Effect of phenolic-rich plant materials on protein and lipid oxidation reactions

Hanna Salminen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry, University of Helsinki, for public criticism in lecture hall B3, Viikki, on April 3rd 2009, at 12 o'clock noon.

Helsingin yliopisto
Soveltavan kemian ja mikrobiologian laitos

University of Helsinki
Department of Applied Chemistry and Microbiology

Helsinki 2009

Custos: Professor Vieno Piironen
Department of Applied Chemistry and Microbiology
University of Helsinki
Helsinki, Finland

Supervisor: Docent Marina Heinonen
Department of Applied Chemistry and Microbiology
University of Helsinki
Helsinki, Finland

Reviewers: Professor Rosario Zamora
Instituto de la Grasa
Spanish National Research Council (CSIC)
Seville, Spain

Docent Maija-Liisa Mattinen
VTT Technical Research Center of Finland
Espoo, Finland

Opponent: Professor Karin Schwarz
Institute of Human Nutrition and Food Science
University of Kiel
Kiel, Germany

ISBN 978-952-10-5371-9 (paperback)
ISBN 978-952-10-5372-6 (pdf; http://ethesis.helsinki.fi)
ISSN 0355-1180

Helsinki University Print
Helsinki 2009

Salminen, H. 2009. Effect of phenolic-rich plant materials on protein and lipid oxidation reactions (dissertation). EKT-series 1444. University of Helsinki. Department of Applied Chemistry and Microbiology.

ABSTRACT

The antioxidant activity of natural plant materials rich in phenolic compounds is being widely investigated for protection of food products sensitive to oxidative reactions. In this thesis plant materials rich in phenolic compounds were studied as possible antioxidants to prevent protein and lipid oxidation reactions in different food matrixes such as pork meat patties and corn oil-in water emulsions. Loss of anthocyanins was also measured during oxidation in corn oil-in-water emulsions. In addition, the impact of plant phenolics on amino acid level was studied using tryptophan as a model compound to elucidate their role in preventing the formation of tryptophan oxidation products. A high-performance liquid chromatography (HPLC) method with ultraviolet and fluorescence detection (UV-FL) was developed that enabled fast investigation of formation of tryptophan derived oxidation products.

Byproducts of oilseed processes such as rapeseed (*Brassica rapa* L.), camelina (*Camelina sativa*) and soy meal (*Glycine max* L.) as well as Scots pine bark (*Pinus sylvestris*) and several reference compounds were shown to act as antioxidants toward both protein and lipid oxidation in cooked pork meat patties. In meat, the antioxidant activity of camelina, rapeseed and soy meal were more pronounced when used in combination with a commercial rosemary extract (*Rosmarinus officinalis*).

Berry phenolics such as black currant (*Ribes nigrum*) anthocyanins and raspberry (*Rubus idaeus*) ellagitannins showed potent antioxidant activity in corn oil-in-water emulsions toward lipid oxidation with and without β-lactoglobulin. The antioxidant effect was more pronounced in the presence of β-lactoglobulin. The berry phenolics also inhibited the oxidation of tryptophan and cysteine side chains of β-lactoglobulin. The results show that the amino acid side chains were oxidized prior the propagation of lipid oxidation, thereby inhibiting fatty acid scission. In addition, the concentration and color of black currant anthocyanins decreased during the oxidation.

Oxidation of tryptophan was investigated in two different oxidation models with hydrogen peroxide (H_2O_2) and hexanal/$FeCl_2$. Oxidation of tryptophan in both models resulted in oxidation products such as 3a-hydroxypyrroloindole-2-carboxylic acid, dioxindolylalanine, 5-hydroxy-tryptophan, kynurenine, *N*-formylkynurenine and β-oxindolylalanine. However, formation of tryptamine was only observed in tryptophan oxidized in the presence of H_2O_2. Pine bark phenolics, black currant anthocyanins, camelina meal phenolics as well as cranberry proanthocyanidins (*Vaccinium oxycoccus*) provided the best antioxidant effect toward tryptophan and its oxidation products when oxidized with H_2O_2. The tryptophan modifications formed upon hexanal/$FeCl_2$ treatment were efficiently inhibited by camelina meal followed by rapeseed and soy meal. In contrast, phenolics from raspberry, black currant, and rowanberry (*Sorbus aucuparia*) acted as weak prooxidants.

This thesis contributes to elucidating the effects of natural phenolic compounds as potential antioxidants in order to control and prevent protein and lipid oxidation reactions. Understanding the relationship between phenolic compounds and proteins as well as lipids could lead to the development of new, effective, and multifunctional antioxidant strategies that could be used in food, cosmetic and pharmaceutical applications.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Applied Chemistry and Microbiology, Food Chemistry Division, at the University of Helsinki. The work was funded by the Academy of Finland (Interaction reactions of functional food components: lipids, proteins and phenolic antioxidants, project 201320), the Finnish Graduate School on Applied Bioscience – Bioengineering, Food and Nutrition, Environment (ABS), the Finnish Cultural Foundation, and the Food Research Foundation (ETS). Their financial support is gratefully acknowledged.

My deepest gratitude goes to my supervisor Docent Marina Heinonen for her support and advice during my work. Working with her has been inspirational. I also wish to express my sincere gratitude to Professor Vieno Piironen for introducing me to the fascinating world of food science. Thank you also for the valuable comments during the writing process of this thesis.

I thank all my co-authors, especially Ph.D. Satu Vuorela, Ph.D. Mario Estévez, Ph.D. Riitta Kivikari and M. Sc. Helena Jaakkola for enjoyable co-operation.

I wish to thank all my former and present colleagues in Viikki D-building for a warm working environment. Especially I want to thank Satu Vuorela, Kaarina Viljanen, and Petri Kylli for sharing the office and many conversations with me.

Part of the data for the thesis was performed at the Department of Food Science, University of Massachusetts Amherst, MA, USA. I am grateful for Professor Eric Decker for the opportunity to work at his laboratory and his invaluable advice as well as acting as a co-author on my paper.

I also want to thank all my friends and colleagues at UMass for a great working atmosphere during my two wonderful years in the USA. Especially I want to thank Ricard Bou, Marianna Iorio, Owen Jones, Young-Hee Cho and Alessandra Arecchi for their friendship and all the great times we shared.

I am very grateful for Professor Rosario Zamora and Docent Maija-Liisa Mattinen for their careful pre-examination of this thesis, their constructive criticism and suggestions for improvements.

I also want to thank my family and friends. Special thanks goes to my mother and sister for their support. Finally, I wish to thank my dear Thrandur. Thank you for sharing your life with me, and understanding both me and my work.

Helsinki, April 2009

LIST OF ORIGINAL PUBLICATIONS

I Vuorela, S., Salminen, H., Mäkelä, M., Kivikari, R., Karonen, M., Heinonen, M. 2005. Effect of plant phenolics on protein and lipid oxidation in cooked pork meat patties. J. Agric. Food Chem. 53, 8492-8497.

II Salminen, H., Estévez, M., Kivikari, R., Heinonen, M. 2006. Inhibition of protein and lipid oxidation by rapeseed, camelina and soy meal in cooked pork meat patties. Eur. Food Res. Technol. 223, 461-468.

III Salminen, H., Heinonen, M. 2008. Plant phenolics affect oxidation of tryptophan. J. Agric. Food Chem. 56, 7472-7481.

IV Salminen, H., Jaakkola, H., Heinonen, M. 2008. Modifications of tryptophan oxidation by phenolic-rich plant materials. J. Agric. Food Chem. 56, 11178-11186.

V Salminen, H., Heinonen, M., Decker, E. A. 2008. Antioxidant effects of berry phenolics incorporated in oil-in-water emulsions with continuous phase β-lactoglobulin. J. Am. Oil Chem. Soc. Submitted.

Contribution of the author to papers I-V

I Hanna Salminen planned the study together with Ph.D. Satu Vuorela, Ph.D. Riitta Kivikari and Docent Marina Heinonen. The experimental work was carried out by M. Sc. student Maija Mäkela. The author had the main responsibility for interpreting the results regarding the part of protein oxidation, and thus she was the second author of the paper.

II Hanna Salminen planned the study together with the other authors. She was also responsible for the experimental work. She had the main responsibility for interpreting the results and hence she was the main author of the paper.

III Hanna Salminen planned the study together with Docent Marina Heinonen. She was responsible for the experimental work and had the main responsibility for interpreting the results. She was the main author of the paper.

IV Hanna Salminen planned the study together with Docent Marina Heinonen. She performed part of the experimental work with M. Sc. student Helena Jaakkola. Hanna Salminen had the main responsibility for interpreting the results, and she was the main author of the paper.

V Hanna Salminen planned the study together with the other authors under supervision of Professor Eric Decker. She was responsible for the experimental work and had the main responsibility for interpreting the results. She was the main author of the paper.

LIST OF ABBREVIATIONS

ABD-F	4-fluoro-7-aminosulfonylbenzofurazan
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
Acetyl-CoA	acetyl-coenzyme A
α	relative retention
a_w	water activity
$BaCl_2$	barium dichloride
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
Brij 35	polyoxyethylene layrylether hydroxyl
BSA	bovine serum albumin
CMC	critical micelle concentration
CO_2	carbon dioxide
CV	coefficient of variation
DAD	diode array detection
DNPH	2,4-dinitrophenylhydrazones
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	ethylenediaminetetraacetic acid
$E^{0'}$	standard reduction potential
EMP-lysine	*Nε*-(5-ethyl-2-methylpyridinium)-lysine
EPR	electron paramagnetic resonance spectroscopy
ESI	electrospray ionization
ESR	electron spin resonance spectroscopy
FDP-lysine	*Nε*-(3-formyl-3,4-dehydropiperidino)-lysine
$FeCl_2$	ferrous dichloride
$FeSO_4$	ferrous sulphate
FI–CL	flow injection with chemiluminescence detection
FTIR	Fourier transform infraded spectroscopy
GC	gas chromatography
GSH	glutathione (tripeptide of glutamine, cysteine and glycine)
H_2O_2	hydrogen peroxide
HACA	hydroxyaminocaproic acid
HAVA	hydroaminovaleric acid
HMW	high molecular weight
HNE	4-hydroxy-2-alkenal
HCl	hydrochloric acid
HOHICA	3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indolol-2-carboxylic acid
HPLC	high-performance liquid chromatography
HSA	human serum albumin
IRS	inactive forms of reactive oxygen species
k’	capacity factor
L.	Linnaeus, used as the authority for species names in botany
LC	liquid chromatography
LDL	low density lipoproteins
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-off-flight mass spectroscopy
MIAC	*N*-(2-acridonyl)-maleimide
MS	mass spectrometry

MW	molecular weight
MWCO	molecular weight cut-off
N	theoretical plate number
$NaBH_4$	sodium borohydride
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance spectroscopy
pK_a	acid dissociation constant
R_s	resolution
RNase	ribonuclease
RNS	reactive nitrogen species
ROS	reactive oxygen species
RP	reverse phase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPE	solid phase extraction
TFA	trifluoroacetic acid
U	enzyme unit i.e the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute,1 U = 1/60 microkatal = 16.67 nano katal
UV	ultraviolet
WHO	The World Health Organization

CONTENTS

1. INTRODUCTION

Oxidative reactions of lipids and proteins are a major cause of chemical deterioration in food. Free radical mediated oxidation of lipids and proteins arise from reactive oxygen species (ROS) generated during food processing and storage (Davies et al., 1995; Stadtman et al., 2003). Free radicals derived from lipid oxidation reactions are easily transferred to other molecules such as proteins, carbohydrates and vitamins, especially in the presence of metal ions (Schaich, 2008). The nature and extent of reactions involved in food processing depend on the ingredients as well as the processing conditions. The oxidative attacks on macromolecules contribute to deterioration of flavor, aroma, color (unwanted browning reactions), and nutritive value. The protein oxidation leads to loss of amino acids and solubility, changes in texture, alterations in protein functionality and may even lead to formation of toxic compounds (Karel et al., 1975; Rice-Evans et al., 1993). Living organisms are also exposed to ROS. Oxidation of proteins in human body has been linked to changes occurring during aging, and particularly in a variety of diseases and disorders, e.g., infectious diseases, autoimmune diseases as well as neuropsychiatric and neurological disorders (Levine et al., 2001; Levine, 2002).

In order to prevent and control lipid and/or protein oxidation, antioxidant compounds can be added to foods. In recent years the consumer demand for “all natural” products has increased. Therefore, natural plant materials could provide an alternative to synthetic food additives. Plant materials rich in phenolic compounds exhibit a wide range of activities such as antioxidant, antimicrobial, antimutagenic, as well as anti-inflammatory activities (Kähkönen et al., 2001; Vuorela et al., 2005a; Heinonen, 2007). Phenolic compounds act as antioxidants by donating electrons and terminating radical chain reactions (Dangles et al., 2006), as well as chelators by binding metal ions (Fernandez et al., 2002). The role of phenolic compounds in prevention of cardiovascular diseases, cancers, diseases mediated by inflammation or pathogens, and neurodegenerescence is still unknown (Sun et al., 2002; Katsube et al., 2003; Howell et al., 2005; Puupponen-Pimia et al., 2005; Ruel et al., 2005; Wang et al., 2005).

Phenolic compounds include flavonoids, phenolic acids, and tannins that originate mainly from fruits, berries and vegetables, and are also relatively abundant in human diet (Heinonen, 2007). Byproducts of deoiling processes of different oilseeds are rich sources of phenolic compounds, proteins and essential fatty acids, and could thus provide an economical source of bioactive compounds for food, cosmetic and pharmaceutical industries. At present, plant ingredients such as

berries are being applied to various food products claimed to be health beneficial (i.e. functional foods) due to their antioxidant or antimicrobial effect.

During the recent years the research on effects of plant phenolics has mainly focused on lipid oxidation reactions, whereas research on protein oxidation remains scarce. Until now, the role of phenolic antioxidants on protein oxidation has been evaluated using phenolics from berries, grapes, red wine, and tea as well as different flavonols, catechins, phenolic acids and anthocyanidins in oxidation models such as oil-in-water emulsions (Almajano et al., 2004; Viljanen et al., 2005b; Almajano et al., 2007), liposomes (Heinonen et al., 1998; Viljanen et al., 2004b) and low density lipoproteins (LDL) (Milde et al., 2004; Viljanen et al, 2004a; Yeomans et al., 2005; Milde et al., 2007). However, these studies have focused on measuring the overall effect of phenolics on protein oxidation i.e. loss of tryptophan fluorescence and formation of carbonyl derivatives, and do not address what individual oxidation products are actually formed. Thus, it is still unclear what functional groups are the targets for the phenolic antioxidants. Therefore, development of accurate measurement methods leads to their applicability to real foods where protein oxidation reactions may result in changes in food quality and in functional properties of proteins and phenolic compounds. By optimizing the use of bioactive ingredients such as plant phenolics as well as the structure of food containing proteins and other food constituents, further benefits may be gained in the food industry developing more stable foods and foods for health benefits. This will benefit also the consumer.

This thesis reviews the literature concerning protein and amino acid oxidation, their reactions and methods as well as protein – phenolic interactions. The experimental part of this thesis is a summary of the research results published in attached papers **I-V**. The oxidation reactions in pork meat patties, corn oil-in-water emulsions and tryptophan models in the presence of phenolic compounds, and the HPLC method developed for detection of tryptophan oxidation products are evaluated in the Discussion section.

2. LITERATURE REVIEW

2.1 Protein oxidation

2.1.1 Protein oxidation pathways

Proteins in food, cosmetics and pharmaceuticals are prone to oxidation reactions. During food processing and storage and *in vivo*, proteins are modified, for example, via oxidation, glycation and glycoxidation reactions. Free radical mediated oxidation of amino acids and proteins arise from ROS generated as byproducts of normal metabolic processes, or external factors such as processing (e.g. heating, fermentation, application of chemicals), photochemical reactions, the presence of oxygen, air pollutants, and irradiation (γ-, x-, and UV) (Davies et al., 1995; Damodaran, 1996; Stadtman et al., 2003). Free radical species can react directly with the protein or they can react with other molecules such as lipids and carbohydrates, forming products that subsequently react with the protein (**Figure 1**). Thus, the oxidation of proteins, peptides and amino acids leads to altered physicochemical and functional properties, and may even result in formation of toxic compounds (Karel et al., 1975; Rice-Evans et al., 1993). Oxidation of proteins has also been linked to changes occurring during aging, particularly with progression of diseases and disorders in humans (Levine et al., 2001; Levine, 2002).

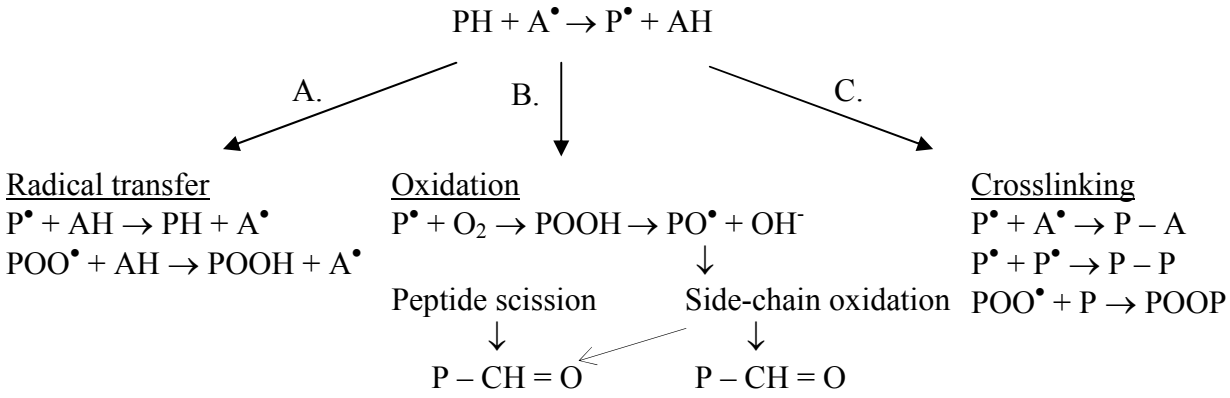


Figure 1. Protein oxidation pathways via A) free radical transfer, B) oxidation, and C) crosslinking (Adapted from Karel et al., 1975, and Schaich, 2008). PH = protein, P• = protein radical, AH = any molecule with abstractable hydrogens, A• = non-protein radical, PO• = alkoxyl radical, POO• = peroxyl radical, POOH = hydroperoxide, P-CH=O = secondary products such as aldehydes.

Free radical transfer

Protein radicals ($P^{\bullet}$) are formed when lipid peroxyl and alkoxy radicals arise from lipid hydroperoxides, and transfer free radicals to proteins by abstracting hydrogens (Karel et al., 1975) (**Figure 1A**). Protein hydroperoxides ($POO^{\bullet}$) and other protein radicals ($P^{\bullet}$) are highly reactive, and thus oxidize to secondary compounds (Davies et al., 1995). The peptide bond in the backbone of the protein or the side-chains of the amino acids may be the target for amino acid modifications. The oxidative modification can cause cleavage of the protein backbone and crosslinking of the side-chains. The reactions are usually highly influenced by redox cycling metals such as iron and copper. In addition, protein radicals can also transfer radicals to other proteins, lipids, carbohydrates, vitamins and other molecules, especially in the presence of metal ions. Radical transfer occurs early in lipid oxidation, and this process underlies the antioxidant effect for lipids. Consequently, it may appear that lipid oxidation is not proceeding whereas the radical transfer to proteins is in its highest (Schaich, 2008).

Oxidation

Backbone fragmentation of proteins occurs via C-C or β-scission that decarboxylates the target amino acid side-chain during exposure to radicals (radiation, oxidizing lipids) in the presence of oxygen as shown in **Figure 2B**. For example, β-scission of alanine, valine, leucine, and aspartic acid side chains generates free formaldehyde, acetone, isobutyraldehyde, and glyoxylic acid, respectively. In each case, cleavage of the side-chain gives α-carbon radical (-NH $^{\bullet}$CHCO-) in the polypeptide chain. This reaction occurs via the formation and subsequent β-scission of the alkoxyl radical (Headlam et al., 2002).

Crosslinking

The general reaction for free radical crosslinking generates usually polymers of intact protein monomers, with and without oxygen bridges (**Figure 1C**) (Schaich, 2008). Oxidative modifications of proteins generating intra- and intermolecular crosslinks can occur by different mechanisms: 1) direct interaction of two carbon-centered radicals, 2) interaction of two tyrosine radicals, 3) oxidation of cysteine sulfhydryl groups, 4) interactions of the carbonyl groups of oxidized proteins with the primary amino groups of lysine side-chains in the same or different protein, 5) reactions of both carbonyl groups of malonaldehyde with two different lysine side-chain in the same or two

different protein molecules, 6) interactions of glycation/glycoxidation derived protein carbonyls with either a lysine or an arginine side-chain of the same or a different protein molecule, 7) interaction of a primary amino group of lysine side-chain with protein aldehydes obtained via Michael addition reactions with the lipid aldehydes such as 4-hydroxy-2-alkenal (HNE) (Stadtman et al., 2003; Stadtman, 2006).

2.1.2 Protein modifications by lipid oxidation

Primary lipid oxidation products

Lipid oxidation products generate multiple reactive species such as hydroperoxides, peroxyl and alkoxyl radicals, carbonyl compounds as well as epoxides which can easily react with non-lipid molecules such as proteins. Lipid hydroperoxyl radicals have low to intermediate reduction potential values ($E^{0\prime}$=1.1-1.5 V, at pH 7) compared to those of hydroxyl radicals ($E^{0\prime}$=2.3 V, pH 7) (Buettner, 1993). Consequently, hydroperoxyl radicals are much more selective in attacking reactive side chains than hydroxyl radicals. Reactions between proteins and free radicals and ROS suggest that proteins could protect lipids from oxidation if they are oxidized preferentially to unsaturated fatty acids. Protein oxidation could be favoured if amino acids are more labile than unsaturated fatty acids, or if the location of the protein enables it to scavenge the free radicals or ROS before they migrate to the lipids (Elias et al., 2008). A study of continuous phase β-lactoglobulin in oil-in-water emulsion showed that tryptophan and cysteine side-chains, but not methionine, oxidized before lipids (Elias et al., 2005). The inaccessibility of methionine to oxidants is probably due to its location in the buried hydrophobic area of β-lactoglobulin.

Transition metal ions can catalyze directly breaking down unsaturated lipids into alkyl radicals but this reaction occurs extremely slowly and is therefore not believed to be important in promoting lipid oxidation. Metal ions catalyzed oxidation of lipid hydroperoxides into formation of reactive radicals (**schemes 1 and 2**) is suggested as the main oxidative pathway in processed foods, especially in oil-in-water emulsions. Redox reactive transition metals such as iron and copper ions are important prooxidants in foods as they are ubiquitous in food ingredients and biological tissues (McClements et al., 2000). The interaction reactions of proteins and lipid radicals are shown in the general reaction pathway in **Figure 1C**. In oil-in-water emulsions iron is a strong prooxidant and it promotes hydroperoxide degradation if it is in close proximity to surface-active lipid hydroperoxides at the emulsion droplet interface. Iron ions (Fe^{2+} and Fe^{3+}) can decompose

hydroperoxides (LOOH) into alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals by the following mechanisms:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\bullet} + OH^{-} \quad \textbf{(Scheme 1)}$$

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO^{\bullet} + H^{+} \quad \textbf{(Scheme 2)}$$

The ability of iron to break down hydroperoxides can depend largely on its physical location relative to the interface of the emulsion droplet. This ability may be slowed down by the presence of large proteins or surfactants on the droplet interphase (McClements et al., 2000). Metal catalyzed oxidation of side chains of lysine, arginine, proline, and threonine yield carbonyl derivatives and histidine side-chains form 2-oxo-histidine (Stadtman et al., 2003). Metalloproteins are especially prone to oxidation due to binding and reducing the lipid hydroperoxides near the ligand site. Most non-metalloproteins have also metal-binding sites, for example on histidine, glutamic acid, or aspartic acid side-chains that enable the metal-catalyzed reactions of hydroperoxides on the protein surfaces. Yuan et al. (2007) showed that iron was bound to the protein surface of β-lactoglobulin oxidized by methyl linoleate.

Lipid epoxides are cyclic products formed by internal reactions of lipid hydroperoxides, peroxyl addition products or alkoxyl radicals, or reaction between alkenals (e.g. hydroxynonenal) and lipid hydroperoxides or hydrogen peroxide. Lipid epoxides exhibit carcinogenic, mutagenic and cytotoxic properties (Chung et al., 1993; Lee et al., 2002). Epoxide adducts are formed when they bind to amino acids such as valine, lysine, serine, histidine and methionine, or to intact proteins such as haemoglobin (Lederer, 1996; Moll et al., 2000). Reactions between epoxides and proteins are most important under anhydrous conditions, e.g., in dry foods and in hydrophobic interior of biomembranes and blood lipoproteins (Lederer, 1996).

Secondary lipid oxidation products

Decomposition of lipid hydroperoxides, via *β*-scission reactions, yields low molecular weight, volatile compounds that are responsible for the off-flavours and aroma in foods. These secondary lipid oxidation products comprise of alkanes, alkenes, aldehydes, ketones, alcohols, esters and acids. Lipid aldehydes are highly reactive and among the most important compounds to contribute to food deterioration, modification of food structure, as well as protein damage via crosslinking

(Schaich, 2008). Lipid aldehydes can react with amino acid side-chains by either Schiff base reactions or Michael additions or by combination of both yielding aldehydic adducts (**Figure 2**). Schiff bases are imines that are formed in complex food systems when the carbonyl group of aldehydic lipid reacts with the functional groups of certain nucleophilic amino acid side-chains (e.g. thiol group of cysteine). Michael addition is the nucleophilic addition of a carbanion to an α,β-unsaturated carbonyl compound. Polyunsaturated aldehydes react faster with proteins than saturated aldehydes, and thus Michael addition reaction is the more preferred pathway (Gardner, 1979). For example, above 99% of the modifications of β-lactoglobulin and human haemoglobin by HNE occurred via Michael addition compared to Schiff base formation (Bruenner et al., 1995). Michael addition products i.e. the protein carbonyls can react further and form cyclic products, especially dihydropyridines and pyrroles. Instead, the formation of intra- and intermolecular crosslinks can occur via Schiff base formation or Michael additions or by complex combinations of both reactions (Schaich, 2008).

Saturated aldehydes such as monofunctional alkanals (e.g. hexanal and nonanal) have low reactivity and high selectivity, and they react with amines exclusively by Schiff base formation with preference for *N*-terminus of protein. In addition, at low aldehyde and oxygen concentration, there are no side reactions (Gardner, 1979; Schaich, 2008). In a study by Fenaille et al. (2003) hexanal modifications occurred only on phenylalanine and lysine side-chains in a B chain of insulin. Bifunctional saturated aldehydes such as glyoxal and malonaldehyde are more reactive due to the second carbonyl and keto-enol tautomerism. These aldehydes have three main reaction mechanisms: 1) Schiff base addition to nucleophilic groups on single amino acids and proteins, 2) formation of cyclic structures (dihydropyridines) with amines, and 3) Michael addition reactions with amines.

Unsaturated aldehydes such as acrolein, crotonaldehyde, alkenals, 4-hydroxy-2-alkenals and 4-oxo-2-alkenals (isoketals) are extremely reactive compounds. Due to α,β-unsaturation, 2-alkenals and their derivatives have three potential reaction sites: Schiff base formation at the carbonyl groups and Michael-type 1,2 and 1,4 additions at the carbocations (Esterbauer et al., 1991b). As described earlier, the Michael additions are preferred over Schiff base formation. Due to these multiple pathways complex reaction mixtures of products are formed. At the moment the research interests are focused on unsaturated aldehydes and their interactions with proteins and amino acids (Yamaki et al., 1992; Uchida et al., 1993; Bruenner et al., 1995; Refsgaard et al., 2000; Chopin et al., 2007; Guilleaguten et al., 2008). The main targets for unsaturated aldehydes are the nucleophilic thiol

groups of cysteine, ε-amine groups of lysine, and imidazole nitrogen of histidine (Esterbauer et al., 1991b). These reactions are discussed in more detail in the section 2.3 ‘Oxidation reactions of other amino acids’.

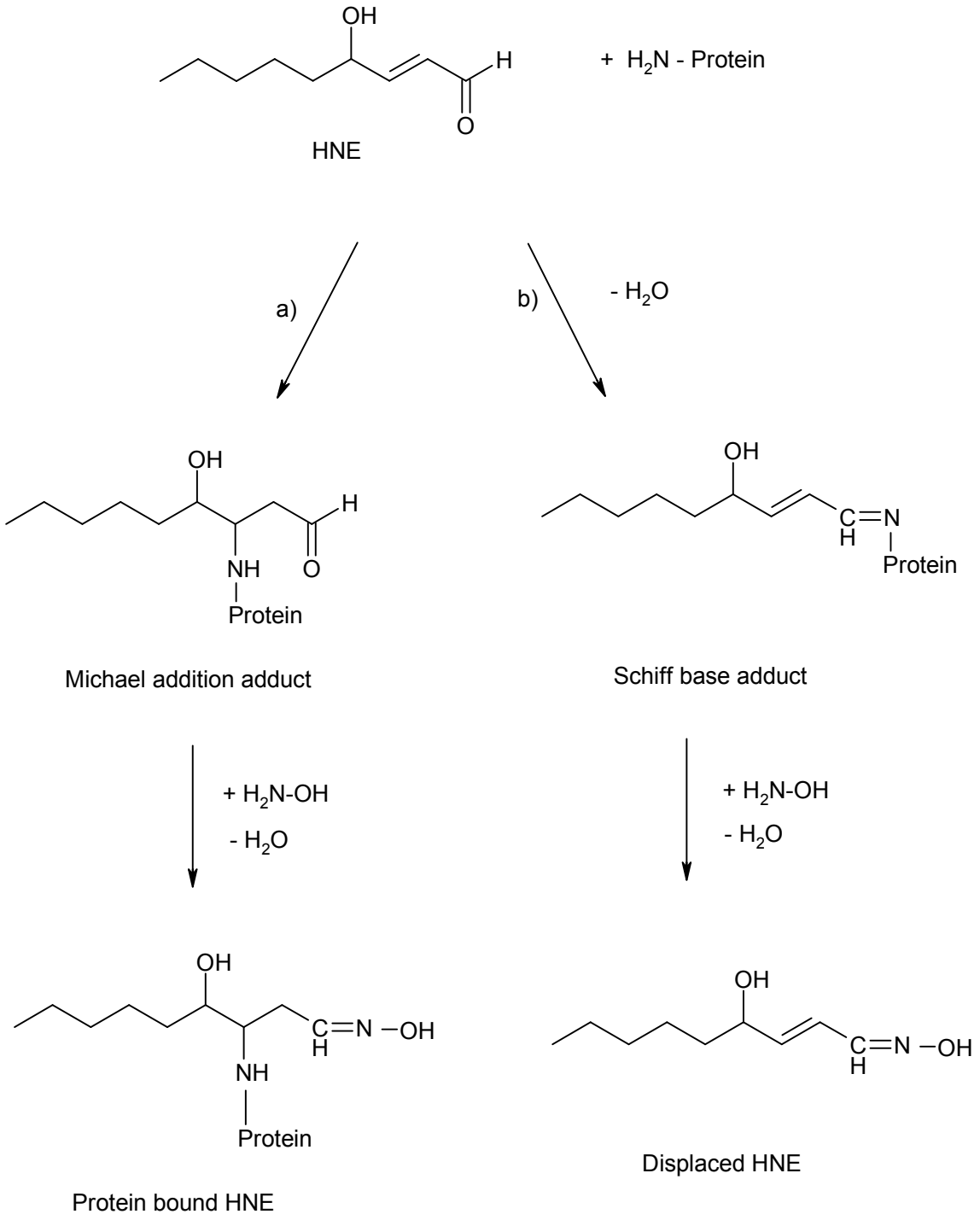


Figure 2. Protein modification by lipid aldehydes via a) Michael addition to cysteine, histidine or lysine side-chains. Carbonyl group undergoes subsequent reaction with hydroxylamine to form oxime derivatives that remain bound to protein; or b) Schiff base formation is followed by displacement of HNE (4-hydroxy-nonenal) from protein (Bruenner et al., 1995).

