties among storage days before digestion. As mentioned above, this can most likely be ascribed to the lower heme iron content and TBARS values in pork. After duodenal digestion, the level of free thiols dropped dramatically in both beef and pork patties, with an average decrease of 92% (Table 4.3). It is reasonable to believe that the proteolytic breakdown of proteins by digestive enzymes exposed initially buried thiol groups, making them susceptible to ROS. Because of the severe thiol oxidation during

digestion, the minor oxidative thiol loss before digestion seemed not to play a significant role on the outcome after digestion.

All thiol samples were subjected to reduction with sodium borohydride in order to measure the amount of total thiols, and thus the reversibility of thiol oxidation (Rysman et al., 2014). The initial total thiol levels in beef and pork were found to be slightly higher than the free thiol levels on day 0, indicating that some thiol oxidation had already taken place *post mortem*, e.g. during manufacturing. No significant differences in total thiols were found among storage days before digestion (Table 4.3). This implies that all thiol oxidation products that were formed in beef and pork patties during storage, were fully reversible independent of the extent of free thiol oxidation. After digestion, all beef and pork patties had significantly lower total thiol levels than their undigested equivalents (Table 4.3). Hence, the reduction step with sodium borohydride was not able to recover the initial total thiol level of fresh beef and pork patties (day 0 before digestion), signifying that up to 27% of the thiol oxidation that took place during digestion was irreversible. Furthermore, total (reducible) thiol levels after digestion tended to be lower for samples that had been stored longer before digestion, though not significantly. This suggests that thiol oxidation before digestion negatively impacts the reversibility of thiol oxidation during digestion. Since the 4-DPS method only gives information on the extent and reversibility of thiol oxidation, the type of irreversible thiol oxidation products and their potential toxicity should be further investigated.

4.3.4.2 Carbonylation

Both total carbonyl measurement (DNPH assay) and GGS and AAS determination with UHPLC-FLD revealed an increase in carbonylation during storage of beef and pork patties (Table 4.3, before digestion). These effects were once again more pronounced in beef patties, however the increase was significant in all cases except for GGS formation in pork. Hence, it is reasonable to assume that ROS, formed through processing, storage and catalyzed by heme iron, attacked not only thiol groups on cysteine residues, but also other amino acid side chains such as lysine, arginine, proline and threonine, converting them to aldehydes and ketones. α-Amino adipic semialdehyde (AAS,

derived from lysine) and γ-glutamic semialdehyde (GGS, derived from arginine and proline) have been proposed as reliable biomarkers for protein carbonylation in bovine serum albumin (BSA), plasma, rat liver, myofibrillar proteins, and meat products (Requena et al., 2001; Akagawa et al., 2006; Armenteros, Heinonen, Illilainen, Toldrá, & Estévez, 2009; Estévez et al., 2009b; Utrera et al., 2011). In the present study, both types of meat patties had similar initial GGS and AAS levels (day 0 before digestion), however the increase of AAS was much more abundant than the GGS increase. Higher levels of AAS in beef and pork patties as compared to GGS were also found by Utrera et al. (2011; 2013b; 2014). According to Souci, Fachmann, and Kraut (2008), bovine and porcine muscle contains on average 4.4 mol% arginine, 5.4 mol% proline, and 7.8 mol% lysine, suggesting that upon complete oxidation the amount of GGS would be similar to or even higher than the amount of AAS. However, the results from the current study and those mentioned above indicate that in common meat matrices, the lysine residues are more prone to oxidation than the arginine and proline residues.

In previous studies on GGS and AAS, the sum of both compounds made up 23 to 60% of total carbonyls (Estévez, 2011), indicating that other carbonyl compounds contribute to the total carbonyl level as measured with the DNPH method. These carbonyls could either be derived from direct (primary) oxidation of amino acid side chains other than lysine, arginine and proline, or could be the consequence of indirect (secondary) carbonylation. The amino acid threonine is known to be oxidatively converted into α-amino-3-ketobutyric acid (Taborsky & McCollum, 1973), and its ketone group could react with DNPH as well. Furthermore, carbonyl groups can be formed by oxidative peptide scission, or in the presence of oxidizing sugars or lipids (Berlett et al., 1997; Xiong, 2000). These sugars or lipids are able to form adducts with e.g. lysine, resulting in Amadori products and MDA or HNE adducts (Xiong, 2000). These secondary protein carbonyls are equally able to be derivatized with DNPH, resulting in an overestimation of primary protein carbonyls. In the present study, the sum of AAS and GGS made up 18 to 44% of total carbonyls in beef and pork patties before digestion. In beef patties, the higher total carbonyl levels could be attributed to protein lipoxidation, as a significant increase in TBARS was observed. Since no significant TBARS

increase was found in pork patties before digestion, it is unlikely that the higher total carbonyl levels can be ascribed to protein lipoxidation. In fact, if protein-MDA adducts would have been significantly formed during storage of the pork patties, this should also be noticeable in TBARS results, since samples were subjected to NaOH hydrolysis prior to TBA derivatization, reducing potentially formed protein-MDA adducts. Therefore, it seems reasonable to assume that certain amino acids are oxidized into primary protein carbonyls other than GGS and AAS. Furthermore, as sugars are naturally present in the water-soluble fraction of meat (Macy, Naumann, & Bailey, 1964), protein carbonylation through reactions with reducing sugars or their oxidation products (glycation and glycoxidation reactions) is worth taking into consideration.

Upon *in vitro* duodenal digestion, total carbonyl levels further increased in all samples, being significant for beef patties on day 4 and 7 (Table 4.3). This increase during digestion is in agreement with Van Hecke et al. (2014a; 2014b; 2015) and indicates that protein carbonylation was further promoted by oxidative enzymes and other pro-oxidants of the digestive tract. Similar to TBARS, the relative increase of total carbonyls was higher when digesting older beef patties (average of 13, 31 and 21% increase after digestion of patties on day 0, 4 and 7, respectively). Hence, this implies that radicals formed during protein carbonylation before digestion intensified ongoing carbonyl formation during digestion. Unlike total carbonyls as measured with DNPH, the levels of GGS and AAS decreased during digestion, with significantly lower levels of both compounds in beef patties on day 4 and 7. This decrease can be ascribed to further oxidative reactions of the aldehyde moiety of GGS and AAS, making the compounds unable to derivatize with ABA prior to UHPLC-FLD analysis. As such, the aldehyde group can further oxidize into a carboxylic acid, as has been shown for the oxidative conversion of AAS into α-amino adipic acid (AAA) (Sell et al., 2007). Furthermore, carbonyl groups can form inter- or intramolecular cross-links by reacting with neighbouring protein-bound amino acids or other carbonyls, forming Schiff base structures and aldol condensation products, respectively (Akagawa & Suyama, 2000). Additionally, Estévez et al. (2011) have highlighted the ability of AAS and GGS to form Strecker aldehydes after reaction with free amino acids. The fact that total carbonyls increased after digestion whereas a decrease in the amount of AAS and GGS was

observed, could be attributed to secondary protein carbonyls and other primary protein carbonyls as described above, which might be more stable than AAS and GGS. Furthermore, it should be taken into account that total carbonyls, AAS and GGS were only measured in proteins which were precipitated by trichloroacetic acid (TCA). Since a profound peptide scission by proteolytic enzymes had occurred during *in vitro* digestion, the digested samples contained mostly small peptides and amino acids. Hence, the molecular weight of some of them might have been too low to precipitate with TCA (Greenberg & Shipe, 1979). Since direct protein carbonylation such as AAS and GGS shortens the amino acid side chain, whereas indirect protein carbonylation increases the mass through adduct formation (Madian & Regnier, 2010), it is reasonable to assume that the protein pellets after digestion contained more secondary protein carbonyls, resulting in higher DNPH values and an underestimation of GGS and AAS.

Finally, it is worth mentioning that caution should be made when comparing absolute values of carbonylation as measured by DNPH and UHPLC-FLD. In the DNPH assay, the amount of all aldehydes and ketones that react with DNPH is calculated based on the extinction coefficient of the DNP hydrazone. In UHPLC-FLD analysis, the GGS-ABA and AAS-ABA peaks are quantified using a ABA standard curve (due to the lack of quantitative GGS and AAS standards), assuming that one mole of ABA emits the same fluorescence as one mole of GGS-ABA or AAS-ABA (Utrera et al., 2011). However, if the emission of derivatized carbonyls were to be lower than that of single ABA, an underestimation of AAS and GGS would be calculated. Nevertheless, the parallel interpretation of both methods offer valuable information about direct and indirect protein carbonylation.

4.3.4.3 Hydroxylation of phenylalanine

Upon inspection of the GGS/AAS chromatograms, the peak with retention time 0.995 minutes drew attention as it appeared to increase in oxidized samples (Figure 4.2), suggesting that the peak corresponded with an oxidation product other than GGS or AAS. UHPLC-FLD analysis of non-derivatized samples revealed the same peak (data not shown), indicating that the compound did

not react with ABA and was fluorescent as such at the selected excitation and emission wavelengths. Subsequently, the samples were subjected to HMRS analysis to determine the identity of the unknown compound. Analysis in negative ESI mode revealed a peak with an m/z-value of 180.0680 and in positive ESI with an m/z value of 182.0808. These two values lead to $C_9H_{11}NO_3$ as proposed molecular formula for the unknown peak with a mass deviation of 10 PPM in ESI- and 5 PPM in ESI+ mode. A ChemSpider search revealed 1109 hits for this molecular formula, however based on the obtained fragmentation pattern in ESI- mode and the presumption that it concerned a fluorescent oxidation product, 4-hydroxyphenylalanine (4-OH-Phe) was proposed as tentative identity. Subsequent UHPLC-FLD analysis of a 4-OH-Phe standard confirmed this identity, and quantification of the concentration in the samples based on a 4-OH-Phe standard curve revealed values as shown in Table 4.3. The compound 4-OH-Phe, also known as tyrosine, is an aromatic amino acid naturally present in meat proteins, however it can also be formed by hydroxylation of phenylalanine (Solar, 1985; Maskos et al., 1992). Hence, it is impossible to make a distinction between endogenous tyrosine and monohydroxylated phenylalanine with the current UHPLC-FLD quantification or any other technique. However, the increase of the total level of 4-OH-Phe during storage of both beef and pork patties suggests that at least part of the measured amount is an oxidation product. Furthermore, even if initial tyrosine would oxidize and thus decrease, the total increase suggests that the formation of 4-OH-Phe from phenylalanine still outnumbered tyrosine loss. Although the rate constant for reaction with hydroxyl radicals was found to be slightly higher for tyrosine than for phenylalanine in aqueous solutions at pH 7 (1.3×10^{10} and 6.5×10^{9} dm^3 mol^{-1} s^{-1}, respectively) (Buxton, Greenstock, Helman, & Ross, 1988; Davies, 2005), to our knowledge no information is available about the oxidation rate of tyrosine and phenylalanine in meat and meat products. Hence, given this possible interference, the relative increase in 4-OH-Phe as a measure of the hydroxylation of phenylalanine rather than the absolute value could be used as a new marker for protein oxidation in meat and meat products. Although oxidative loss of aromatic amino acids in meat has previously been determined spectrophotometrically (Gatellier et al., 2009;

Utrera et al., 2012a), the specificity of such measurements can be questioned, whereas our UHPLC-FLD method provides a more accurate and more specific determination.

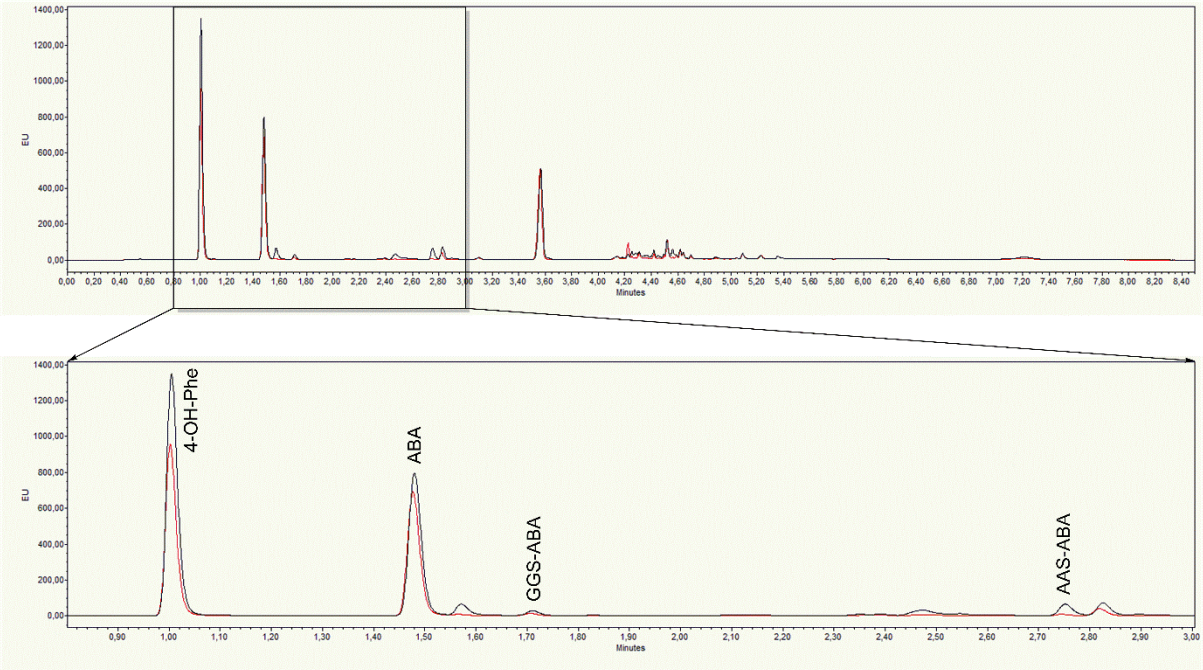

Figure 4.2: Overlay of UHPLC-FLD chromatograms of beef samples before digestion on day 0 (red) and day 7 (black).

As mentioned above, an increase of 4-OH-Phe was found during storage of both beef and pork patties, however the increase was only significant in beef patties. This pattern is similar to all other oxidation products that were identified and is likely due to the higher heme iron content, and thus Fenton-like reactions, in beef. After digestion, all samples had significantly lower 4-OH-Phe levels than their undigested equivalents (Table 4.3), therefore it is likely that further oxidation of 4-OH-Phe has taken place during digestion. It is known that 4-OH-Phe, or tyrosine, is able to oxidize into various compounds, of which the most important are dityrosine, a cross-link between two tyrosine residues (Davies, Delsignore, & Lin, 1987), and dihydroxyphenylalanine (DOPA), which is formed after secondary hydroxylation of phenylalanine (Maskos et al., 1992). However, when interpreting the decrease of 4-OH-Phe levels in digested samples, the remark about proteolysis and TCA pellet formation as explained in Section 4.3.4.2 must also be taken into consideration: if

most 4-OH-Phe residues were located on the small peptides and amino acids, it is likely that those were not included in the analysis.