Epoxyalkenals are also common secondary products of lipid oxidation, and they can modify amino acids and proteins as well. In general, the oxidation of *n*-6 polyunsaturated fatty acids (e.g. linoleic acid) leads to formation of an intermediate 12,13-(*E*)-epoxy-9-hydroperoxy-10-octadecanoic acid which decomposes into 4,5-(*E*)-epoxy-2(*E*)-decenal (Gardner et al., 1984). On the other hand, the decomposition of *n*-3 fatty acids leads to the formation of 4,5-epoxy-2-heptenal (Frankel et al., 1981). For example, the formation of various epoxyalkenals has been detected in oxidized sunflower oil (Guillen et al., 2005). Several studies have confirmed that pyrrolization of proteins (e.g., BSA, bovine plasma, bovine α-globulins, bovine γ-globulins) occurs after reaction with epoxyalkenals such as 4,5(*E*)-epoxy-2(*E*)-decenal and 4,5(*E*)-epoxy-2(*E*)-heptenal (Hidalgo et al., 1998; Hidalgo et al., 2000). Reaction with 4,5-(*E*)-epoxy-2(*E*)-heptenal can lead to changes in the primary, secondary and tertiarty structures of BSA which have been observed as lysine losses, formation of ε-*N*-pyrrolylnorleucine, increase in fluorescence and protein polymerization (Hidalgo et al., 2000). In addition to ε-*N*-pyrrolylnorleucine resulting as a final product of oxidative stress (Hidalgo et al., 1998), it is also a normal compound found in many fresh food products such as fishes, meats, nuts, seeds and vegetables (Schieberle, 1996; Zamora et al., 1999a). Amino acid degradation with epoxyalkenals can occur via Strecker-type mechanism (Hidalgo et al., 2004) or by chemical conversion into α-keto acids (Zamora et al., 2006) which will lead to formation of flavor compounds.

2.1.3 Impact on protein functionality

The oxidation of proteins leads to damage of amino acids and decreased solubility resulting in aggregation of proteins (e.g. in myosin, egg albumin, γ-globulin and albumin, cytochrome c, casein, β-lactoglobulin, and soy protein) (Schaich, 2008), changes in food texture, alterations in tissue and membrane structures, changes in protein functions such as inactivation of enzymes, and formation of toxic products. Oxidative modifications in foods leading to the deterioration of structure, flavor, aroma, loss of nutritive value and alterations in protein functionality are a great concern to food industry (Damodaran, 1996). In addition, oxidation reactions of proteins are also important in other fields such as chemistry, biochemistry and medicine. Processing-induced changes leading to denaturation of proteins may improve the digestibility, especially of plant proteins containing antinutritional compounds (Mithen et al., 2000). Processing can also impair the digestibility and biological bioavailability by destruction of essential amino acids, conversion of amino acids into nonmetabolizable derivatives, and by intra- and intermolecular crosslinking (Karel et al., 1975; Rice-Evans et al., 1993).

The damage of amino acids in proteins, decrease in digestibility and inhibition of proteolytic and glycolytic enzymes leads to subsequent loss of nutritive quality of proteins. For example, oxidation of BSA with 4,5(*E*)-epoxy-2(*E*)-heptenal led to inhibition of the proteolysis, which was suggested to be due to the formation and accumulation of pyrrolized amino acid side-chains (Zamora et al., 2001). Protein oxidation reactions lead to conformational changes in the protein structure by altering surface charges, increasing hydrophobicity, inducing denaturation, or complexing lipids to the protein. Some of these reactions lead to polymerization of proteins. Functional properties important to food processing such as gelling, foaming, water-holding capacity and ability to act as surfactant are greatly affected by lipid oxidation products (Damodaran, 1996).

2.2 Oxidation of amino acids

2.2.1 Tryptophan oxidation

2.2.1.1 Tryptophan oxidation pathways

Tryptophan has been shown to be highly susceptible to many oxidizing agents, e.g., to oxidizing lipids, H_2O_2, H_2O_2/peroxidase, γ-irradiation, ROS such as singlet oxygen, ozone, heat/O_2, light/O_2, Fe^{3+}/ascorbic acid/O_2, hypoxanthine/xanthine oxidase/Fe^{3+}-EDTA, visible light and photosensitizers (Friedman et al., 1988; Steinhart et al., 1993; Itakura et al., 1994; Simat et al., 1998; Ronsein et al., 2008). Although tryptophan in proteins is relatively low in abundance, it has the highest molar absorption coefficients, which makes it one of the most important amino acids in the photodegradation pathways (Kerwin et al., 2007), and therefore the reactions of tryptophan are more throrouhgly reviewed than those of other amino acids.

Oxidation of tryptophan is shown in **Figure 3**. First initiator (I*) (metal ion or some other initiators such as UV light) converts tryptophan to nitrogen (**1**) and carbon (**2**) centered radicals, which can react with oxygen (O_2) yielding tryptophan-peroxyl radicals (**3**). Thus, tryptophan-peroxyl radicals can react with lipids (RH) or photooxidize by singlet oxygen yielding unstable *cis*- and *trans*-3-hydroxyperoxyindolenine (tryptophan hydroperoxide) (**4**), which can then decompose rapidly to yield *N*-formylkynurenine and kynurenine as major end-products (Friedman et al., 1988; Davies et al., 1995; Davies, 2003; Ronsein et al., 2008). However, the transformation from this hydroperoxide into *N*-formylkynurenine has been postulated to occur by three pathways. First, a hydrated indolenine (**5**) and its rearranged product (**6**) were suggested as the intermediates (Hamilton, 1969).

This mechanism is suggested to involve the heterolysis of the O–O hydroperoxide bond, followed by alkyl migration to yield (**6**), which subsequently breaks down into *N*-formylkynurenine. Another proposed pathway is degradation of the tricyclic hydroperoxide (**7**), which occurs through homolysis or heterolysis of its O–O bond, and is transformed into eight-membered hydroxyketone intermediate (**8**), which then decomposes to *N*-formylkynurenine. Finally, a dioxetane (**9**) derived from a ring chain tautomerism between (**4**) and (**7**) is also suggested as a likely intermediate. The reduction of the 3-hydroxyperoxyindolenine (**4**) or its ring tautomer (**7**) leads to concurrent formation of another degradation product, an alcohol (**10**) (Nagakawa et al., 1979; 1981).

End-products such as *N*-formylkynurenine and kynurenine are formed also during irradiation and interactions with lipid oxidation products. Other degradation products such as 3-hydroxykynurenine, anthranilic acid, aspartic acid, carbon dioxide and ammonia have been also detected during oxidation of both free tryptophan amino acid alone and from tryptophan side-chains in peptides and proteins (Davies, 2003). In addition, the indole ring of tryptophan is susceptible to irreversible oxidation producing 3a-hydroxypyrroloindole-2-carboxylic acid, β-oxindolylalanine, and dioxindolylalanine that can be further transformed to *N*-formylkynurenine and kynurenine and, dioxindolylalanine and kynurenine, and kynurenine, respectively (Itakura et al., 1994; Simat et al., 1998). This is because the tryptophan oxidation products are more prone to oxidation that tryptophan is itself. Photodegradation of tryptophan in the proteins may also arise from formation of tryptophan radical cation that rapidly deprotonates to yielding a neutral indolyl radical. The tryptophan indolyl radical may extract hydrogen from a nearby tyrosine repairing the tryptophan, thus forming a tyroxyl phenoxy radical. In the presence of oxygen, tyroxyl phenoxy radical will form a peroxy radical on the tryptophan, or react with nearby amino acids. This phenomenon has been reported in goat α-lactalbumin with a formation of a thioether bond with indole between cysteine(73) and tryptophan(118) (Vanhooren et al., 2002). Increasing the pH, temperature or ionic strength of the solution increases tryptophan oxidation due to changes in tryptophan excitation states (Lee et al., 1988; Steinhart et al., 1993).

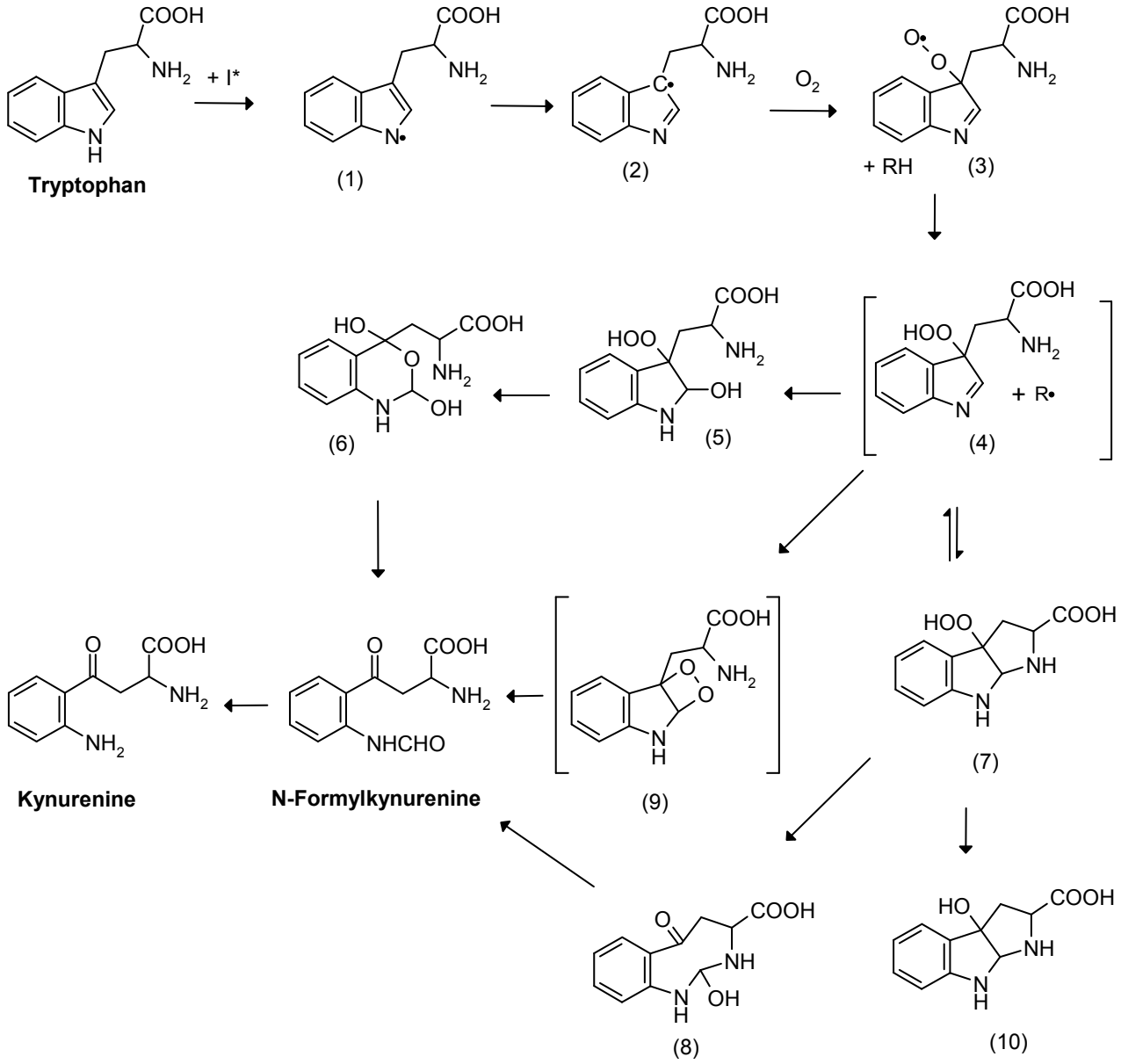


Figure 3. Proposed reactions pathways for oxidation of tryptophan to kynurenine via *N*-formylkynurenine. Tryptophan is converted to radicals by initiator. Further reactions with oxygen generate tryptophan-peroxyl radicals and tryptophan hydroperoxides, which via different pathways lead to the formation of *N*-formylkynurenine and kynurenine. I* = initiator, RH = lipid, $R^{\bullet}$ = lipid radical.

Modification of amino acid tryptophan and tryptophan side-chains in proteins can arise also by reactive nitrogen species (RNS). RNS catalyze pathophysiological and physiological conditions via nitric oxide radical ($NO^{\bullet}$) and its derivatives such as peroxynitrate ($ONOO^{-}$). Free tryptophan (amino acid) can be modified to several nitrated products such as (1-,4-,5-,6-, and 7-)1-*N*-nitroso compounds, and several oxidized products by reaction with various RNS, depending on the conditions used. The most common products formed, 1-*N*-nitrosotryptophan and 6-

nitrosotryptophan (6-NO_2-tryptophan), have been found in the reaction with peroxynitrate. In proteins, the modifications by RNS suggest that interactions with tryptophan are more limited than with tyrosine side-chains. This may be because tryptophan side-chains are more likely to be buried inside the protein (Yamakura et al., 2006). 6-NO_2-tryptophan is the most abundant product of reactions between tryptophan side-chains and peroxynitrate in BSA, hemoglobin, human Cu, Zn/superoxide dismutase as well as in peroxidase/H_2O_2/nitrite and myeloperoxidase/H_2O_2/nitrite systems (Stadtman et al., 2003; Yamakura et al., 2006).

2.2.1.2 Metabolic routes of tryptophan

Tryptophan is an essential amino acid for humans and it functions as a precursor for series of metabolic reactions. Tryptophan is degraded primarily by a complex enzymatic cascade known as the kynurenine pathway (**Figure 4**). Two enzymes, indolamine 2,3-dioxygenase (EC 1.13.11.42) (Hirata et al., 1975) and tryptophan 2,3-dioxygenase (EC 1.13.11.11) (Batabyal et al., 2007), catalyze the irreversible cleavage of the indole ring of tryptophan leading to the formation *N*-formylkynurenine, which is then metabolized by kynurenine aminotransferases (EC 3.5.1.9; EC 2.6.1.7) (Okuno et al., 1991) into kynurenine. Kynurenine is metabolized by three different enzymes: (1) kynurenine hydroxylase (EC 1.14.13.9) with the formation of 3-hydroxy-kynurenine; (2) kynurenine aminotransferase (EC 2.6.1.7) with formation of kynurenic acid; (3) kynurenine hydrolase (EC 3.7.1.3) with the formation of anthranilic acid. 3-Hydroxy-kynurenine is then further transformed into 3-hydroxy-anthranilic acid and 2-amino-3-carboxy-muconate semialdehyde, which can transform into quinolic acid participating in nicotinamide metabolism, or acetyl-CoA via several enzymatic steps contributing to glycolysis (Moroni, 1999; Schwarcz, 2004).

Tryptophan is also metabolized via another biochemical route into neurotransmitter serotonin (5-hydroxytryptamine) and neurohormone melatonin (**Figure 5**). First tryptophan is converted to 5-hydroxytryptophan by tryptophan-5-monooxygenase (EC 1.14.16.4) and subsequent decarboxylation by aromatic-L-amino acid decarboxylase (EC 4.1.1.28) converts it to the serotonin (5-hydroxytryptamine) (Hirata et al., 1972). Serotonin is further metabolized into *N*-acetylserotonin by serotonin-*N*-acyltransferase (EC 2.3.1.5) and into melatonin by hydroxyindol-*O*-methyltransferase (EC 2.1.1.4) (Boutin et al., 2005).

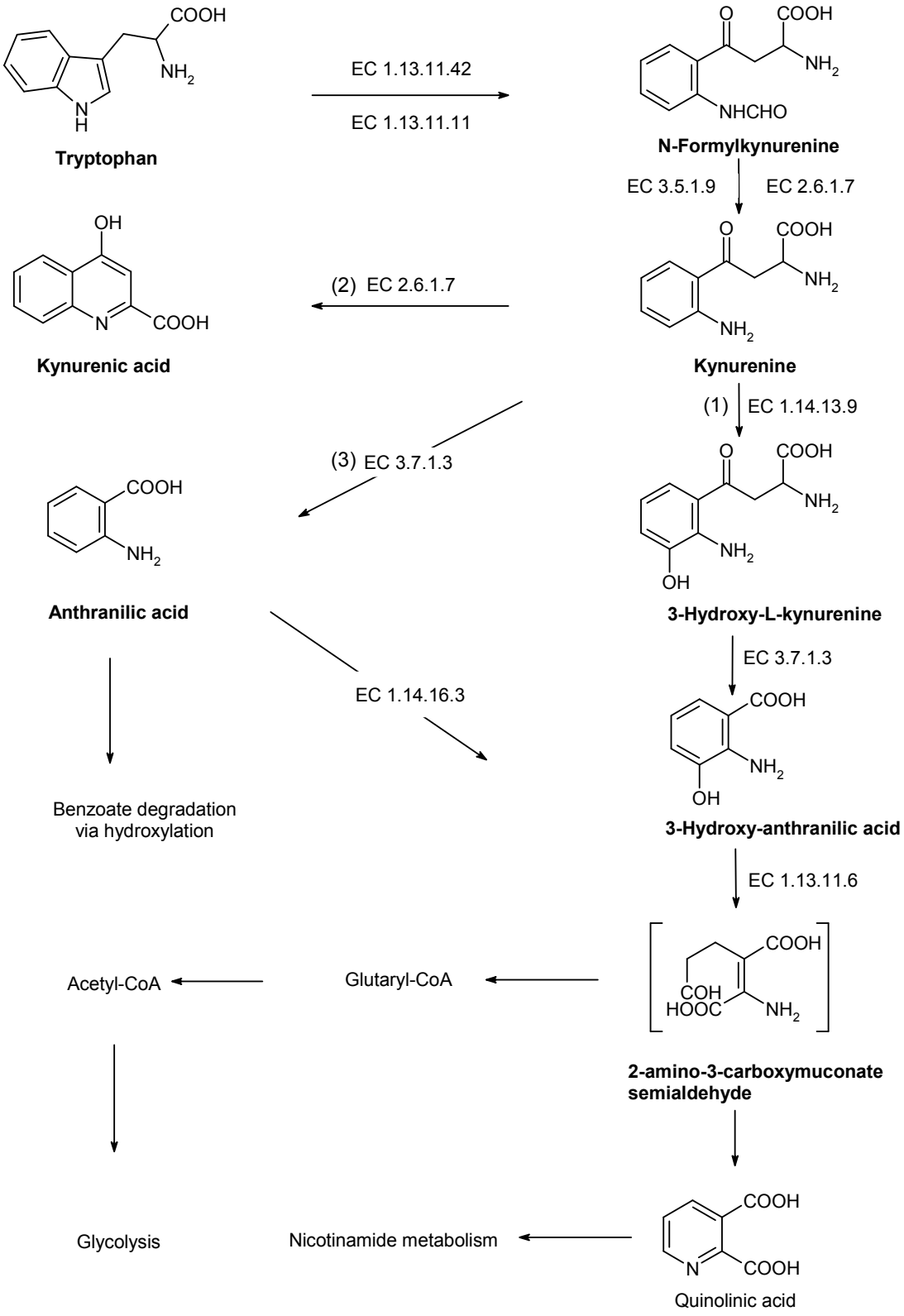


Figure 4. The kynurenine pathway of tryptophan metabolism.
EC 1.3.11.42 = indolamine 2,3-dioxygenase, EC 1.13.11.11 = tryptophan 2,3-dioxygenase, EC 3.5.1.9 and EC 2.6.1.7 = kynurenine aminotransferases, EC 1.14.13.9 = kynurenine hydroxylase, EC 2.6.1.7 = kynurenine aminotransferase, EC 3.7.1.3 = kynurenine hydrolase.

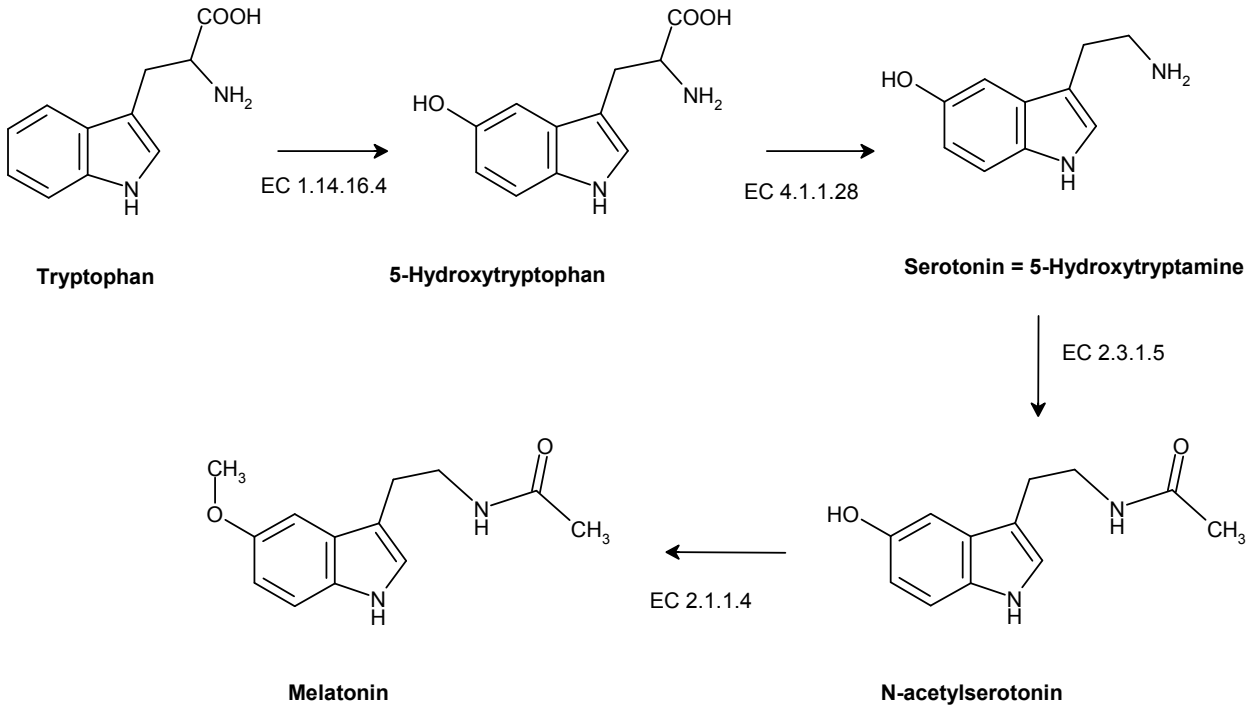


Figure 5. Metabolism of tryptophan into serotonin and further to melatonin via *N*-acetylserotonin. EC 1.14.16.4 = tryptophan-5-monooxygenase, EC 4.1.1.28 = aromatic-L-amino acid decarboxylase, EC 2.3.1.5 = serotonin-*N*-acyltransferase, EC 2.1.1.4 = hydroxyindol-*O*-methyltransferase.

Serotonin and melatonin metabolisms play an important role e.g. in the regulation of mood, anger, aggression, sleep and appetite (Boutin et al., 2005). Enhanced tryptophan degradation is observed during e.g. pregnancy (Schrocksnadel et al., 2003) and in a variety of diseases and disorders concomitant with cellular immune activation, e.g., infectious diseases, autoimmune diseases (Widner et al., 2000) as well as diabetes (Ahmed et al., 2005a) and cataract (Parker et al., 2004; Vazquez et al., 2004). In addition, neuropsychiatric (Tourette's syndrome, depression, schizophrenia), and neurological disorders (e.g. Alzheimer's, Parkinson's and Huntington's diseases, dementia, multiple sclerosis, rheumatoid arthritis) are observed with disturbed serotonin and/or tryptophan metabolism (Widner et al., 2000; 2002a; 2002b).

2.2.1.3 Reactions in foods

Tryptophan is essential amino acid for human nutrition and it is common constituent in most protein-based foods and dietary proteins. However, the amount of tryptophan is usually smaller in food proteins compared to the other essential amino acids. In general, most plant proteins are nutritionally incomplete due to their deficiency in several essential amino acids. For example, some cereal proteins are under the WHO recommendation of tryptophan content (1.0 %, calculated as

ratio of tryptophan to protein). High content of tryptophan (≥ 1%) is found e.g. in bovine milk, eggs, soya, wheat flour, rice, red meat, fish, poultry, turkey, sesame, chickpeas, sunflower seeds, pumpkin seeds, and peanuts whereas bananas, and potatoes have a lower content of tryptophan (0.8-0.9%) (Belitz et al., 2004). Tryptophan is also available as dietary supplements (Delgado-Andrade et al., 2006).

Reactions of tryptophan with carbonyl compounds have been widely studied (Culp et al., 1990; Damodaran, 1996; Herraiz, 1996; 2000a; Diem et al., 2001a; 2001b; Herraiz et al., 2003b; Papavergou et al., 2003; Herraiz et al., 2004). Lipid carbonyl compounds such as aldehydes can be formed during food processing and storage due to lipid oxidation. In addition, naturally existing aromatic and phenolic aldehydes such as cinnamic aldehyde, benzaldehyde, anisaldehyde, salicylaldehyde, syringaldehyde, vanillin and trans-2-hexenal are used as flavouring agents (Culp et al., 1990; Herraiz et al., 2003b). Tryptophan has been shown to react with aldehydes or α-keto acids forming tetrahydro-β-carboline-3-carboxylic acids via Pictet-Spengler condensation (Diem et al., 2001b) (**Figure 6**). The decarboxylation of tetrahydro-β-carboline-3-carboxylic acids forms β-carboline alkaloids. Generally, β-carbolines are naturally formed during production, processing and storage. In addition, the levels of β-carbolines in the food depend on the composition of food and the amount of precursors present, food processing conditions (heating, cooking, fermentation, smoking, and ripening), temperature, pH as well as the presence of oxygen, application of chemicals and antioxidants (Herraiz, 2000a). For example, broiling or grilling of meat products yields β-carbolines (Damodaran, 1996). In fruit and vegetable – derived products such as juices, jams and tomato sauces, glycoconjucates of β-carbolines are formed from a condensation reaction between D-glucose and tryptophan at low pH and high temperature (Papavergou et al., 2003). Carbohydrate-derived β-carbolines have also been identified in reactions between tryptophan and ribose (Diem et al., 2001a; Diem et al., 2001b). Tetrahydro-β-carboline-3-carboxylic acids and β-carbolines have been found in variety of food products such as cocoa, chocolate (Herraiz, 2000b), fermented alcoholic and non-alcoholic beverages such as wines, beers, ciders, distillates, soy sauces, and vinegar (Herraiz, 1996) as well as in meat, cured ham, fermented and cooked sausages (Herraiz et al., 2004), smoked sausages, smoked cheeses and smoked fish (Papavergou et al., 2003). In smoked foodstuffs the formaldehyde-derived formation of tetrahydro-β-carbolines is favored, and the concentration are higher than those of unsmoked (Herraiz et al., 2003b). Therefore, the intake of tetrahydro-β-carbolines in the diet may be several milligrams per day.

Tryptophan-derived tetrahydro-β-carbolines are biologically active alkaloids that occur and accumulate in mammalian tissues, fluids, and brain, and thus function as potential neuromodulators (Herraiz et al., 2003a). Studies have reported the possibility of tetrahydro-β-carbolines having toxic or mutagenic properties (Ostergren et al., 2004; Bringmann et al., 2006; Herraiz et al., 2007; Wernicke et al., 2007). However, it has been suggested that tetrahydro-β-carbolines might act as antioxidants when absorbed and accumulated in the body, contributing to the antioxidant effect of fruit products containing these compounds (Herraiz et al., 2003a). The study was performed by 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) free radical scavenging assay. However, the hydrogen-donating ability of antioxidants is a simple test model that does not necessarily indicate their activity in a more complex food models or *in vivo*.

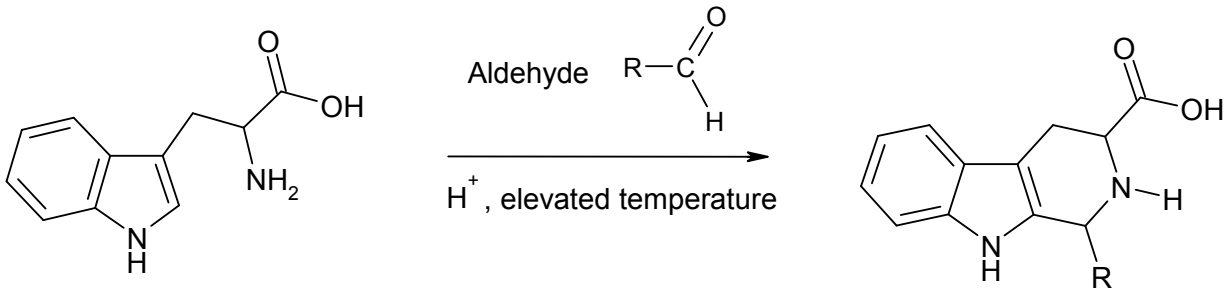


Figure 6. Condensation of tryptophan with aldehydes yields tetra-hydro-β-carboline-3-carboxylic acids. Aliphatic aldehydes: formaldehyde (R = H) and acetaldehyde (R = CH_3). Phenolic aldehydes: benzaldehyde, salicylaldehyde, anisaldehyde, vanillin, and syringaldehyde (R = phenol ring consisting of functional groups such as –H, –OH and/or –OCH_3 at different positions).

Maillard reaction initiated by reaction between amino acids and carbonyl compounds at elevated temperatures has also a great impact on organoleptic and nutritional properties of proteins (Damodaran, 1996). One study reported that half of the tryptophan was lost when α-NH_2 of the free tryptophan reacted with reducing sugars such as glucose. The rate of the tryptophan loss depends on the water activity (a_w). Higher a_w increases the reaction. The indole ring of tryptophan can also react with Maillard derivatives (Leahy et al., 1983). Oxidation of tryptophan has been detected in α-lactalbumin and β-lactoglobulin when they were incubated with lactose (Meltretter et al., 2007). Decomposition of free tryptophan in cookies was also reported to be more severe with glucose than with sucrose (Morales et al., 2007).

2.2.2 Oxidation reactions of other amino acids

The susceptibility of amino acid side-chains in proteins to oxidation depends on their location in the protein, the exposure to the aqueous medium, the nearby amino acids in the primary amino acid

sequence and the three dimensional structure (whether buried or exposed). The conformational changes due to pH, temperature, salts, or binding ligands are major factors in the degradation reactions (Kerwin et al., 2007). Amino acids located mainly on protein surfaces (cysteine, tryptophan, histidine, lysine, arginine, tyrosine, and methionine) are primary targets for ROS mediated oxidation with their readily abstractable hydrogens (except methionine) and hydrogen-bonding properties (Schaich, 2008). Most of these amino acids (cysteine, histidine, lysine, tryptophan and arginine) form stable radicals upon oxidation with lipids. Side-chain thiol and amine groups of these amino acids react readily with carbonyl compounds derived from lipid oxidation and form Shiff bases, Michael adducts, and their cyclic products. Therefore, these amino acids are first modified during oxidation, and can remain reactive through propagation and termination stages. Buried hydrophobic side-chains (glycine, alanine, valine, proline, leucine and isoleucine) have no easily abstractable hydrogen atoms and they do not participate in hydrogen bonding. These amino acids need to be exposed by denaturation in order to react with oxidizing lipids.

Cysteine

Cysteine, and peptides, proteins and enzymes containing a thiol group (e.g. glutathione (GSH), *N*-acetylcysteine, thioredoxin, peroxiredoxins, and tyrosinase phosphatase) are highly reactive with various ROS. The reactions can be divided into two categories: one electron and two electron oxidations (**Figure 7**) (Winterbourn et al., 2008). Radicals and transition metal ions (**one electron oxidants**) oxidize the cysteine side-chains in the protein yielding thiol radical (P-S•). These radicals can further react with amino acids resulting in crosslinking (Kerwin et al., 2007). Under aerobic conditions its favored reaction is with a thiolate anion (in protein or GSH), thereby forming disulfide anion radical ($PSSG^-$ for GSH). Further reactions with oxygen generate superoxide and thus amplify the oxidative reactions. Alternatively, the thiol radical can propagate radical reactions or be quenched by scavengers. Dimerization of two thiol radicals is usually a minor pathway. Most thiol radical reactions are reversible. In addition, as the level of oxygenation of the sulphur increases, pK_a decreases, and the thiols are present as thiolates thus forming also ionized oxidation products. **Two electron oxidation** of protein thiol group (P-SH) first forms an intermediate product of sulfenic acid (P-SOH) that will give rise to several secondary reactions. Sulfenic acid can form mixed disulfides with GSH (P-SS-G), intramolecular disulfides (favored in vicinal thiols), and intermolecular disulfides between protein molecules. In protein tyrosinase phosphatase, reaction with an adjacent amide forms a sulfenylamide (Winterbourn et al., 2008). Cysteine side-chains in whey proteins such as α-lactalbumin and β-lactoglobulin were shown to oxidize into sulfenic acid

(Meltretter et al., 2008b). Measuring the modifications to cysteine and other thiol compounds during oxidation can be evaluated by derivatization into fluorescent compounds (see chapter 2.3).

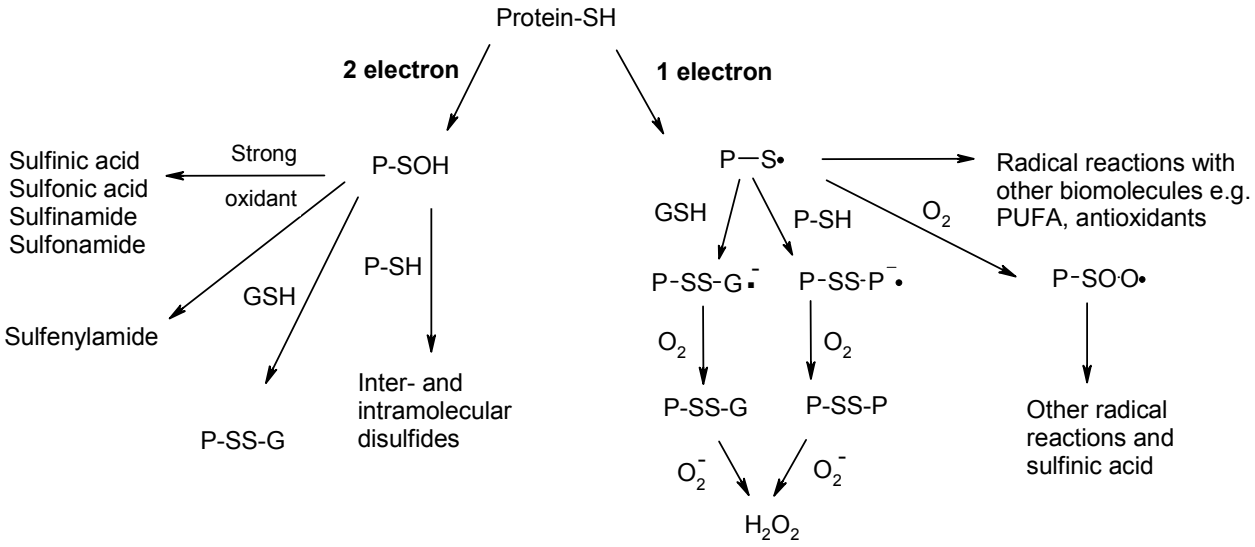


Figure 7. Oxidation pathways for protein thiols (Winterbourn et al., 2008).
P = protein, P-SH = thiol group of cysteine, P-S$^{\bullet}$ = thiol radical, GSH = glutathione (tripeptide of glutamine, cysteine and glycine), P-SS-G$^{\bullet-}$ and P-SS-P$^{\bullet-}$ = disulfide anion radicals, P-SOH = intermediate product of sulfenic acid, P-SS-G = disulfide between GSH and protein, PUFA = polyunsaturated fatty acids.

Methionine

Methionine side-chains in proteins are readily oxidized to methionine sulphoxide via zwitterionic intermediate that undergoes subsequent reaction with a second molecule of methionine (Sysak et al., 1977). Only pure methionine sulphoxide oxidizes into methionine sulphone, however, in a mixture of methionine and methione sulphoxide this reaction does not occur (Karel et al., 1975) (**Figure 8**). Methionine side-chains of α-lactalbumin and β-lactoglobulin have been shown to yield methionine sulphoxide and methionine sulphone (Meltretter et al., 2007).

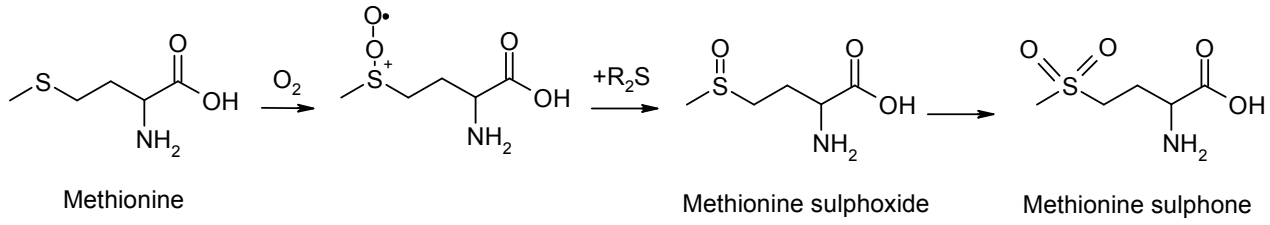


Figure 8. Oxidation of methionine.

The susceptibility of sulphur atom to oxidize is due to the rapid delocalization of electrons (Sysak et al., 1977). This reaction, unlike the oxidation of other amino acids (except cysteine), is reversible. Oxidation of methione side-chains by ROS yields inactive forms of ROS (IRS) (**scheme 3**). Methione sulphoxide can be reduced back to methione by methionine sulphoxide reductases (EC 1.8.4.6). These enzymes use thioredoxin $Th(SH)_2$ as reducing agents (**scheme 4**). Furthermore, the oxidized form of thioredoxin (ThS-S) in the presence of NADPH can be converted back to its reduced form by the enzyme thioredoxin reductase (**scheme 5**). Finally, the coupling of these previous reactions (**schemes 3-5**) leads to conversion of ROS to inactive forms (**scheme 6**), and thus provides a mechanism for radical scavenging (Stadtman, 2006). Methionine can thus act as antioxidant and protect other amino acid side-chains from oxidation (Levine et al., 1999).

Methione + ROS $\rightarrow$ Methione sulphoxide + IRS (**Scheme 3**)

Methionine + $Th(SH)_2$ $\rightarrow$ Methione + ThS-S (**Scheme 4**)

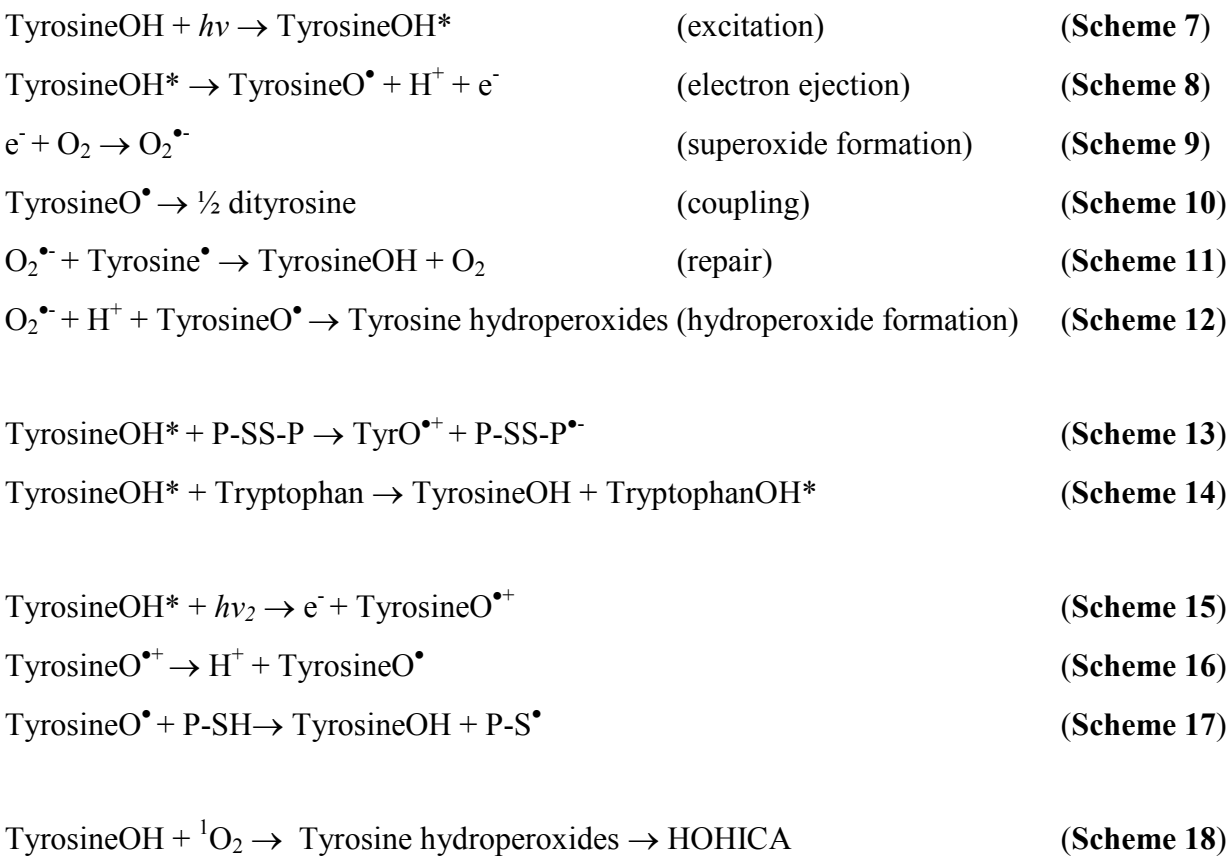
ThS-S + NADPH + H^+ $\rightarrow$ $Th(SH)_2$ + $NADP^+$ (**Scheme 5**)

ROS + NADPH + H^+ $\rightarrow$ IRS + $NADP^+$ (**Scheme 6**)

Tyrosine

Photolytic excitation of tyrosine side-chains (TyrosineOH) in protein leads to formation of tyrosyl radicals (TyrosineO$^{\bullet}$). The triplet state Tyr (TyrOH*) can a) form a neutral tyrosyl radical via electron transfer mechanism, b) further react with oxygen yielding peroxides (**schemes 7-12**), or c) react with disulfides (P-SS-P) or tryptophan thereby forming disulfide-base radical anion (P-SS-P$^{\bullet-}$) and tyrosyl radical cation (TyrosineO$^{\bullet+}$) (**schemes 13-14**), respectively. In **schemes 7-12**, the ejected electrons during the excitation (e.g. UV irradiation) of tyrosine react with molecular oxygen to yield superoxide radical anion ($O_2^{\bullet-}$), which in turn participates with tyrosyl radical that compete coupling (**schemes 7-10**). The repair process and formation of hydroperoxides in the presence of superoxide radical anion are presented in **schemes 11-12**. The excited tyrosine can further generate tyrosyl radicals, which can react with thiol groups of proteins (P-SH) (**schemes 15-17**). Free tyrosine, upon reaction with $^1O_2$, can also form cyclised products such as 3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indolol-2-carboxylic acid (HOHICA) through formation of endoperoxides and C1-hydroperoxides (**scheme 18**). HOHICA can be further oxidized, thereby forming decarboxylated keto compounds. In peptides, however, tyrosine oxidation products yield C1-hydroperoxides, which subsequently decomposes to the corresponding dienone alcohol (Davies, 2003). The tyrosyl radical is important precursor in forming degradation byproducts such as

dityrosines (**Figure 9**). Dityrosines can be formed via intramolecular or intermolecular crosslinking (Davies, 2003; Kerwin et al., 2007). Dityrosine formation has been observed in both purified proteins *in vitro* and intact red blood cells during oxidation (Giulivi et al., 1993). The dityrosine generated remains in the intact protein unless it is released by the action of proteolytic enzymes. Other tyrosine oxidation products such as dopamine, dopamine quinone, and 5,6-dihydroxyindol are also formed during exposure to oxidants and are also only released following proteolytic digestion (Giulivi et al., 1993; Davies, 2003). In general, since dityrosine can only be formed by reaction of two tyrosyl radicals, dityrosine is a specific marker for free radical modification of proteins and protein oxidation. Although the levels of dityrosines are not high, the sensitivity of fluorometric detection methods (particularly when coupled with the specificity of HPLC separation) makes dityrosine measurement a relatively simple and powerful procedure (Davies, 2003). More comprehensive discussion of these methods is given in chapter 2.3.



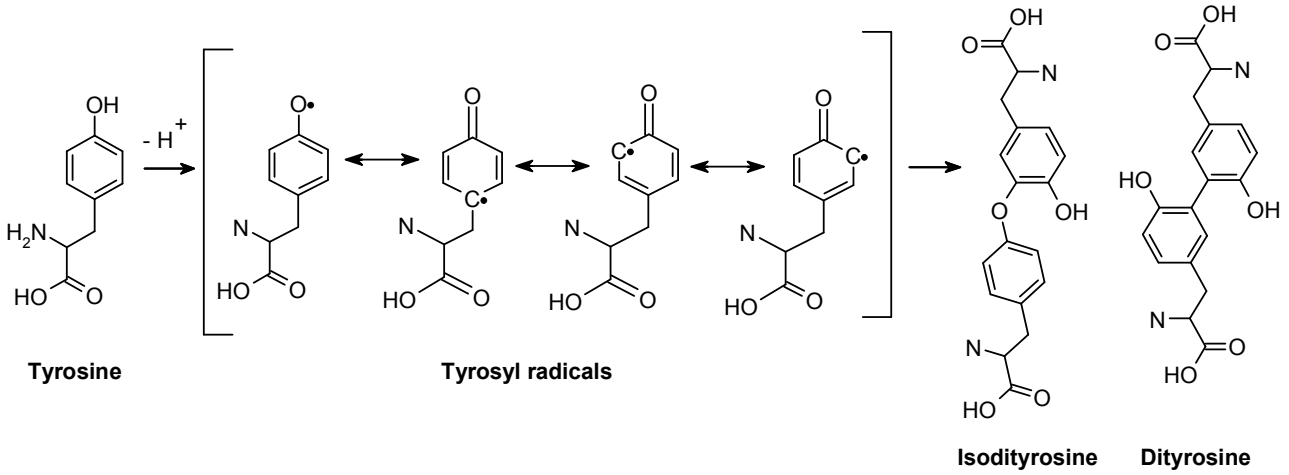


Figure 9. Formation of tyrosyl radicals. Crosslinking tyrosine molecules yields isodityrosine and dityrosine (Kerwin et al., 2007).

Phenylalanine

Oxidation of phenylalanine side-chains is initiated by photolysis (**Figure 10A**). First, excited triplet state of phenylalanine (Phe*) can form phenyl radicals (Phe$^{\bullet}$) that react readily with water forming *ortho*-, *meta*, or *para*-tyrosine derivatives (**Figure 10A and B**) (Stadtman et al., 2003). Reaction of excited phenylalanine competes also with oxygen to produce superoxide anion and the phenyl radical cation (Phe$^{+\bullet}$) which subsequently undergoes further reactions. Another pathway for excited phenylalanine is photolytic cleavage to form benzyl and glycyl radicals (Kerwin et al., 2007). Strecker-type degradation of amino acid phenylalanine by 4,5-(*E*)-epoxy-2(*E*)-alkenals has been reported (Hidalgo et al., 2004). This reaction yielded *N*-substituted pyrroles and *N*-substituted 2-(1-hydroxyalkyl)pyrroles as well as Strecker aldehyde phenylacetaldehyde and 2-alkylpyridines. In addition, epoxyalkenals have been reported to convert phenylalanine into phenylpuryvic acid, a corresponding α-keto acid (Zamora et al., 2006). These compounds are flavor compounds analogous to Maillard reactions. Therefore, flavors traditionally connected to Maillard reactions may also be produced as a result of lipid oxidation.

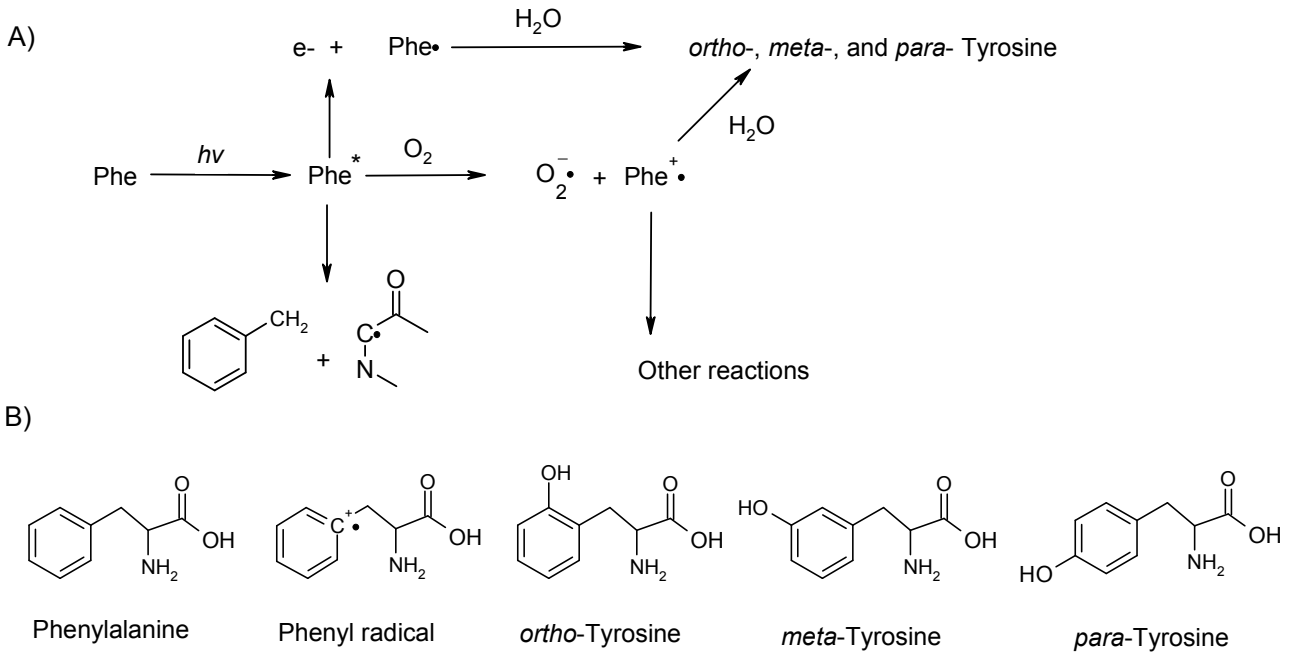


Figure 10. A) Phenylalanine reaction pathways (Kerwin et al., 2007). B) Structures of phenylalanine, phenylalanine radical as well as *ortho*-, *meta*-, and *para*-tyrosines.
Phe = phenylalanine, Phe* = excited phenylalanine, $Phe^{\bullet}$ = phenyl radical, $O_2^{\bullet-}$ = superoxide radical anion, $Phe^{+\bullet}$ = phenyl radical cation.

Histidine

Histidine is especially sensitive for oxidative degradation. Photocatalytic oxidation of the imidazole group by singlet oxygen ($^1O_2$) forms endoperoxides (**Figure 11**). These products can decompose rapidly to various products such as aspartic acid- and asparagine-derivatives and urea (Tomita et al., 1969; Davies, 2003). Metal ion -catalyzed oxidation of histidine, however, was found to be the main pathway in the formation of 2-oxo-histidine. This reaction pathway also yields aspartate, aspartylurea, and formylasparagine (Uchida et al., 1986). Radical reactions may also induce formation of histidine-histidine and histidine-lysine crosslinks (Davies, 2003). The formation of 2-oxo-histidine has been detected in oxidized proteins such as low density lipoprotein (LDL) (Retsky et al., 1999; Yamada et al., 2004), BSA (Yamada et al., 2004) and Cu, Zn-superoxide dismutase (Uchida et al., 1994). Histidine is also prone to specific modifications by secondary lipid oxidation products such as 2-alkenals, 4-hydroxy-2-alkenals, and ketoaldehydes (Hashimoto et al., 2003; Uchida, 2003) (**Figure 12**). These reactions between histidine and reactive aldehydes occurs via Michael addition of the imidazole nitrogen of histidine to the α,β-unsaturated bond. The oxidation product such as 4-hydroxy-2-alkenal-histidine with its free aldehyde group undergoes cyclization

and forms cyclic hemiacetal derivatives (Uchida et al., 1993). Histidine side-chains can also be modified by lipid peroxidation product 4,5-epoxy-2-alkenals (Zamora et al., 1999b). For example, the reaction of BSA with 4,5(*E*)-epoxy-2(*E*)-heptenal leads to histidine losses and formation of carbonyl groups in the protein as well as formation of 4,5(*E*)-epoxy-2(*E*)-heptenal – histidine adducts. This data revealed that in BSA, histidine side-chains can compete with lysine side-chains for scavenging these reactive aldehydes (Zamora et al., 1999b).

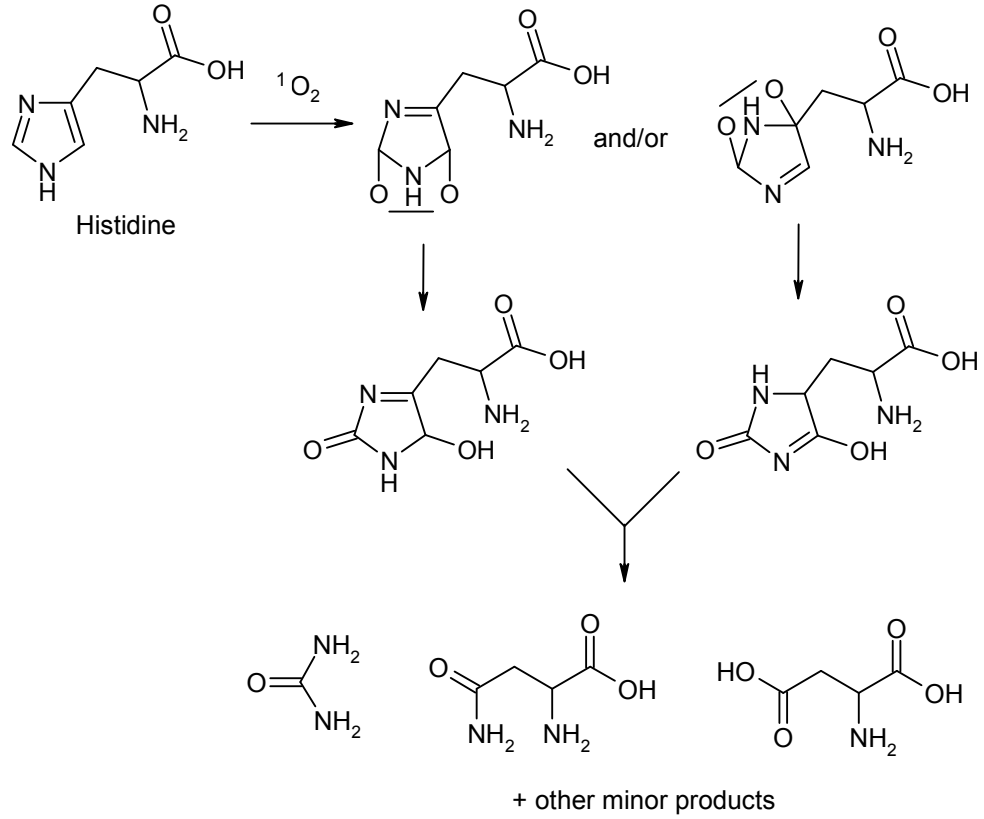


Figure 11. Oxidation of histidine in the presence of singlet oxygen yields endoperoxides, which decompose to various products (Uchida, 2003).

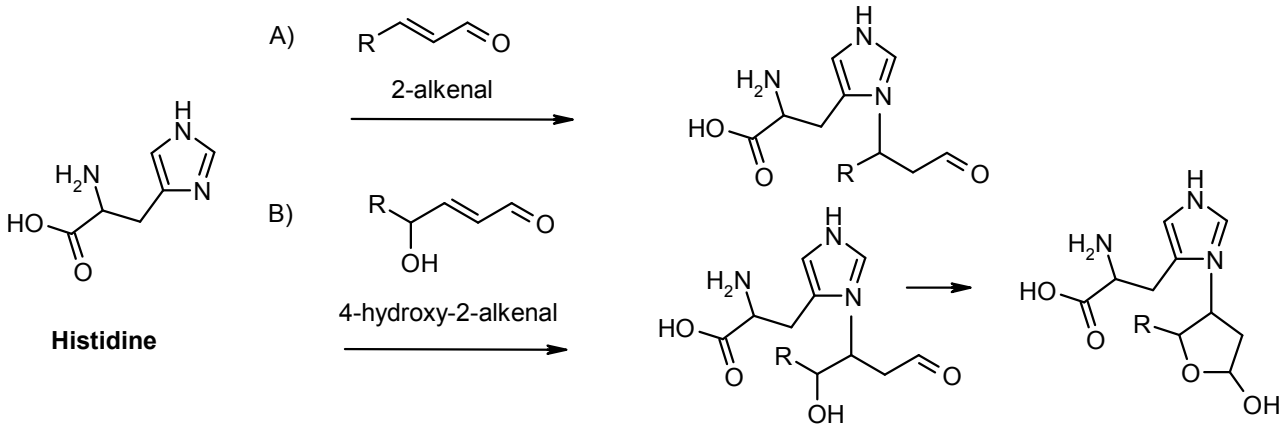


Figure 12. Modifications of histidine with aldehydes (Uchida, 2003).

Lysine

Metal ion catalyzed oxidation of lysine yields aminoadipic semialdehydes. In addition, lysine reacts easily with aldehydes such as 2-alkenals and 4-hydroxy-2-alkenals, as well as ketoaldehydes and epoxyalkenals forming various oxidation products (**Table 1**). Lysine side-chains in proteins in the presence of 2-alkenals such as well-known air pollutants acrolein and crotonaldehyde, and 2-hexenal yield *Nε*-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine), *Nε*-(2,5-dimethyl-3-formyl-3,4-dehydropiperidino)lysine (dimethyl-FDP-lysine), and diethyl-FDP-lysine and dipropyl-FDP-lysine, respectively. However, FDP adducts are not the end products but the electrophilic intermediates that potently react with thiol compounds. Reaction with crotonaldehyde forms also minor oxidation products with pyridinium ring such as *Nε*-(5-ethyl-2-methylpyridinium)-lysine (EMP-lysine) (Ichihashi et al., 2001). HNE reacts with lysine forming Michael adducts, and pyrrolo derivatives. Study on HNE treated LDL suggested that HNE may be a major contributor to fluorescence properties of oxidized LDL (Yamada et al., 2004). Epoxyalkenals such as 4,5-(*E*)-epoxy-2(*E*)-heptenal has been shown to react with lysine generating pyrrole derivatives and polymers (Zamora et al., 1994). In addition, reaction between 4,5-(*E*)-epoxy-2(*E*)-decenal and lysine leads to formation of *N*-substituted pyrroles and *N*-substituted 2-(1-hydroxyalkyl)pyrroles which in turn can lead to polymerization and formation of polypyrroles (Zamora et al., 1995; Hidalgo et al., 2004). Oxidation of carbohydrates and lipids followed by reaction with lysine side-chains generates carboxymethyllysine and glyoxal-derived lysine-lysine dimers. Reaction of lysine with malonaldehyde forms *Nε*-(2-propenal)lysine (Uchida, 2003). Oxidation in lysine side-chains of whey proteins has been reported (Meltretter et al., 2007; Meltretter et al., 2008b). The modifications in α-lactalbumin and β-lactoglobulin revealed also glycation/glyoxidation of lysine in the precence of lactose into Amadori product (lactulosyllysine) and N^{ε}-carboxymethyllysine. Lysine was also oxidized to lysine aldehydes (Meltretter et al., 2007). These and other reactions with other amino acids such as tryptophan, cysteine and methionine have been shown to occur also in dairy products such as raw, high-temperature, ultra-high temperature, sterilized, and condensed milk as well as in infant formulas (Meltretter et al., 2008a). Most of the lysine oxidation products are also carbonyl compounds or they have fluorescent properties. This makes them easy to detect (Uchida, 2003). More detailed methodology of semialdehydes is discussed in the following chapter 2.3.

Table 1. Oxidation products of lysine side-chains in proteins in the presence of reactive lipids.