As phenylalanine is an essential amino acid, its oxidative degradation emphasizes the deleterious effect of protein oxidation on the nutritional value of meat and meat products. Furthermore, its hydroxylation product 4-OH-Phe or tyrosine is known to be metabolized into phenol and *p*-cresol during bacterial fermentation in the colon (Hughes et al., 2000). These potentially mutagenic metabolites are largely absorbed from the colonic lumen, detoxified in the liver and urinarily excreted (Evenepoel et al., 1998), however an increase of residual fecal *p*-cresol has been observed in rats fed high protein diets (Toden, Bird, Topping, & Conlon, 2005). Winter et al. (2011) found a correlation between fecal *p*-cresol and promutagenic DNA adducts in the colon, however the exact mechanisms remain to be clarified. In the present study, digests from oxidized meat samples (day 7) contained up to 57% more residual 4-OH-Phe as compared to digests from fresh meat patties (day 0) (P_{beef} = 0.145 and P_{pork} = 0.108) (Table 4.3). Hence, consumption of oxidized meat, leading to higher duodenal levels of 4-OH-Phe, may negatively impact health by formation of phenol and *p*-cresol in the colon.

4.3.5 Proteolysis

No significant changes in proteolysis were observed during storage of meat patties (Figure 4.3), indicating no detectable endogenous protease activity in the current meat matrix. As expected, significantly more proteolysis was measured after digestion, confirming protein breakdown by the proteolytic enzymes in the gastric and duodenal juices. However, *in vitro* digestion of oxidized meat samples resulted in slightly fewer free amino groups in the digests (significant for pork patties), suggesting that protein oxidation decreases the digestibility of meat. Equivocal results have been reported about the effect of protein oxidation on the digestibility in meat model systems, depending on the extent of oxidation and the *in vitro* digestion conditions applied (Liu & Xiong, 2000b; Santé-Lhoutellier et al., 2007; Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier,

2008a; Bax et al., 2012). It is generally believed that mild oxidative conditions enhance enzyme digestibility by minor modifications and partial unfolding, increasing the availability for proteases, while severe protein oxidation impairs the digestibility by protein polymerization and aggregation, decreasing the protease susceptibility (Davies, 2001; Grune et al., 2004; Bax et al., 2012). In the present study, the decrease in proteolysis suggests that the latter has occurred. However, a critical remark should be made on the TNBS assay that was applied in the present paper to measure proteolysis. TNBS is a widely used reagent to quantify primary amino acids, but no distinction is made between α- and ε-amino groups (Cayot & Tainturier, 1997). Hence, the ε-amino group on the lysine residue could lead to an overestimation of proteolysis. On the other hand, when lysine is oxidized and the ε-NH_2 group is lost, this will lead to a decrease in the estimate of proteolysis. Since pork and beef contain on average 7.8 mol% lysine (Souci et al., 2008), complete oxidation of all lysine residues will theoretically result in a 7.2% decrease of proteolysis. In our results, a 7.1% and 15.2% decrease of proteolysis was measured in beef and pork digests due to a 7-day storage, respectively. Hence, these results do not allow to conclude whether proteolysis was decreased due to lysine oxidation, or due to protein polymerization before digestion. However, it is unlikely that all lysine residues would be oxidized, and the results for pork suggest that even if all ε-amino groups were lost, there should still be an additional loss in α-amino groups, most likely due to protein polymerization prior to digestion. Finally, it is worth noting that although more protein oxidation was observed in beef patties, no significant differences in proteolysis were found between pork and beef. Most likely the differences between beef and pork in terms of oxidation and proteolysis would be enhanced if the samples would be subjected to heat treatment prior to digestion. Upon heating, heme-iron is released from the porphyrin moiety, and can subsequently take part in Fenton-like reactions (Kanner, 1994; Lombardi-Boccia et al., 2002). Furthermore, heating inactivates endogenous antioxidant enzymes in meat, disrupts muscle cells and denatures proteins, making both proteins and lipids more susceptible to oxidation (Nuñez de Gonzalez et al., 2008; Wen et al., 2015). Van Hecke et al. (2015) observed significantly more lipid and protein oxidation during gastric and duodenal digestion of cooked and overcooked pork compared to raw pork.

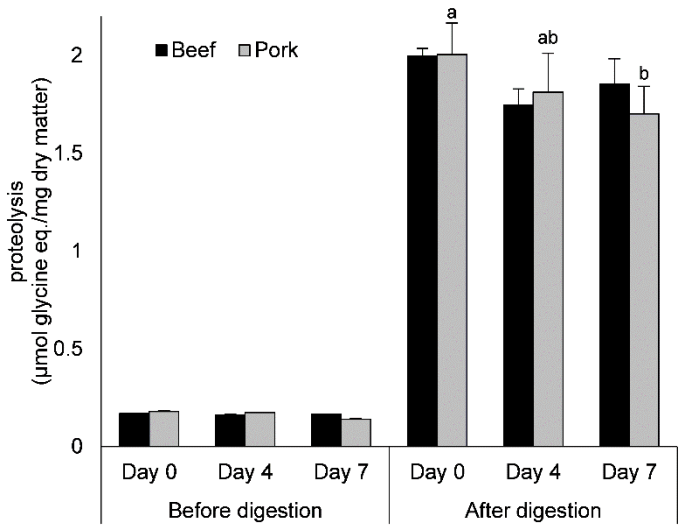

Figure 4.3: Proteolysis in beef and pork patties during chilled illuminated storage and *in vitro* digestion. [a-b] Significant difference between storage days within the same type of meat and digestive state ($P < 0.05$).

The *in vitro* digestibility studies mentioned above were often conducted on meat protein isolates with addition of pro-oxidative chemicals (Liu et al., 2000b; Morzel et al., 2006; Santé-Lhoutellier et al., 2007). Even if pro-oxidative conditions such as meat cooking, mincing and aging were mimicked (Santé-Lhoutellier et al., 2008a; Bax et al., 2012), the subsequent *in vitro* digestions were conducted on protein isolates and after addition of gastric pepsin and pancreatic trypsin and α-chymotrypsin only. The *in vitro* tool used in the present study was applied to the whole meat product and consisted of static incubations with digestive juices simulating the *in vivo* situation as close as possible (Van Hecke et al., 2014a; Van Hecke et al., 2014b). Therefore, it can be expected that the results obtained in the present study better reflect the relationship between protein oxidation and proteolysis during digestion of meat.

4.4 CONCLUSIONS

Several oxidative changes were observed during illuminated storage and subsequent *in vitro* digestion of beef and pork patties. Oxidation of free thiols was the most abundant protein oxidation mechanism during digestion (average of 92% free thiol loss), emphasizing that thiol groups are highly reactive and sensitive to oxidative damage. Measurement of carbonylation with DNPH (total carbonyls) and UHPLC-FLD (AAS/GGS) indicate the formation of other direct protein carbonyls, as well as indirect protein carbonylation through protein lipoxidation or glycation and glycoxidation reactions. The hydroxylation pathway of the essential amino acid phenylalanine in meat during exposure to air and light has been shown through the identification of 4-hydroxyphenylalanine. Protein oxidation prior to digestion had a reducing effect on proteolysis during digestion, which can be ascribed to polymerization.

To the best of our knowledge, this is the first study to use a specific combination of protein oxidation markers (thiol oxidation, total and specific carbonylation, and hydroxylation) in meat products and their simulated digests. The meat had been subjected to relevant oxidative conditions and the applied *in vitro* tool closely simulated *in vivo* conditions, which should have allowed to properly evaluate the effects of protein oxidation on proteolysis during meat digestion. Additionally, the identification of 4-hydroxyphenylalanine in oxidized meat arises questions about its role in the colonic metabolism when it is converted into phenol and *p*-cresol. The combination of the current *in vitro* digestion model and protein oxidation markers thus forms a valuable tool to evaluate the consequences of oxidation during digestion of meat, and could be applied to heated meat and meat products in future. Subsequent *in vitro* colonic fermentations of duodenal digests can provide information on protein fermentation derived metabolites.

Chapter 5

Ascorbate and apple phenolics affect protein oxidation in emulsion-type sausages during storage and *in vitro* digestion

Redrafted after

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ABSTRACT

The effect of sodium ascorbate and apple phenolics on the oxidative stability of emulsion-type sausages during storage and digestion was investigated. Emulsion-type sausages containing 0.05% sodium ascorbate or 3% freeze dried apple pomace were subjected to chilled illuminated storage and subsequent *in vitro* digestion. Lipid oxidation was assessed as TBARS, and protein oxidation was evaluated as thiol oxidation, total carbonyls, and γ-glutamic and α-amino adipic semialdehyde. Proteolysis was measured after digestion to evaluate protein digestibility. The results suggest the presence of protein-ascorbate and protein-phenol interactions, which may decrease protein digestibility and may interfere with spectrophotometric methods for measuring oxidation.

5.1 INTRODUCTION

During aging and processing, meat and meat products are exposed to various reactive oxygen species (ROS) which are able to target both the lipid and protein fraction. Lipid oxidation is marked for the typical rancidity and off-flavour in meat products, and the mechanisms and inhibition by antioxidants have been studied extensively (Velasco et al., 2010). The consequences of protein oxidation are perhaps more subtle, which is probably why this phenomenon has for a long time been ignored in food science. However, ROS can damage proteins by attacking the amino acid side chains or the peptide backbone (Lund et al., 2010). Consequently, protein oxidation can be detrimental for meat quality in many ways, e.g., by altering protein functionality such as the emulsifying, gel-forming and water holding capacity (Decker et al., 1993; Ooizumi et al., 2004; Bertram et al., 2007), and by decreasing sensory aspects such as meat tenderness and juiciness through cross-link formation (Rowe et al., 2004; Lund et al., 2007b). Furthermore, oxidative modification of proteins may affect their nutritional value and digestibility (Xiong, 2000).

Ascorbic acid or its salt, sodium ascorbate, is a reducing agent which is often added to meat products. It acts as a chain-breaking antioxidant by donating electrons or hydrogen atoms to free radicals, thereby inhibiting further oxidation (Buettner & Jurkiewicz, 1996). Furthermore, ascorbate can react with atmospheric oxygen and is able to regenerate other endogenous and exogenous antioxidants (Feiner, 2006a). In nitrite cured meat products such as emulsion-type sausages, ascorbate also acts as a colour enhancer by accelerating the reduction of nitrite into nitric oxide. The latter reacts with myoglobin to form nitrosomyoglobin, which upon heating turns into the typical pink cured meat colour (Totosaus, 2009). Over the last decades, phenolic-rich fruit and plant material have been considered as an alternative to conventional antioxidants. Like ascorbate, polyphenols have reducing capacities and can scavenge free radicals by either donating hydrogen atoms or capturing radicals into their phenolic ring structure. Furthermore, some polyphenols are

able to chelate metal ions, enabling Fenton-like reactions in which ROS are formed (Shahidi et al., 2015).

In the present study, emulsion-type sausages were prepared with the addition of either sodium ascorbate or freeze dried apple pomace, and their oxidative stability was investigated during chilled illuminated storage and subsequent *in vitro* digestion. Lipid oxidation was evaluated as thiobarbituric acid reactive substances (TBARS), and protein oxidation was evaluated as thiol oxidation and carbonylation (total carbonyls, γ-glutamic semialdehyde and α-amino adipic semialdehyde). Furthermore, the proteolytic degradation after digestion was evaluated in order to shed light on the potential correlation between protein oxidation and protein digestibility.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of freeze dried apple pomace

Apples of the cultivar 'Jonagold' were obtained from a local fruit distributor. The apples were vacuum pressed (VaculIQ 1000, VaculIQ, Hamminkeln, Germany) and the retained apple pomace was immediately vacuum packed and frozen to -20 °C. After being freeze dried (Epsilon 2-10 D LSC, Martin Christ, Osterode am Harz, Germany), the apple pomace was homogenized into a powder using a food processor, vacuum packed and stored in the dark at room temperature until manufacturing of the emulsion-type sausages.

5.2.2 Preparation and sampling of emulsion-type sausages

Lean pork and pork fat were obtained from a local meat processing plant. Three treatments were prepared, each containing 2.8 kg of lean pork, 2.45 kg of pork fat, 1.75 kg of crushed ice, 133 g of nitrite salt (Solina group, Eke-Nazareth, Belgium), and 35 g of emulsifier (Emulsioninne AB, Solina group, Eke-Nazareth, Belgium). No other ingredients were added to the first treatment, which was included as a control. The ascorbate and apple treatments were enriched with 3.5 g of sodium ascorbate and 210 g of freeze dried apple pomace to obtain a final concentration of 0.05% and

3%, respectively. A concentration of 0.05% (500 ppm) of sodium ascorbate is commonly applied for meat products (Sahoo & Anjaneyulu, 1997; Feiner, 2006a). The 3% apple concentration was chosen on the basis of the work of Nuñez de Gonzalez et al. (2008), who tested 3% and 6% of dried plum puree and dried plum and apple puree as natural antioxidants in pork sausages. After cooking and dark storage (21 days), these authors reported TBARS inhibition in the 6% plum and apple puree treatment, however this puree had a moisture content of 50% to 56%. The meat batters were prepared in a laboratory blender (Stephan Prime Cut 12, Stephan Machinery GmbH, Hameln, Germany) and transferred to a vacuum filler (Robby Type 136, VEMAG, Verden, Germany) to make 1.5 kg sausages in artificial casings with 105 mm diameter (F plus rot, Walsroder Casings GmbH, Walsrode, Germany). The sausages were heated (Baskett PRIV 130 M, Firex, Sedico, Italy) to a core temperature of 68 °C and subsequently cooled to 4 °C. Sausages were automatically sliced (VS12 D, Bizerba, Balingen, Germany) into 1.5 mm slices and displayed in portions of three stacked slices at 4 °C (atmospheric air) under fluorescent light of 1000 lux, which was turned on for 12 h per day. Upon sampling time (day 0, 4 and 7), portions were homogenized using a food processor, vacuum packed, and stored at -80 °C until analysis.

5.2.3 Physico-chemical characterization of meat samples

Moisture, crude protein and crude fat content were determined according to ISO 1442:1997, ISO 937:1978 and ISO 1444:1996, respectively. Colour measurements (L^*, a^* and b^*) were performed using a UV–Vis spectrophotometer (Sensing Unveils CM-5, Konica Minolta Sensing, Osaka, Japan).

5.2.4 *In vitro* digestion

The *in vitro* digestion of the meat products was performed according to a previously described protocol (Van Hecke et al., 2014a), as described in Chapter 4, Section 4.2.3.

5.2.5 Quantification of lipid oxidation

Malondialdehyde (MDA) concentrations in meat products and digests were measured colorimetrically as TBARS by a modified method in accordance with Grotto et al. (2007). Results were expressed as nmol MDA equivalents per mg free fat.

5.2.6 Quantification of free and total thiols

Free and total thiols were quantified according to Rysman et al. (2014) with slight modification as described in Chapter 4, Section 4.2.5. However, 1 g instead of 0.5 g of meat was taken to level out the protein concentration in emulstion-type sausages as compared to meat patties. The thiol content was expressed as nmol thiol per mg protein.

5.2.7 Quantification of total carbonyls

Total carbonyls were determined according to Vossen et al. (2015) with slight modifications as described in Chapter 4, Section 4.2.6. However, 200 μL (instead of 100 μL) and 400 μL (instead of 200 μL) were taken from the meat homogenates and digests, respectively, to level out the protein concentration in emulstion-type sausages as compared to meat patties. Protein carbonyl concentration was expressed as nmol carbonyl per mg protein.

5.2.8 Quantification of γ-glutamic semialdehyde (GGS) and α-amino adipic semialdehyde (AAS)

Samples were prepared for ultra high performance liquid chromatography with fluorescence detection (UHPLC-FLD) analysis of γ-glutamic semialdehyde (GGS) and α-amino adipic semialdehyde (AAS) according to Utrera et al. (2011) with modifications as described in Chapter 4, Section 4.2.7. However, 200 μL (instead of 100 μL) and 400 μL (instead of 200 μL) were taken from the meat homogenates and digests, respectively, to level out the protein concentration in

emulstion-type sausages as compared to meat patties. Results were expressed as nmol of GGS or AAS per mg of protein.

5.2.9 Quantification of proteolysis

Proteolysis in digested samples was determined according to Polychroniadou (1988) with modifications as described in Chapter 4, Section 4.2.9. Results were expressed as μmol glycine equivalents per mg of dry weight.

5.2.10 Statistical analysis

All analyses were done in triplicate. Statistical analyses were carried out using SAS® software (SAS Institute Inc., Cary, NC, USA). Colour values (L^*, a^* and b^*) were analyzed using a linear model with treatment as fixed effect. Data before and after digestion were analyzed separately. Results for TBARS, free thiols, total thiols, total carbonyls, GGS, AAS, and proteolysis in the meat samples and the digests were analyzed using a linear model with 'storage day' and 'treatment', as well as their interaction, as categorical fixed effects. Tukey-adjusted *post hoc* tests were performed for pairwise comparisons, and the significance level was selected as $P < 0.05$.

5.3 RESULTS AND DISCUSSION

5.3.1 Characteristics of apple pomace

Apple pomace contains approximately 3.1 – 4.5% protein, 1.6 – 4.5% fat, 0.6 – 1.9% ash, 10.8 – 15.0% sugars and 60.7 – 89.8% dietary fibers, of which 56.5 – 81.6% insoluble dietary fibers (dry weight basis) (Figuerola, Hurtado, Estévez, Chiffelle, & Asenjo, 2005; Dhillon, Kaur, & Brar, 2013; Younis & Ahmad, 2015).

In 2015, De Paepe et al. quantified the phenolic profile in the peel and flesh of 47 Belgian apple cultivars. Table 5.1 shows the major phenolics in apple fruit and their content in Jonagold (the apple cultivar used in this Chapter) as determined by these authors. Considering that apple peel

accounts for approximately 3% of the apple (on dry weight basis), the phenolic content in the whole apple can be calculated (Table 5.1). In a comparative study between spiral-filter pressing (the technology used to obtain the pomace in this Chapter) and belt pressing in apple juice production, these same authors determined the extraction efficiency for each of these phenolics (De Paepe et al.), allowing to calculate the phenolic content in apple pomace. Hence, the concentrations as shown in Table 5.1 are a good representation of what the phenolic profile in the apple pomace from the current Chapter would look like.

Table 5.1: Proximate phenolic content in Jonagold apple.

		Concentration (mg/kg dry weight)			
Phenolic compound	**E_e (%)[1]**	**Peel[2]**	**Flesh[2]**	**Whole[3]**	**Pomace[3]**
Chlorogenic acid	79.2%	122.4	15.1	18.3	3.8
Isoquercitrin	4.5%	459.5	0.5	14.2	13.6
Hyperin	3.5%	644.1	0.1	19.5	18.8
Rutin	10.1%	53.3	0.0	1.6	1.5
Avicularin	1.8%	339.4	2.3	12.5	12.2
Quercitrin	10.2%	331.7	10.4	20.0	18.0
(+)-Catechin	64.9%	19.5	4.2	4.7	1.6
(-)-Epicatechin	58.5%	295.1	57.2	64.3	26.7
Phloridzin	10.5%	43.6	13.3	14.2	12.7
Procyanidin B2	45.9%	248.8	80.8	85.9	46.5

E_e, extraction efficiency during spiral-filter pressing of apple (% phenolic transfer to juice).
[1]De Paepe et al. (2015a). [2]De Paepe et al. (2015b). [3]By calculation.

Some critical remarks should be made when interpreting this data. The Jonagold apples used for the determination of the phenolic content in Table 5.1 are from a different growing year than the ones used in this Chapter. As it concerns the same cultivar, the phenolic profile would be comparable, however the growing year might influence the phenolic content (De Paepe, 2014). Furthermore, to determine the phenolic content in the apple peel and flesh as shown in Table 5.1, the apples were cored (De Paepe et al., 2015b). Hence, phenolics that are mainly present in the apple seeds, such as phloridzin (Li, 2012), are not taken into account. Moreover, only a selection of

phenolics were tested on their extractability during apple juice production, however there are more phenolics in Jonagold than presented in Table 5.1. Especially those that are abundantly present in the apple peel, are strongly retained in the apple pomace (De Paepe et al., 2015a). Hence, it would be incorrect to simply add up the phenolic content as shown in Table 5.1 to obtain a total phenolic content. Finally, not only the total phenolic content, but also the effects of synergism and antagonism among phenolic antioxidants, as explained in Chapter 3 (Section 3.3.4.4), should be considered when evaluating the antioxidant capacity of phenolics in the apple pomace.

Diñeiro García et al. (2009) determined the antioxidant capacity of apple pomaces after hydraulic pressing of six cultivars by means of spectrophotometric methods. On average, they found a total phenolic content of 7.3 ± 1.8 g GAE per kg dry weight according to the Folin-Ciocalteu assay. The radical scavenging activity according to the DPPH$^{\bullet}$ assay was on average 12.6 ± 1.8 g ascorbic acid equivalents per kg dry weight, and the mean ferric reducing antioxidant power (FRAP) was 10.7 ± 1.6 g ascorbic acid equivalents per kg dry weight.

With regard to ascorbic acid (vitamin C), few literature can be found on its content in apple pomace. According to Lee et al. (2003), whole apple contains on average 12.8 mg per 100 g fresh weight (ca. 0.64 g per kg dry weight). Considering that the majority of vitamin C is located in the apple flesh, it is reasonable to assume that most of the vitamin C will be transferred to the apple juice. Hence, compared to phenolic compounds, vitamin C would have only a minor contribution to the antioxidant capacity of apple pomace.

5.3.2 Characteristics of emulsion-type sausages

The composition (moisture, protein and fat content) and colour values (L^*, a^* and b^*) of the emulsion-type sausages are represented in Table 5.2. The redness (a^*) significantly increased in the order control < apple pomace < ascorbate treatment. The high a^* value in the ascorbate treatment can be ascribed to the role of ascorbate as a colour enhancer. The intermediate redness in emulsion-type sausages treated with freeze dried apple pomace suggests that phenolic

compounds are able to reduce nitrite to nitric oxide, subsequently contributing to the formation of nitrosomyoglobin, however the phenolics do not seem to be as efficient as ascorbate. The lightness (L^*) and yellowness (b^*) significantly increased in the order control < ascorbate < apple pomace. The brighter and more yellow colour of the sausages in the apple treatment is due to the bright, yellowish green colour of the apple pomace powder.

Table 5.2: Moisture, protein and fat content and colour values (L^*, a^* and b^*) in emulsion-type sausages (control, 0.05% ascorbate and 3% freeze dried apple pomace).

	Moisture (%)	**Protein (%)**	**Fat (%)**	***L****	***a****	***b****
Control	60.7	10.7	25.7	$68.0^{a} \pm 0.2$	$4.2^{a} \pm 0.2$	$8.5^{a} \pm 0.1$
Ascorbate	60.1	10.1	26.6	$69.1^{b} \pm 0.2$	$5.3^{b} \pm 0.2$	$9.5^{b} \pm 0.1$
Apple	59.7	9.7	25.5	$70.8^{c} \pm 0.1$	$4.8^{c} \pm 0.1$	$12.4^{c} \pm 0.2$
P values				<0.001	<0.001	<0.001

5.3.3 Lipid oxidation

During storage, a small though significant decrease in TBARS expressed on fat basis was measured in the control treatment (Figure 5.1), while an increase was expected. Since the TBARS assay is based on reaction of MDA with thiobarbituric acid (TBA), an underestimation or decrease of TBARS can be ascribed to instability of MDA. As such, MDA can bind to proteins through Schiff base formation with lysine residues (Domingues et al., 2013). However, the hydrolysis step in our TBARS assay should have reduced such MDA-protein adducts, making the MDA molecule available again for detection with TBA. Lebepe, Molins, Charoen, Farrar, and Skowronski (1990) suggested that MDA was metabolized by spoilage bacteria, causing a decrease of TBARS in vacuum packed pork loin. Bax et al. (2012) proposed a breakdown of MDA into volatile compounds, together with MDA-protein adducts, to explain their TBARS decrease in porcine meat cooked at high temperatures. Another likely explanation for the TBARS decrease in the present study is interference of residual nitrite. Since the emulsion-type sausages were cured with nitrite salt, the residual nitrite could lead to partial nitrosation of MDA during storage, making it unreactive to TBA (Raharjo & Sofos, 1993).

Sindelar, Cordray, Sebranek, Love, and Ahn (2007) used curing conditions in emulsion-type sausages similar to our production, and measured a decrease of residual nitrite in the ppm range during vacuum storage. Hence, it is plausible that some of the residual nitrite reacted with MDA that was formed during processing and storage of the emulsion-type sausages, making it undetectable with the TBARS assay.

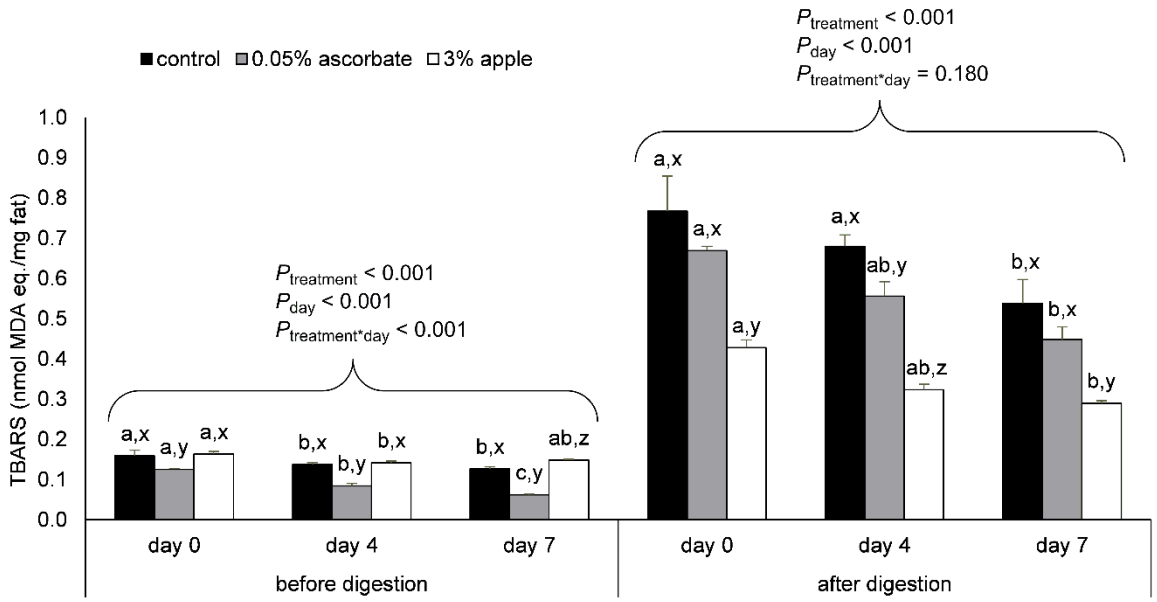

Figure 5.1: Lipid oxidation in emulsion-type sausages (control, 0.05% ascorbate and 3% freeze dried apple pomace) during illuminated storage (4 °C) and after *in vitro* digestion. Results are shown as mean ± standard deviation (N=3). [a-c] Significant difference between days within the same treatment ($P < 0.05$). [x-z] Significant difference between treatments within the same day ($P < 0.05$).

Although TBARS values in the ascorbate treatment also decreased significantly during storage, they were significantly lower than the control treatment on all sampling days (Figure 5.1). This indicates that ascorbate worked effectively as an antioxidant against lipid oxidation. The apple treatment did not significantly differ from the control during the first 4 days of storage, however by day 7, TBARS values increased slightly and were significantly higher than the control (Figure 5.1). These results clearly indicate that the freeze dried apple pomace did not act as an inhibitor of lipid oxidation during storage of the emulsion-type sausages. Contradictory results were reported by Sun et al. (2010), where both ascorbic acid and apple polyphenols significantly inhibited lipid

oxidation in sliced cooked cured ham. However, differences in concentration and purification of the apple extracts, as well as in the meat matrix (cooked ham vs. emulsion-type sausages), impede comparisons between their and our study.

After digestion, a 2- to 7-fold increase of TBARS values was found (Figure 5.1), which is in agreement with Van Hecke et al. (2014a; 2014b; 2015) and confirms the ongoing lipid oxidation during digestion. The trends of decreasing TBARS over light exposure time and lower TBARS in the ascorbate treatment were similar to those before digestion, suggesting that MDA reactions with residual nitrite still interfered with the TBARS assay, yet ascorbate remained an inhibitor of lipid oxidation during digestion. Interestingly, the TBARS results after digestion in the apple treatment were significantly lower than in all other digests, indicating that the phenolic-rich apple pomace effectively inhibited lipid oxidation during digestion. Possibly, the physiological conditions of the gastro-intestinal tract positively influenced the antioxidative capacities of the apple phenolics. As redox reactions are pH dependent, it is possible that the pH decrease in the stomach and/or the subsequent increase in the duodenum affects the antioxidant activity of phenolics. Curiously, Nuñez de Gonzalez et al. (2008) observed that 3% dried plum and apple puree only significantly inhibited TBARS formation in pork sausages after frozen storage and not during manufacturing, cooking, or refrigerated storage. This suggests that apple polyphenols are slower to act as an antioxidant than ascorbate. However, possible interactions of phenolics or other apple compounds with MDA, interfering with the TBARS assay and resulting in an underestimation after digestion, should not be excluded.