Oxidant	Oxidation products	Reference
Acrolein	*Nε*-(FDP)-lysine	Ichihashi et al., 2001
Crotonaldehyde	*Nε*-(2,5-dimethyl-FDP)-lysine *Nε*-(EMP)-lysine	Ichihashi et al., 2001
2-hexenal	Diethyl-FDP-lysine, dipropyl-FDP-lysine	Ichihashi et al., 2001
4-hydroxy-2-alkenals	Micheal adducts, pyrrolo derivatives	Yamada et al., 2004
4,5-epoxy-2-heptenal	Pyrrole derivatives, polymers	Zamora et al., 1994
4,5-epoxy-2-decenal	*N*-substituted pyrroles, *N*-substituted 2-(1-hydroxyalkyl)pyrroles, polypyrroles	Zamora et al., 1995; Hidalgo et al., 2004
Malonaldehyde	*Nε*-(2-propenal)lysine	Uchida, 2003
glycation/glyoxidation	Lactulosyllysine, N^{ε}-carboxymethyllysine	Meltretter et al., 2007

FDP = 3-formyl-3,4-dehydropiperidino, EMP = 5-ethyl-2-methylpyridinium

2.3 Analyses of protein, peptide and amino acid oxidation

2.3.1 Protein carbonyls

The oxidative modification of proteins by reactive species, especially by ROS, has been widely studied in food products such as meat as well as in human tissues. Formation of carbonyl compounds has been shown to be one of the salient changes in oxidized proteins. Therefore, the concentration of carbonyl groups is highly indicative of protein oxidation. The extent of modifications in proteins can be quantified by measurement of protein carbonyl content (Levine et al., 1994). The carbonyl compounds can be detected by converting them to 2,4-dinitrophenylhydrazones (DNPH) by reaction with 2,4-dinitrophenylhydrazine and measuring the derivatives by spectrophotometric or immunochemical methods (Oliver et al., 1987). In addition, the DNPH –derivatives can also be analyzed by using HPLC gel filtration or electrophoresis such as Western blot (Levine et al., 1994). In foods, DNPH method has been implemented for example in studies of oxidation in pork meat patties and frankfurters (Vuorela et al., 2005b; Salminen et al., 2006, Estevez et al., 2007).

Carbonyl compounds can also be measured by fluorescence spectroscopy. The protein – lipid interactions give rise to carbonyl complexes. They have a specific fluorescence at excitation wavelength around 350 nm. However, the maximum emission wavelength varies from 400 to 500 nm depending on the different carbonyl compounds due to differences in the interacting amino acids and lipid oxidation products (Aubourg et al., 1992; Yamaki et al., 1992). In order to measure

only protein carbonyls, the removal of lipid carbonyl compounds and other interfering compounds is important for the success of the analysis. This can be done for example by precipitating the proteins or centrifuging with specific molecular weight cut-off membranes.

2.3.2 Oxidized tryptophan

HPLC - methods have been used widely in research of oxidation reactions of amino acids and peptides. Oxidation of amino acid tryptophan and tryptophan side-chains of proteins and the formation of tryptophan derived oxidation products have been assessed with RP-HPLC combined with UV- and fluorescence detection (Simat et al., 1994; 1998). RP-HPLC was also used in detecting tyrosine dimerization of hen and turkey egg-white lysozymes induced by irradiation (Audette et al., 2000).

Protein mass spectrometry techniques have been proven to be effective method in identifying and monitoring the major protein modifications. The advantages of mass spectrometry are the potential to analyze intact proteins without major sample preparation, thus avoiding artifact formation, and the simultaneous detection of all modification types independent of their structure (Meltretter et al., 2008b). Oxidation and nitration reaction of tryptophan has been studied by HPLC combined with electrochemical detection (EC) and mass spectrometry (MS) (Bregere et al., 2008). Protein glycation has been successfully studied by using matrix-assisted laser desorption/ionization time-off-flight mass spectrometry (MALDI-TOF-MS) or electrospray ionization mass spectrometry (ESI-MS) (Kislinger et al., 2002; 2004; Ahmed et al., 2005b). These methods have made it possible to detect Amadori adducts of glycated proteins in model solution, milk samples and *in vivo* in diabetic patients (Traldi et al., 1997; Ahmed et al., 2005a). The modification site of proteins can be also determined by applying first enzymatic hydrolysis and then peptide mapping (Humeny et al., 2002; Kislinger et al., 2005). MALDI-TOF-MS has also been applied to studying the oxidation of 5-hydroxytryptamine by tyrosinase, which resulted in enzymatic oligomerization of 5-hydroxytryptamine (Favretto et al., 1997). In another study, LC-ESI-MS analysis was used to conclude that tryptophan and methionine side-chains were oxidized in myoglobin modified by hypochlorous acid (Szuchman-Sapir et al., 2008). Tryptophan derived oxidation products and free radicals under Fenton reaction conditions i.e. in the presence of H_2O_2 and Fe^{2+} have been studied with ESI-MS and ESI-MS/MS. These methods allowed the identification of mono- and dihydroxytryptophans and *N*-formylkynurenine as well as 3-methyl derivatives and tryptophan dimers and monohydroxy-dimers (Dominques et al., 2003).

In addition to HPLC –methods, a fast and simple method of isothermal microcalorimetry can be applied to studying the modifications of amino acids in aqueous media in the presence of hydrogen peroxide. This technique was used to study the degradation of tryptophan, cysteine, methionine and tyrosine as well as ascorbic acid following the heat flow curves from the calorimetry (Roskar et al., 2008).

Fluorescence is exceptionally sensitive method to detect intermolecular interactions, and it is inexpensive and easy to implement (Lakowicz, 1999). Tryptophan fluorescence has been widely implemented in research of protein structures and functions. Tryptophan fluorescence is measured at specific excitation wavelength of 280-283 nm with emission wavelength around 330 nm. Tryptophan fluorescence techniques have been found invaluable in studying the interactions of modified proteins and peptides especially in membranes (Ladokhin et al., 2000), and in food components such as milk proteins (Dalsgaard et al., 2007; Elmnasser et al., 2008), in oil-in-water emulsions (Viljanen et al., 2005a; 2005b), liposomes (Viljanen et al., 2004a; 2004b) and LDL (Griessauf et al., 1995; Ferroni et al., 2004). In addition, the protein conformations in human eye lens protein D-crystallin have been evaluated by fluorescence techniques (Chen et al., 2006).

2.3.3 Polymers

Formations of high molecular weight dimers and polymers of proteins due to oxidation have been analyzed with electrophoresis. For example, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot immunoassay was applied to identify formation of 3-nitrotyrosine in human LDL (Ferroni et al., 2004) and formation of nitrotyrosine and carbonyl compounds in hypochlorous acid-treated human serum albumin (HSA) and nitrated HSA (Capeillere-Blandin et al., 2004). These methods are also easy to implement.

2.3.4 Free radicals and paramagnetic species

Lipid – protein interactions can be studied by electron paramagnetic resonance (EPR), sometimes also referred as electron spin resonance (ESR) spectroscopy. This is a technique for detection and quantification of chemical species that have one or more unpaired electrons, such as organic and inorganic free radicals or inorganic complexes possessing a transition metal ion. This results in high specifity of the EPR technique, since ordinary chemical solvents and matrices do not give rise to EPR spectra. Free radicals and paramagnetic species can be detected directly or indirectly by spin

trapping or immuno-spin trapping, or by chemical modifications to proteins (Marsh et al., 2004; Megli et al., 2005; Tsuchiya et al., 2005). However, the high water content of food and biological samples interferes with the measurements. This can be resolved by optimizing the instrument frequency for each sample type. Oxidative modifications in food samples with low moisture content such as wheat grains have been studied using EPR techniques (Schaich et al., 1999; Partridge et al., 2003). Lipid – protein interactions causing oxidative modifications have been studied by EPR, e.g., in models with methyl linoleate and lysozyme, BSA, α-lactalbumin, myoglobin or different amino acids (Karel et al., 1974; 1975; Schaich et al., 1976), myoglobin (Irwin et al., 1999), and in LDL (Hazell et al., 1999; Pietraforte et al., 2002). In addition, the effects of phenolic antioxidants as inhibiting radical formation by using EPR have been reported (Kanski et al., 2002; Liu et al., 2003; Ferroni et al., 2004).

2.3.5 Thiol compounds

Various methods have been reported for the determination of cysteine in biological and pharmaceutical samples. Oxidation of cysteine side-chains can be determined by derivatization with fluorescent dye 4-fluoro-7-aminosulfonylbenzofurazan (ABD-F), and then measuring directly with fluorescence spectroscopy at excitation 365 nm with emission set at 492 nm. This method has been applied in determining the oxidation of cysteine in menhaden oil-in-water emulsions with continous phase β-lactoglobulin (Elias et al., 2007). Biologically active thiols in pharmaceuticals have been determined by a simple and highly sensitive spectrophotometric method based on the fading of eosin-silver(I)-adenine ternary complex (Fujita et al., 2002). A compound having a disulfide bond (S-S-), such as cystine, could also be determined by the conversion of disulfides to free thiols with the sulfite ion.

A method for the simultaneous quantitation of total GSH and total cysteine in wheat flour by a stable isotope dilution assay using HPLC/tandem mass spectrometry (MS/MS) has been reported (Reinbold et al., 2008). The method procedure consisted of the protection of free thiol groups with iodoacetic acid, derivatization of free amino groups with 1-dimethylaminonaphathlene 5-sulfonyl chloride (dansyl chloride), and determination by HPLC-MS/MS. Sano et al. (1998) showed that thiols can be determined by precolumn derivatization with *o*-phthalaldehyde and *N*-(4-aminobutyl)-*N*-ethylisoluminol to form isoindole derivatives, separation by RP-HPLC, followed by successive postcolumn chemiluminescence reactions with H_2O_2 and hematin. Another derivatization reagent *N*-(2-acridonyl)-maleimide (MIAC) has been used for determination of thiol groups such as

homocysteine, cysteine and glutathione (Benkova et al., 2008). The reaction of MIAC with aminothiols is specific, very fast and yields highly fluorescent products, which can be detected by HPLC. In addition, flow injection with chemiluminescence detection (FI–CL) has been used for the determination of cysteine, glutathione and acetylcysteine in pharmaceuticals and synthetic amino acid mixtures by using various chemiluminescent reagents, e.g., luminol-persulphate (Waseem et al., 2008). A flow injection spectrophotometric method using Fe^{3+}-1,10-phenanthroline complex has been used in glutathione detection with ascorbic acid (Teshima et al., 2008).

2.3.6 Dityrosine

Formation of dityrosines in proteins as a result of oxidation or normal physiological processes can be used as a biomarker for assessing oxidative damage. Dityrosine has a specific fluorescence at emission wavelength of 400 nm with either excitation at 315 nm (for alkaline solutions) or at 284 nm (acidic solutions) (Malencik et al., 1996). In addition, absorbtion spectra of dityrosine exhibit isosbestic points at 315 nm and at 283 nm. In a study of Capeillere-Blandin et al. (2004) dityrosine formation in hypochlorous acid oxidized HSA was determined by using fluorescence measurement with excitation wavelength set at 320 nm and emission maximum set at 410 nm. Detection of 3-nitrotyrosine as an *in vivo* marker for the production of the cytotoxic species peroxynitrite ($ONOO^-$) has been reported using continuous flow photodiode array spectrophotometry (Eiserich et al., 1996). This reaction occurs when phenolic subtrate such as tyrosine reacts with nitrate and hypochlorous acid. In an another study, the excitation wavelength was set at 317 nm and emission at 407 nm for detection the formation of *o*-tyrosine and dityrosine which acted as indicators of oxidative damage in ribonuclease (RNase) and lysozyme exposed to radiolytic and metal-catalyzed (H_2O_2/Cu^{2+}) oxidation (Huggins et al., 1993).

2.3.7 Semialdehydes

The metal-catalyzed oxidation of proteins generates oxidation products of proline i.e. glutamic semialdehydes, and oxidation products of arginine and lysine i.e. aminoadipic semialdehydes. These products are carbonyl containing compounds. Glutamic and aminoadipic semialdehydes have been analyzed by using a specific isotope dilution by selected ion monitoring gas chromatography – mass spectrometry (GC-MS). The procedure involves the reduction of glutamic and aminoadipic semialdehyde side-chains into acid resistant hydroaminovaleric (HAVA) and hydroxyaminocaproic (HACA) acids, respectively by $NaBH_4$. By using hydrolysis in the presence of deuterated internal

standards, HAVA and HACA and their deuterated counterparts are converted to volatile trifluoroacetyl-methyl ester derivatives which can be analyzed by GC-MS (Requena et al., 2001).

2.4 Interactions between proteins and phenolic compounds

2.4.1 Natural sources and structures of phenolic compounds

Phenolic compounds are abundant in various fruits, berries, vegetables and their peels, tree materials, oilseeds, beverages and medicinal plants. **Tables 2 and 3** present some of the phenolic compounds present in different plant materials. Black currants, bilberries, cranberries, and strawberries mainly comprise of anthocyanins whereas cloudberries and red raspberries contain primarily ellagitannins. Cranberries and lingonberries are rich in flavanols and proanthocyanidins (Kähkönen et al., 2001: Viljanen et al., 2004b; Määtta-Riihinen et al., 2005). Many fruits and vegetables are predominated by flavonols such as quercetin, kaempferol and myricetin as well as phenolic acids (Rice-Evans et al., 1996; Justesen et al., 1998; Aherne et al., 2002). Medicinal plants such as aloe vera leaves are also rich in flavonols (Sultana et al., 2008). Tea is a source for flavanols such as epigallocatechin. Tea also has many polymerized phenolics present: theaflavins and theaflavin 3-gallate (dimers) and thearubigins (tannin oligomers) (Peterson et al., 2005). Oilseeds such as rapeseed, camelina, olive, and corn are rich in phenolic acids (Vuorela et al., 2005b; Tuberoso et al., 2007). Lignans are present especially in oilseeds, nuts, and grains, but also in *Brassica* vegetables such as broccoli, curly cale, and Brussels sprouts. In addition, other vegetables, fruits and beverages have low amounts of lignans (Milder et al., 2005). Soy bean and kudzu root are high in isoflavones (Dixon, 2004).

Table 2. Some flavonoids and phenolic acids present in plants.

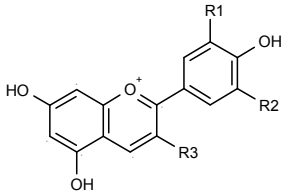
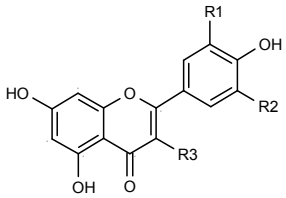
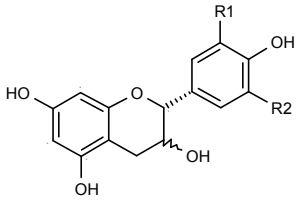
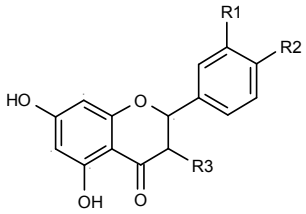
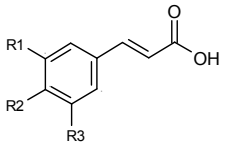
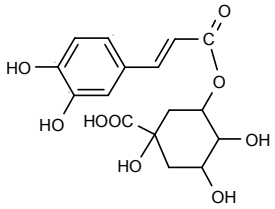
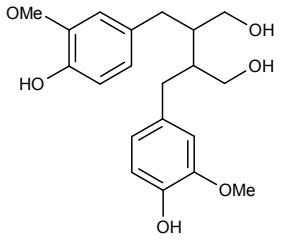
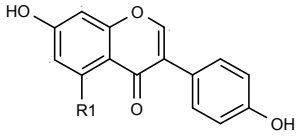
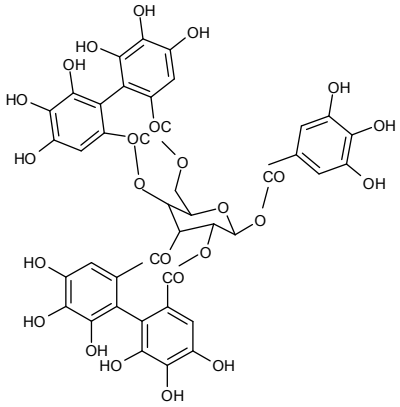
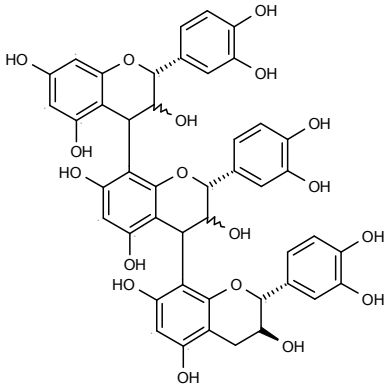
Phenolic group	Compounds		Examples of sources	References
Flavonoids				
Anthocyanidins				
	Cyanidin Delphinidin Pelargonidin Peonidin Malvidin Kuromanin	R_1=OH, R_2=R_3=H R_1=R_2=OH, R_3=H R_1=R_2=R_3=H R_1=OCH_3,R_2=R_3=H R_1= R_2=OCH_3, R_3=H R_1=OH,R_2=H, R_3=glucose	Raspberry, strawberry, black currant, bilberry, lingonberry, cranberry, cherry, grapes, red wine	Rice-Evans et al., 1996; Kähkönen et al., 2001; Viljanen et al., 2004b; Määtta-Riihinen et al., 2005;
Flavonols				
	Quercetin Myricetin Kaempferol Rutin Isoquercitrin	R_1=OH, R_2=R_3=H R_1=OH, R_2=OH, R_3=H R_1=R_2=R_3=H R_1=H, R_2=OH, R_3=rutinose R_1=OH, R_2=H, R_3=β-D-glucose	Onion, lettuce, broccoli, cranberry, apple skin, cranberry, bilberry, cloudberry, lingonberry, aloe vera, strawberry, olive, tea, red wine, grapes, endive, leek, radish, grapefruit, camelina	Rice-Evans et al., 1996; Justesen et al., 1998; Aherne et al., 2002; Kris-Etherton et al., 2002; Sultana et al., 2008;
Flavanols (Catechins)				
	(±)-Catechin Epigallo-catechin	R_1=OH, R_2=H R_1=OH, R_2=OH	Green and black teas, red wine, black currant, cranberry, lingonberry, strawberry, rapeseed, camelina, soy, pine bark	Rice-Evans et al., 1996; Kris-Etherton et al., 2002; Peterson et al., 2005; Vuorela et al., 2005b; Tuberoso et al., 2007
Flavanones				
	Naringenin Hesperitin Taxifolin	R_1=H, R_2=OH, R_3=H R_1=OH, R_2=OCH_3, R_3=H R_1=OH, R_2=OH, R_3=OH	Peel of citrus fruits, citrus fruits, pine bark	Rice-Evans et al., 1996; Justesen et al., 1998
Phenolic acids				
	Caffeic acid Ferulic acid Sinapic acid	R_1=H, R_2=OH, R_3=OH R_1=H, R_2=OH, R_3=OCH_3 R_1=OCH_3, R_2=OH, R_3=OCH_3	White grapes, white wine, olives, olive oil, spinach, cabbage, asparagus, coffee, tomatoes, wheat, corn, rice, rapeseed, camelina	Rice-Evans et al., 1996; Kris-Etherton et al., 2002; Vuorela et al., 2005b; Tuberoso et al., 2007
	Chlorogenic acid		Apples, pears, cherries, plums, peaches, apricots, blueberries, tomatoes, anis, rapeseed, camelina	Kris-Etherton et al., 2002; Vuorela et al., 2005b

Table 3. Some lignans, isoflavones and phenolic polymers present in plants.

Phenolic groups	Compounds	Examples of sources	References
Lignans			
	Secoisolariciresinol (shown) Other: Syringaresinol Pinoresinol Lariciresinol Matairesinol	Soybean, flaxseed, sesame seed, sunflower seed, cashew, oat bran, wheat bran, rye bran, barley bran, *Brassica* vegetables, garlic, apricot	Kris-Etherton et al., 2002; Dixon, 2004; Milder et al., 2005
Isoflavones			
	Daizdein R_1=H Genistein R_1=OH	Soybean, kudzu root, fava bean, chick pea, lupine, alfalfa, peanut	Kris-Etherton et al., 2002; Dixon, 2004
Phenolic polymers			
Ellagitannins			
	Casuarictin (shown) Other: Sanguiin H-10 Sanguiin H-6 Lambertianin C	Raspberry, strawberry, cloudberry	Kähkönen et al., 2001; Määtta-Riihinen et al., 2004b; Mullen et al., 2003; Kähkönen et al., unpublished results;
Proanthocyanidins			
	Procyanidin trimer (flavanol) (shown) Other: Procyanidin B1 and B2 Prodelphinidin B2	Cranberry, lingonberry, bilberry, bog whortleberry, grapes	Kris-Etherton et al., 2002; Määtta-Riihinen et al., 2005

2.4.2 Antioxidant function of phenolics toward protein oxidation

Phenolic compounds act as antioxidants by donating electrons and terminating radical chain reactions (Dangles et al., 2006), and by binding metals as metal chelators (Fernandez et al., 2002). In flavonoid molecule (**Figure 13**), the antioxidant and metal chelating properties are mainly due the 3',4'-dihydroxy group located on the B ring, the 3-hydroxy or 5-hydroxy and the 4-carbonyl groups in the C-ring. In addition, the antioxidant activity increases with the number of hydroxyl groups in rings A and B. For example, complexation of metal ions with oxidized quercetin is proposed to involve a ketone structure in the 3-hydroxyl group. In addition, the five hydroxyl groups in quercetin contribute to the antioxidant properties. Catechin with no 4-carbonyl group may chelate metal ions through the *ortho*-catechol group in the positions 3' and 4' in the B-ring (Fernandez et al., 2002).

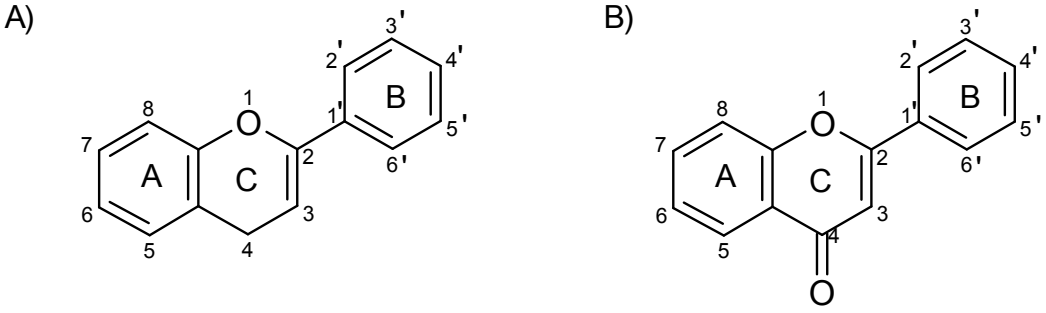


Figure 13. A) The flavan nucleus, and B) 4-oxo-flavonoid nucleus.

The overall antioxidant mechanism of flavonoids is recognized as a combination of a direct reaction with free radicals and chelation of metal ions (Fernandez et al., 2002; Dangles et al., 2006). Modifications of proteins and lipids are frequently influenced by redox cycling transition metal ions. Flavonoids primarily chelate prooxidant transition metal ions such as Fe^{2+}, Fe^{3+} and Cu^{2+} and alter their redox potentials rendering them inactive in generating free radicals (Fernandez et al., 2002). Same flavonoids can behave as both antioxidants and prooxidants, depending on the concentration and free radical source. In a study by Cao et al. (1997), flavonoids acted as antioxidants against free radicals but demonstrated prooxidant activity when transition metal ions were available. This is because the flavonoid may react directly with the transition metal ions and affect the rate of related free radical generation. It has been shown that if the metal-flavonoid –complex still undergoes redox reactions, the free radicals generated can be scavenged by the ligand itself since metal-flavonoid chelates are considerably more potent free radical scavengers than the parent flavonoids (Fernandez et al., 2002).

The role of phenolic antioxidants on protein oxidation has been studied in different oxidation models such as oil-in-water emulsions, meat, liposomes and LDL (Heinonen et al., 1998; Almajano et al., 2004; Rababah et al., 2004; Viljanen et al., 2004a; 2005a; 2005b; Yeomans et al., 2005; Almajano et al., 2007). However, the extent of protein oxidation studies is still limited as research has been mostly focused on lipid oxidation reactions. Berry phenolics have been shown to act as antioxidants in oil-in-water emulsions. A study by Viljanen et al. (2005b) reported that anthocyanins isolated from black currant, raspberry and lingonberry showed protection toward protein oxidation in whey protein stabilized rapeseed oil-in-water emulsion. In another study, the antioxidant activity of raspberry and blackberry juices were evaluated also in whey protein stabilized rapeseed oil-in-water emulsions (Viljanen et al., 2005a). Raspberry juice provided a better overall antioxidant protection towards lipid and protein oxidation compared to blackberry juice. In all of these studies, the antioxidant activity of berry phenolics was shown to increase with the increasing concentration of the anthocyanins (Viljanen et al., 2005a; 2005b). Different berry phenolics such as anthocyanins, ellagitannins, and proanthocyanidins from raspberry, bilberry, lingonberry, and black currant, as well as pure compounds of procyanidins, anthocyanins and their aglycons were found to be potent in inhibiting protein oxidation in α-lactalbumin-lecithin liposome system (Viljanen et al., 2004a; 2004b).

Depending on the concentration, almond skin polyphenolics showed 6.7-76% inhibitions toward tryptophan oxidation in human LDL (Chen et al., 2007). Red wine phenolics and phenolic compounds with catechin structure (catechin, epicatechin, and quercetin) protected LDL tryptophan from oxidation by 30-40% under peroxynitrite-mediated oxidation of LDL. The same phenolics showed, however, a more pronounced effects on inhibiting 3-nitrotyrosine formation (Ferroni et al., 2004). EPR studies confirmed that red wine phenolics are potent scavengers of radicals formed by peroxynitrite/CO_2 due to increased formation of *o*-semiquinone radicals. Combination of quercetin and caffeic acid were potent in inhibiting the tryptophan oxidation and formation of protein carbonyls in copper-induced oxidation of human LDL (Yeomans et al., 2005). Oxidation of proteins (measured as protein carbonyls) in porcine liver pâté was shown to be inhibited by sage and rosemary essential oils (Estévez et al., 2006).

The impact of oxidation and berry phenolics on the location of tryptophan side-chains in oil-in-water emulsions have been reported (Viljanen et al., 2005a). Most of the tryptophan side-chains in whey proteins located in the buried hydrophobic areas were protected by berry phenolics during

oxidation due to non-covalent i.e. hydrophobic interactions. Tryptophan side-chains in the aqueous phase, however, were oxidized, leading to conformational changes due to covalent bond formations.

2.4.3 Binding properties of phenolic compounds

Phenolic – protein interactions influence the characteristics of food, thus affecting the technological processes involved in food manufacturing. In food products, the phenolic compounds influence the taste such as bitterness or astringency, appearance such as color as well as nutritional value. Phenolic bioavailability and phenolic-mediated cell effects may involve the interactions of phenolic compounds with specific biological targets, mainly proteins, and thus contribute to potential health effects. In addition, possible participation of phenolic compounds in the regulation of gene expression for specific proteins may also play an important role in health effects (Dangles et al., 2006). The interactions of tannins (oligomeric procyanidins) with proline-rich salivary proteins developing sensation of astringency is currently the sole binding process with clear *in vivo* biological significance (Baxter et al., 1997). Tannin – protein interactions occur via two-phase mechanis: 1) tannin binds to protein forming soluble complexes, and 2) soluble tannin – protein complexes aggregate forming insoluble complexes (Charlton et al., 2002). Tannin – protein interactions due to astringency contribute to desirable qualities in many foods and beverages, e.g., in tea and red wine. However, another food technological aspect is haze formation in beer, fruit juices and wine. Tannin – protein haze is unwanted in these products, but the affinity of tannins for proteins can also be used as a mechanism to reduce the content of phenolics during wine making. Relative concentrations of tannin and proteins have a large influence on the amount of haze formed (Siebert et al., 1996a; 1996b; Siebert, 1999).

The properties of polyphenols affect their binding and quenching (i.e. the process which decreases the fluorescence intensity of a given substance) in BSA and human salivary α-amylase (Soares et al., 2007). The binding affinity increases with increasing molecular weight of the phenolic compounds and in the presence of galloyl groups (procyanidin oligomers > procyanidin B2 gallate > epicatechin gallate > catechin > procyanidin B4). The binding affinities of flavanols increase with the number of hydroxyl groups in the B-ring. This was revealed in a study with BSA with myricetin showing the highest binding affinity followed by quercetin, kaempherol and galangin. The glycoside substitute at the C-ring, however, decreased the binding (Xiao et al., 2008). Isolated mixtures of chesnut and myrabolan ellagitannins exhibited 3-4 fold greater binding affinities for interaction with gelatin than for BSA, whereas tara and sumac gallotannins bound with equal

strength to both gelatin and BSA. The differences in the binding constants are dependent on the structural flexibility of the tannin molecule. As the loss of conformational freedom of ellagitannins appears not to interfere with binding to a flexible protein such as gelatin, however, the flexibility of the protein plays also an important role in the protein - phenolic binding (Deaville et al., 2007).

It has been suggested, that association between flavonoids (quercetin, rutin, epicatechin, catechin) and BSA do not change molecular conformation of BSA (Papadopoulou et al., 2005). Hydrogen bonding, ionic, and hydrophobic interactions are equally important in protein-flavonoid associations (Papadopoulou et al., 2005; Dangles et al., 2006). Reactions of *m*-, *o*-, *p*-dihydroxybenzenes, ferulic and gallic acid with lysozyme indicated reduction in tryptophan and lysine side-chains, proceeding to the formation of complex polymeric species (Rawel et al., 2001). In a study of Tsai et al. (2006), coumaric acid and caffeic acid were shown to bind with pea protein (26 and 23%, respectively), whereas catechin (13%) and ferulic acid (3%) had less effect, and gallic acid had no binding. It was concluded that the binding between phenolics and protein matrix might account for the enhancement of antioxidant capacity, since protein – phenolic interaction is able to stabilize the protein and its antioxidant capacity is increased during heating.

Absorption studies show that a LDL particle has the capacity to bind ca. 10 quercetin molecules through interaction with apolipoprotein B. LDL-bound quercetin has been shown to efficiently repair oxidative damage of at least tryptophan and tyrosine side-chains in apolipoprotein B in LDL via intramolecular electron transfer. In contrast, rutin which does not bind to LDL, cannot repair oxidized apolipoprotein B (Filipe et al., 2002). Binding of olive oil phenolics to food proteins such as BSA, β-lactoglobulin, sodium caseinate, and gelatin was found to be relatively weak (Pripp et al., 2005). It has been reported that binding of hexanal to soy glycinin and β-conglycinin induced structural modifications such as increase in surface hydrophobicity and turbidity as well as loss of lysine (O'Keefe et al., 1991). Phenolic compounds such as flavonols, flavones, flavanones and isoflavones can bind to bovine and reindeer β-lactoglobulin (Riihimäki et al., 2008). Flavonols (myricitrin, myricetin and morin), flavanones (hesperidin and naringenin), flavones (baicalin), and isoflavones (daidzein) were shown to have higher affinities to both bovine and reindeer β-lactoglobulin than phenolic acids and their derivatives. In addition, it was found that phenolic – β-lactoglobulin complex was stable at acidic and basic conditions, but not during thermal treatment, which would implicate that β-lactoglobulin could act as a binder or carrier for phenolic compounds, and protect them from degradation during non-heating processing.