5.3.4 Protein oxidation

5.3.4.1 Thiol oxidation

The levels of free and total thiols in the three treatments of emulsion-type sausages (control, 0.05% ascorbate and 3% apple) during storage are shown in Table 5.3. During 7 days of chilled illuminated storage, the free thiol content decreased significantly in all treatments, indicating that

thiols were lost to oxidation. After 7 days, a free thiol decrease of 32% was observed in the control treatment, however free thiol loss was more abundant in the presence of ascorbate and apple (62% and 46%, respectively). This suggests that more thiol oxidation took place and thus, that ascorbate and apple were not effective as antioxidants against protein thiol oxidation. Next to its free radical scavenging properties, ascorbate is able to reduce free metal ions, such as ferric iron which can be released upon heating of myoglobin. Its reduced form, ferrous iron, can subsequently take part in Fenton-like reactions producing ROS. Hence, ascorbate can indirectly act as a pro-oxidant (Carr & Frei, 1999). However, the applied 4-DPS method only gives information about the extent of thiol loss and not about the types of thiol oxidation products formed. Therefore, it is unknown whether the observed thiol loss is caused by direct thiol oxidation such as disulfide cross-link formation, or by thiol reactions with ascorbate or apple phenolics. As such, reactions of thiols with degradation products of ascorbate have been reported. Upon oxidation of ascorbate, e.g. by donating hydrogen atoms to free radicals, dehydroascorbate (DHA) is formed. This reaction is reversible, however under severe oxidative stress the ascorbate:DHA ratio will decrease. Since DHA is unstable under physiological conditions, it will degrade into numerous products, one of which was identified as a five-carbon derivative reacting with thiol groups of glutathione, insulin B-chain, α-lactalbumin, and hemoglobin, forming protein-DHA* adducts (Figure 5.2A) (Regulus, Desilets, Klarskov, & Wagner, 2010; Kay, Wagner, Gagnon, Day, & Klarskov, 2013). These types of adducts could explain the significantly lower thiol levels in the ascorbate treatment on day 4 and 7. Similar adduct formation and interactions have been reported about phenolic compounds and protein thiols. Upon oxidation of the hydroxyl groups on the phenolic ring, quinone structures are formed, which in turn can react with thiol groups on cysteine side chains. After this covalent bonding, the phenol moiety is regenerated and is able to reoxidize and bind with another cysteine side chain, forming a protein-phenol-protein cross-link (Figure 5.2B) (Ozdal et al., 2013). Hence, these protein-phenol interactions would lead to a decrease in free thiols as detected with 4-DPS. The fact that in the present study, the free thiols in the apple treatment on day 0 were already significantly lower than in the control treatment, suggests that some of these protein-phenol

reactions had already taken place during manufacturing of the emulsion-type sausages. Similar results were observed by Jongberg et al. (2015b), who studied the dose-dependent effects of green tea extract as an antioxidant in emulsion-type sausages, and found significantly fewer free thiols in the high dose treatment (1500 ppm of extract). Hence, the concentration of 3% freeze dried apple pomace in the present study is possibly too high, resulting in significant protein-phenol interactions.

Table 5.3: Protein oxidation in emulsion-type sausages (control, 0.05% ascorbate and 3% freeze dried apple pomace) during illuminated storage (4 °C).

		storage				P values		
	treatment	**day 0**	**day 4**	**day 7**	**RMSE**	**T**	**D**	**T×D**
free thiols	control	$21.8^{a,x}$	$20.4^{a,x}$	$14.8^{b,x}$				
(nmol/mg protein)	ascorbate	$19.5^{a,x}$	$11.4^{b,y}$	$7.3^{c,y}$	1.0	<0.001	<0.001	<0.001
	apple	$16.0^{a,y}$	$11.9^{b,y}$	$8.6^{c,y}$				
total thiols	control	52.3^{x}	53.7^{x}	49.3^{x}				
(nmol/mg protein)	ascorbate	$50.1^{a,x}$	$47.6^{a,y}$	$34.3^{b,y}$	2.0	<0.001	<0.001	<0.001
	apple	$44.1^{a,y}$	$40.6^{a,z}$	$32.1^{b,y}$				
total carbonyls	control	$2.36^{a,x}$	$2.89^{ab,x}$	$3.35^{b,x}$				
(nmol/mg protein)	ascorbate	$3.02^{a,xy}$	$5.41^{b,y}$	$6.30^{c,y}$	0.25	<0.001	<0.001	<0.001
	apple	$3.12^{a,y}$	$3.86^{b,z}$	$3.93^{b,x}$				
GGS	control	0.23^{xy}	0.28	0.28				
(nmol/mg protein)	ascorbate	$0.16^{a,x}$	0.29^{b}	0.33^{b}	0.04	0.005	<0.001	0.133
	apple	0.29^{y}	0.36	0.34				
AAS	control	1.47	1.57^{xy}	1.48^{xy}				
(nmol/mg protein)	ascorbate	1.09^{a}	$1.89^{b,x}$	$1.98^{b,x}$	0.26	<0.001	0.014	0.040
	apple	0.85	1.04^{y}	0.91^{y}				

GGS, γ-glutamic semialdehyde; AAS, α-amino adipic semialdehyde; RMSE, root mean square error; T, treatment; D, day. a-c Significant difference between days within the same treatment ($P < 0.05$). x-z Significant difference between treatments within the same day ($P < 0.05$).

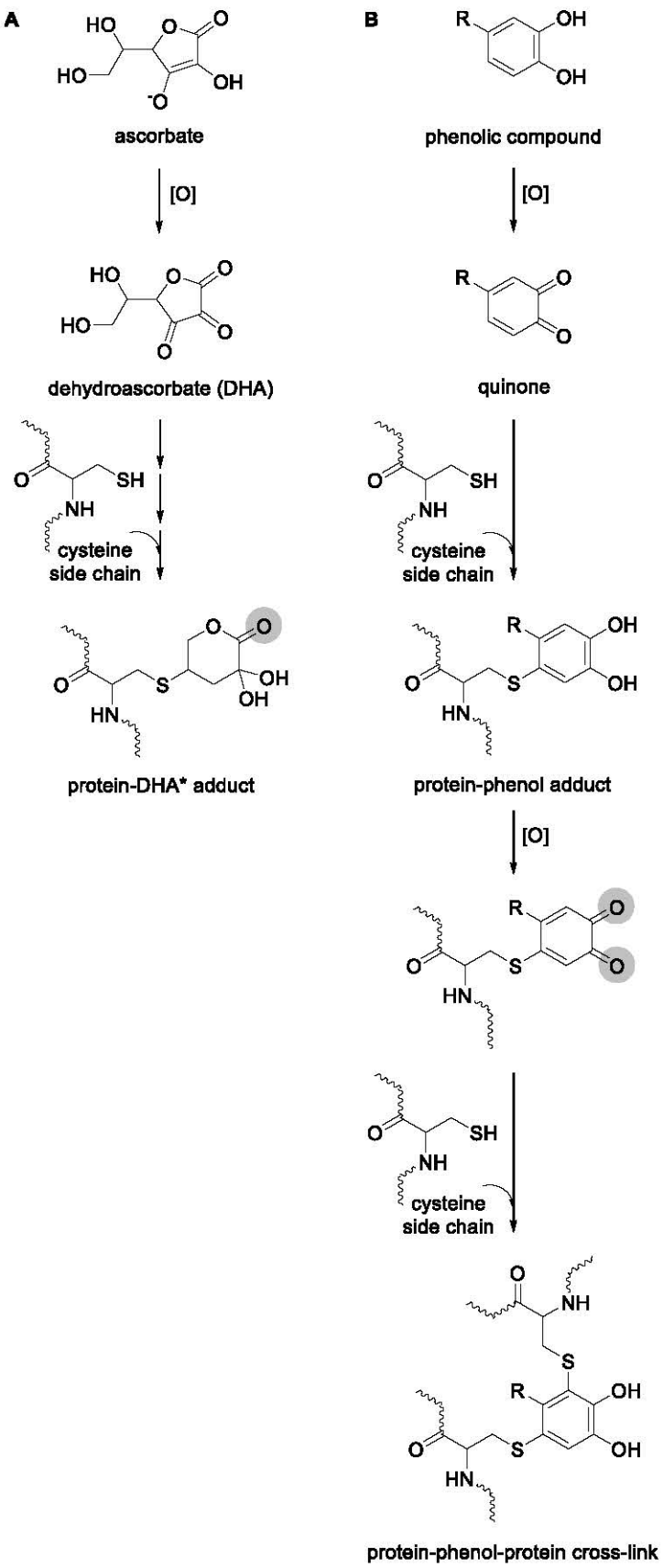

Figure 5.2: Proposed mechanisms of ascorbate (scheme A) and phenol (scheme B) interactions with protein thiols. Gray spheres indicate protein-bound carbonyl groups susceptible to DNPH derivatization. Schemes modified from Kay et al. (2013) and Jongberg, Lund, and Otte (2015a).

Upon reduction with sodium borohydride, the total thiol level in the fresh control sample (day 0) was 2.4 fold higher than the free thiol level (Table 5.3). This indicates that approximately 30.5 nmol of reducible thiol oxidation products per mg of protein were already formed during manufacturing of the emulsion-type sausages. No significant changes in total thiols were observed during storage of the control sample, indicating that the free thiol loss was due to reducible oxidation reactions. The total thiol content in the ascorbate treatment was similar to that of the control treatment on day 0, however by day 7 a significant 32% decrease was observed, indicating that part of the thiol reactions were irreversible. According to Kay et al. (2013), the reaction between DHA* and thiols likely involves Michael addition. Since the reversibility of these type of adducts are dependent on temperature and structure (Allen & Humphlett, 1966), it is likely that in this study the proposed protein-DHA* adducts are irreversible as determined with the 4-DPS assay. Analogue to the free thiol content, the total thiol content in the apple treatment was significantly lower than the control on day 0 and continued to decrease during storage. This could again be attributed to the formation of protein-phenol adducts as described above. Jongberg et al. (2015a) studied the effects of dithiothreitol, tris(2-carboxyethyl)phosphine, and sodium sulfite on quinone-β-lactoglobulin adducts using mass spectrometry, and found that these three reducing agents were able to dissociate the adducts for 68, 36 and 75%, respectively. Although no information is available on the reducing effect of sodium borohydride on protein-phenol adducts, it is reasonable to assume that part of these covalent bonds were not dissociated during the reduction step in the 4-DPS analysis, resulting in a decrease in total thiols.

After digestion, lower thiol contents were measured (Table 5.4), indicating that further thiol oxidation took place. In the control treatment, the free thiol content decreased ca. 50% on each sampling day compared to the undigested equivalents. Although free thiols in the ascorbate and apple treatment were lower than the control before digestion, no significant differences from the control were found after digestion. Hence, if the lower values before digestion were caused by protein-DHA* and protein-phenol adducts as hypothesized, these interactions did apparently not occur during digestion, nor did they promote further thiol oxidation. No significant differences in

total thiols were found among storage days or treatment (Table 5.3), with an average of 31.5 nmol of total thiols per mg of protein. Compared to the initial total thiol level after production of the emulsion-type sausages (control day 0 before digestion), approximately 40% of the thiol oxidation became irreversible during digestion, disregarding the treatment. These data suggest that formation of protein-DHA* and protein-phenol adducts took place faster than thiol oxidation, however these interactions did not influence the ultimate thiol oxidation and its reversibility after digestion.

Table 5.4: Protein oxidation in emulsion-type sausages (control, 0.05% ascorbate and 3% freeze dried apple pomace) after *in vitro* digestion.

		storage				P values		
	treatment	**day 0**	**day 4**	**day 7**	**RMSE**	**T**	**D**	**T×D**
free thiols	control	11.9^{a}	$9.1^{b,xy}$	7.7^{b}				
(nmol/mg protein)	ascorbate	13.3^{a}	$10.5^{b,x}$	8.1^{c}	0.8	<0.001	<0.001	0.718
	apple	11.2^{a}	$8.0^{b,y}$	6.7^{b}				
total thiols	control	29.3	31.0	32.8				
(nmol/mg protein)	ascorbate	29.7	26.8	34.1	3.6	0.239	0.277	0.306
	apple	33.4	33.6	32.3				
total carbonyls	control	2.11	2.20	2.30				
(nmol/mg protein)	ascorbate	2.68	2.77	2.19	0.34	0.009	0.091	0.280
	apple	2.87	3.02	2.40				
GGS	control	0.22^{x}	0.19^{x}	0.20				
(nmol/mg protein)	ascorbate	0.18^{x}	0.25^{x}	0.19	0.05	<0.001	<0.001	<0.001
	apple	$0.58^{a,y}$	$0.60^{a,y}$	0.26^{b}				
AAS	control	0.20	0.21	0.24				
(nmol/mg protein)	ascorbate	0.15	0.21	0.22	0.04	0.304	0.295	0.385
	apple	0.19	0.20	0.17				

GGS, γ-glutamic semialdehyde; AAS, α-amino adipic semialdehyde; RMSE, root mean square error; T, treatment; D, day. a-c Significant difference between days within the same treatment ($P < 0.05$). x-y Significant difference between treatments within the same day ($P < 0.05$).

5.3.4.2 Carbonylation

Results for carbonylation as determined with DNPH (total carbonyls) and with UHPLC-FLD (GGS and AAS) are presented in Table 5.3 (during storage) and Table 5.4 (after digestion). Although GGS and AAS have been highlighted as the main carbonyls in meat proteins (Estévez, 2011), it is worth interpreting all results simultaneously. Table 5.5 shows the sum of the GGS and AAS results expressed relative to the total carbonyl level. In the control treatment on day 0, GGS+AAS accounted for 72% of total carbonyls. Total carbonyls in the control increased significantly during storage, however no significant changes were observed in GGS or AAS, decreasing the GGS+AAS percentage to 52% by day 7. Hence, the higher initial total carbonyl level, as well as the increase during storage, should be ascribed to other carbonyls than GGS and AAS, which are also detectable with DNPH. Such carbonyls could be derived from direct carbonylation of other amino acids, or from reactions of proteins with oxidizing sugars or lipids containing an aldehyde (Estévez, 2011).

Table 5.5: Amount of GGS and AAS, relative to the total carbonyl level. Results calculated as the sum of GGS and AAS (as determined with UHPLC-FLD) divided by total carbonyls (as determined with DNPH) (%).

	before digestion			**after digestion**		
treatment	**day 0**	**day 4**	**day 7**	**day 0**	**day 4**	**day 7**
control	72%	64%	52%	20%	18%	19%
ascorbate	41%	40%	37%	12%	17%	18%
apple	37%	36%	32%	27%	27%	18%

In the ascorbate treatment, a significant increase in total carbonyls, GGS and AAS was observed during storage. Total carbonyls were significantly higher and almost double of the total carbonyl level in the control on day 4 and 7. This suggests that ascorbate acted as a pro-oxidant through iron reduction as described in Section 5.3.4.1, especially since protein carbonylation is believed to be metal-catalyzed (Estévez, 2011). However, GGS and AAS levels did not significantly differ from the control, indicating that the spectacular increase in total carbonyls was caused by other

carbonyls. As such, the carbonyl moieties on ascorbate and DHA are able to react with DNPH in a similar way as protein carbonyls. If these molecules were covalently bound to the protein, e.g., through protein-DHA* adducts, they would not be removed by washing of the protein pellet, therefore leading to an overestimation of total protein carbonyls (Figure 5.2A) (Srinivasan, Xiong, & Decker, 1996). In the ascorbate treatment, GGS+AAS initially made up 41% of total carbonyls, which suggests that part of these DNPH interfering DHA adducts were already formed during processing.

The apple treatment contained slightly higher total carbonyl and GGS levels compared to the control, however these differences were only significant for total carbonyls during the first 4 days of storage. Similar to the proposed ascorbate interference, covalent protein-phenol interactions could contribute to higher total carbonyls through reaction of DNPH with carbonyl moieties on the quinone structure (Figure 5.2B). After production (day 0), GGS+AAS made up 37% of total carbonyls in the apple treatment compared to 72% in the control, which suggests phenol adduct formation during processing, and thus DNPH overestimation from the beginning of the experiment. Total carbonyls in the apple treatment no longer significantly increased nor differed from the control by day 7, which could suggest that some sort of equilibrium between phenol interference and carbonyl inhibition was reached. Interestingly, apple phenolics seemed to inhibit AAS formation, though not significantly. This suggests that phenolics specifically protected the lysine residue from oxidizing into AAS. Most likely this is not by radical scavenging, because results for thiol oxidation, total carbonyls and GGS formation indicate otherwise. Possibly some apple phenolics interacted with the lysine residue, e.g. through ionic bonds between the positively charged ε-amino group from lysine and the negatively charged hydroxyl groups from the phenol structure (Le Bourvellec et al., 2012), thereby preventing lysine from further oxidation.

Total carbonyl levels in the digested samples (Table 5.4) were 8 to 65% lower than in their undigested equivalents. This decrease can be ascribed to further reactions of carbonyls during digestion, in which the carbonyl moiety is lost and no longer can be detected with DNPH. As such, carbonyls can further degrade into carboxylic acids, Schiff base structures, aldol condensation

products, and Strecker aldehydes (Estévez, 2011). The total carbonyl decrease during digestion of the ascorbate and apple samples suggests that if the high total carbonyl levels before digestion would be due to ascorbate and phenol interference, at least part of the carbonyl groups on these adducts also took part in further reactions. In all treatments, the total carbonyl decrease during digestion was more extensive in older samples (average of 10, 31 and 45% decrease in samples from day 0, 4 and 7, respectively), signifying that more secondary carbonyl reactions took place in severely oxidized samples. Due to this further degradation of carbonyls, no significant differences in total carbonyls could be observed among digested samples. Results for GGS formation indicate a similar pattern as for total carbonyls in the control and ascorbate treatments, however the GGS content in the presence of apple nearly doubled during digestion of samples on day 0 and 4, whereas a decrease was observed after digestion of the 7 day old apple sample. This could indicate that the apple phenolics were able to delay the secondary oxidation of GGS during digestion on day 0 and 4, but that by day 7, GGS degradation could no longer be prevented. Results for AAS formation revealed an average decrease of 85% after digestion, without significant differences between storage days or treatments. As a result, GGS+AAS only accounted for an average of 19% of total carbonyls after digestion (Table 5.5). This spectacular decrease indicates that AAS undergoes excessive ongoing reactions during digestion, and therefore might not be an optimal marker for carbonylation during severe oxidative stress, especially for treatment comparison. However, it is worth taking into consideration that both methods for carbonylation measurement include protein precipitation steps. Since proteins were exposed to proteases in the simulated digestive juices, digests contained mostly small peptides and amino acids, of which the molecular weight might be too low to precipitate. Direct carbonylation, such as AAS or GGS formation, shortens the amino acid side chain and thus lowers the molecular weight, whereas adduct formation increases it. Hence, the precipitated protein pellet might contain more carbonyl containing protein adducts, resulting in more DNPH derivatization.

5.3.5 Proteolysis

Both increased and decreased proteolytic susceptibility have been reported with oxidized meat proteins, depending on the degree and nature of oxidation (Liu et al., 2000b; Santé-Lhoutellier et al., 2007). An increase in proteolysis can be ascribed to protein unfolding due to oxidative degradation, making the protease recognition site more available. Severe protein oxidation however might lead to protein aggregation, preventing access by proteolytic enzymes (Bax et al., 2012). Results for proteolysis in the digested samples after increasing storage days are shown in Figure 5.3. Statistical analysis revealed a significant effect of treatment ($P < 0.001$) and storage day ($P = 0.026$), whereas the interaction was not significant ($P = 0.139$). A decreasing trend was observed in all treatments, signifying that the oxidative modifications that had occurred during storage led to impaired proteolytic susceptibility, and thus impaired digestibility. It is worth noting that some gastric and duodenal proteases such as trypsin preferentially cleave proteins at arginine and lysine residues (Gasteiger et al., 2005). Hence, carbonylation of these residues, as was observed in our results, could impair protease recognition. Furthermore, it is likely that aggregation caused by protein cross-linking occurred during storage and digestion of the emulsion-type sausages. As thiol loss was observed in all treatments during storage and especially after digestion (Table 5.3 and 5.4), it is likely that part of these thiols were oxidized into intra- or intermolecular disulfide bonds. Furthermore, as stated in Section 5.3.4.2, the decrease in carbonyls after digestion suggests ongoing reactions such as carbonyl mediated cross-link formation. These results indicate that the radical attack during digestion, resulting in thiol and carbonyl mediated cross-links, happened faster than the proteolytic attack by the digestive enzymes.

Proteolysis in the ascorbate treatment was slightly lower than the control, though not significantly. This indicates that ascorbate did not act as an antioxidant by inhibiting carbonylation and cross-link formation, and even slightly induced cross-linking. As such, Reihl, Lederer, and Schwack (2004) characterized a number of lysine-arginine cross-links derived from degradation products of DHA.

Hence, these ascorbate mediated cross-links, on top of thiol and carbonyl mediated cross-links, could account for more aggregation and thus less digestibility. Similarly, phenol (quinone) mediated cross-links as described in Section 5.3.4.1 (Figure 5.2) might possibly lead to a impaired proteolytic susceptiblity in the apple treatment.

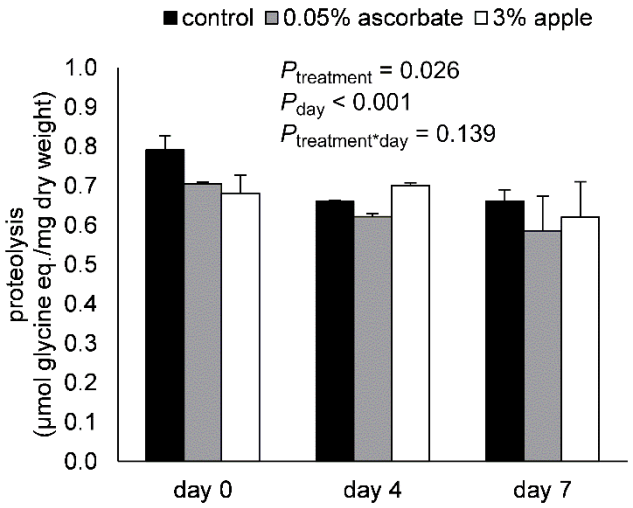

Figure 5.3: Proteolysis in cooked sausage (control, 0.05% ascorbate and 3% freeze dried apple pomace) after *in vitro* digestion. Results are shown as mean ± standard deviation (N=3).

5.4 CONCLUSIONS

In the present study, no significant protection of proteins against oxidation was observed during storage and *in vitro* digestion when 0.05% of sodium ascorbate or 3% of freeze dried apple pomace was added to emulsion-type sausages, even though a significant inhibition of lipid oxidation was found in the ascorbate treatment during storage and digestion, and in the apple treatment after digestion. The addition of ascorbate and apple phenolics led to significant more thiol loss, and results for total carbonyls and GGS and AAS formation were inconsistent. The results suggest that degradation products of ascorbate and phenolics reacted with protein moieties, resulting in more free thiol loss and non-protein carbonyls. Indications for interactions with ascorbate only appeared after prolonged storage and digestion, whereas phenolic interactions were noticeable right after manufacturing, however they changed slower and were less pronounced. These interactions might also impair the digestibility of proteins. More accurate methods with mass spectrometry are necessary to confirm and investigate these interactions.

Chapter 6

General discussion and future prospects

Meat and meat products are highly susceptible to quality loss due to oxidation of both the lipid and protein fraction (Xiong, 2000). Lipid and protein oxidation go hand in hand, and are first and foremost expressed as off-flavours and off-odours. However, protein oxidation may also influence texture and technological properties, and the impact on health upon consumption has been a topic of great interest, though still not fully elucidated (Soladoye et al., 2015). The use of antioxidants in meat products can delay such reactions, and consumers' growing interest in natural ingredients and so called 'clean label' products have prompted meat researchers to explore natural antioxidants derived from plant material rich in phenolic compounds (Hygreeva et al., 2014). In this dissertation, the mechanisms of protein oxidation in meat products and the effects of apple phenolics as a source of natural antioxidants during storage and digestion were studied.

6.1 PROTEIN OXIDATION MARKERS IN MEAT AND MEAT PRODUCTS

Thiol oxidation and carbonylation are reported to be the most abundant oxidative changes in meat proteins (Estévez, 2011; Lund et al., 2011), and therefore are the most widely used protein oxidation markers. In this PhD thesis, a new method for thiol measurement was introduced and methods for carbonylation measurement were optimized. Schiff base measurement was applied for the evaluation of the carbonylation pathway. Furthermore, hydroxylation of phenylalanine was introduced as an additional oxidation marker, contributing to the understanding and evaluation of protein oxidation in meat. However, all methods have certain drawbacks and limitations which should always be borne in mind when evaluating protein oxidation.

6.1.1 Free and total thiols

In most meat research, thiol oxidation into disulfides is analyzed as thiol loss, measured spectrophotometrically with the thiol detection agent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent), and/or as cross-linked myosin heavy chain (CL-MHC) formation, semi-

quantitatively measured by means of SDS-PAGE (e.g. Lund et al., 2007b; Jongberg et al., 2011b). In Chapter 2 of this thesis, a new spectrophotometric method was introduced for the quantification of free and total thiols in meat with 4,4'-dithiodipyridine (4-DPS). This thiol detection agent is suggested to be more sensitive and reliable than DTNB due to its smaller size and thus higher accessibility, its higher extinction coefficient, and its stability at low pH ranges (Riener et al., 2002; Hansen et al., 2007). The latter was an important feature when including a reduction step in thiol analysis, since acidification was needed at the end of this step. While the aforementioned DTNB method only measures thiol loss, the reduction step with sodium borohydride in the 4-DPS assay allows to gain information on the reducibility of thiol oxidation in meat. Compared to the SDS-PAGE method, more reducible thiol oxidation products are measured with 4-DPS in ground beef during high-oxygen storage. This can be explained by several aspects: (1) two MHC molecules may be cross-linked by multiple disulfide bridges; (2) intramolecular disulfide cross-linking are not visualized by gel electrophoresis, but are measured with the 4-DPS assay; (3) severely cross-linked MHC molecules are not able to migrate through the gel, leading to an underestimation; (4) thiol oxidation in meat proteins other than myosin, as well as reducible thiol oxidation products other than disulfides, are only measured with 4-DPS and not with SDS-PAGE. While SDS-PAGE visualizes thiol oxidation as disulfide formation on a molecular level, the measurement of free and total thiols in meat with 4-DPS offers valuable information about thiol oxidation on a functional group level (**H1 accepted**). However, the 4-DPS method only analyses thiol loss and its reversibility, but it does not provide information on the type of thiol oxidation products formed. For that, more accurate (U)HPLC or LC-MS/MS methods are necessary. Furthermore, it is worth noting that the 4-DPS assay is carried out on a filtrate, after meat proteins were solubilized with a denaturant. Hence, proteins that adhere to insoluble particles through cross-linking or other reactions, might be retained by the filter paper and excluded from the assay. This potential protein loss is leveled out by measuring the protein concentration of the filtrate, but should be considered when interpreting 4-DPS results. Finally, some optimization to the 4-DPS method should be considered. During the method development, the reduction procedure was tested on oxidized glutathione,

and an average recovery percentage of 86% was obtained. Including a spike addition step in the 4-DPS assay, by adding a known amount of GSSG to the meat at the beginning of sample preparation, allows to calculate the recovery percentage in meat, which subsequently can be taken into account for thiol content calculations.

6.1.2 Total carbonyls

Measurement of total carbonyls with 2,4-dinitrophenylhydrazine (DNPH) is by far the most common method for evaluating protein carbonylation in meat. As with all spectrophotometric assays, the specificity of the DNPH method can be questioned, however measures are taken to ensure a reliable quantification of protein carbonyls. As DNPH and DNP hydrazone exhibit the same yellow colour, it is important to include a thorough washing step after derivatization to remove all excess DNPH. This is done with ethanol:ethyl acetate, which at the same time washes away lipid particles. These lipid particles might contain secondary lipid oxidation products such as malondialdehyde (MDA), which are equally able to derivatize with DNPH, thus leading to an overestimation of protein carbonyls. During the DNPH derivatization and washing procedure, it is unavoidable that some of the protein fraction is washed away. To allow to correct for protein concentration differences, a blank derivatization (without DNPH) should thus be included for all samples. When following this procedure, the DNPH method can be considered as a rapid and reliable method for measuring total protein carbonyls in meat (**H2 accepted**). Nonetheless, the DNPH method does not provide information on which amino acids were carbonylated. Furthermore, this method does not allow to make a distinction between primary and secondary protein carbonylation. This is especially important in complex meat products, in which secondary protein carbonyls cannot only be derived from other meat components (e.g. oxidizing lipids), but also from non-meat ingredients such as ascorbate and phenolic compounds. Finally, it is worth noting that carbonyls on small proteins, peptides and free amino acids are not included in the DNPH assay, since they cannot be precipitated by TCA and are washed away.