3. AIMS OF THE STUDY

The hypothesis is that interactions between proteins and dietary phenolic compounds have impact on the quality of food and functional properties of proteins and phenolic compounds, that in turn may influence the bioavailability i.e. the fraction of the administered bioactive compound that is absorbed in the intestinal tract, and health related effects of dietary phenolics. The overall aims of the study were to address the oxidation of proteins and amino acids as well as the interactions between other constituents of foods such as lipids in the presence of phenolic compounds from berries and byproducts of oilseed processes. In order to prevent and control oxidation reactions, the formation of individual oxidation products must be elucidated. Therefore, the oxidation was also studied at the level of single amino acid such as tryptophan.

The specific objectives of the study were:

- To investigate the effect of different plant phenolics toward tryptophan oxidation in different oxidation environments (**III, IV**).

- To investigate the antioxidant activity of different plant phenolics toward protein and lipid oxidation in food models such as meat and emulsions (**I, II, V**).

4. MATERIALS AND METHODS

This section summarizes the materials and methods presented in more detail in the original papers (**I-V**).

4.1 Oxidation models

The meat model (**I, II**) was performed according to the method of Vuorela et al. (2005b) using red meat from *longissimus dorsi* muscle of pork, and fat from pork back (2:1) with added water (10%), salt (1.4%), and different phenolic compounds (**Table 4**). After homogenization the samples were vacuum-packed and heated in a water bath (80 °C, 10 min). After heating, the bags were opened, and the samples were transferred to decanter flasks and oxidized for 9 days (**I**) or 10 days (**II**) at 5 °C under light.

The tryptophan model solution (2 mM) using 0.1 M borate (pH 6.3) in the presence of selected extracts of phenolic compounds was oxidized with H_2O_2 (resulting in final pH of 4.6) added hourly (final concentration of 1.05 M) for 6 h at 37 °C in dark (**III**) or oxidized with 50 mM hexanal and 0.1 mM $FeCl_2$ (resulting in final pH of 5.5) for 8 days at 37 °C in the dark (**IV**) (**Table 4**).

The corn oil-in-water emulsions (**V**) with continuous phase β-lactoglobulin with added phenolic compounds were used in all oxidation studies. Oil-in-water emulsions were prepared using 5% corn oil (containing tocopherols 14.4 mg/100 g) and 0.5% surfactant (Brij 35) in sodium phosphate buffer (10 mM, pH 7.0) containing 0.02% sodium azide as an antimicrobial agent. Emulsions were oxidized for 24 days at 55 °C in the dark (**Table 4**).

Table 4. Design of studies I-V.

STUDY	OXIDATION MODEL	EXPERIMENTAL CONDITIONS	ANTIOXIDANTS	CONCENTRATIONS	METHODS
I	Meat	+5 °C under light 9 days	Rapeseed meal Rapeseed meal extract [a] Rapeseed meal extract [b] Rapeseed oil extract [c] Pine bark extract [d] Sinapic acid, vinylsyringol, taxifolin	282.4 mg/100 g meat 29.4 mL/100 g meat 29.4 mL/100 g meat 4.7 mL/100 g meat 10.6 mL/100 g meat 23.5 mg/100 g meat	GC (hexanal) DNPH-method
II	Meat	+5 °C under light 10 days	Rapeseed meal, camelina meal, soy meal and flour Rapeseed meal, camelina meal, soy meal and flour + rosemary extract Quersetin, chlorogenic acid, genistein	0.3, 0.5, and 0.7 g/100 g meat 0.5 g/100 g + 0.04 g/100 g meat 0.01 g/100 g meat.	GC (hexanal, propanal, pentanal) DNPH-method
III	Tryptophan	+37 °C in the dark 6 hours pH 4.6	Camelina meal [e], rapeseed meal [a], soy meal and flour [e], pine bark [d], raspberry and black currant anthocyanins, raspberry ellagitannins, cranberry proanthocyanidins Cyanidin-3-glucoside, delphinidin-3-glucoside, caffeic acid, chlorogenic acid, catechin, daidzein, ellagic acid, ferulic acid, genistein, procyanidin B1, quercetin, sinapic acid, taxifolin, vinylsyringol	10, 50, 100 µM	HPLC-UV-FL
IV	Tryptophan	+37 °C in the dark 8 days pH 5.5	Camelina meal [e], rapeseed meal [a], soy meal [e], pine bark [d], raspberry and black currant anthocyanins, raspberry ellagitannins, rowanberry Cyanidin-3-glucoside, chlorogenic acid, catechin, ellagic acid, genistein, procyanidin B2, quercetin, sinapic acid, taxifolin	50, 100 µM	HPLC-UV-FL
V	5% Corn oil-in-water emulsion	+55 °C in the dark 24 days pH 7.0	Black currant anthocyanins Rasperry ellagitannins	20, 50 µM	Ammonium thiocyanate –method (lipid hydroperoxides) GC (hexanal) Trp fluorescence Cys fluorescence Stability of anthocyanins

[a] Aqueous ethanolic (70%) extract. [b] Extract obtained by enzymatic treatment with Ultraflo L. [c] Aqueous methanolic (80%) extract of crude rapeseed oil. [d] Aqueous water extract containing 30% pine bark and phloem. [e] Aqueous methanolic (80%) extract. Trp = tryptophan, Cys = cysteine.

4.2 Plant materials

The rapeseed (*Brassica rapa* L., L. = Linnaeus) meal (**I-IV**) used was the byproduct of rapeseed deoiling process in which the oil was expelled by pressing the seeds at an elevated temperature by Mildola Ltd. (Finland). Camelina (*Camelina sativa*) meal (**II-IV**) was the byproduct of cold pressed camelina oil obtained from Raisio Ltd. (Finland). Soy (*Glycine max* L.) meal (**II-IV**) (Risetti®) was obtained from Risetti Ltd. (Finland) and soy flour (**II-III**) (Soyolk) was obtained from Cereform Ltd. (Northampton, England). Scots pine (*Pinus sylvestris* L.) bark drink (**I-IV**) was obtained by extraction with water so that it contained 30% pine bark and phloem (Ravintorengas Ltd., Siikainen, Finland). All berries, raspberry (*Rubus idaeus*) (**III-V**), black currant (*Ribes nigrum*) (**III-V**), cranberry (*Vaccinium oxycoccus*) (**III**) and rowanberry (*Sorbus aucuparia*) (**IV**), were purchased from a market place. Protein, fatty acid, and tocopherol compositions as well as isoflavone and lignan contents of the oilseed processing byproducts were measured (**II**). The protein concentration was measured by determination of nitrogen according to the Kjehldahl procedure (AOAC International, 1995), and calculated with a 6.25 nitrogen conversion factor. The total fat of plant materials was determined by using a Soxtec Avanti 2050 automatic extraction system. The fatty acid composition was measured by GC after hydrolyzing and methylating the fat extracts. Nonadecanoic acid (C 19:0 fatty acid) and a methyl ester mixture (Nu Chek Prep, GLC-63A) were used as an internal standard and standard, respectively. The results were expressed as methyl ester equivalents of fatty acids. The content of tocopherols in camelina, rapeseed, and soy meals, soy flour, and rosemary extract were analyzed according to a method of Ryynänen et al. (2004). Isoflavones and lignans were analyzed in soy meal and flour according to the methods by Nurmi et al. (2003a) and Nurmi et al. (2003b), respectively.

4.2.1 Extraction of oilseed phenolics (I-IV)

Plant phenolics were extracted with 70% methanol (rapeseed meal **I**), 80% methanol (camelina and soy meals **II-IV**, soy flour **III**), 70% ethanol (rapeseed meal **I-IV**), and enzyme-assisted extraction with Ultraflo L enzyme preparation in 0.02 M ammonium diphosphate buffer solution at pH 5.5 (**I**).

Plant materials (0.8 g in studies **I, III, IV**, 1-2 g in study **II**) and 20 mL of respective solvent were put in a centrifuge tube, which was then shaken in a water bath at 75 °C for 1 h (**I-IV**), or at 37 °C for 2 h (enzyme extraction in study **I**). The enzymatic reaction was stopped by boiling the mixture for 10 min (**I**). After centrifugation (3500 rpm, 20 min is study **I,** 15 min in studies **II-IV**), the clear

phenolic extract was collected. The Ultraflo L enzyme preparation was checked by RP-HPLC not to contain phenolic compounds. Rapeseed oil phenolics (**I**) were extracted with 80% methanol according to the method outlined by Koski et al. (2003).

4.2.2 Isolation of berry phenolics (III-V)

Extraction and isolation of raspberry anthocyanins (**III, IV**), black currant anthocyanins (**III-V**) and raspberry ellagitannins (**III-V**) were carried out as described by Kähkönen et al. (2003). The berry anthocyanin fractions were further purified by preparative RP-HPLC and the interfering sugars were removed by solid phase extraction (SPE) as described by Kähkönen et al. (2003). A method by Määtta-Riihinen et al. (2004b) was followed to isolate cranberry proanthocyanidins (**III**). Rowanberry phenolics (**IV**) were extracted and isolated as described by Kylli et al. (unpubl. data).

4.2.3 Characterization of plant phenolics (I-V)

The amount of total polyphenols (**I-IV**) in plant materials was measured colorimetrically according to the Folin–Ciocalteau procedure (Singleton et al., 1965). The RP-HPLC analysis of phenolics was performed according to the method outlined by Koski et al. (2003) for phenolic acids and their derivatives (**I-IV**), by Kähkönen et al. (2001) for other phenolic compounds (**II-V**), by Kylli et al. (unpubl. data) for rowanberry phenolics (**IV**), and by Karonen et al. (2004) for pine bark phenolics (**I**). Phenolic compositions of berries and plant materials are shown in **Tables 5** and **6**, respectively.

Table 5. Phenolic composition of berry isolates (mg/g ± SD) used in studies III-V [a].

Concentration (mg/g)	Raspberry anthocyanins	Raspberry ellagitannins	Black currant anthocyanins	Cranberry proanthocyanidins	Rowanberry extract
Total phenolics [b]	NA	NA	NA	NA	18.7 ± 0.8
Anthocyanins [c]	534 ± 25	18 ± 0	314 ± 44	ND	9.6 ± 0.3
Ellagitannins [d]	ND	369 ± 44	ND	ND	ND
Proanthocyanidins [e]	ND	ND	ND	554	ND
Ellagic acid [d]	ND	16 ± 2	ND	ND	0.2 ± 0.04
Flavanols [f]	ND	ND	12 ± 1	ND	ND
Catechin [f]	NA	NA	NA	NA	52 ± 2
Hydroxybenzoic acids [b]	ND	ND	ND	ND	0.1 ± 0.02
Hydroxycinnamic acids and derivatives [g]	ND	ND	ND	ND	341 ± 23
Flavonols [h]	ND	8 ± 1	ND	1	64 ± 2

[a] NA = not analyzed, and ND = not detected/concentration under detection limit. [b] Gallic acid as the standard. [c] Cyanidin 3-glucoside as the standard. [d] Ellagic acid as the standard. [e] Procyanidin B1 as the standard. [f] Catechin as the standard. [g] Chlorogenic acid as the standard. [h] Rutin as the standard.

Table 6. Phenolic composition (μg/g ± SD) of plant extracts used in studies I-IV [a].

Material	Total phenolics [h]	Flavonoids and phenolic acids								
		Flavanols [i]	Hydroxybenzoic acids [h]	Hydroxycinnamic acids and derivatives [j]	Sinapine [k]	Sinapic acid [k]	Vinylsyringol [k]	Flavonols [l]	Isoflavones [m]	Lignans
Rapeseed meal										
I [b]	4751 ± 114	NA	NA	NA	2861 ± 7	275 ± 2	ND	NA	NA	NA
I [c]	5885 ± 109	NA	NA	NA	275 ± 2	2831 ±76	ND	NA	NA	NA
I (oil) [d]	785 ± 28	NA	NA	NA	3 ± 0	22 ± 1	463 ± 5	NA	NA	NA
II [b]	5100 ± 155	35 ± 4	14 ± 3	3096 ± 330	2861 ± 7	275 ± 2	ND	ND	NA	NA
III [b]	6730 ± 290	ND	ND	8030 ± 1400	1800 ± 180	140 ± 20	64 ± 7 [n]	ND	NA	NA
IV [b]	5865 ± 547	ND	ND	3773 ± 1063	765 ± 134	74 ± 9	ND	ND	NA	NA
Camelina meal										
II [e]	6200 ± 490	236 ± 37	ND	747 ± 54	1437 ± 47	427 ± 10	ND	1325 ± 196	NA	NA
III [e]	3940 ± 110	2110 ± 410	ND	3020 ± 160	650 ± 40	30 ± 3	ND	2150 ± 70	NA	NA
IV [e]	9791 ± 993	3233 ± 818	ND	3590 ± 383	450 ± 52	9 ± 1	ND	6029 ± 677	NA	NA
Soy meal										
II [e]	2600 ± 195	92 ± 22	ND	ND	ND	ND	ND	ND	1310 ± 6	15 ±1
III [e]	2770 ± 65	760 ± 10	ND	ND	ND	ND	ND	ND	NA	NA
IV [e]	1761 ± 133	449 ± 127	ND	ND	ND	ND	ND	ND	NA	NA
Soy flour										
II [e]	1800 ± 150	99 ± 14	ND	ND	ND	ND	ND	ND	1400 ± 51	23 ± 2
III [e]	1650 ± 50	790 ± 70	ND	ND	ND	ND	ND	ND	NA	NA
Pine bark										
I [f]	762 ± 10	336 ± 8[h]	70 ± 4	ND	ND	ND	ND	ND	NA	83 ± 2
III [f]	3400 ± 250	80 ± 10	3 ± 0	ND	ND	ND	ND	ND	NA	NA
IV [f]	3400 ± 250	80 ± 10	3 ± 0	ND	ND	ND	ND	ND	NA	NA
Rosemary										
II [f, g]	15600 ± 520	ND	ND	ND	ND	ND	ND	ND	NA	NA

[a] NA = not analyzed, and ND = not detected/concentration under detection limit. [b] Aqueous ethanolic (70%) extract. [c] Extract obtained by enzymatic treatment with Ultraflo L. [d] Aqueous methanolic (80%) extract of crude rapeseed oil. [e] Aqueous methanolic (80%) extract. [f] Commercial extract. [g] According to manufacturer, extract contains 9 ± 1% phenolic diterpenes: carnosic acid, carnosol and rosmanol. [h] Gallic acid as the standard. [i] Catechin as the standard. [j] Chlorogenic acid as the standard. [k] Sinapic acid as the standard. [l] Rutin as the standard. [m] As aglycone calculated from total 7-*O*-glucoside and aglycone. [n] Vinylsyringol as the standard.

4.3 Analyses of protein oxidation products

4.3.1 Protein carbonyls (I, II)

Protein oxidation in meat (**I, II**) was followed by measuring the formation of protein carbonyls by converting them to 2,4-dinitrophenylhydrazones (DNPH) and the derivatives were measured spectrophotometrically according to method outlined by Oliver et al. (1987). Two different measurements were made for protein oxidation: quantification of (a) carbonyls and (b) protein. Meat samples of 1 g (amount of protein = 0.7-1 mg of a sample) were homogenized with 10 mL of 0.15 M KCl with an UltraTurrax homogenizor for 60 s. One hundred microliters of homogenate was transferred into a 2 mL Eppendorf vial, where 1 mL of 10% trichloroacetic acid was added. The sample was centrifuged for 5 min at 5000 rpm, and the supernatant was removed. For sample (a) 1 mL of 2 M HCl with 0.2% DNPH and for sample (b) 1 mL of 2 M HCl was added. After an incubation of 1 h (shaken every 20 min), 1 mL of 10% trichloroacetic acid was added. The sample was vortexed and centrifuged for 5 min at 5000 rpm. Supernatant was removed carefully without damaging the pellet with a Pasteur pipet. The pellet was washed with 1 mL of ethanol/ethyl acetate (1:1), shaken, and centrifuged for 5 min at 10000 rpm; this procedure was repeated two to three times. After this, the pellet was completely dried with nitrogen. The pellet was dissolved in 1.5 mL of 20 mM sodium phosphate buffer with 6 M guanidine hydrochloride, final pH 6.5, shaken, and centrifuged for 2 min at 5000 rpm. Carbonyls (sample a) and protein concentration (sample b) were measured spectrophotometrically at 370 nm and 280 nm, respectively. Concentration of carbonyls was calculated as [$Abs_{370\ nm}$/21.0 mM^{-1} cm^{-1}) x 1000], where 21.0 mM^{-1} cm^{-1} is the molar extinction coefficient of carbonyls. Protein quantification was determined using a standard curve made from BSA.

The inhibitions of plant phenolics (**I, II**) against formation of protein carbonyls was calculated from the equation: $(C_0-C_1)/C_0\times100$, where C_0 is the concentration (nM) of protein carbonyls per mg of protein in the control sample and C_1 is the concentration (nM) of protein carbonyls per mg of protein in the tested sample. The inhibitions were expressed as percentages.

4.3.2 Tryptophan derived oxidation products (III, IV)

The tryptophan model solutions were prepared first by pipetting solutions of phenolic extracts (at selected concentration levels) into 4 mL (**III**) or 10 mL (**IV**) screw-top vials, and the solvent was

evaporated under nitrogen flow. In the control sample, no phenolic antioxidant was added. In study **III**, the 2 mM tryptophan solution (2 mL) and 0.19 M H_2O_2 (40 μL) were then added into the vials resulting in final tryptophan concentration of 317 μg/mL. Every hour during the incubation, an aliquot of 40 μL of H_2O_2 was added to samples to keep the catalyst abundant. Samples were agitated on a vortex mixer after additions of H_2O_2, and the mixtures in sealed vials were stirred during incubation. After incubation, the reaction was stopped by 30 min of treatment with 100 μL of catalase (about 300 U = 5000 nkat). The absence of H_2O_2 was subsequently checked by potassium iodide test. After that, the sample was acidified with 164 μL of 2 M acetic acid. In study **IV**, the 2 mM tryptophan solution (5 mL) followed by 50 mM hexanal (31 μL) and 0.1 mM $FeCl_2$ (25 μL) were added to the vials. All the samples (studies **III** and **IV**) were filtered with 0.45 μm Acrodisc syringe filters with hydrophilic polypropylene membrane (Pall Life Sciences, Ann Arbor, MI, USA) prior to HPLC analysis.

Quantitative determination of tryptophan and its oxidation products (**III**, **IV**) was carried out using HPLC combined with diode array and fluorescence detection. The HPLC system (Waters, Milford, MA, USA) consisted of 717 plus autosampler, 515 and 510 pumps equipped with a control module, a column oven with a temperature control module, a PDA 996 diode array and a 2475 multi λ fluorescence detectors (**IV**) or scanning fluorescence detector (Hewlett Packard 1046) (**III**).

Analysis of H_2O_2 oxidized tryptophan and its oxidation products (**III**) were determined by method outlined by Simat et al. (1998) with some modifications. Analysis of hexanal/$FeCl_2$ oxidized tryptophan and its oxidation products (**IV**) was carried out by developing and validating a new HPLC method. The experimental conditions are summarized in **Table 7**. Tryptophan oxidation products were identified by retention times and UV-spectra. The quantification of tryptophan and its oxidation products were made using tryptophan, kynurenine and 5-hydroxy-tryptophan (**III, IV**) as well as tryptamine and β-oxindolylalanine (**III**) as external standards. The quantifications of oxidation products 3a-hydroxypyrroloindole-2-carboxylic acid A/B (i.e. diastereomers of A and B), dioxindolylalanine A/B, and *N*-formylkynurenine were calculated as equivalents to 5-hydroxy-tryptophan (**III**) or kynurenine (**IV**) as external standard since these compounds were not commercially available. The method of quantification with standard compounds was as followed: 5-hydroxy-tryptophan, kynurenine, β-oxindolylalanine, tryptamine, and kynurenic acid were dissolved in 0.1 M borate (pH 6.3) to a concentration of approximately 0.6 mg/25 mL except for tryptophan to 6 mg/25 mL (stock solutions) followed by dilutions of 1:4 and 1:40. Samples of these

three concentrations were analyzed with injection volumes of 10, 15, and 20 µL. Consequently, a nine-point calibration curve was determined from standard solutions for each compound.

The percent inhibition of plant materials against formation of tryptophan oxidation products was calculated from the equation: $(A_0-A_1)/A_0$ x 100, where A_0 is the area of tryptophan oxidation product in control sample and A_1 is the tryptophan oxidation product in antioxidant sample (**III, IV**). The percent inhibition of plant materials against formation of tryptophan oxidation was calculated from the equation: $[(C_0-C_t)-(S_0-S_t)/(C_0-C_t)]$ x 100, where C_0 is the area of tryptophan in control sample at day 0, C_t is the area of tryptophan at day 6, S_0 is tryptophan with antioxidant at day 0, and S_t is tryptophan with antioxidant at day 6 (**IV**). In study **III** the same time points of day 0 and day 6 corresponded to 0 h and 6 h, respectively. The inhibitions were expressed as percentages.

Table 7. Conditions of HPLC-methods (III, IV).

	STUDY III	**STUDY IV**
Column	Nova-Pak C18 column (3.9 mm x 150 mm, 4 µm) Spherisorb S5 ODS2 precolumn (20 mm x 2 mm, 5 µm) (Waters, Milfrod, MA)	C8 Discovery® BioWide Pore column (100 mm x 4.6 mm i.d., 5 µm, 300 Å) C8 Discovery® BioWide Pore guard column (20 mm x 40 mm i.d., 5 µm) (Supelco, Bellefonte, PA)
Solvents	0.1% TFA (solvent A) Methanol (solvent B) Acetonitrile (solvent C)	0.1% TFA (solvent A) Acetonitrile (solvent B)
Gradient program	0 min: 95% A 5% B 10 min: 86% A 14% B 40 min: 46% A 14% B 40% C 42 min: 26% A 14% B 60% C 46 min: 95% A 5% B	0 min: 98% A 2% B 30 min: 70% A 30% B 30.1 min: 50% A 50% B 32 min: 75% A 25% B 40 min: 98% A 2% B
FL-detector	0-13 min: Ex 230 nm, Em 342 nm 13-46 min: Ex 280 nm, Em 335 nm	0-7 min: Ex 230 nm, Em 340 nm 7-11 min: Ex 230 nm, Em 435 nm 11-40 min: Ex 270 nm, Em 350 nm
DAD-detector	260 nm	260 nm
Temperature	35 °C	25 °C
Flow rate	1.0 mL/min	1.0 mL/min
Injection volume	20 µL	10 µL

TFA = trifluoroacetic acid

4.3.2.1 HPLC method validation (IV)

The tryptophan oxidation product standard compounds (3-hydroxy-tryptophan, 5-hydroxy-tryptophan, kynurenine, tryptamine, and kynurenic acid) were dissolved in 0.1 M borate (pH 6.3) to a concentration of approximately 0.6 mg/25 mL except for tryptophan where ten times higher concentration was used (6 mg/25 mL) (stock solutions). Limit of detection (LOD) was defined as the concentration of analyte that resulted in a peak height three times higher as the noise. The limit of quantification (LOQ) was defined as the concentration of analyte that resulted in a peak height three times higher than the limit of detection. Standard samples (tryptophan, kynurenine, 5-hydroxy-tryptophan) of three different concentrations (1:1, 1:4, and 1:40 diluted from stock solutions) were analyzed at the beginning and at the end of every HPLC run to calculate the precision and accuracy. The accuracy was determined as the mean of the measure relative to the theoretical value and is reported in percentage (%). The precision is denoted by the intra- and inter-day coefficient of variation (CV). System suitability parameters such as capacity factor (k'), relative retention (α), resolution (R_s), and theoretical plate number (N) of the column were also tested.

4.3.3 Tryptophan and cysteine fluorescence (V)

Loss of amino acid side-chains such as tryptophan and cysteine in aqueous phase β-lactoglobulin in oil-in-water emulsions during oxidation were measured by fluorescence spectroscopy. All amino acid oxidation assays were performed on continuous phase proteins that were free of Brij-stabilized oil droplets. To remove oil droplets, emulsions were centrifuged (1000*g*) for 30 minutes in Amicon Ultra centrifugal tubes (MWCO 100000, Millipore, Billerica, MA, USA). Berry phenolics caused quenching of tryptophan and cysteine fluorescence. Consequently, the clear sample after the ultracentrifugation was passed through a Econo-Pac® 10 DG - desalting column with exclusion limit at 6000 Da (= g/mol) (Bio-Rad, Richmond, CA, USA) equilibrated with sodium phosphate buffer (pH 7.0). The protein β-lactoglobulin was separated from phenolics by elution with the buffer. A protein assay (Bradford – Coomassie Blue, Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to adjust all the samples to same concentrations. Tryptophan fluorescence was measured directly by fluorescence with excitation at 283 nm and emission at 331 nm. Cysteine side-chains were measured directly using a sensitive fluorescent probe ABD-F (Sigma Chemical Co., St. Louis, MO, USA) (Carr et al., 2000). Before the measurement the protein was denaturated with 8 M urea at room temperature for one hour to expose the buried cysteine side-chains. Then, 100 mM sodium phosphate buffer (pH 8.0) containing 1 mM diethylenetriaminepenta

acetic acid (300 µL) was added to the mixture. ABD-F thiol probe (10 µL in 10 mM stock solution in buffer) was added to the protein solution, mixed by vortexing, and incubated in a 60 °C water bath for 20 min. Derivatized cysteine was measured by using excitation wavelength at 365 nm and emission at 492 nm. Concentrations were expressed relative to day 0 for tryptophan and cysteine concentrations, because small differences in their concentrations occurred in the different emulsions.

4.4 Analyses of lipid oxidation products

4.4.1 Lipid hydroperoxides (V)

Primary lipid oxidation products, lipid hydroperoxides, were determined according to method from Nuchi et al. (2001). Emulsion samples of 0.3 mL were mixed with 1.5 mL isooctane/2-propanol (3:1, v/v) by vortexing (10 s, 3 times) and isolating of the organic solvent phase by centrifugation at 1000g for 2 min. The organic solvent phase (100 µL) was added to 2.8 mL of methanol/1-butanol (2:1, v/v) followed by addition of thiocyanate/ferrous solution (30 µL) [prepared by mixing equal volumes of 0.132 M $BaCl_2$ with 0.144 M $FeSO_4$, centrifuging, and mixing equal volumes of the clear ferrous solution with 3.970 M ammonium thiocyanate]. The absorbance of the solution was measured 20 min after the addition of the iron at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA, USA). Hydroperoxide concentrations were quantified by using a standard curve made from cumene hydroperoxide (80%). Cumene hydroperoxide was used as a standard because it is relatively stable organic peroxide and commercially available. Though linoleic acid hydroperoxide could be used as a standard and obtained relatively easily by treatment with lipoxygenase, cumene hydroperoxide is more convienient to use because of its greater stability.

4.4.2 Volatile aldehydes (I, II, V)

Lipid oxidation was followed by measuring the formation of volatile aldehydes: hexanal (**I, II, V**), pentanal (**II**) and propanal (**II**) by headspace gas chromatography. Sample (2 g of meat in studies **I-II,** 1 mL of emulsion in study **V**) was put in a headspace vial. Formation of volatile aldehydes were measured in study **I-II** by using a static-headspace gas chromatography (Autosystem XL gas chromatograph, Perkin-Elmer, Waltham, MA, USA) equipped with capillary column (Nordibond NB-54, 25 m, 0.32 mm, HNU-Nordion Ltd., Helsinki, Finland), and a flame ionization detector.

Samples were injected with an automatic sampler (Perkin Elmer HS 40XL, Waltham, MA, USA), and sample vials were thermostated for 20 min at 80 °C. Oven temperature was held constant at 60 °C.

In study **V**, the formation of hexanal was measured by using a GC-17A Shimadzu gas chromatography equipped with an AOC-5000 Autosampler (Shimadzu, Kyoto, Japan), and a flame ionization detector (at 250 °C). A 30 m x 0.32 mm Equity DB-1 column (Supelco, Bellafonte, PA, USA) with a 1 μm film thickness was used for chromatographic separations. Samples were thermostated at 55 °C in the autosampler heating block for 13 min. A 50/30 μm DVB/Carboxen/PDMS solid-phase microextraction (SPME fiber needle, Supelco, Bellafonte, PA, USA) was injected into the sample vial for 1 min to absorb volatiles and then analytes were injected into the 250 °C injector port for 3 min. The gas chromatographic run was 10 min at 65 °C for each sample. Hexanal concentrations were determined from peak areas using a standard curve made from hexanal (**I, II, V**).

The inhibitions of plant phenolics (**I, II**) against formation of different aldehydes was calculated after 6 days of incubation using equation: $(A_0-A_1)/A_0\times100$, where A_0 is the area of aldehyde in control sample and A_1 is the area of aldehyde in tested sample. The inhibitions were expressed as percentages.

4.5 Determination of stability of anthocyanins in oil-in-water emulsion (V)

The concentrations of black currant anthocyanins were quantitatively determined by the absorbance of black currant anthocyanin – containing emulsions. Periodically (0, 0.5, 1, 2, 3, 6, 8, 17, and 24 days), 7 mL sample volumes were placed in open glass fluorometer cells (20 × 10 mm) and measured using a Shimadzu UV-2101 PC UV–vis Scanning Spectrophotometer equipped with an ISR-Integrating Sphere Assembly (Shimadzu, Kyoto, Japan) at 520 nm. Integrating sphere is used for measurements of the total reflectance or transmittance from diffuse or scattering materials. The measurements are almost always performed spectrally, as a function of wavelength. Black currant anthocyanin content was determined using a standard curve created using various concentrations of corn oil-in-water emulsions with and without added black currant anthocyanins. In addition, the concentration of 20 μM black currant anthocyanins in sodium phosphate buffer (pH 7.0) was measured by using a Ultrospec 3000 pro spectrophotometer equipped with thermostated cuvette

holder (Biochrom Ltd., Cambridge, Great-Britain) at 520 nm with temperature set at 55 °C. The concentrations were calculated by using a standard curve made of black currant anthocyanins.

4.6 Statistical analysis (I-V)

In studies **I-V** analyses were performed at least once in triplicate. Differences between results were analysed by multivariance (ANOVA) analysys using Statgraphics Plus (**I-III**) software (STCC Inc., Rockville, MD, USA). Duncan's multiple range tests (**IV-V**) and t-tests (**IV**) were performed using SAS® 9.1 (SAS Institute Inc., Cary, NC, USA).

5. RESULTS

This section summarizes the results presented in studies **I-V**.

5.1 Tryptophan oxidation in different models (III, IV)

Oxidation of tryptophan was investigated in two different model systems: with H_2O_2 (**III**), and with hexanal/$FeCl_2$ (**IV**). Tryptophan oxidized with H_2O_2 was degraded by 50% after 6 hours of oxidation (**III**: Table 4), and tryptophan oxidized with hexanal/$FeCl_2$ was degraded by 77% after 6 days of oxidation (**IV**: Figure 2). In both H_2O_2 and hexanal/$FeCl_2$ modified tryptophan the following oxidation products were formed: β-oxindolylalanine, *N*-formylkynurenine, kynurenine, 5-hydroxy-tryptophan, dioxindolylalanine A/B (i.e. the diastereomers), and 3a-hydroxypyrroloindole-2-carboxylic acid B (**III, IV**). However, formation of oxidation products such as tryptamine and 3a-hydroxypyrroloindole-2-carboxylic acid A were only detected in H_2O_2 oxidized tryptophan (**III**: Table 3). In both studies (**III, IV**) only ~ 20% of the oxidation products could be elucidated by HPLC determination.