6.1.3 Specific carbonyls: γ-glutamic and α-amino adipic semialdehyde

In 2011, Utrera, Morcuende, Rodríguez-Carpena, and Estévez introduced an HPLC method for the quantification of two specific carbonylation products in meat: γ-glutamic semialdehyde (GGS, derived from arginine and proline) and α-amino adipic semialdehyde (AAS, derived from lysine). The method is based on reductive amination of these aldehydes with the fluorescent 4-aminobenzoic acid (ABA), followed by acid hydrolyzation and HPLC separation coupled to a fluorescence detector (FLD). In Chapter 4 of this thesis, the HPLC-FLD procedure was adapted to a more rapid and accurate UHPLC-FLD procedure, reducing analysis time from 40 to 8.5 minutes per sample. Furthermore, a blank derivatization (without ABA) for protein concentration measurement was included for all samples, similar to the DNPH method. Up to now, AAS and GGS concentrations were expressed as nmol carbonyl per mg protein, and the protein concentration was measured in the meat samples before starting the derivatization procedure. However, as with the DNPH procedure, protein loss during sample preparation for carbonyl measurement is inevitable. Since severely oxidized meat proteins are often very fragile and harder to precipitate with TCA, the protein concentration in these samples at the end of the derivatization procedure can be considerably lower. Hence, including a blank derivatization and measuring protein concentration at the end of the derivatization procedure instead of before, corrects for protein loss during sample preparation and avoids an underestimation of carbonyls. With these procedure improvements, the suggested UHPLC-FLD method for AAS and GGS determination can be considered as a good and reliable way to evaluate direct protein carbonylation in meat (**H3 accepted**). Its specificity towards AAS and GGS can however at the same time be a limitation, since no information is given on carbonylation of amino acids other than arginine, proline or lysine, or any other oxidative changes. Another shortcoming of the method that cannot be overcome at present, is the absence of quantitative GGS and AAS standards. The use of an ABA standard curve to quantify GGS-ABA and AAS-ABA peaks, with the assumption that one mole of ABA emits the

same fluorescence as one mole of GGS-ABA or AAS-ABA (Utrera et al., 2011), could contribute to an over- or underestimation if the emission of derivatized carbonyls would be higher or lower than that of single ABA. Finally, as with the DNPH assay, AAS and GGS in small proteins, peptides and free amino acids are excluded from the assay since TCA only precipitates larger proteins.

6.1.4 Schiff base structures

Schiff base (SB) structures can be formed from cross-linking between protein carbonyl compounds and the ε-amino group of a lysine residue in the same or another protein (Xiong, 2000). Spectrophotometric measurement of their natural fluorescence can thus contribute to the understanding of the carbonylation pathway in meat proteins (e.g. see Chapter 3). However, SB structures can also be formed from other reactions where protein carbonyls are not involved, such as cross-linking between lipid oxidation aldehydes and lysine residues (Guyon, Meynier, & de Lamballerie, 2016). Furthermore, SB cross-linking can contribute to protein polymerization, aggregation, and in severe cases insolubility (Xiong & Decker, 1995), which can interfere with the spectrophotometric measurement. The use of a high ionic strength buffer might be sufficient to bring myofibrillar protein isolates into suspension for fluorescent SB measurement (e.g. see Chapter 3), however when analyzing meat and meat products for SB structures, a purification and solubilization step should be included in sample preparation to avoid the interference of other meat components and insoluble (protein) fractions (**H4 undecided**). In any case, it should always be considered that fluorescent molecules other than carbonyl-amino SB structures might be measured at the given excitation and emission wavelengths. Furthermore, the lack of SB standards prevents the exact quantification of SB structures in the samples; SB measurement is expressed as fluorescence intensity units, and can only be evaluated in comparison with other samples.

6.1.5 4-Hydroxyphenylalanine

In Chapter 4, a peak in the UHPLC-FLD chromatograms for GGS/AAS was identified as 4-hydroxyphenylalanine (4-OH-Phe) or tyrosine, an aromatic amino acid which is naturally present in meat, or can be formed by oxidation of phenylalanine. Thus far, oxidative loss of aromatic amino acids in meat was measured spectrophotometrically (Gatellier et al., 2009; Utrera et al., 2012a), while this UHPLC determination provides more accurate and more specific determination of 4-OH-Phe. Although it is impossible to make a distinction between endogenous tyrosine and monohydroxylated phenylalanine, the progression of the amount of 4-OH-Phe in meat during storage and/or digestion can be used as a new protein oxidation marker (**H5 accepted**). Hence, the UHPLC-FLD method described in this thesis does not only provide information about specific carbonylation (GGS and AAS formation), but also about hydroxylation of phenylalanine in meat. Since 4-OH-Phe or tyrosine is commercially available as a standard, the peaks in the chromatograms can easily be quantified. However, as with interpreting DNPH and GGS/AAS results, protein loss during sample preparation should be considered. With regards to 4-OH-Phe, sample preparation for UHPLC-FLD analysis could be simplified, since 4-OH-Phe emits a natural fluorescence at the applied wavelengths, and therefore derivatization with ABA is not necessary,

In conclusion, all of the described methods offer valuable information on protein oxidation in meat, however their limitations should always be considered when analyzing results. When evaluating protein oxidation in meat, it is advisable to use at least two oxidation markers, because no single oxidation marker is all inclusive. Depending on the research objectives and the type of oxidative modification of interest (carbonylation, thiol oxidation, hydroxylation, ...), a combination of specific and general (spectrophotometric) markers should be chosen carefully. Spectrophotometric methods such as the 4-DPS, DNPH and Schiff base assays are relatively rapid and inexpensive. However in these assays all chromogenic compounds at a given wavelength are

measured, and therefore the specificity towards the target molecules cannot be assured. On the other hand, UHPLC-FLD analysis gives specific information on GGS, AAS and 4-OH-Phe formation, however other oxidation products are overlooked. Furthermore, it is known that both carbonyls and thiols are highly reactive compounds that are able to react with other amino acid residues, with secondary lipid oxidation products such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), or with other meat components (Xiong, 2000; Estévez, 2011). Therefore, the parallel interpretation of several protein and lipid oxidation markers is of the utmost importance to comprehend oxidative reactions. Finally, it should be noted that even when quantification is possible by means of a standard, results from oxidation assays should not stand alone. Oxidation is a complex and dynamic phenomenon, and results from two different laboratories or even two different studies within the same laboratory may vary considerably (Pegg, 2001; Stadtman et al., 2003). Therefore, trends in oxidized samples should always be compared to suitable controls (e.g. before and after storage or digestion).

6.2 PROTEIN OXIDATION DURING STORAGE AND DIGESTION OF MEAT AND MEAT PRODUCTS

All of the applied oxidation systems in this PhD thesis (metal-catalyzed oxidation (MCO), O_2 and light exposure) caused significant oxidative deterioration of amino acid side chains in myofibrillar protein isolates, ground beef, pork and beef patties, and emulsion-type sausages. Ongoing oxidative reactions were observed during *in vitro* gastric and duodenal digestion of meat patties and emulsion-type sausages, and higher levels of protein oxidation were found in digests from meat samples that had been oxidized prior to digestion. Oxidation during both storage and digestion was more abundant in beef than pork patties. This can be ascribed to the higher heme iron content in beef, which is known to contribute to ROS formation through Fenton-like reactions.

6.2.1 Thiol oxidation

The 4-DPS method for quantification of free and total thiols was applied on ground beef during high-oxygen storage in Chapter 2, and results revealed a significant decrease of free thiols during the course of the experiment, suggesting that thiol oxidation took place. The decrease in total thiols suggested that in oxidized meat samples, the borohydride reduction step was not able to restore the total thiol level from fresh meat, signifying that not all thiol loss was reversible. Similar results were found in Chapter 4 and 5 during illuminated storage under atmospheric air and subsequent *in vitro* digestion of patties (pork and beef) and emulsion-type sausages, respectively. Here, the level of free thiols decreased significantly during storage and dropped dramatically after digestion (decrease up to 92%), while the total thiol level decreased only after digestion. This partly irreversible thiol loss, either during storage or digestion, suggests that other thiol oxidation mechanisms than disulfide formation alone took place (**H6 partly rejected**). Although thiol loss in meat was previously ascribed mainly to reversible disulfide formation, the results from this thesis suggest the formation of other, irreversible thiol oxidation products, such as sulfinic or sulfonic acid and thiosulfinates (Nagy et al., 2010), or irreversible reactions with other meat components. The reversibility of thiol oxidation in meat is an important observation with regard to further development of strategies to delay or repair thiol oxidation. LC-MS/MS analysis could provide more information on the type of thiol oxidation products that are formed during storage and digestion of meat and meat products.

6.2.2 Carbonylation

Carbonylation was evaluated in myofibrillar proteins (subjected to MCO), and meat patties and emulsion-type sausages (subjected to illuminated storage under atmospheric air and subsequent *in vitro* digestion). Similar patterns were observed during the experiments; first an increase in carbonyl compounds was measured, followed by a decrease at the end of storage or after

digestion. This progression demonstrates that oxidation is a dynamic process that should be evaluated in the course of time. Measurements at a single time point do provide limited information on the kinetics of protein oxidation and the state of oxidation of different samples. The decrease in protein carbonyl compounds suggests that ongoing reactions with carbonyl groups took place, and this was in fact shown in the myofibrillar proteins where a significant increase in Schiff base structures was measured simultaneously with protein carbonyl decrease. It is however likely that also other carbonyl reactions than SB cross-linking alone took place, such as formation of carboxylic acids (e.g. α-amino adipic acid or AAA), aldol condensation products, and Strecker aldehydes (Estévez, 2011). Furthermore, reactions of protein carbonyl groups with non-protein compounds are likely to occur in complex matrices such as meat products.

In Chapter 4 and 5 of this thesis, both total and specific (GGS/AAS) carbonylation was analyzed on the same meat samples and digests. All protein carbonylation markers followed the familiar pattern of increase and decrease during storage and digestion, however the slope and peak of the carbonylation curves differed among total carbonyls, GGS and AAS. This suggests that amino acids such as arginine, proline and lysine are not equally susceptible to carbonylation, possibly because of their accessibility within the protein core. Furthermore, the amount of total carbonyls was considerably higher than the sum of GGS and AAS (**H7 rejected**). Although direct carbonylation of amino acid side chains is suggested to be the main route for protein carbonylation, and GGS and AAS are often highlighted as the most abundant protein carbonyls (Estévez, 2011), The formation of other direct protein carbonyls, as well as indirect protein carbonylation through protein lipoxidation or glycation and glycoxidation reactions warrants consideration.

6.2.3 Hydroxylation

In Chapter 4, a significant increase of 4-OH-Phe was found during storage of beef patties, suggesting that the aromatic amino acid phenylalanine was oxidized. After *in vitro* digestion of the samples, 4-OH-Phe levels were significantly lower, signifying that ongoing reactions took place,

such as formation of dityrosine or dihydroxyphenylalanine (DOPA) (Maskos et al., 1992; Stadtman et al., 1997). Hydroxylation of phenylalanine may influence the nutritional value of meat and meat products, since phenylalanine is an essential amino acid. Furthermore, when reaching the colon, 4-OH-Phe is fermented into phenol and *p*-cresol by colonic bacteria. These potentially mutagenic metabolites are believed to be largely detoxified in the liver and urinarily excreted (Evenepoel et al., 1998). However residual faecal *p*-cresol has been correlated with promutagenic DNA adducts in the colon (Winter et al., 2011). In Chapter 4, duodenal digests from oxidized meat samples contained up to 57% more 4-OH-Phe than digests from fresh meat. Further research should determine whether this influences bacterial fermentation in the colon and the level of residual faecal *p*-cresol, and ultimately its possible impact on human health.

6.3 APPLE PHENOLICS AS ANTIOXIDANTS AGAINST PROTEIN OXIDATION IN MEAT AND MEAT PRODUCTS

In this PhD research, apples were chosen as a source of natural antioxidants because of their high phenolic content, which is preserved very good after spiral-filter pressing for juice production. The press residue or apple pomace, which in this regard is considered as a by-product, could be valorized as a phenolic-rich meat ingredient to inhibit oxidation. The effects of apple phenolics (in the form of pure phenolic compounds, an apple peel extract or freeze dried apple pomace) as a source of natural antioxidants against protein oxidation were investigated in myofibrillar proteins (Chapter 3) and emulsion-type sausages (Chapter 5).

6.3.1 Apple phenolics in myofibrillar proteins and emulsion-type sausages

In myofibrillar proteins, the selected pure phenolic compounds and the apple peel extract showed significant antioxidative effects against GGS, AAS and SB formation. (-)-Epicatechin inhibited the carbonylation pathway most efficiently, while phloridzin exhibited the weakest antioxidant effect. The higher concentrations of apple peel extract showed inhibition of AAS, GGS and SB formation

similar to the (-)-epicatechin treatment. These results demonstrate that certain apple phenolics can be a good source of natural antioxidants in meat protein suspensions when applied in the right concentration, suggesting the potential role of apple by-products as natural inhibitors of protein oxidation in meat and meat products.

When enriching emulsion-type sausages with freeze dried apple pomace, contradictory results for protein oxidation were found. Results for free and total thiols (4-DPS assay) and total carbonyls (DNPH assay) revealed that more (irreversible) thiol oxidation and carbonylation took place in the presence of freeze dried apple pomace as compared to a control treatment (without antioxidants added), suggesting that apple phenolics stimulated oxidation. However, as mentioned in Section 6.1, caution must be exercised with interpreting spectrophotometric methods. Like thiols and carbonyls, phenolics are highly reactive compounds; upon oxidation of the hydroxyl group on the phenolic ring (e.g. by donating hydrogen atoms to stabilize radicals), a quinone structure is formed, which in turn is able to react with protein thiols (Ozdal et al., 2013; Jongberg et al., 2015a). Hence, these covalent protein-phenol interactions would lead to a decrease in free and total thiols as detected with 4-DPS. What is more, such protein-phenol interactions might interfere with total carbonyl measurement, since carbonyl moieties on protein bound quinone may react with DNPH, resulting in an overestimation of primary protein carbonylation. This hypothesis was strengthened upon UHPLC-FLD analysis, revealing no significant increase of GGS or AAS during storage of the apple treatments, and no significant differences from the control treatment. Hence, the higher carbonyl levels that were measured with DNPH, were caused either by other primary carbonyls than GGS or AAS, or by secondary protein carbonyls (e.g. from protein bound phenols). It is important to notice that this increased thiol loss and total carbonylation in the presence of freeze dried apple pomace was already observed at the start of the experiment (day 0 before digestion), suggesting that protein-phenol interactions had already taken place during manufacturing of the emulsion-type sausages. Although phenol interactions might prevent further direct protein oxidation, it has been suggested that protein bound phenol is able to re-oxidize and bind with another amino acid side chain, forming a protein-phenol-protein cross-link (Ozdal et al., 2013;

Jongberg et al., 2015a). In this regard, apple phenolics could contribute to the formation of phenol mediated cross-links, which could negatively influence sensory aspects and technological properties. Such interfering phenol mediated cross-links were in fact suggested to occur in emulsion-type sausages treated with high doses of green tea extract (1500 ppm) (Jongberg et al., 2015b). It is likely that the concentration of freeze dried apple applied in this PhD research (3% w/w) was too high, resulting in protein-phenol interactions instead of (measurable) antioxidant mechanisms.

In conclusion, the selected pure phenolic compounds and apple peel extract showed inhibition against protein oxidation in myofibrillar protein suspensions (Chapter 3), however there appeared to be interfering protein-phenol interactions when applying freeze dried apple pomace in emulsion-type sausages (Chapter 5), impeding the evaluation of protein oxidation (**H8 undecided**). To understand and possibly avoid or inhibit such protein-phenol interactions, their kind and dose-dependency could be clarified by means of LC-MS/MS analysis in model systems, before gradually extrapolating the protein and phenol matrix to a meat product enriched with apple phenolics as a source of natural antioxidants.

6.3.2 Finding the optimal phenolic concentration

Table 6.1 represents the phenolic content in the apple treatments of myofibrillar proteins and emulsion-type sausages, expressed on weight basis. As the 200 µM apple peel extract was effective in the MPI model system (Chapter 3), theoretically a concentration of 6.5 g GAE/kg protein should be achieved in emulsion-type sausages as well. Considering that (1) according to Diñeiro García et al. (2009), the average total phenolic content of apple pomace is 7.3 g GAE/kg pomace (dry weight), and (2) emulsion-type sausages have an average protein content of 10%, apple pomace powder should be added to the emulsion-type sausages in a concentration of 89 g/kg meat, or 8.9%. This is thrice the amount that was added in the experiment from Chapter 5, and would in practice be hardly feasible without altering technological and sensory properties of the meat product.

Table 6.1: Proximate phenolic content in myofibrillar proteins treated with 200 μM apple peel extract, and emulsion-type sausages treated with 3% freeze dried apple pomace.

	Myofibrillar proteins (Chapter 3)	**Emulsion-type sausages (Chapter 5)**	
	mg/kg protein	**mg/kg meat**	**mg/kg protein**
Gallic acid equivalents	6531[1]	219[3]	2258
Chlorogenic acid	121.6[2]	0.1[4]	1.2
(-)-Epicatechin	175.7[2]	0.8[4]	8.3
Phloridzin	25.9[2]	0.4[4]	3.9

[1]Calculated from the total phenolic content (Folin-Ciocalteu) in apple peel extract treatment (200 μM GAE). [2]Calculated from phenolic content (LC-MS quantification) in apple peel extract (Chapter 3). [3]Calculated from the mean total phenolic content (Folin-Ciocalteu) of six apple pomaces (Diñeiro García et al., 2009). [4]Calculated from phenolic content of apple pomace derived from LC-MS quantification by De Paepe et al. (2015b) (Chapter 5).

One should however be very cautious when comparing these phenol:protein ratios, and when using them to predict the antioxidant outcome. The oxidative conditions in both experimental designs differed greatly. The model system in Chapter 3 was specifically designed to promote metal-catalyzed oxidation by adding iron and H_2O_2 at 37 °C. The concentrations and temperature in this model were chosen to enlarge and accelerate the effects of oxidation and antioxidants, allowing to elucidate the underlying mechanisms. In Chapter 5, the oxidative conditions did simulate retail display (refrigerator temperature and exposed to light). Moreover, protein oxidation was studied in a different matrix. In Chapter 3, the isolation of myofibrillar proteins minimized matrix interference, whereas in the emulsion-type sausages from Chapter 5, other meat components such as oxidizing lipids are likely to influence protein oxidation and antioxidant mechanisms. Finally, some caution should be made in interpreting the phenolic content in apple extract or powder. In order to determine the phenolic content, either by spectrophotometric methods or by LC-MS, an extract must first be made. As illustrated in Chapter 3, the choice of solvent, as well as other factors such as sample preparation, solvent:sample ratio, and extraction time, temperature and pressure, will influence the extraction rate. It is unlikely that all phenolics will be extracted and included in the assay, leading to an underestimation when expressed on

weight basis. On the other hand, when adding apple (pomace) powder to meat products (as a whole instead of as an extract), little is known about the availability of the phenolic compounds. The severe comminution during cuttering of the meat batters is likely to improve the release and availability of phenolics as a source of antioxidants, however microscopic images of the cell integrity should confirm this.

6.3.3 Valorization of apple by-products as a source of natural antioxidants in meat and meat products

As with all antioxidants, the correct dosage is of utmost importance to ensure an optimal functionality. Because phenolics are only a small fraction of apple pomace, it will likely be necessary to make an extract, to concentrate the phenolics and limit interactions from other apple compounds such as fibers. After all, antioxidants are a group of food additives which are used to extend the shelf-life of food products without altering their sensory or nutritional properties (Shahidi et al., 2015), so they should be added in small, concentrated amounts. Considering that fruit and vegetable by-products are often processed in animal feed or used for renewable energy, a cost-benefit analysis should be made when valorizing apple by-products as natural antioxidants in meat and meat products; costs for extraction procedures and safety regulations must be taken into account.

6.4 EFFECTS OF PROTEIN OXIDATION AND APPLE PHENOLICS ON DIGESTION OF MEAT AND MEAT PRODUCTS

Protein oxidation in meat has been suggested to influence proteolytic breakdown during gastric and duodenal digestion in several ways. Mild protein oxidation is believed to enhance enzyme accessibility by partial unfolding, thereby increasing proteolysis. Severe oxidation, on the other hand, reduces enzyme accessibility by polymerization and aggregation, thereby decreasing proteolysis (Bax et al., 2012). Furthermore, some gastric and duodenal proteases such as trypsin preferentially cleave proteins at arginine and lysine residues (Gasteiger et al., 2005), and oxidation of these amino acids may impair protease recognition. In this PhD thesis, fresh and oxidized pork and beef patties and emulsion-type sausages (enriched with freeze dried apple pomace) were subjected to an *in vitro* digestion model, specifically designed for studying oxidation processes during passage in the gastrointestinal system (Van Hecke et al., 2014a). Quantification of proteolysis in the duodenal digests provided information about the effect of protein oxidation on proteolysis during digestion.

6.4.1 Effect of protein oxidation and apple phenolics on proteolysis during *in vitro* digestion of meat and meat products

After *in vitro* digestion of fresh and oxidized beef and pork patties (Chapter 4) and emulsion-type sausages (Chapter 5), a decrease in proteolysis was observed as the samples were more oxidized. This suggests that the proteolytic enzymes in the gastric and duodenal juices (1) were structurally hindered to reach the peptide backbone in the oxidized meat samples because of protein cross-linking, polymerization and aggregation, and/or (2) did not recognize their cleavage sites because of oxidative modification of amino acid side chains. Hence, protein oxidation during storage results in impaired proteolysis during digestion (**H9 accepted**). As mentioned above, ongoing protein oxidation was observed in the meat digests. The severe thiol loss after digestion suggested

that at least part of these thiols were oxidized into intra- and intermolecular disulfide bonds. Furthermore, the decrease in carbonyls that was measured after digestion could be caused by carbonyl mediated cross-link formation. These results suggest that radical attack during digestion, resulting in thiol and carbonyl mediated cross-links, happened faster than the proteolytic attack by digestive enzymes.

After *in vitro* digestion of emulsion-type sausages enriched with freeze dried apple pomace, slightly less proteolysis was observed as compared to the control treatment (without apple phenolics). It is likely that protein-phenol interactions in the apple treatment impaired proteolytic attack by forming phenol mediated cross-links or altering recognition sites. As discussed above, the protein-phenol interactions most likely are dose-dependent, and until this has been clarified, no conclusions can be made about the effect of apple phenolics as antioxidants on impaired proteolysis caused by protein oxidation (**H10 undecided**).

6.4.2 Health impact of protein oxidation and apple phenolics in meat and meat products

It has been reported that when proteins are poorly hydrolyzed in the small intestine, they are intensely fermented by colonic bacteria (Evenepoel 1998). Impaired proteolysis of oxidized meat proteins in the stomach and duodenum could therefore increase the amount of potentially mutagenic or carcinogenic bacterial fermentation metabolites in the colon (e.g. phenol and *p*-cresol from 4-OH-Phe). Such metabolites could be analyzed in colonic digests if a colonic fermentation stage would be added to the *in vitro* digestion model. In this aspect, it is important to note that with a static *in vitro* digestion model such as the one applied in this PhD research, the absorption of amino acids and metabolites through the lumen of the small and large intestine is not taken into account. It is likely that part of the oxidized proteins are still hydrolyzed into small peptides and amino acids and absorbed in the upper gastrointestinal tract, escaping colonic fermentation. If so, it is unknown what effect the oxidative modifications of these dietary amino

acids have on protein synthesis in the human body (Soladoye et al., 2015). If oxidized proteins do reach the colon and are fermented, further research should determine whether the formed metabolites are harmful for the human body, or simply detoxified and excreted. Hence, the bioavailability of oxidized meat proteins and their possible colonic metabolites remains to be elucidated.

Adding apple phenolics to meat products as a source of natural antioxidants arises more questions about the effects on digestion, bioavailability and health. In medical science, (natural) phenolic antioxidants are often praised as being beneficial for human health by protecting the human body from ROS damage (Shahidi et al., 2015). It is however more complicated than that, since some antioxidants, either synthetic or natural, can exhibit pro-oxidant and even carcinogenic activity under certain conditions (Boudet, 2007). Furthermore, the health impact of dietary phenolic compounds also depends on the composition of the diet and interactions with other dietary components (Shahidi et al., 2015). Moreover, ingested polyphenols are hydrolyzed and degraded in the colon, and these degradation products should also be taken in consideration (Boudet, 2007). Hence, the bioavailability and lack of toxicity of natural antioxidants remains to be confirmed.

6.5 LIMITATIONS AND FUTURE PROSPECTS

In this PhD thesis, some final thoughts and issues came forward that deserve further consideration and research.

i. When evaluating protein oxidation in meat, the choice of protein oxidation markers must be considered well. To do so, the basic principles of each assay should be taken into account: free and total thiols (4-DPS assay) are determined in soluble proteins after denaturation, carbonyls and 4-OH-Phe (DNPH and UHPLC-FLD) are determined in proteins after TCA precipitation.

ii. To optimize the 4-DPS method for free and total thiol quantification, a spike addition with oxidized glutathione could be included in order to take the recovery of the reduction procedure into account.

iii. Other peaks in the UHPLC-FLD chromatograms for GGS/AAS analysis, especially those that increase upon oxidation, require identification with GC/MS. For now, all that is known is that the compounds representing those peaks either derivatize with ABA during sample preparation, or that they are fluorescent as such at the given wavelengths. They might be other oxidation products that are not necessarily carbonyl compounds.

iv. As with protein oxidation, a single marker does not allow to fully comprehend the manifestations of lipid oxidation. Additional to the TBARS assay, the peroxide value could be determined as a measure for the formation of hydroperoxides (primary lipid oxidation), and specific secondary lipid oxidation products such as 4-HNE and MDA could be quantified using HPLC.

v. Potential synergistic or antagonistic interactions among phenolic antioxidants towards protein oxidation can be studied in more detail by adding combinations of pure phenolic compounds to model systems.

vi. Microscopic analysis of the apple pomace would enable to evaluate the cell integrity, helping to understand the availability of the phenolics for antioxidant activities or for protein interactions.

vii. The nature and dose-dependency of protein-phenol interactions require further investigation by means of LC-MS/MS.

viii. Heating during meat processing might affect the functionality of phenolics. Although phenolics are known to have good heat resistance *in vitro*, they might act differently to high temperatures when incorporated in meat batters, resulting in decreased antioxidant activity or increased protein-phenol interactions.

ix. The effect of apple phenolics on colour formation in cured meat products, as well as their effect as an antioxidant against colour oxidation, remains to be elucidated.

x. Studies on the role of nitrite towards protein oxidation in meat were thus far inconclusive. As nitrite is a highly reactive compound, its potential synergistic or antagonistic antioxidant effect should be considered when evaluating oxidation in cured meat products.

xi. The potential loss of essential amino acids because of protein oxidation should be evaluated by determination of the amino acid composition.

xii. Finally, it is worth emphasizing the importance of the origin of the meat when studying and comparing oxidation. Diversity with regard to animal species, muscle type, redox status of the muscle, and the presence of naturally occurring prooxidants and antioxidants (e.g. through animal feed) may have great influence on the progression of oxidation. A similar comment can be made about the origin of apple phenolics, because the phenolic profile and content depends on several factors such as variety, cultivar, geographic origin, growing year, weather conditions, maturity stage, crop load, and fruit position within the canopy. This biological variability is inevitable when studying natural products, and requires consideration when standardizing antioxidant treatments.

Summary

Protein oxidation has been studied in medical science for many decades, however in food science, it is a relatively new research topic. Research on oxidation in meat was thus far mainly focused on the lipid fraction, resulting in the typical rancid off-odours and off-flavours. However, oxidation of meat proteins can be detrimental for meat quality as well, since oxidative damage of amino acid side chains and the peptide backbone may affect technological, sensory or nutritional properties. A literature review on the possible consequences of protein oxidation in meat, and the inhibition hereof by means of antioxidants, was provided in **Chapter 1** of this PhD thesis.

Oxidation of the thiol group on the cysteine side chain in meat was investigated in **Chapter 2**. A new method for the quantification of free and total thiols in meat was introduced, based on thiol detection with 4,4'-dithiodipyridine (4-DPS) and reduction with sodium borohydride. This method was conducted on ground beef during storage under high-oxygen atmosphere at 4 °C in two independent trials. In trial 1, only reversible thiol oxidation was observed, with 30% thiol loss after 9 days of storage. In trial 2, 33% thiol loss occurred after 12 days of storage, of which ca. half was caused by irreversible thiol oxidation during the first days of storage, suggesting the presence of thiol oxidation products other than reversible disulfides. The results were compared with SDS-PAGE analysis of cross-linked myosin heavy chain formed by disulfide bonding. Both methods confirmed increasing disulfide formation due to thiol oxidation in meat during storage, but the 4-DPS method showed higher disulfide percentages than the SDS-PAGE method (22.2 ± 0.3% and 8.5 ± 1.2%, respectively). It was concluded that the 4-DPS assay provides an accurate method to evaluate the thiol oxidation and its reversibility in meat, and can be useful in the development of strategies to avoid or repair thiol oxidation.

In **Chapter 3**, the carbonylation pathway and the effect of apple phenolics were investigated in myofibrillar proteins during *in vitro* metal-catalyzed oxidation (Fe^{3+}/H_2O_2, 37 °C, 10 days). Three pure phenolic compounds (chlorogenic acid, (-)-epicatechin and phloridzin) and an apple peel extract were added to myofibrillar protein suspensions in three concentrations (50, 100 and 200 μM), and a blank treatment was included as a control. Protein oxidation was evaluated as specific carbonylation (α-amino adipic and γ-glutamic semialdehydes, or AAS and GGS) and Schiff

base cross-link formation. Significant inhibition of protein carbonylation was observed in most of the phenolic treatments, depending on the chemical structure of the pure phenolic compound ((-)-epicatechin > chlorogenic acid > phloridzin), and the applied concentration (200 μM > 100 μM > 50 μM). The higher concentrations of the apple peel extract showed significant inhibition similar to the (-)-epicatechin treatments. Hence, phenolic compounds in apple by-products could be introduced as a source of natural antioxidants against protein oxidation in meat and meat products.