The oxidation kinetics of hexanal/$FeCl_2$ modified tryptophan was followed in more detail (0.1, 2, 6 and 8 days) (**IV**). In study **IV**, the amount of primary oxidation product, 3a-hydroxypyrroloindole-2-carboxylic acid B, reached a plateau already after two days of oxidation, whereas the amount of dioxindolylalanine A/B, 5-hydroxy-tryptophan, kynurenine and *N*-formylkynurenine increased during the oxidation period. However, the amount of β-oxindolylalanine began to decrease after second day of oxidation, and continued decreasing over the entire study period.

5.1.1 Analysis method of tryptophan oxidation products (III, IV)

Tryptophan and its oxidation products were detected by two different HPLC-UV-FL methods as described in studies **III** and **IV**. In study **III** the HPLC method was modified from a method outlined by Simat and Steinhart (1998). In order to improve the quality of the separation of the oxidation products, a new HPLC method was developed (**IV**). Tryptophan oxidation products such as kynurenic acid, 3-hydroxy-kynurenine, 3a-hydroxypyrroloindole-

2-carboxylic acid A/B, dioxindolylalanine A/B, *N*-formylkynurenine, kynurenine, and β-oxindolylalanine were detectable by the UV –detector, and 3a-hydroxypyrroloindole-2-carboxylic acid A, 5-hydroxy-tryptophan, and tryptamine were detectable by the FL – detector. Tryptophan itself could be detected with both UV and FL – detectors.

Separation of tryptophan and its oxidation products was achieved within 16 min (**III**) or 13 min (**IV**) by using the selected columns and other parameters such as temperature and flow rate. The best separation between tryptophan as well as tryptophan oxidation products of dioxindolylalanine A/B, 3a-hydroxypyrroloindole-2-carboxylic acid B, 5-hydroxy-tryptophan, kynurenine, *N*-formylkynurenine, β-oxindolylalanine A/B was achieved by using a gradient of 0.1% TFA and acetonitrile (**IV**: Figure 1). Dioxindolylalanine and 3a-hydroxypyrroloindole-2-carboxylic acid each gave two peaks and β-oxindolylalanine a double peak (**III**) or two peaks (**IV**), referring to their diastereomers. Some peaks remained unidentified. The identification of tryptophan and its oxidation products were confirmed by their UV spectra (**III**: Figure 4; **IV**).

The developed HPLC method was validated (**IV**). In general, the chromatographic separation was good; the resolution value $R_s \geq 1.5$ and capacity factor $k' \geq 1.0$ were obtained for all compounds studied in this work, including also the standard compounds. The relative retention (α) was between 1.1 and 1.5 for all compounds. It was best for dioxindolylalanine B and kynurenic acid (i.e. 1.5). For tryptophan it was 1.4 and the most critical (i.e. 1.1) for *N*-formylkynurenine, tryptamine and 3a-hydroxypyrroloindole-2-carboxylic acid B. Number of theoretical plates (N) obtained for all compounds was > 2000: 3900 (3-hydroxy-kynurenine), 4100 (dioxindolylalanine A), 4300 (kynurenic acid), 5800 (dioxindolylalanine B), 7400 (3a-hydroxypyrroloindole-2-carboxylic acid B), 11000 (kynurenine), 17000 (*N*-formylkynurenine and β-oxindolylalanine), 17400 (5-hydroxy-tryptophan), 44000 (tryptamine), and 46000 (tryptophan). Repeatability (intra-day precision) of the method was good for tryptophan (CV 6.4%, n=17), kynurenine (CV 5.7%, n=17) and 5-hydroxy-tryptophan (CV 9.8%, n=16). In addition, inter-day precision was excellent for tryptophan (CV 6.3%, n=23) and kynurenine (CV 6.9%, n=23), and the most critical for 5-hydroxy-tryptophan (14.5%, n=21).

5.2 Impact of plant phenolics on oxidation of tryptophan (III, IV)

Different plant phenolics affected the oxidation or tryptophan either by acting as antioxidants or as prooxidants (**III, IV**). The antioxidant activity toward oxidation of tryptophan was dependent on the oxidation model applied. In addition, the content of phenolic compounds varied among different plant materials (**Table 6**).

5.2.1 Effects of phenolics from oilseed byproducts on tryptophan oxidation

Camelina meal phenolics exhibited the best inhibitions (50-52%) among the plant phenolics against loss of tryptophan oxidized by hexanal/$FeCl_2$ (**Figure 14**) (**IV**). In study **III** in the H_2O_2 catalyzed oxidation of tryptophan, camelina meal phenolics were shown to inhibit the loss of tryptophan only by 6-32%, where camelina meal phenolics at concentration level of 10 μM were the most potent (**III**: Table 4). Thus camelina meal phenolics revealed a more pronounced effect in inhibiting the hexanal/$FeCl_2$ induced tryptophan oxidation than H_2O_2 induced oxidation of tryptophan. The camelina meal phenolics comprise of flavonols, hydroxycinnamic acids and flavanols (**Table 6)**.

Camelina meal phenolics showed excellent i.e. over 95% inhibitions towards dioxindolylalanine A and β-oxindolylalanine, and good i.e. over 80% inhibitions toward *N*-formylkynurenine and kynurenine. Moderate inhibitions over 50% towards dioxindolylalanine B and 5-hydroxy-tryptophan in hexanal/$FeCl_2$ modified tryptophan were obtained (**IV**: Table 3). In H_2O_2 oxidized tryptophan, camelina meal phenolics exhibited over 95% inhibitions toward formation of tryptamine and dioxindolylalanine B, over 80% toward *N*-formylkynurenine, kynurenine, and over 50% toward β-oxindolylalanine (**III**: Table 5).

Rapeseed meal phenolics at 50 and 100 μM showed 25-34% inhibitions toward degradation (i.e. loss) of tryptophan in hexanal/$FeCl_2$ - oxidized tryptophan (**IV**: Table 2). Similarly to the effect of camelina meal phenolics, the effect of rapeseed meal phenolics in inhibiting tryptophan loss was more pronounced in hexanal/$FeCl_2$ modified tryptophan than to that of tryptophan oxidized with H_2O_2 (8-23%) (**III**: Table 4). Rapeseed meal phenolics provided protection toward tryptophan oxidation products such as tryptamine (≥ 95%, **III**), β-oxindolylalanine (≥ 95% in **IV;** ≥ 50% in **III**), kynurenine, *N*-formylkynurenine and

dioxindolylalanine B (70-80% in **III**; 20-50% in **IV**) and dioxindolylalanine A (16-55% in **III**; 30-50% in **IV**) (**III**: Table 5, **IV**: Table 4). The rate of oxidation was dependent on the concentration level of applied rapeseed phenolics in both oxidation models: By increasing the concentration of the rapeseed meal, the inhibitions were more pronounced toward tryptophan loss and oxidation compounds (**Figure 14**) (**III, IV**). Rapeseed meal phenolics comprised mainly of hydroxycinnamic acids and derivatives (**III**: Table 2). The main compounds dominating were sinapine and sinapic acid (**Table 6**).

Soy phenolics exhibited weak inhibitions toward tryptophan loss (26% in **IV**, 7-18% in **III**). **Figure 14** shows the differences in inhibitions of tryptophan losses with soy meal and soy flour phenolics. Soy meal phenolics were potent in inhibiting the formation of oxidation products such as tryptamine (≥ 95%, **III**), β-oxindolylanine (≥ 95% in **III**; 100% in **IV**), dioxindolylalanine B (≥ 95% in **III**; 33% in **IV**), *N*-formylkynurenine (63-73% **III**; 81% in **IV**) and kynurenine (60-100% in **III**; 77% in **IV**). The soy meal and soy flour consisted flavanols (**Table 6**).

Pine bark phenolics protected from the loss of tryptophan in both hexanal/$FeCl_2$ - (**IV**) and H_2O_2 -oxidized (**III**) tryptophan. The effect of pine bark phenolics were more pronounced in tryptophan oxidized by H_2O_2 than in tryptophan oxidized by hexanal/$FeCl_2$ (**Figure 14**). Pine bark phenolics were able to inhibit the formation of secondary tryptophan oxidation products and/or inhibiting the further oxidation of primary oxidation products of tryptophan (**III, IV**). The main phenolics in the pine bark extract were comprised of flavanols (**Table 6**) with catechins dominating (**I**: Table 1).

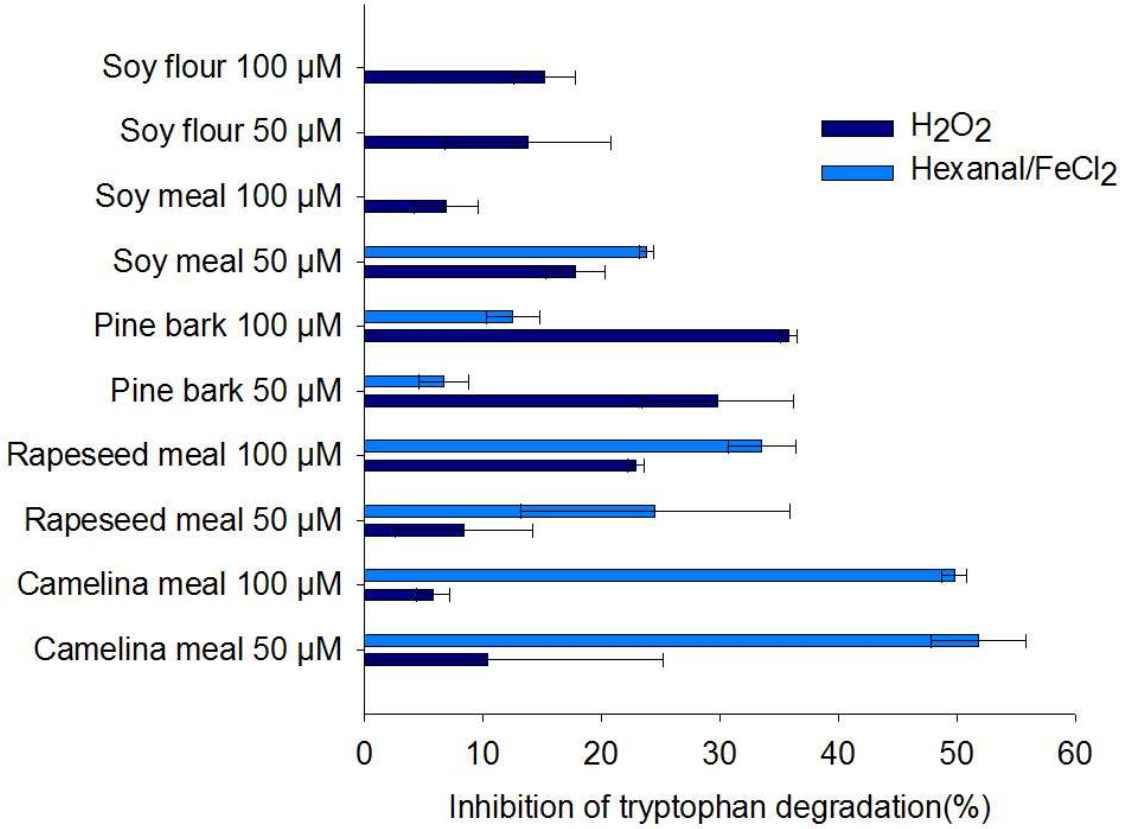


Figure 14. Inhibition of tryptophan degradation (i.e. loss) (%) with plant phenolics in H_2O_2 – oxidized tryptophan (*dark blue*) (**III**) and in hexanal/$FeCl_2$ – oxidized tryptophan (*light blue*) (**IV**). Data points represent means (n = 3) ± standard deviations. *Note*. Soy meal phenolics at 100 µM and soy flour phenolics at 50 and 100 µM were only employed in the presence of H_2O_2 in study III.

5.2.2 Effects of berry phenolics on tryptophan oxidation

Berry phenolics were more potent in inhibiting the oxidation of tryptophan in H_2O_2 modified tryptophan than in hexanal/$FeCl_2$ modified tryptophan. Black currant anthocyanins followed by cranberry proanthocyanidins exhibited the best inhibition (42% and 33%, respectively) among plant phenolics against tryptophan loss in H_2O_2 oxizided tryptophan (**III:** Table 4). In hexanal/$FeCl_2$ modified tryptophan, however, black currant anthocyanins slightly increased the degradation of tryptophan (**IV:** Table 2). Raspberry ellagitannins provided only a very modest (11-17%) protection against oxidation of tryptophan by H_2O_2 (**Figure 15**). In contrast, raspberry ellagitannins showed weak prooxidant activities in hexanal/$FeCl_2$ oxidized tryptophan (**Figure 15**). Other berry phenolics such as raspberry anthocyanins and rowanberry extract at all concentrations were not able to inhibit tryptophan loss.

Although most of the berry phenolics were not very effective in inhibiting the degradation of tryptophan i.e. tryptophan loss, the formation of tryptophan oxidation products was inhibited. All the berry phenolics were able to inhibit almost totally tryptamine indicating that the alanyl moiety oxidized products derived from tryptophan were highly affected by them (**III**). Black currant and raspberry anthocyanins, raspberry ellagitannins and cranberry proanthocyanidins showed antioxidant activity toward the formations of both primary and secondary tryptophan oxidation products in H_2O_2 oxidized tryptophan (**III**: Table 5), whereas in hexanal/$FeCl_2$ oxidized tryptophan, only the formation of β-oxindolylalanine and *N*-formylkynurenine were inhibited (**IV:** Table 3). Cranberry proanthocyanidins were also able to inhibit the formation of tryptophan-derived oxidation products (**III**). Rowanberry phenolics in tryptophan modified by hexanal/$FeCl_2$ were good at inhibiting only formation of β-oxindolylalanine (**IV**).

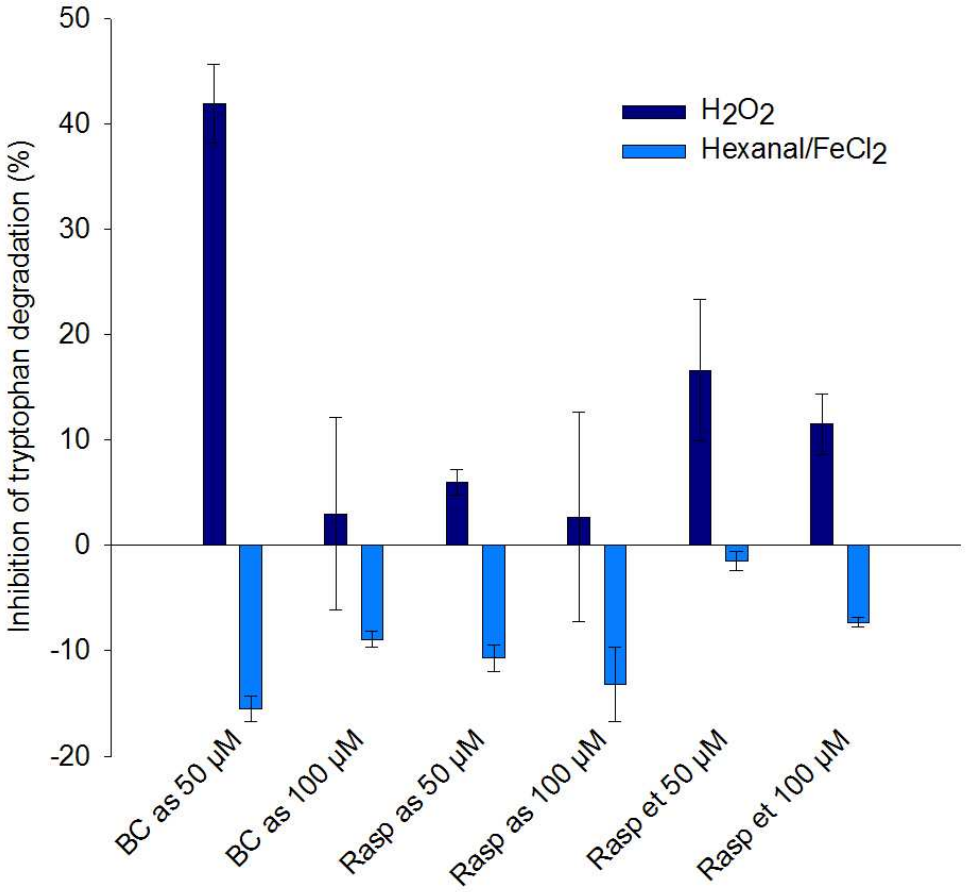


Figure 15. Inhibition of tryptophan degradation (%) with berry phenolics in H_2O_2 – oxidized tryptophan (*dark blue*) (**III**), and in hexanal/$FeCl_2$ – oxidized tryptophan (*light blue*) (**IV**). Data points represent means (n = 3) ± standard deviations. Raspberry ellagitannin isolate concentrations at 50 µM and 100 µM correspond to 58 and 115 µM, respectively. Negative values indicate prooxidant activity. BC as = black currant anthocyanins, Rasp as = raspberry anthocyanins, Rasp et = raspberry ellagitannins.

5.3 Effect of plant phenolics on the oxidation reactions in meat (I, II)

Plant materials rich in phenolic compounds were effective in inhibiting both protein and lipid oxidation reactions in meat (**I, II**). The concentration levels of phenolics from different rapeseed meal extracts exhibiting antioxidant activity of ≥ 80% (**I**: Figure 2) were selected for further experiments to study protein oxidation. Rapeseed meal extracts were able to inhibit the formation of protein carbonyls by 40-72% (**I**: Table 2). The amount of rapeseed and pine bark phenolics present in the samples varied between 1.3 mg and 8.1 mg (**I**: Table 2). The antioxidant activity increased with increasing concentration of phenolic compounds present in the samples. For example, the enzyme-assisted extract with Ultraflo L of rapeseed meal, with concentrations of 3.5 mg and 6.9 mg of total phenolics present, showed inhibitions of 47% and 90% toward formation of hexanal, respectively.

In addition to rapeseed meal, other byproducts of deoiling processes such as camelina meal, soy meal and flour were investigated in meat model at different concentration levels (**II**). A commercial supercritical CO_2 extract from rosemary (*Rosmarinus officinalis*) was used as a reference material alone and in combination with the other dry plant materials. Rapeseed and camelina meal were the most effective antioxidants in inhibiting the formation of protein carbonyls and hexanal (**II**: Table 1). In comparison to the antioxidant activity of rapeseed and camelina meal, soy meal and flour were only effective in inhibiting the protein and lipid oxidation in combination with the rosemary extract (**II**: Table 1). The antioxidant effect of all the phenolics toward protein and lipid oxidation was more pronounced with increasing concentration of plant material present (**II**: Table 1).

5.4 Effect of berry phenolics in oil-in-water emulsions (V)

Berry phenolics such as black currant anthocyanins and raspberry ellagitannins provided moderate protection toward both lipid and protein oxidation reactions in oil-in-water emulsions with and without continuous phase β-lactoglobulin. The berry phenolics were more effective in inhibiting the formation of hexanal (the secondary oxidation product) than lipid hydroperoxides (the primary oxidation products) (**Figure 16**). The black currant anthocyanins and raspberry ellagitannins alone (at 20 and 50 μM) were able to inhibit the lipid hydroperoxides by 31-50% and 34-49%, respectively, whereas with combination with

the aqueous phase β-lactoglobulin, the inhibitions were 29-39% and 38-44%, respectively. The antioxidant activity toward hexanal formation was 26-89% in the presence of black currant anthocyanins and 34-95% in the presence of raspberry ellagitannins alone at concentration levels of 20 and 50 μM. However, when β-lactoglobulin was present with the berry phenolics the antioxidant effects were more pronounced toward formation of hexanal. In the presence of aqueous phase β-lactoglobulin, black currant anthocyanins and raspberry ellagitannins (at 20 and 50 μM) exhibited antioxidant activities of 56-96% and 94-97%, respectively.

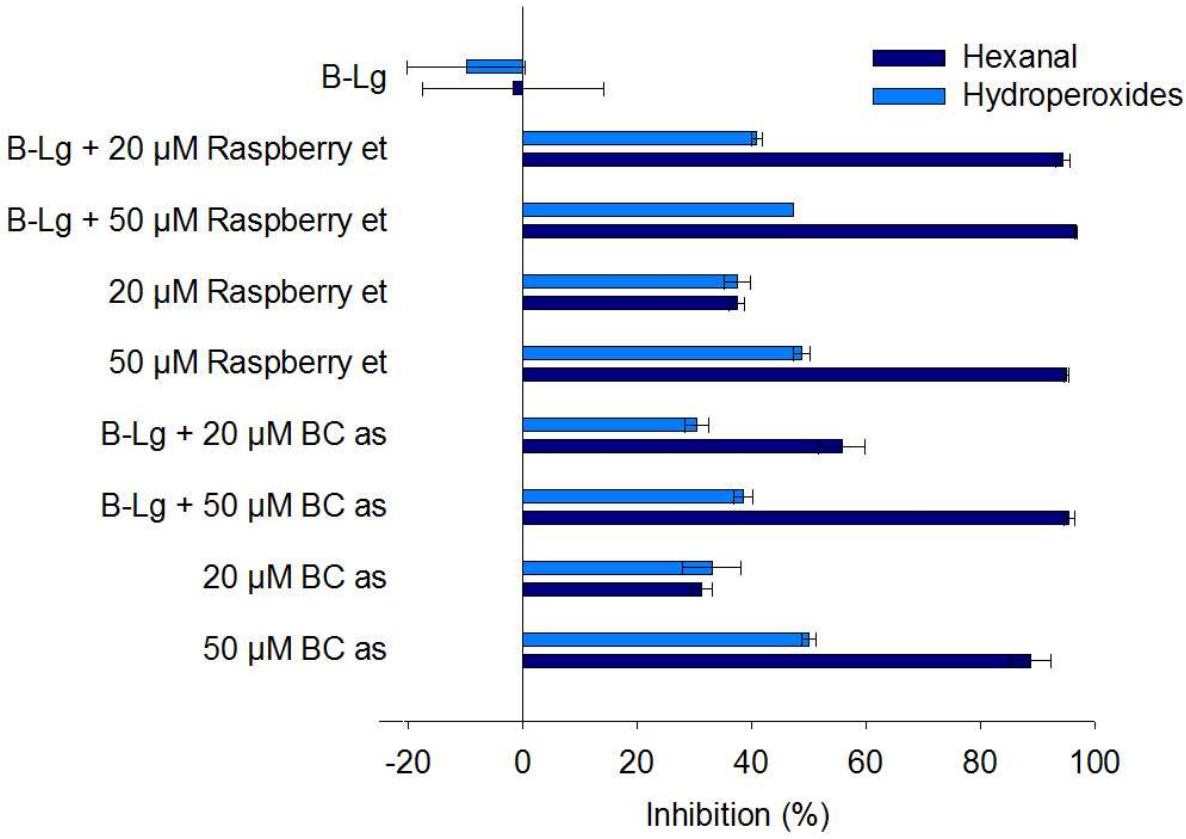


Figure 16. Antioxidant activity of raspberry ellagitannins and black currant anthocyanins in 5% corn oil-in-water emulsion (pH 7.0) with and without continuos phase β-lactoglobulin (0.5 mg/g oil) toward lipid hydroperoxides (*light blue*) and hexanal (*dark blue*) after 24 days of oxidation at 55 °C in the dark (**V**). Data points represent means (n = 3) ± standard deviations. Negative values indicate prooxidant activity. B-Lg = β-lactoglobulin, et = ellagitannins, BC as = black currant anthocyanins.

The loss of amino acid side-chains of continuous phase β-lactoglobulin in oil-in-water emulsions was monitored during oxidation. Berry phenolics inhibited the loss of tryptophan fluorescence after the first day of oxidation by 32-38% compared to the control (**V**: Table 5). Only raspberry ellagitannins at 50 μM continued to inhibit the loss of tryptophan fluorescence by 10% after the second day of oxidation. Berry phenolics were more active in inhibiting the loss of cysteine side-chains compared to the tryptophan side-chains (**V**:Table 6). They provided protection toward the loss of cysteine fluorescence at days 1, 2, 3, and 6 during the oxidation by 20-27%, 32-45%, 24-40%, and 0-24%, respectively. The results showed that the amino acid side-chains were oxidized prior propagation of lipid oxidation.

5.4.3 Stability of anthocyanins in oil-in-water emulsion

The black currant anthocyanins in emulsion samples were predominantly degraded within the first 12 hours (**V**: Figure 8). There were no differences between the emulsion samples with and without β-lactoglobulin. After 12 h of oxidation approximately 30% of 20 μM black currant, and 38% of 50 μM black currant isolate were left. After 24 days, there was still about 25% left of the 20 μM black currant isolate compared to 19% of the 50 μM black currant. In addition, to study if the rate of the anthocyanin loss was different in aqueous solution (sodium phosphate buffer, pH 7.0) compared with anthocyanins incorporated in an emulsion, the stability of black currant anthocyanins at 20 μM was tested during the 24 h of storage as shown in (**V**: Figure 9). It was observed that the concentration of anthocyanins decreased rapidly in both oil-in-water emulsion and in buffer solution with degradation being faster in the aqueous solution.

6. DISCUSSION

6.1 Oxidation of tryptophan

Tryptophan losses were shown to be more pronounced when oxidized with hexanal/$FeCl_2$ (**IV**) compared to H_2O_2 (**III**). In a previous study, tryptophan losses after 6 h of oxidation with H_2O_2 were reported to be 18-68% depending on the reaction conditions used (Simat et al., 1998). Simat and Steinhart (1998) also observed that the amount of tryptophan degradation products formed (13%) with 68% degradation of tryptophan was similar to our results with 50% of tryptophan degradation (11%). The major experimental differences were the temperature and pH used; instead of temperature 40 °C and pH of 8.3, temperature of 37 °C and pH 6.3 were used in our study. Therefore, the oxidation of tryptophan depends on the oxidative conditions such as incubation time, the amount and type of oxidants, and temperature (Friedman et al., 1988; Simat et al., 1998). The formation of individual tryptophan oxidation products by both hexanal/$FeCl_2$ and H_2O_2 followed a similar pattern as in reported earlier (Simat et al., 1998), However, the formation of tryptamine and 3a-hydroxypyrroloindole-2-carboxylic acid A were only detected in tryptophan oxidized with H_2O_2. Tryptophan oxidation products (3a-hydroxypyrroloindole-2-carboxylic acid A, β-oxindolylalanine, dioxindolylalanine, kynurenine, and 5-hydroxy-tryptophan) can be even more susceptible to oxidation than tryptophan is itself, and thus yield even more kynurenine, *N*-formylkynurenine, and dioxindolylalanine (Itakura et al., 1994; Simat et al., 1998).

The results (**III, IV**) show that the measured oxidation products explained only 10-20% of tryptophan loss. These results were in accordance with the results of Simat and Steinhart (1998) who observed that only ~20% of the total tryptophan loss could be elucidated by the determined degradation compounds. Therefore, it still remains unclear to what other compounds tryptophan is degraded into. The appearance of less polar UV-active and fluorescent compounds was detected after tryptophan was eluted. However, formation of putative tryptophan aggregation was not investigated in these studies as described by Dominques et al. (2003). They reported crosslinking of two tryptophan radicals forming a dimer, and monohydroxy-dimer due to crosslinking between tryptophan and hydroxy-tryptophan or due to the hydroxylation of the tryptophan dimer, and other new adducts resulting from reaction of tryptophan and oxidized tryptophan and 3-methyl indole

derivatives. Intra- and intermolecular crosslinks have been shown to form in aldehyde modified proteins (Schaich, 2008). It has been reported that *trans*-2-hexenal and hexanal had an effect on covalent binding leading to aggregation, and formation of fluorescent compounds in whey proteins and sodium caseinate (Meynier et al., 2004). Dalsgaard et al. (2007) reported polymerization of milk proteins such as α- and β-casein, and lactoferrin upon photo-oxidation. However, the reaction mechanisms for polymerization in α- and β-casein were not substantiated. In lactoferrin (contains cysteine) disulfide bonds were responsible for polymer formation. Therefore, secondary tryptophan oxidation products are not necessarily final products of the tryptophan oxidation process. Some of these products can either suffer further oxidations or react with other components to produce new compounds. In conclusion, the results show that tryptophan is extensively oxidized in the presence of both H_2O_2 and hexanal/$FeCl_2$ which leads to formation of various primary and secondary tryptophan derived oxidation products. Based on our results and literature, the employed procedure of observing only ~20% of the total tryptophan derived oxidation products is consistent when based on the determined oxidation products. However, more basic research is needed to investigate the still unkown compounds formed during tryptophan oxidation.

The oxidation system of empoying hexanal and $FeCl_2$ (**IV**) is not a very common one. This oxidation system of hexanal as the reactive lipid oxidation product was chosen because hexanal is a major contributor to oxidation reactions and commonly used as a marker of food quality. Iron ($FeCl_2$) was chosen to the model because it is ubiquitous in food ingredients and biological tissues and an important pro-oxidant (McClements et al., 2000). The effect of hexanal and iron ($FeCl_2$) has been previously investigated only in an aqueous glucose/phenylalanine model system (Fallico et al., 1999). This study showed that an addition of whether hexanal, iron or hexanal/iron equally inhibited the formation of coloured compounds. However, the formation of a thermal decomposition product, 5-hydroxymethylfurfural, was more pronounced in the presence of hexanal/iron than hexanal or iron alone. Different mechanism routes were proposed to be involved when hexanal is added to the glucose/phenylalanine system: a) reaction between phenylalanine and hexanal can form a Schiff base, b) addition of hexanal can prevent the reaction of dicarbonyl compounds with an amino group which would lead to formation of heterocyclic compounds and Strecker degradation products; instead the formation of 5-hydroxymethylfurfural is favored, c) hexanal can react with the Strecker aldehyde (phenylacetaldehyde) and prevent its

condensation with 5-hydroxymethylfurfural (Fallico et al., 1999). Other studies on the effects of hexanal on protein oxidation have been done in few studies. Ishino et al. (2008) suggested that saturated aldehydes such as hexanal, in combination with H_2O_2 or to a lesser extent alkyl hydroperoxides, can mediate covalent modification of proteins by binding to the lysine side-chains and thus forming N^{ε}-hexanoyllysine via a Baeyer-Villiger-like reaction mechanism (peroxide addition to the aldehyde Schiff base). Hexanal has been shown to react with tryptophan, both in model systems and in protease-digested soybeans (Arai et al., 1971), to give condensation product involving a crosslink between two tryptophan molecules (Kaneko et al., 1989). Exposure to hexanal can modify also ε-amino groups of lysine side-chains of proteins into alkyl-substituted pyridinium betaine (Kato et al., 1986). In addition, hexanal has been shown to modify lysozyme (Tashiro et al., 1985; Kato et al., 1986), BSA, chicken serum albumin (Smith et al., 1999), whey proteins, sodium caseinate (Meynier et al., 2004) as well as soy glycinin and β-conglycinin (O'Keefe et al., 1991). In conclusion, the research of the effects of hexanal and iron on protein oxidation is still scarce, and more studies are needed. This study (**IV**) only focused on the effects of both hexanal and iron, however, their effects should also be investigated separately in the future studies.

6.1.1 Evaluation of the validated HPLC method

The developed HPLC method was shown to be selective and fast enough to be able to study oxidation products of tryptophan. There were no significant differences (p<0.05) between inter-day precision and repeatability for each reference compound (tryptophan, kynurenine, and 5-hydroxy-tryptophan). Values of precision were below 15% of the CV. This indicates that the oxidation experiments were repeatable. The biggest challenge was identification and quantification of tryptophan oxidation products that were not commercially available. Therefore, identification of those compounds was based on UV spectra found in literature (Simat et al., 1996). In addition, kynurenine as external standard was used for quantification of these oxidation products. It can be concluded that the method was consistent for detection of main primary and secondary tryptophan oxidation products. However, once proceeding to identification of the still unknown tryptophan oxidation products, it could be useful to combine the method with MS, NMR (nuclear magnetic resonance) as well as FTIR (Fourier transform infraded spectroscopy) techniques.

6.2 Effects of antioxidative plant phenolics on oxidation of tryptophan (III, IV)

6.2.1 Tryptophan oxidation in the presence of oilseed byproducts

Camelina, rapeseed and soy meal phenolics revealed a more pronounced effect in inhibiting the hexanal/$FeCl_2$ induced tryptophan oxidation (**IV**) than H_2O_2 induced oxidation of tryptophan (**III**). In general, since tryptophan was less oxidized in the presence of camelina meal, rapeseed meal, and soy phenolics in both studies (**III**, **IV**), the formation of tryptophan oxidation products was delayed, which consequently resulted in more pronounced inhibitions toward primary and secondary oxidation products. In contrast, pine bark phenolics were more effective antioxidants in H_2O_2 oxidized (**III**) tryptophan than when oxidized with hexanal/$FeCl_2$ (**IV**). Therefore, also the ability of pine bark phenolics in hexanal/$FeCl_2$ model to inhibit the formation of tryptophan derived oxidation products was weakened.

The camelina meal phenolics comprise of flavonols, hydroxycinnamic acids and flavanols, which presumably have a synergistic effect. For example, in study **IV** the antioxidant activity of the principal reference compounds in camelina meal such as sinapic acid and catechin toward loss of tryptophan was weaker than that of the camelina meal extract. In addition, quercetin and chlorogenic acid showed either no effect or prooxidant activity toward tryptophan loss. However, in study **III**, it was shown that individual reference compounds such as catechin, quercetin, sinapic acid and chlorogenic acid at different concentration levels were able to inhibit the oxidation of tryptophan with similar or even better activities than the extracts themselves. According to study **II**, quercetin contributed to the antioxidant effect of camelina meal. As rapeseed meal contained sinapine as the main component, it may also be the effective form contributing to antioxidant activity. This would be in accordance with studies **I** and **II**, where rapeseed phenolics inhibited both protein and lipid oxidation in cooked pork meat patties. Rapeseed phenolics have been reported to show moderate radical scavenging activity (DPPH test) and inhibit oxidation of liposomes (Vuorela et al., 2005a). Flavanols and isoflavones are the most important phenolic compounds present in soy. The effect of individual isoflavones such as genistein and daidzein on tryptophan oxidation was more pronounced in H_2O_2 oxidized tryptophan than in hexanal/$FeCl_2$ model. This data shows

that the antioxidant activity of individual phenolic compounds and oilseed byproducts toward oxidation tryptophan is dependent on the oxidant present.

The comparison between the total phenolic content measured by Folin-Ciocalteau method and the total amount of specific phenolic groups or phenolic compounds measured by HPLC methods showed that there are differences between the methods (**Table 6**). It is known that Folin-Ciocalteau method reacts strongly with all reducing hydroxyl groups present not only in phenolic compounds but also in some proteins and sugars (Singleton and Rossi, 1965). These other highly reactive compounds include tertiary aliphatic amines, primary, secondary, and tertiary aromatic amines, tryptophan, hydroxylamine, hydrazine, certain purines, and other miscellaneous organic and inorganic reducing agents (Ikawa, 2003). Therefore, this procedure usually leads to an overestimation of the total polyphenolic content. Therefore, the separation and identification carried out by HPLC methods gives a more specific data on phenolic groups and phenolic compounds. The results also showed that there were variations between the phenolic compositions among similar extracts used in different studies (**Table 6**). This is due to that the materials used in different studies originated from different batches. Therefore, the variations in composition (phenolics, protein, lipids) are most likely due to environmental factors such as light, temperature and humidity as well as genetics of the plant. It can be concluded that the results of phenolic profiles obtained by HPLC methods give a more accurate and reliable data on the phenolic compositions compared to total phenolic content obtained by Folin-Ciocalteau procedure. In addition, the processing (extraction) methods have a great impact on the phenolic composition as have been described in other studies (Matthaus, 2002; Vuorela et al., 2003; 2004).

Pine bark phenolics consist mainly of flavanols (~80 μg/g) with catechin dominating. In study **IV**, catechin at 100 μM was able to inhibit tryptophan loss by 10%, whereas taxifolin, another phenolic compound reported in pine bark, showed either no effect or weak prooxidant activity. However, in study **III** when tryptophan was oxidized by H_2O_2, catechin and taxifolin protected tryptophan from oxidation. Flavonols (e.g. quercetin) and flavanones (e.g. taxifolin) have been shown to have higher metal-initiated prooxidant activity (Cao et al., 1997). This may explain why reference compounds quercetin and taxifolin as such exhibited prooxidant or no effect toward loss of tryptophan when iron was added to the model (**IV**). Based on this data, pine bark phenolics showed either antioxidant or prooxidant effects toward tryptophan oxidation depending on the oxidant.

In conclusion, for the first time camelina, rapeseed and soy meal as well as pine bark rich in diverse phenolics were shown to be potential antioxidant towards oxidation of amino acid tryptophan. However, as the results showed, this is highly dependent on the oxidant used in the model. As the data showed, the pure phenolic compounds used as reference compounds did not explicate the exact activity of certain plant extract. It can be hypothized that the network of phytochemicals is essential for the activity of plant materials, particularly when considering that a plant antioxidant may become a pro-oxidant if suitable and sufficient co-antioxidants are missing. Most of the active antioxidants are likely to be pro-oxidants when they lie beyond the optimum. In addition, the antioxidant activity of certain compound(s) contributing to activity is difficult to demonstrate since there may also be synergistic effects between the different phenolics present in the extracts.

6.2.2 Tryptophan oxidation in the presence of berry phenolics

Black currant anthocyanins showed the best protection toward tryptophan loss when oxidized with H_2O_2 (**III**). In contrast, when oxidized with hexanal/$FeCl_2$, black currant was not able to inhibit the oxidation of tryptophan. In addition, raspberry anthocyanins were not effective in either model (**III, IV**). Rowanberry phenolics were not able to inhibit the oxidation of tryptophan (**IV**). Berry phenolics that were not able to protect tryptophan from oxidation yielded also more oxidation products regardless of the oxidant used. Black currant contains four major anthocyanins: the 3-glucosides and 3-rutinosides of cyanidin (7 and 38%) and delphinidin (16 and 39%), whereas raspberry consists mainly of the 3-sophorisides (59%), 3-glucosides (16%), and 3-glucosylrutinosides (16%) of cyanidin with minor amounts of pelargonidin (4%) with different 3-glucosyl substituents (Viljanen et al., 2005b). Cyanidin-3-glucoside and delphinidin-3-glucoside were able to inhibit the tryptophan loss by 30% (**III**). Effects of cyanidin-3-glucoside and delphinidin-3-glucoside (although with less effect) showed a consistency in the pattern of oxidation products formed with black currant and raspberry anthocyanins (**III**). Therefore, according to these results, it seems that cyanidin- and delphinidin-3-glucosides present are the main compounds responsible for the antioxidant activities in black currant isolates. In study **IV**, however, cyanidin-3-glucoside showed prooxidant activity toward tryptophan loss.

It has been reported that black currant consists also of procyanidins (43% of total proanthocyanidins), and prodelphinidins (57%) with low molecular weight (LMW) (1-10

μg/g) and insoluble high molecular weight (HMW) (100 μg/g) proanthocyanidins, which may also contribute to the antioxidant activity (Ferreira et al., 2006). Prodelphinidins in the form of trimeric gallocatechins (Ferreira et al., 2006) and flavan-3-ols such as catechin (8 μg/g) and epicatechin (11 μg/g) have been identified from black currant (Määtta-Riihinen et al., 2004a). This suggests that the amount of 3.7% of flavanols in black currant isolate may also affect the oxidation of tryptophan, which is in accordance with the results that catechin as a reference compound inhibited the tryptophan oxidation (**III, IV**).

It has been shown that 3-glucosides and 3-rutinosides of cyanidin and dephinidin can act as lipid and protein antioxidants in liposomes and oil-in-water emulsions (Viljanen et al., 2004a; 2005b). In addition, black currant and raspberry anthocyanin isolates have been shown to inhibit protein and lipid oxidation in liposomes (Viljanen et al., 2004b). In another study, black currant anthocyanins were reported to be better antioxidants toward protein and lipid oxidation in oil-in-water emulsions than raspberry anthocyanins (Viljanen et al., 2005b). The antioxidant properties of flavonoids are mainly due to the 3′, 4′-dihydroxy group located on the B ring, the 3-hydroxy or 5-hydroxy and the 4-carbonyl groups in the C-ring (Fernandez et al., 2002). In addition, the antioxidant activity increases with the number of hydroxyl groups in rings A and B. The inability to protect tryptophan from oxidation may be due to that anthocyanins have a very low oxidation potential (spontaneous oxidation) which renders them into either pro-oxidants by redox-cycling, or good antioxidants depending on the reaction conditions (Van Acker et al., 1996).

Raspberry ellagitannins showed a weak antioxidant activity toward tryptophan oxidation when oxidized with H_2O_2 (**III**), but a prooxidant activity when oxidized with hexanal/$FeCl_2$ (**IV**). The main compounds in ellagitannin fraction consist of mixture of monomers (MW 936 g/mol), dimers (sanguiin H6), trimers (lambertianin C), and polymers (Kähkönen et al., unpublished results). Raspberry is reported to contain minor amounts of flavonols such as 3-glucosides and 3-glucuronides of quercetin (Määtta-Riihinen et al., 2004b), which was in accordance with our results (**III, IV**). Ellagic acid and raspberry ellagitannins have been attributed with antioxidative properties (Vuorela et al., 2005a). Ellagic acid with increasing concentration exhibited the best activity against oxidation of tryptophan (**III**) by decreasing the tryptophan loss by 50%. Ellagic acid, however, was not able to inhibit tryptophan loss when oxidized with hexanal/$FeCl_2$ (**IV**). It is known that the affinities of tannins for binding,

crosslinking and consequently precipitating proteins are dependent on the structural flexibility of both the tannin and protein molecule (Deaville et al., 2007). The loss of conformational freedom of ellagitannins significantly affects their binding capability (Deaville et al., 2007). In addition, it has been shown that metal ions catalyze the oxidation and polymerization of the phenolic compounds therefore reducing their available binding-sites (Dangles et al., 2006). It may be concluded that the effects of raspberry ellagitannins and ellagic acid toward oxidation of tryptophan depends on the oxidant used. The presence of hexanal/$FeCl_2$ in the tryptophan solution renders both ellagitannins and ellagic acid to act as prooxidants. Ellagic acid, however, acted as antioxidant when H_2O_2 is present. In addition, based on this data as well as knowledge on literature it may be that the ability of ellagic acid as superior antioxidant compared to ellagitannins may be due to their structural differences, or perhaps synergistic properties with other compounds.

Functions of cranberry proanthocyanidins were investigated only when tryptophan was oxidized by H_2O_2 (**III**). In this model cranberry proanthocyanidins were among the best phenolics that inhibited the oxidation of tryptophan. Cranberry procyanidin fractions have been found to be effective antioxidants when using DPPH test and toward lipid oxidation inhibiting the oxidation of methyl linoleate emulsion and LDL (Määttä-Riihinen et al., 2005). Cranberry, blueberry, and grape seed extracts alone and in combinations showed antioxidant activity assayed by using a DPPH radical inhibition test (Vattem et al., 2005a). In addition, cranberry juice powder and its synergies with ellagic and rosmarinic acids have been shown to reduce oxidative stress and mediate antioxidant enzyme responses in porcine muscle tissue induced by H_2O_2 oxidation (Vattem et al., 2005b). This data suggests that cranberry proanthocyanidins are effective in inhibiting the oxidation of tryptophan. In addition, according to literature the antioxidant activity of cranberries have also been proven in other oxidation models. Therefore, cranberries could be used in many different food applications to improve the oxidative stability.

In conclusion, based on the data obtained combinations of antioxidants i.e. extracts of berry and oilseed phenolics are more effective in preventing oxidative degradation in tryptophan than single compounds. However, the effectiveness is dependent on the oxidation model used as was also concluded before. The concentration ratios of different phenolic compounds in the plant extracts may be critical for their activity. This may explain the differences in efficacy for camelina and rapeseed meals compared to rowanberry phenolics even though

they comprised of similar phenolics. In addition, it may be that the concentration levels of the phenolics used in studies **III** and **IV** were not optimal for the antioxidant activity, especially when the concentrations of the phenolics (10, 50 or 100 μM) were very low compared to the tryptophan concentration (2 mM). More systematic research is needed to optimize the levels of phenolics to be the most effective and to further explicate their antioxidant effect toward protein oxidation by identifying the unknown compounds formed during the oxidation.

The indole i.e. pyrrole moiety of tryptophan is the most likely group to be involved in the reaction with the phenolic compounds since the oxidation of the indole structure yielding *N*-formylkynurenine and kynurenine was effectively inhibited by oilseed byproducts in studies **III** and **IV,** and by berry phenolics in study **III**. This is in accordance with a study (Rawel et al., 2001) proposing that the semiquinones or quinones of phenolic compounds may react with the heterocyclic nitrogen-atom of tryptophan. The antioxidant activity of plant phenolics may be due to the ability of flavonoid semiquinones or quinones in binding directly to tryptophan, and thereby preventing it from further reactions. Further oxidation of this product can lead to formation of tryptophan dimers or longer polymers. Another possibility is the reaction between flavonoid radical and tryptophyl radical. However, flavonoid termination reactions do not necessary lead to termination of radical scavenging since oxidation products (dimers or quinones) and their degradation products may still be reactive (Seyoum et al., 2006). Based on the results, it may be concluded that the most important target for antioxidant action of plant phenolics was preventing the cleavage of the indole moiety of tryptophan. Consequently, the overall oxidation of tryptophan was then inhibited. The exact antioxidant mechanism, however, remains unclear, and needs to be investigated in the future studies.

6.3 Antioxidant activity of plant phenolics in meat (I, II)

In study **I**, the different extracts of rapeseed meal (water, ethanolic or enzyme-assisted extracts) and pine bark showed no statistically significant difference inhibiting the formation of hexanal or protein carbonyls, even though the different extracts of rapeseed meal had different amounts of phenolics present. Therefore, the antioxidant effects of the rapeseed meal extracts may be more dependent on the individual phenolic compounds present in the extracts than of the amount of total phenolics. In ethanolic extract of rapeseed meal the main

phenolic compound was sinapine, and in enzymatic extract of rapeseed meal it was sinapic acid. Therefore, both sinapic acid and sinapine, the choline ester of sinapic acid, contributed to the antioxidant effect of rapeseed meal extracts. This is accordance with earlier studies showing the effectiveness of rapeseed phenolics as lipid antioxidants (Vuorela et al., 2004; 2005a). Lipid oxidation has been shown to be the main reason to quality losses in meat and other muscle foods (Frankel, 1996). Oxidation of meat leads to flavor deterioration, discolorization, destruction of nutrients, and possible formation of toxic compounds (Kanner, 1994). In study **I**, the antioxidant effect of rapeseed and pine bark extracts toward lipid oxidation was also more pronounced than toward protein oxidation. The formation of protein-carbonyl compounds is a secondary sign of protein oxidation due to proteins interacting with secondary lipid oxidation products (Griessauf et al., 1995; Rampon et al., 2001). Sinapic acid as a reference compound was effective inhibiting both protein and lipid oxidation in cooked pork meat patties. In crude rapeseed oil, vinylsyringol was the principal phenolic compound, and it provided the best protection against both protein and lipid oxidation reactions. These results showed that rapeseed meal was an effective antioxidant toward protein and lipid oxidation reactions in cooked pork meat patties. It can be concluded that the antioxidant activity of rapeseed meal is due to its sinapic acid and sinapine content.

Pine bark extract is a mixture of several phenolic compounds, which may contribute to the antioxidant activity. Taxifolin was as potent as sinapic acid against lipid and protein oxidation, but also flavonoids and lignans present in pine bark extract may be responsible for the antioxidant activity. Pine bark phenolics have been reported to provide protection toward oxidation in liposomes (Vuorela et al., 2005a). It was also shown, that the lignans matairesinol and pinoresinol, which are the major phenolic compounds present in pine bark, were excellent in inhibiting oxidation. The effectiveness of catechins toward protein oxidation has been described in irradiated raw chicken meat (Rababah et al., 2004). Therefore, lignans and flavonoids may in part explain the antioxidant activity of pine bark.

In studies **I** and **II**, there were no statistically significant differences between the antioxidant activities of dry rapeseed meals at addition level of 0.3 g/100 g meat toward formation of hexanal and protein carbonyls. In study **II**, it was observed that the antioxidant activity toward both lipid and protein oxidation increased with increasing concentration (0.5 and 0.7 g/100 g meat) of dry byproducts of oilseed plants. The phenolic profile of the rapeseed meal showed that the antioxidant activity of phenolics may be due to the hydroxycinnamic acids

and sinapine, whereas the antioxidant activity of camelina meal is probably a combination of hydroxycinnamic acids and sinapine as well as flavonols. The weak antioxidant effect of soy is mainly due to isoflavones and lignans as they are the main phenolics present in soy. Similar antioxidant activity of soy protein hydrosylates toward lipid oxidation has been reported earlier in cooked pork meat patties (Peña-Ramos et al., 2003). In addition, other studies have shown that different plants or their phenolic extracts such as potato peel (Kanatt et al., 2005), tea catechins (He et al., 1997; McCarthy et al., 2001; Tang et al., 2001; 2002; Rababah et al., 2004), sage and oregano (McCarthy et al., 2001; Fasseas et al., 2008), garlic (Mariutti et al., 2008), cloudberry, beetroot, willow herb (Rey et al., 2005), dried plums (de Gonzalez et al., 2008), and pomegranate (Naveena et al., 2008) can act as lipid antioxidants in meat.

The differences in antioxidant activities between dry oilseed materials may also be due to their composition of different amounts of proteins, fatty acids, and tocopherols. Dietary vitamin E has been shown to inhibit lipid oxidation in precooked meat products made from pork, beef and poultry (Mercier et al., 1998). However, addition of α-tocopherol to meat products has not been shown to be very effective as lipid antioxidant (Georgantelis et al., 2007). Thus, based on the data obtained from studies **I** and **II**, it can be conluded that antioxidant effect of dry oilseed byproducts increased with increasing concentration. The overall antioxidant effect of dry oilseed byproducts is also suggested to be due to their diverse composition of phenolic compounds, however, the role of other molecules (proteins, fatty acids and tocopherols) cannot be ruled out.

In study **II**, rosemary was very effective only in combinations with rapeseed and camelina meals as well as soy. Rosemary extract has been shown to act as a potent antioxidant among herbs (Karpinska et al., 2000; McCarthy et al., 2001) and it is widely used in food industry. The phenolic diterpenes, carnosic acid and carnosol, account for over 90% of antioxidant activity of rosemary. In meat and meat products, rosemary extract has been shown to be effective usually in combinations with chelators such as phosphates (Murphy et al., 1998), in combination with α-tocopherol or chitosan (Georgantelis et al., 2007), and in combination with butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) with citric acid (Barbut et al., 1985).

Phenolic compounds can act as antioxidants by terminating radical reactions and by metal chelation. During cooking temperatures, iron is released from myoglobin (Moller et al., 2006). This nonheme iron is a known pro-oxidant of lipid hydroperoxides. The addition of sodium chloride, an important food additive in meat industry, also increases the pro-oxidant activity of iron (Kanner et al., 1991). Based on their structures, the oilseed phenolics are also able to chelate metals, and thus directly retard oxidation of proteins.

In conclusion, phenolic-rich oilseed byproducts were shown to be potent antioxidants toward both protein and lipid oxidation reactions in cooked pork meat patties. Plant phenolics containing various bioactive compounds could provide an alternative to synthetic antioxidants, especially when the consumers demand for natural products increases. In addition, the use of different byproducts of oilseed processes would be an important in developing functional foods and also provide economical benefit to food industry.

6.4 Antioxidant activity of berry phenolics in oil-in-water emulsion (V)

Berry phenolics such as black currant anthocyanins and raspberry ellagitannins were efficient in protecting lipid oxidation in corn oil-in-water emulsions. The antioxidant effect of berry phenolics in combination with the aqueous phase β-lactoglobulin was more pronounced than without β-lactoglobulin. Continuous phase β-lactoglobulin, however, did not have an effect on the initial lag phase (i.e. the time period between exposure to the employed treatment, and the apparent formation of oxidation products) in the current study since there were no differences in formation of lipid hydroperoxides between emulsion samples with and without the protein. Most likely this is due to natural tocopherols present in corn oil (14.4 mg/100 g oil) acting as free radical scavengers. The ability of α-tocopherol to act as chain-breaking antioxidant scavenging lipid peroxyl radicals by donating hydrogen to a lipid peroxyl radical that otherwise would propagate the radical chain reaction of lipid peroxidation has been shown in LDL (Esterbauer et al., 1991a; Yeomans et al., 2005). Proteins such as BSA and ovalbumin can increase the stability of oil-in-water emulsions in the presence of phenolic antioxidants even though BSA itself does not act as antioxidant (Almajano et al., 2004; 2007). This synergistic effect is due to the changed structure of BSA, with a loss of tryptophan groups, and the formation of BSA-antioxidant adducts, which concentrate at the oil-water interface due to the surface-active properties of the protein (Almajano et al., 2004;

2007). In addition, sodium caseinate with lactose (Velasco et al., 2004), casein hydrolysates (Diaz et al., 2003), and whey proteins (Hu et al., 2003a) as well as β-lactoglobulin (Kellerby et al., 2006) have been found to increase the stability of oil-in-water emulsions. Soy protein isolate, sodium caseinate and whey protein isolate in the continuos phase or as emulsifiers can enhance the oxidative stability of oil-in-water emulsions (Hu et al., 2003b; Faraji et al., 2004). Increasing the concentration of proteins such as whey proteins in the presence of berry phenolics (blackberry and raspberry juices) have been shown to enhance the stability of the emulsion toward lipid and protein oxidation (Viljanen et al., 2005a).

β-lactoglobulin alone in the continuous phase of oil-in-water emulsion was shown to be able to extend the lag phase of hexanal compared to the control (without protein). This suggests that it may be able to quench alkoxy radicals to inhibit the β-scission reactions to produce hexanal. This result is in accordance with previous studies that showed β-lactoglobulin alone exhibited antioxidant activity toward lipid oxidation (Elias et al., 2005; 2006; 2007). Thermal treatment of β-lactoglobulin has been described to be more effective in inhibiting lipid oxidation reactions and scavenging peroxyl radicals, however, the ability to chelate iron ions have been shown to decrease compared to native β-lactoglobulin (Elias et al., 2007). The heat treated β-lactoglobulin in this study may still able to chelate prooxidant metal ions (iron) to some extent and remove them from the vicinity of the emulsion droplet surface, thereby retarding the oxidation reactions. Proteins and peptides have been shown to act as antioxidants in lipid oxidation reactions in oil-in-water emulsions (Elias et al., 2005; 2006; 2007). The antioxidant activity of these proteins is suggested to be combination of free radical scavenging by amino acid side-chains and chelation of prooxidative metal ions. It has been described that heat treated and enzymatic hydrolysates of β-lactoglobulin were more potent in inhibiting lipid and protein oxidation than the native protein. This is due to the conformational changes attributable to the exposure of buried amino acid side-chains that are able to scavenge free radicals (Levine et al., 1996).

The antioxidant activity of raspberry ellagitannins (MW = 936-3740 g/mol) and black currant anthocyanins (MW = 449-625 g/mol) (Kähkönen et al., 2003) increased with increasing concentration. The main compounds in raspberry ellagitannin fraction consist of mixture of monomers (MW = 936 g/mol), dimers (sanguiin H6, MW = 1870 g/mol), trimers (lambertianin C, MW = 2804 g/mol), and polymers (lambertianin D, MW = 3740 g/mol)

(Mullen et al., 2002; 2003; Kähkönen et al., unpublished results). The antioxidant effect of berry phenolics was more pronounced with β-lactoglobulin. This may be due to that the larger molecular weight phenolics such as ellagitannins have the ability to bind to the protein more efficiently due to the proximity of many aromatic rings and hydroxyl groups, and increase association of antioxidants at the surface of the emulsion droplets, and thus inhibit oxidation (Almajano et al., 2004). Anthocyanins, however, have been suggested to bind only to specific glutathione S-transferase – proteins in grape berries (*Vitis vinifera* L.) (Conn et al., 2008) and petunia (*Petunia hybrida*) (Mueller et al., 2000) – in the transport of anthocyanins from the cytosol to the plant vacuole. Natural plant materials rich in phenolics such as extracts from berries (Kähkönen et al., 2003; Viljanen et al., 2005a; 2005b), green tea (Almajano et al., 2007), raisins (Zhao et al., 2007), olives (Paiva-Martins et al., 2006), grape seeds (Hu et al., 2004), and cactus pear fruits (Siriwardhana et al., 2004) have been used as antioxidants in studies of lipid oxidation in different oil-in-water emulsions.

In conclusion, the inability of β-lactoglobulin and berry phenolics to decrease lipid hydroperoxide formation suggests that these additives were increasing lipid hydroxide decomposition possibly by increasing the solubility and/or reactivity of iron ion (via reduction). However both β-lactoglobulin and the berry phenolics were able to inhibit hexanal formation suggesting that they were able to scavenge alkoxyl radicals to decrease fatty acid scission. As with many antioxidants, combinations of β-lactoglobulin and berry phenolics were found to be more effective at inhibiting fatty acid scission than individual antioxidants.

In this study, also amino acid oxidation in the continuous phase β-lactoglobulin was investigated. The results showed that tryptophan and cysteine side-chains in β-lactoglobulin were oxidized prior to lipid oxidation. This suggests that these amino acids are able to act as antioxidants. These results are consistent with previous studies (Elias et al., 2005) where it was observed that cysteine and tryptophan side-chains were oxidized before lipid oxidation was detected. Consequently, these results corroborate that the radical transfer to proteins is high in the beginning of oxidation when lipid oxidation appears to be low. Berry phenolics contributed to retarding the oxidation of the amino acid side-chains in β-lactoglobulin. It has been reported that berry phenolics in whey protein stabilized rapeseed oil-in-water emulsions, where the oil was purified from tocopherols, were able to inhibit protein oxidation measured

as the loss of tryptophan fluorescence and formation of protein carbonyls (Viljanen et al., 2005a; 2005b).

β-lactoglobulin consists of 162 amino acid side-chains (18.3 kDa) and contains two difulfide bonds and a free thiol (cysteine121) as well as two tryptophan side-chains (tryptophan19 and tryptophan61). The reactivity of tryptophan side-chains in β-lactoglobulin usually limited to 50%. This is due to that tryptophan19 is completely buried and tryptophan61 is exposed. The limited fluorescence of tryptophan19 has been explained by the nearby arginine124 side-chain quenching its signal (Brownlow et al., 1997). The reduction in tryptophan fluorescence by ~40% in β-lactoglobulin during oxidation was consistent with previous studies (Elias et al., 2005), and is most likely due to oxidation of tryptophan61. An increase in the fluorescence may indicate that β-lactoglobulin is denaturated, as has been suggested by others (Brownlow et al., 1997; Manderson et al., 1999), thereby exposing the tryptophan19 side-chain. Further decrease in tryptophan fluorescence indicates a conformational change in the protein structure, destruction of the tryptophan side-chains or aggregation of the protein. In native β-lactoglobulin, free cysteine121 is buried within the hydrophobic core, and therefore it is low in reactivity (Brownlow et al., 1997). However, a heat treatment of β-lactoglobulin has been shown to increase the solvent accessibility of sulfhydryl groups (Elias et al., 2007).

Based on the data of amino acid oxidation, it may be concluded that both tryptophan and cysteine in the continuous phase β-lactoglobulin were substantially oxidized prior to the decomposition of fatty acids to form hexanal. This suggests that these amino acids are able to inhibit fatty acid scission. In addition, both berry phenolics were able to inhibit the oxidation of tryptophan and cysteine side-chains of β-lactoglobulin in the beginning of oxidation. More research is, however, needed to elucidate the exact interactions between the individual amino acid side-chains and phenolic compounds as well as lipids.

6.4.1 Stability of black currant anthocyanins in emulsion during oxidation

The concentration of black currant anthocyanins in emulsion was predominantly degraded within the first 12 hours. There were no differences between the emulsion samples with and without β-lactoglobulin. It was observed that the concentration of anthocyanins decreased

rapidly in both oil-in-water emulsion and in buffer solution with degradation being faster in the aqueous solution. The stability of anthocyanins have been shown to increase with increasing lipid and protein content (Viljanen et al., 2005a). It is known, that the intensity and stability of anthocyanin pigments is dependent on various factors including structure and concentration of the pigments, pH, temperature, light intensity, quality and presence of other pigments together, metal ions, enzymes, oxygen, ascorbic acid, sugar and sugar metabolites, and sulfur oxide. Anthocyanins exhibit the highest stability as the red flavylium cation around pH 1.0 – 2.0, whereas the other forms are unstable and eventually lead to degradation of the anthocyanins (von Elbe et al., 1996). At pH 7.0 the flavylium salts loose the proton and transform into quinoidal base, which is an unstable pigment, and immediately bond to water and form colourless chalcone. If the pH value is too high and unstable chalcones have already been formed, the color loss becomes irreversible. It has been reported that in oil-in-water emulsion studies carried out at pH 5.4 – 7.0, berry phenolics showed antioxidant activity toward lipid and protein oxidation (Viljanen et al., 2005a; 2005b). In conclusion, the fact that the black currant anthocyanins were lost faster in buffer (without lipid) than emulsions at pH 7.0 suggests that the loss of color was primarily dependent on pH. The ability of the black currant to inhibit lipid oxidation after decolorization suggests that the decolorized compounds were still able to inhibit lipid oxidation, which has also been confirmed by literature.

7. CONCLUSIONS

Oxidative reactions of lipids and proteins are a major cause of chemical deterioration in food. Therefore, the antioxidant activity of plant materials rich in phenolic compounds is being widely investigated for protection of food components sensitive to oxidative reactions. In addition, phenolic compounds are involved in still incompletely understood mechanisms related to prevention of food deterioration as well as diseases and disorders in humans. This study showed that phenolic-rich plant materials can provide protection toward protein and lipid oxidation reactions in different food models.