To investigate the effects of protein oxidation on meat digestibility, beef and pork patties were subjected to illuminated storage (4 °C, atmospheric air) and subsequent *in vitro* digestion in **Chapter 4**. Protein oxidation was evaluated as thiol oxidation, total carbonylation, and specific carbonylation (AAS/GGS). Furthermore, 4-hydroxyphenylalanine (4-OH-Phe), a hydroxylation product of phenylalanine, was identified and quantified as a new protein oxidation marker. Proteolysis was measured after digestion to evaluate protein digestibility. After 7 days of storage, significant oxidative modifications were quantified and the oxidative degradation was continued during *in vitro* digestion. The observed effects were more abundant in beef patties. An average of 92% of free thiol loss was measured after digestion, and the decrease in total thiols indicates the formation of irreversible thiol oxidation products during digestion. Results for total and specific carbonyls suggest the presence of other direct protein carbonyls than AAS and GGS, as well as indirect protein carbonylation through protein lipoxidation or glycation and glycoxidation reactions. Protein oxidation before digestion resulted in impaired digestibility, which can be ascribed to thiol and carbonyl mediated cross-links, as well as the oxidative modification of recognition sites of proteolytic enzymes. The identification of 4-OH-Phe in oxidized meat arises questions about its role in colonic metabolism when it is converted into phenol and *p*-cresol.

In **Chapter 5**, the effect of sodium ascorbate and apple phenolics on the oxidative stability of emulsion-type sausages during storage and digestion was investigated. Emulsion-type sausages containing 0.05% sodium ascorbate or 3% freeze dried apple pomace were subjected to illuminated storage (4 °C, atmospheric air) and subsequent *in vitro* digestion. Protein oxidation

was evaluated as thiol oxidation, total carbonylation, and specific carbonylation (AAS/GGS). Proteolysis was measured after digestion to evaluate protein digestibility. During storage and digestion, more thiol loss was observed in the presence of ascorbate and freeze dried apple pomace as compared to the control treatment. This can be ascribed to interactions of degradation products of ascorbate and phenol with the protein thiol group. Results for total and specific carbonylation were inconsistent, strengthening the hypothesis of protein-ascorbate and protein-phenol interactions interfering with spectrophotometric methods. Furthermore, these interactions appeared to decrease protein digestibility, because of ascorbate and phenol mediated cross-links and alteration of proteolytic recognition sites.

In conclusion (**Chapter 6**), protein oxidation in meat and meat products is a complex and dynamic phenomenon which should be evaluated by means of at least two markers, since no single protein oxidation marker is all inclusive. Protein oxidation during storage and subsequent digestion was shown to impair meat digestibility. The impact of decreased digestibility in the upper intestinal tract on the colonic metabolism requires further research. Apple phenolics were able to inhibit protein carbonylation in myofibrillar protein suspensions, however in emulsion-type sausages there appeared to be interfering protein-phenol interactions, impeding the evaluation of protein oxidation. Further research should elucidate the exact nature and dose-dependency of such interactions, before optimizing (extracts of) apple by-products as a source of natural antioxidants in meat products.

Samenvatting

Eiwitoxidatie wordt al decennialang bestudeerd in de medische wetenschappen, maar het is een relatief nieuw onderzoeksthema in de voedingswetenschappen. Onderzoek omtrent oxidatie in vlees was tot nu toe vooral gericht op de vetfractie, in relatie tot de typische ranzige geur- en smaakafwijking bij vetoxidatie. Oxidatie van vleeseiwitten kan echter ook nadelig zijn voor de vleeskwaliteit, aangezien oxidatieve schade van aminozuurzijketens en de peptideruggengraat de technologische, sensorische en nutritionele eigenschappen kan beïnvloeden. **Hoofdstuk 1** van deze doctoraatsthesis omvat een literatuurstudie over de mogelijke gevolgen van eiwitoxidatie in vlees en de remming hiervan door gebruik te maken van antioxidanten.

Oxidatie van de thiolgroep op de cysteïnezijketen in vlees werd bestudeerd in **Hoofdstuk 2**. Er werd een nieuwe methode ontwikkeld voor de kwantificatie van vrij en totaal thiol in vlees, gebaseerd op thioldetectie met 4,4'-dithiodipyridine (4-DPS) en reductie met natriumborohydride. Deze methode werd toegepast op rundsgehakt tijdens bewaring in een zuurstofrijke verpakking bij 4 °C in twee onafhankelijke proeven. In proef 1 werd enkel omkeerbare thioloxidatie waargenomen, met 30% thiolverlies na 9 dagen bewaring. In proef 2 werd een verlies van 33% thiol vastgesteld na 12 dagen bewaring, waarvan ongeveer de helft veroorzaakt werd door onomkeerbare thioloxidatie gedurende de eerste dagen van bewaring. Dit suggereert de aanwezigheid van andere thioloxidatieproducten dan omkeerbare disulfidebindingen. De resultaten werden vergeleken met SDS-PAGE-analyse van gecrosslinkte myosine zware ketens gevormd door disulfidebinding. De beide methodes bevestigden een toename in disulfidebindingen veroorzaakt door thioloxidatie in vlees tijdens bewaring, maar de 4-DPS-methode leverde hogere disulfide-percentages op dan de SDS-PAGE-methode (respectievelijk 22.2 ± 0.3% en 8.5 ± 1.2%). Er werd geconcludeerd dat de 4-DPS-methode een accurate methode is om (de omkeerbaarheid van) thioloxidatie in vlees te evalueren. De methode kan worden toegepast bij de ontwikkeling van strategieën om thioloxidatie te voorkomen of herstellen.

In **Hoofdstuk 3** werd carbonylatie en het effect van appelfenolen bestudeerd in myofibrillaire eiwitten tijdens *in vitro* metaalgekatalyseerde oxidatie (Fe^{3+}/H_2O_2, 37 °C, 10 dagen). Drie zuivere fenolische componenten (chlorogeenzuur, (-)-epicatechine en phloridzin) en een appelschilextract

werden in drie concentraties (50, 100 en 200 μM) toegevoegd aan myofibrillaire eiwitsuspensies en een blanco behandeling werd toegevoegd ter controle. Eiwitoxidatie werd geëvalueerd aan de hand van specifieke carbonylatieproducten (α-amino adipic en γ-glutamic semialdehyden, of AAS en GGS) en Schiff base crosslinking. Er werd een significante remming van eiwitcarbonylatie waargenomen in de meeste behandelingen met fenolische componenten, afhankelijk van de chemische structuur van de zuivere fenolische component ((-)-epicatechine > chlorogeenzuur > phloridzin) en de toegepaste concentratie (200 μM > 100 μM > 50 μM). De hogere concentraties aan appelschilextract vertoonden een significante remming die gelijkaardig was aan de (-)-epicatechinebehandelingen. Fenolische componenten in nevenstromen van appel kunnen dus mogelijks dienen als een bron van natuurlijke antioxidanten tegen eiwitoxidatie in vlees en vleesproducten.

Om het effect van eiwitoxidatie op de vertering van vlees te onderzoeken, werden runds- en varkensburgers bewaard onder licht (4 °C) en vervolgens onderworpen aan een *in vitro* vertering in **Hoofdstuk 4**. Eiwitoxidatie werd geëvalueerd als thioloxidatie, totale carbonylatie en specifieke carbonylatie (AAS/GGS). Bovendien werd 4-hydroxyfenylalanine (4-OH-Phe), een hydroxylatieproduct van fenylalanine, geïdentificeerd en gekwantificeerd als een nieuwe merker voor eiwitoxidatie. Na de vertering werd proteolyse gemeten om de eiwitverteerbaarheid te evalueren. Na 7 dagen bewaring werden significante oxidatieve wijzigingen waargenomen die werden verder gezet tijdens *in vitro* vertering. De waarnemingen waren meer uitgesproken in rundsburgers. Na vertering werd gemiddeld 92% verlies aan vrij thiol gemeten en de daling in totaal thiol wijst op de vorming van onomkeerbare thioloxidatieproducten tijdens vertering. De resultaten voor totale en specifieke carbonylatie suggereren de aanwezigheid van andere directe eiwitcarbonylproducten dan AAS en GGS en/of indirecte eiwitcarbonylatie via oxiderende vetten en suikers. Eiwitoxidatie voor vertering resulteerde in een verminderde verteerbaarheid. Dit kan worden toegeschreven aan thiol- en carbonylgemedieerde crosslinks en de oxidatieve wijziging van herkenningsplaatsen voor proteolytische enzymen. Door de identificatie van 4-OH-Phe in

geoxideerd vlees kunnen vragen worden gesteld over de gezondheidsimpact wanneer 4-OH-Phe in de dikke darm wordt gemetaboliseerd in fenol en *p*-cresol.

In **Hoofdstuk 5** werden de effecten van natriumascorbaat en appelfenolen op de oxidatieve stabiliteit van kookworst onderzocht tijdens bewaring en vertering. Kookworsten met 0.05% natriumascorbaat of 3% gevriesdroogde appelpomace werden bewaard onder licht (4 °C) en vervolgens onderworpen aan een *in vitro* vertering. Eiwitoxidatie werd geëvalueerd als thioloxidatie, totale carbonylatie en specifieke carbonylatie (AAS/GGS). Na de vertering werd proteolyse gemeten om de eiwitverteerbaarheid te evalueren. Tijdens bewaring en vertering werd, vergeleken met de controle, meer thiolverlies waargenomen in de aanwezigheid van ascorbaat en gevriesdroogde appelpomace. Dit kan worden verklaard door interacties van degradatieproducten van ascorbaat en fenolen met de thiolgroep in vleeseiwitten. De resultaten van totale en specifieke carbonylatie waren tegenstrijdig, wat de hypothese versterkt over eiwit-ascorbaat- en eiwit-fenol-interacties die interfereren met de spectrofotometrische methoden. Dergelijke interacties leken bovendien de verteerbaarheid van vleeseiwitten te verminderen, door ascorbaat- en fenolgemedieerde crosslinks en de verandering van proteolytische herkenningsplaatsen.

Er kan worden geconcludeerd (**Hoofdstuk 6**) dat eiwitoxidatie in vlees en vleesproducten een complex en dynamisch fenomeen is dat met minstens twee testen moet worden geëvalueerd, aangezien geen enkele test alomvattend is. Eiwitoxidatie tijdens bewaring en vertering zorgt voor een verminderde verteerbaarheid van vlees. De impact van dergelijke verminderde verteerbaarheid in de maag en dunne darm op het metabolisme in de dikke darm moet verder worden onderzocht. Appelfenolen waren in staat om eiwitcarbonylatie te remmen in myofibrillaire eiwitten, maar in kookworst belemmerden interfererende eiwit-fenol-interacties de evaluatie van eiwitoxidatie. Verder onderzoek zal de aard en dosisafhankelijkheid van zo'n interacties moeten achterhalen om (extracten van) nevenstromen van appel te optimaliseren als een bron van natuurlijke antioxidanten in vlees en vleesproducten.

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Curriculum Vitae

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WORK EXPERIENCE

2012 – 2016 **Doctoral researcher in applied biological sciences**
Institute for Agricultural and Fisheries Research (ILVO), Melle, Belgium

Focus on amino acid oxidation in meat proteins (thiol oxidation, carbonylation, hydroxylation) and effects of phenolic antioxidants

2013 **International internship: Method development**
University of Copenhagen, Copenhagen, Denmark

Development of spectrophotometric method for quantification of thiol oxidation in meat proteins

2012 **International internship: Collaborative study**
University of Extremadura, Cáceres, Spain

Training of HPLC method and experiment on effects of apple phenolics on carbonylation of myofibrillar proteins

2011 - 2012 **Research associate**
ILVO, Melle, Belgium

Flanders' FOOD project on correlation between physico-chemical and sensory analyses of meat products

2010 – 2011 **QA assistant**
Dekeyzer-Ossaer nv, Koekelare, Belgium

2009 **International internship: Food safety implementation**
Amdo Food Company, New Delhi, India

Internship for master thesis in cooperation with Oxfam (Belgium) and Fair Trade Original (The Netherlands)

EDUCATION

2012 – 2017 **Doctor of Applied Biological Sciences**
University Ghent, Ghent, Belgium

Thesis: Protein oxidation in meat products: Effects of apple phenolics during storage and digestion
Promotors: Prof. dr. ir. Stefaan De Smet
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2006 – 2010 **Master of Applied Bio-engineering: Food-industry**
Hogeschool Gent (now University Ghent), Ghent, Belgium

Thesis: Implementation of a food safety system in a noodle factory in India
Promotor: Prof. dr. ir. Mia Eeckhout

2004 – 2006 **Bachelor of Medical Imaging**
EHSAL (now Odisee), Brussels, Belgium

1998 – 2004 **Sciences – Mathematics (8h)**
IHK Ardooie, Belgium

COURSES

2013 **Advanced Meat Science and Technology**
Ege University Food Engineering Department, Izmir, Turkey

2012 **Multivariate statistics**
Institute for Continuing Education in Science (ICES), Ghent, Belgium

2011 **Statistics: basics + multivariate statistics and sampling**
Agentschap voor Overheidspersoneel (AgO), Brussels, Belgium

2011 **Science communication**
Kluwer & Flanders' FOOD, Ghent, Belgium

2011 **Sensory evaluation**
Flanders' FOOD, Kruishoutem, Belgium

PUBLICATIONS

A1 articles

Berardo, A., De Maere, H., Stavropoulou, D.A., Rysman, T., Leroy, F., & De Smet, S. (2016). Effect of sodium ascorbate and sodium nitrite on protein and lipid oxidation in dry fermented sausages. *Meat Science, 121*, 359-364.

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Conference proceedings

Rysman, T., Van Hecke, T., Van Weyenberg, S., De Smet, S., & Van Royen, G. (2015). Potential mechanisms of protein oxidation in cooked sausage as affected by sodium ascorbate and apple phenolics. BAMST Symposium, 2 December 2015, Melle, Belgium. (oral presentation)

Berardo, A., De Maere, H., Stavropoulou, D. A., Rysman, T., Leroy, F., & De Smet, S. (2015). Differential effects of the curing agents sodium ascorbate and sodium nitrite on protein oxidation in dry fermented sausages. 61st International Congress of Meat Science and Technology, 23-28 August 2015, Clermont-Ferrand, France, Short paper 7.43. (poster presentation)

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