Phenolic-rich byproducts of oilseed processes such as rapeseed meal (*Brassica rapa* L.), camelina meal (*Camelina sativa*) and soy (*Glycine max* L.) as well as Scots pine bark (*Pinus sylvestris*) and several reference compounds were shown to act as antioxidants toward both protein and lipid oxidation in cooked pork meat patties. In meat, the antioxidant activity of camelina, rapeseed and soy meal were more pronounced when used in combination with a commercial rosemary extract (*Rosmarinus officinalis*). The antioxidant activity of rapeseed and camelina phenolics is mainly due to sinapic acid and sinapine. Flavanols and flavanols also contribute to the antioxidant activity of camelina meal. The active compounds in pine bark are flavanols. In meat, metal chelation is very likely to promote antioxidant effect of plant phenolics.

Berry phenolics such as black currant (*Ribes nigrum*) anthocyanins and raspberry (*Rubus idaeus*) ellagitannins showed potent antioxidant activity in corn oil-in-water emulsions with and without aqueous phase β-lactoglobulin. The antioxidant effect was more pronounced with β-lactoglobulin. The berry phenolics were also able to inhibit the oxidation of tryptophan and cysteine side-chains of β-lactoglobulin. The results show that the amino acid side-chains were oxidized prior the propagation of lipid oxidation. This suggests that these amino acids are able to inhibit fatty acid scission. Consequently, these results corroborate that the radical transfer to proteins is high in the beginning of oxidation when lipid oxidation appears to be low. In addition, the concentration and color of black currant anthocyanins in the emulsion decreased during the oxidation confirming that decolorized compounds can still act as antioxidants.

The impact of plant phenolics on amino acid level was studied in tryptophan to elucidate their role in preventing the formation of specific oxidation products. Tryptophan oxidation was investigated in two different oxidation models with either H_2O_2 or hexanal/$FeCl_2$. The results show that hexanal/$FeCl_2$ and H_2O_2 increased the oxidation of tryptophan and the formation of tryptophan derived oxidation compounds. The extent of oxidation is dependent on the oxidative conditions such as the type and amount of oxidant, incubation time and temperature. The oilseed byproducts such as camelina, rapeseed and soy meal as well as pine bark phenolics inhibited oxidation of tryptophan in both H_2O_2 and hexanal/$FeCl_2$ induced oxidation models. Berry phenolics such as black currant anthocyanins, raspberry ellagitannins and cranberry proanthocyaninds showed antioxidant activity toward tryptophan loss and on individual oxidation compounds only when oxidized with H_2O_2. In contrast, when hexanal/$FeCl_2$ was used as an oxidant, berry phenolics showed prooxidant effects. The oxidative attack on tryptophan (side-chains) occurs first on the indole moiety. Therefore, the ability of semiquinones or quinones of phenolic compounds to react with the nitrogen-atom in indole moiety may prevent tryptophan from further reactions. Another possibility is the reaction between flavonoid and tryptophan radicals. Further oxidation of these protein – phenolic complexes can lead to formation of tryptophan dimers or polymers. However, more scientific research is needed to optimize the levels of phenolics to be the most effective and to further explicate their antioxidant effect toward protein oxidation by investigating the unknown compounds formed during the oxidation.

The effects of plant phenolics varied from antioxidant to prooxidant depending on the choice of phenolic compound. Therefore, optimal ratios between the phenolic compound and protein/amino acid for antioxidant action should be further elucidated. Our results contribute to elucidating the effects of natural phenolic compounds as potential antioxidants in order to control and prevent protein oxidation reactions. Understanding the relationship between phenolic compounds and proteins as well as lipids could lead to the development of new, effective, and multifunctional antioxidant strategies that could be used in food, feed, cosmetic and pharmaceutical applications.

8. REFERENCES

AOAC International. 1995. Official methods of analysis. AOAC International: Arlington, VA.

Aherne, S. A. and O'Brien, N. M. 2002. Dietary flavonols: chemistry, food content, and metabolism. Nutr. 18: 75-81.

Ahmed, N., Babaei-Jadidi, R., Howell, S. K., Beisswenger, P. J. and Thornalley, P. J. 2005a. Degradation products of proteins damaged by glycation, oxidation and nitration in clinical type 1 diabetes. Diabetologia. 48: 1590-1603.

Ahmed, N., Dobler, D., Dean, M. and Thornalley, P. J. 2005b. Peptide mapping identifies hotspot site of modification in human serum albumin by methylglyoxal involved in ligand binding and esterase activity. J. Biol. Chem. 280: 5724-5732.

Almajano, M. P. and Gordon, M. H. 2004. Synergistic effect of BSA on antioxidant activities in model food emulsions. J. Am. Oil Chem. Soc. 81: 275-280.

Almajano, M. P., Delgado, M. E. and Gordon, M. H. 2007. Albumin causes a synergistic increase in the antioxidant activity of green tea catechins in oil-in-water emulsions. Food Chem. 102: 1375-1382.

Arai, S., Abe, M., Yamashita, M., Kato, H. and Fujimaki, M. 1971. Applying proteolytic enzymes on soybean. Part VIII. Formation of an indole derivative by condensation between tryptophan and n-hexanal. Agric. Biol. Chem. 35: 552-559.

Aubourg, S. P., Perez-Martin, R. I., Medina, I. and Gallardo, J. M. 1992. Fluorescence formation by interaction of albacore (*Thunnus alalunga*) muscle with acetaldehyde in a model system. J. Agric. Food Chem. 40: 1805-1808.

Audette, M., Blouquit, Y. and Houee-Levin, C. 2000. Oxidative dimerization of proteins: Role of tyrosine accessibility. Arch. Biochem. Biophys. 376: 217-220.

Barbut, S., Josephson, D. B. and Maurer, J. 1985. Antioxidant properties of rosemary oleoresin in turkey sausage. J. Food Sci. 50: 1356-1359.

Batabyal, D. and Yeh, S. R. 2007. Human tryptophan dioxygenase: A comparison to indoleamine 2,3-dioxygenase. J. Am. Chem. Soc. 129: 15690-15701.

Baxter, N. J., Lilley, T. H., Haslam, E. and Williamson, M. P. 1997. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. Biochem. 36: 5566-5577.

Belitz, H.-D., Grosch, W. and Schieberle, P. 2004. Food Chemistry. 3rd ed.; Springer-Verlag: Berlin Heidelberg.

Benkova, B., Lozanov, V., Ivanov, I. P., Todorova, A., Milanov, I. and Mitev, V. 2008. Determination of plasma aminothiols by high performance liquid chromatography after

precolumn derivatization with N-(2-acridonyl)maleimide. J. Chromatogr. B - Anal. Technol. Biomed. Life Sci. 870: 103-108.

Boutin, J. A., Audinot, V., Ferry, G. and Delagrange, P. 2005. Molecular tools to study melatonin pathways and actions. Trends Pharmacol. Sci. 26: 412-419.

Bregere, C., Rebrin, I. and Sohal, R. S. 2008. Detection and characterization of in vivo nitration and oxidation of tryptophan residues in proteins. In: *Nitric Oxide, Pt G: Oxidative and Nitrosative Stress in Redox Regulation of Cell Signaling*. pp 339-349 Vol. 441.

Bringmann, G., Feineis, D., Munchbach, M., God, R., Peters, K., Peters, E. M., Mossner, R. and Lesch, K. P. 2006. Toxicity and metabolism of the chloral-derived mammalian alkaloid 1-trichloromethyl-1,2,3,4-tetrahydro-beta-carboline (TaClo) in PC12 cells. J. Biosci. 61: 601-610.

Brownlow, S., Cabral, J. H. M., Cooper, R., Flower, D. R., Yewdall, S. J., Polikarpov, I., North, A. C. T. and Sawyer, L. 1997. Bovine beta-lactoglobulin at 1.8 angstrom resolution - still an enigmatic lipocalin. Structure. 5: 481-495.

Bruenner, B. A., Jones, A. D. and German, J. B. 1995. Direct characterization of protein adducts of the lipid peroxidation product 4-hydroxy-2-nonenal using electrospray mass spectrometry. Chem. Res. Toxicol. 8: 552-559.

Buettner, G. R. 1993. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. Arch. Biochem. Biophys. 300: 535-543.

Cao, G., Sofic, E. and Prior, R. L. 1997. Antioxidant and prooxidant behavior of flavonoids: Structure-activity relationships. Free Radical Biol. Med. 22: 749-760.

Capeillere-Blandin, C., Gausson, V., Descamps-Latscha, B. and Witko-Sarsat, V. 2004. Biochemical and spectrophotometric significance of advanced oxidized protein products. Biochim. Biophys. Acta – Molecul. Basis Disease. 1689: 91-102.

Carr, A. C., Tijerina, T. and Frei, B. 2000. Vitamin C protects against and reverses specific hypochlorous acid- and chloramine-dependent modifications of low-density lipoprotein. Biochem. J. 346: 491-499.

Charlton, A. J., Baxter, N. J., Khan, M. L., Moir, A. J. G., Haslam, E., Davies, A. P. and Williamson, M. P. 2002. Polyphenol/peptide binding and precipitation. J. Agric. Food Chem. 50: 1593-1601.

Chen, J., Flaugh, S. L., Callis, P. R. and King, J. 2006. Mechanism of the highly efficient quenching of tryptophan fluorescence in human γD-crystallin. Biochem. 45: 11552-11563.

Chen, C.-Y., Milbury, P. E., Chung, S.-K. and Blumberg, J. 2007. Effect of almond skin polyphenolics and quercetin on human LDL and apolipoprotein B-100 oxidation and conformation. J. Nutr. Biochem.. 18: 785-794.

Chopin, C., Kone, M. and Serot, T. 2007. Study of the interaction of fish myosin with the products of lipid oxidation: The case of aldehydes. Food Chem. 105: 126-132.

Chung, F.-L., Chen, H.-J. C., Guttenplan, J. B., Nishikawa, A. and Hard, G. C. 1993. 2,3-Epoxy-4-hydroxynonenal as a potential tumor-initiating agent of lipid peroxidation. Carcinogenesis. 14: 2073-2077.

Conn, S., Curtin, C., Bezier, A., Franco, C. and Zhang, W. 2008. Purification, molecular cloning, and characterization of glutathione S-transferases (GSTs) from pigmented *Vitis vinifera* L. cell suspension cultures as putative anthocyanin transport proteins. J. Experiment. Botany. 59: 3621-3634.

Culp, R. A. and Noakes, J. E. 1990. Identification of isotopically manipulated cinnamic aldehyde and benzaldehyde. J. Agric. Food Chem. 38: 1249-1255.

Dalsgaard, T. K., Otzen, D., Nielsen, J. H. and Larsen, L. B. 2007. Changes in structures of milk proteins upon photo-oxidation. J. Agric. Food Chem. 55: 10968-10976.

Damodaran, S. 1996. Amino acids, peptides, and proteins. In: *Food Chemistry*. Fennema, O. R., Ed. Marcel Dekker, NY, USA.

Dangles, O. and Dufour, C. 2006. Flavonoid-protein interactions. In: *Flavonoids: chemistry, biochemistry and applications*. Andersen, Ø. M.,Markham, K. R., Eds., CRC Press, Boca Raton, FL, USA.

Davies, M. J., Fu, S. and Dean, R. T. 1995. Protein hydroperoxides can give rise to reactive free radicals. Biochem. J. 305: 643-649.

Davies, M. J. 2003. Singlet oxygen-mediated damage to proteins and its consequences. Biochem. Biophysic. Res. Comm. 305: 761-770.

Deaville, E. R., Green, R. J., Mueller-Harvey, I., Willoughby, I. and Frazier, R. A. 2007. Hydrolyzable tannin structures influence relative globular and random coil protein binding strengths. J. Agric. Food Chem. 55: 4554-4561.

De Gonzalez, M. T. N., Boleman, R. M., Miller, R. K., Keeton, J. T. and Rhee, K. S. 2008. Antioxidant properties of dried plum ingredients in raw and precooked pork sausage. J. Food Sci. 73: H63-H71.

Delgado-Andrade, C., Rufian-Henares, J. A., Jimenez-Perez, S. and Morales, F. J. 2006. Tryptophan determination in milk-based ingredients and dried sport supplements by liquid chromatography with fluorescence detection. Food Chem. 98: 580-585.

Diaz, M., Dunn, C. M., McClements, D. J. and Decker, E. A. 2003. Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. J. Agric. Food Chem. 51: 2365-2370.

Diem, S., Albert, J. and Herderich, M. 2001a. Reactions of tryptophan with carbohydrates: Identification of pentose-derived tryptophan glycoconjugates in food. Eur. Food Res. Technol. 213: 439-447.

Diem, S. and Herderich, M. 2001b. Reaction of tryptophan with carbohydrates: Mechanistic studies on the formation of carbohydrate-derived beta-carbolines. J. Agric. Food Chem. 49: 5473-5478.

Dixon, R. A. 2004. Phytoestrogens. Annu. Rev. Plant Biol. 55: 225-261.

Dominques, M. R. M., Dominques, P., Reis, A., Fonseca, C., Amado, F. M. L. and Ferrer-Correia, A. J. V. 2003. Identification of oxidation products and free radicals of tryptophan by mass spectrometry. J. Am. Soc. Mass Spectrom. 14: 406-416.

Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B. and vanderVliet, A. 1996. Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid - A novel mechanism for nitric oxide-mediated protein modification. J. Biol. Chem. 271: 19199-19208.

Elias, R. J., McClements, D. J. and Decker, E. A. 2005. Antioxidant activity of cysteine, tryptophan, and methionine residues in continuous phase beta-lactoglobulin in oil-in-water emulsions. J. Agric. Food Chem. 53: 10248-10253.

Elias, R. J., Bridgewater, J. D., Vachet, R. W., Waraho, T., McClements, D. J. and Decker, E. A. 2006. Antioxidant mechanisms of enzymatic hydrolysates of beta-lactoglobulin in food lipid dispersions. J. Agric. Food Chem. 54: 9565-9572.

Elias, R. J., McClements, D. J. and Decker, E. A. 2007. Impact of thermal processing on the antioxidant mechanisms of continuous phase beta-lactoglobulin in oil-in-water emulsions. Food Chem. 104: 1402-1409.

Elias, R. J., Kellerby, S. S. and Decker, E. A. 2008. Antioxidant Activity of Proteins and Peptides. Crit. Rev. Food Sci. Nutr. 48: 430 - 441.

Elmnasser, N., Dalgalarrondo, M., Orange, N., Bakhrouf, A., Haertle, T., Federighi, M. and Chobert, J. M. 2008. Effect of pulsed-light treatment on milk proteins and lipids. J. Agric. Food Chem. 56: 1984-1991.

Esterbauer, H., Dieber-Rotheneder, M., Striegl, G. and Waeg, G. 1991a. Role of vitamin E in preventing the oxidation of low-density lipoprotein. Am. J. Clin. Nutr. 53: 314S-321.

Esterbauer, H., Schaur, R. J. and Zollner, H. 1991b. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11: 81-128.

Estévez, M., Ventanas, S. and Cava, R. 2006. Effect of natural and synthetic antioxidants on protein oxidation and colour and texture changes in refrigerated stored porcine liver pâté. Meat Sci. 74: 396-403.

Estévez, M., Ventanas, S. and Cava, R. 2007. Oxidation of lipids and proteins in frankfurters with different fatty acid compositions and tocopherol and phenolic contents. Food Chem. 100: 55-63.

Fallico, B. and Ames, J. M. 1999. Effect of hexanal and iron on color development in a glucose/phenylalanine model system. J. Agric. Food Chem. 47: 2255-2261.

Faraji, H., McClements, D. J. and Decker, E. A. 2004. Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. J. Agric. Food Chem. 52: 4558-4564.

Fasseas, M. K., Mountzouris, K. C., Tarantilis, P. A., Polissiou, M. and Zervas, G. 2008. Antioxidant activity in meat treated with oregano and sage essential oils. Food Chem. 109: 173-173.

Favretto, D., Bertazzo, A., Costa, C., Allegri, G. and Traldi, P. 1997. A study of the enzymatic oligomerization of 5-hydroxytryptamine using matrix-assisted laser desorption/ionization mass spectrometry. Rapid Comm. Mass Spectrom. 11: 1038-1042.

Fenaille, F., Guy, P. A. and Tabet, J.-C. 2003. Study of protein modification by 4-hydroxy-2-nonenal and other short chain aldehydes analyzed by electrospray ionization tandem mass spectrometry. J. Am. Soc. Mass Spectrom. 14: 215-226.

Fernandez, M. T., Mira, M. L., Florêncio, M. H. and Jennings, K. R. 2002. Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. J. Inorg. Biochem. 92: 105-111.

Ferreira, D., Slade, D. and Marais, J. P. J. 2006. Flavans and proanthocyanidins. In: *Flavonoids: Chemistry, Biochemistry and Applications*. Anderson, O. M.,Markham, K. R., Eds., CRC Press, Boca Raton, FL.

Ferroni, F., Maccaglia, A., Pietraforte, D., Turco, L. and Minetti, M. 2004. Phenolic antioxidants and the protection of low density lipoprotein from peroxynitrite-mediated oxidations physiologic CO_2. J. Agric. Food Chem. 52: 2866-2874.

Filipe, P., Morliere, P., Patterson, L. K., Hug, G. L., Maziere, J. C., Maziere, C., Freitas, J. P., Fernandes, A. and Santus, R. 2002. Repair of amino acid radicals of apolipoprotein B100 of low-density lipoproteins by flavonoids. A pulse radiolysis study with quercetin and rutin. Biochem. 41: 11057-11064.

Frankel, E. N., Neff, W. E. and Selke, E. 1981. Analysis of autoxidized fats by gas chromatography-mass spectrometry: VII. Volatile thermal decomposition products of pure hydroperoxides from autoxidized and photosensitized methyl oleate, linoleate and linolenate. Lipids. 16: 279-285.

Frankel, E. N. 1996. Antioxidants in lipid foods and their impact on food quality. Food Chem. 57: 51-55.

Friedman, M. and Cuq, J. L. 1988. Chemistry, analysis, nutritional-value, and toxicology of tryptophan in food - a review. J. Agric. Food Chem. 36: 1079-1093.

Fujita, Y., Mori, I. and Yamaguchi, T. 2002. Spectrophotometric determination of biologically active thiols with eosin, silver(I) and adenine. Anal. Sci. 18: 981-985.

Gardner, H. W. 1979. Lipid hydroperoxide reactivity with proteins and amino acids: a review. J. Agric. Food Chem. 27: 220-229.

Gardner, H. W. and Selke, E. 1984. Volatiles from thermal-decomposition of isomeric methyl(12S,13*S*)-(*E*)-12,13-epoxy-9-hydroperoxy-10-octadecenoates. Lipids. 19: 375-380.

Georgantelis, D., Ambrosiadis, I., Katikou, P., Blekas, G. and Georgakis, S. A. 2007. Effect of rosemary extract, chitosan and [alpha]-tocopherol on microbiological parameters and lipid oxidation of fresh pork sausages stored at 4 °C. Meat Sci. 76: 172-181.

Giulivi, C. and Davies, K. J. A. 1993. Dityrosine and tyrsoine oxidation products are endogenous markers for the selective proteolysis of oxidatively modified red blood cell hemoglobin by (the 19 S) proteasome. J. Biol. Chem. 268: 8752-8759.

Griessauf, A., Steiner, E. and Esterbauer, H. 1995. Early destruction of tryptophan residues in apolipoprotein B is a vitamin E-independent process during copper-mediated oxidation of LDL. Biochim. Biophysic. Acta 1256: 221-232.

Guilleaguten, M. and Goicoechea, E. 2008. Toxic oxygenated alfa-beta-unsaturated aldehydes and their study in foods: A review. Crit. Rev. Food Sci. Nutr. 48: 119 - 136.

Guillen, M. D., Cabo, N., Ibargoitia, M. L. and Ruiz, A. 2005. Study of both sunflower oil and its headspace throughout the oxidation process. Occurrence in the headspace of toxic oxygenated aldehydes. J. Agric. Food Chem. 53: 1093-1101.

Hamilton, G. A. 1969. Mechanisms of two- or four-electron oxidations catalyzed by some metalloenzymes. Adv. Enzymol. Relat. Areas Mol. Biol. 32: 55-96.

Hashimoto, M., Sibata, T., Wasada, H., Toyokuni, S. and Uchida, K. 2003. Structural basis of protein-bound endogenous aldehydes - Chemical and immunochemical characterizations of configurational isomers of a 4-hydroxy-2-nonenal-histidine adduct. J. Biol. Chem. 278: 5044-5051.

Hazell, L. J., Davies, M. J. and Stocker, R. 1999. Secondary radicals derived from chloramines of apolipoprotein B-100 contribute to HOCl-induced lipid peroxidation of low-density lipoproteins. Biochem. J. 339: 489-495.

He, Y. and Shahidi, F. 1997. Antioxidant activity of green tea and its catechins in a fish meat model system. J. Agric. Food Chem. 45: 4262-4266.

Headlam, H. A. and Davies, M. J. 2002. Beta-scission of side-chain alkoxyl radicals on peptides and proteins results in the loss of side-chains as aldehydes and ketones. Free Radic. Biol. Med. 32: 1171-1184.

Heinonen, M., Rein, D., Satue-Gracia, M. T., Huang, S. W., German, J. B. and Frankel, E. N. 1998. Effect of protein on the antioxidant activity of phenolic compounds in a lecithin-liposome oxidation system. J. Agric. Food Chem. 46: 917-922.

Heinonen, M. 2007. Antioxidant activity and antimicrobial effect of berry phenolics - a Finnish perspective. Mol. Nutr. Food Res. 51: 684-691.

Herraiz, T. 1996. Occurrence of tetrahydro-beta-carboline-3-carboxylic acids in commercial foodstuffs. J. Agric. Food Chem. 44: 3057-3065.

Herraiz, T. 2000a. Analysis of the bioactive alkaloids tetrahydro-beta-carboline and beta-carboline in food. J. Chromatogr. A. 881: 483-499.

Herraiz, T. 2000b. Tetrahydro-beta-carbolines, potential neuroactive alkaloids, in chocolate and cocoa. J. Agric. Food Chem. 48: 4900-4904.

Herraiz, T. and Galisteo, J. 2003a. Tetrahydro-beta-carboline alkaloids occur in fruits and fruit juices. Activity as antioxidants and radical scavengers. J. Agric. Food Chem. 51: 7156-7161.

Herraiz, T., Galisteo, J. and Chamorro, C. 2003b. L-tryptophan reacts with naturally occurring and food-occurring phenolic aldehydes to give phenolic tetrahydro-beta-carboline alkaloids: Activity as antioxidants and free radical scavengers. J. Agric. Food Chem. 51: 2168-2173.

Herraiz, T. and Papavergou, E. 2004. Identification and occurrence of tryptamine- and tryptophan-derived tetrahydro-beta-carbolines in commercial sausages. J. Agric. Food Chem. 52: 2652-2658.

Herraiz, T., Guillen, H. and Galisteo, J. 2007. N-methyltetrahydro-beta-carboline analogs of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin are oxidized to neurotoxic beta-carbolinium cations by heme peroxidases. Biochem. Biophysic. Res. Comm. 356: 118-123.

Hidalgo, F. J., Alaiz, M. and Zamora, R. 1998. A spectrophotometric method for the determination of proteins damaged by oxidized lipids. Anal. Biochem. 262: 129-136.

Hidalgo, F. J. and Zamora, R. 2000. Modification of bovine serum albumin structure following reaction with 4,5(*E*)-epoxy-2(*E*)-heptenal. Chem. Res. Toxicol. 13: 501-508.

Hidalgo, F. J. and Zamora, R. 2004. Strecker-type degradation produced by the lipid oxidation products 4,5-epoxy-2-alkenals. J. Agric. Food Chem. 52: 7126-7131.

Hirata, F. and Hayaishi, O. 1972. New degradative routes of 5-hydroxytryptophan and serotonin by intestinal tryptophan 2,3-dioxygenase. Biochem. Biophysic. Res. Comm. 47: 1112-&.

Hirata, F. and Hayaishi, O. 1975. Studies on indolamine 2,3-dioxygenase. 1. Superoxide anion as substrate. J. Biol. Chem. 250: 5960-5966.

Howell, A. B., Reed, J. D., Krueger, C. G., Winterbottom, R., Cunningham, D. G. and Leahy, M. 2005. A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity. Phytochem. 66: 2281-2291.

Hu, M., McClements, D. J. and Decker, E. A. 2003a. Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. J. Agric. Food Chem. 51: 1435-1439.

Hu, M., McClements, D. J. and Decker, E. A. 2003b. Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. J. Agric. Food Chem. 51: 1696-1700.

Hu, M., McClements, D. J. and Decker, E. A. 2004. Antioxidant activity of a proanthocyanidin-rich extract from grape seed in whey protein isolate stabilized algae oil-in-water emulsions. J. Agric. Food Chem. 52: 5272-5276.

Huggins, T. G., Wellsknecht, M. C., Detorie, N. A., Baynes, J. W. and Thorpe, S. R. 1993. Formation of o-tyrosine and dityrosine in proteins during radiolytic and metal-catalyzed oxidation. J. Biol. Chem. 268: 12341-12347.

Humeny, A., Kislinger, T., Becker, C. M. and Pischetsrieder, M. 2002. Qualitative determination of specific protein glycation products by matrix-assisted laser desorption/ionization mass spectrometry peptide mapping. J. Agric. Food Chem. 50: 2153-2160.

Ichihashi, K., Osawa, T., Toyokuni, S. and Uchida, K. 2001. Endogenous formation of protein adducts with carcinogenic aldehydes - Implications for oxidative stress. J. Biol. Chem. 276: 23903-23913.

Irwin, J. A., Ostdal, H. and Davies, M. J. 1999. Myoglobin-induced oxidative damage: Evidence for radical transfer from oxidized myoglobin to other proteins and antioxidants. Arch. Biochem. Biophys. 362: 94-104.

Ishino, K., Shibata, T., Ishii, T., Liu, Y. T., Toyokuni, S., Zhu, X. C., Sayre, L. M. and Uchida, K. 2008. Protein N-acylation: H_2O_2-mediated covalent modification of protein by lipid peroxidation-derived saturated aldehydes. Chem. Res. Toxicol. 21: 1261-1270.

Itakura, K., Uchida, K. and Kawakishi, S. 1994. Selective formation of oxindole-type and formylkynurenine-type products from tryptophan and its peptides treated with a superoxide-generating system in the presence of iron(III) EDTA - a possible involvement with iron oxygen complex. Chem. Res. Toxicol. 7: 185-190.

Justesen, U., Knuthsen, P. and Leth, T. 1998. Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection. J. Chromatogr. A. 799: 101-110.

Kähkönen, M. P., Hopia, A. I. and Heinonen, M. 2001. Berry phenolics and their antioxidant activity. J. Agric. Food Chem. 49: 4076-4082.

Kähkönen, M. P., Heinämäki, J., Ollilainen, V. and Heinonen, M. 2003. Berry anthocyanins: isolation, identification and antioxidant activities. J. Sci. Food Agric. 83: 1403-1411.

Kähkönen, M., Kylli, P., Ollilainen, V., Salminen, J.-P. and Heinonen, M. Unpublished results. Ellagitannins from red raspberries (*Rubus idaeus*) and cloudberries (*Rubus chamaemorus*) - Isolation, identification and antioxidant activity.

Kanatt, S. R., Chander, R., Radhakrishna, P. and Sharma, A. 2005. Potato peel extract - a natural antioxidant for retarding lipid peroxidation in radiation processed lamb meat. J. Agric. Food Chem. 53: 1499-1504.

Kaneko, S., Okitani, A., Hayase, F. and Kato, H. 1989. Novel crosslinking compounds formed through the reaction of N-alfa-acetyltryptophan with hexanal. Agric. Biol. Chem. 53: 2679-2685.

Kanner, J., Harel, S. and Jaffe, R. 1991. Lipid peroxidation of muscle food as affected by sodium chloride. J. Agric. Food Chem. 39: 1017-1021.

Kanner, J. 1994. Oxidative Proxesses in meat and meat-products - quality implications. Meat Sci. 36: 169-189.

Kanski, J., Aksenova, M., Stoyanova, A. and Butterfield, D. A. 2002. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure-activity studies. J. Nutr. Biochem. 13: 273-281.

Karel, M., Roy, R. B. and Schaich, K. M. 1974. Interaction of peroxidizing methyl linoleate with some proteins and amino-acids. Abstracts Papers Am. Chem. Soc. 36-36.

Karel, M., Schaich, K. and Roy, R. B. 1975. Interaction of peroxidizing methyl linoleate with some proteins and amino-acids. J. Agric. Food Chem. 23: 159-163.

Karonen, M., Hämälainen, M., Nieminen, R., Klika, K. D., Loponen, J., Ovcharenko, V. V., Moilanen, E. and Pihlaja, K. 2004. Phenolic extractives from the bark of *Pinus sylvestris* L. and their effects on inflammatory mediators nitric oxide and prostaglandin E2. J. Agric. Food Chem. 52: 7532-7540.

Karpinska, M., Borowski, J. and Danowska-Oziewicz, M. 2000. Antioxidative activity of rosemary extract in lipid fraction of minced meat balls during storage in a freezer. Nahrung/Food. 44: 38-41.

Kato, H., Kaneko, S., Okitani, A., Ito, M. and Hayase, F. 1986. Modification of lysine residues to alkyl-substituted pyridiniums on exposure of proteins to vaporized hexanal. Agric. Biol. Chem. 50: 1223-1228.

Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K. and Kobori, M. 2003. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. J. Agric. Food Chem. 51: 68-75.

Kellerby, S. S., McClements, D. J. and Decker, E. A. 2006. Role of proteins in oil-in-water emulsions on the stability of lipid hydroperoxides. J. Agric. Food Chem. 54: 7879-7884.

Kerwin, B. A. and Remmele Jr., R. L. 2007. Protect from light: Photodegradation and protein biologics. J. Pharmaceut. Sci. 96: 1468-1479.

Kislinger, T., Humeny, A., Seeber, S., Becker, C. M. and Pischetsrieder, M. 2002. Qualitative determination of early Maillard-products by MALDI-TOF mass spectrometry peptide mapping. Eur. Food Res. Technol. 215: 65-71.

Kislinger, T., Humeny, A. and Pischetsrieder, M. 2004. Analysis of protein glycation products by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Curr. Med. Chem. 11: 2185-2193.

Kislinger, T., Humeny, A., Peich, C. C., Becker, C. M. and Pischetsrieder, M. 2005. Analysis of protein glycation products by MALDI-TOF/MS. In: *Maillard Reaction: Chemistry at the Interface of Nutrition, Aging, and Disease*. pp 249-259 Vol. 1043.

Koski, A., Pekkarinen, S., Hopia, A., Wähälä, K. and Heinonen, M. 2003. Processing of rapeseed oil: effects on sinapic acid derivative content and oxidative stability. Eur. Food Res. Technol. 217: 110-114.

Kris-Etherton, P. M., Hecker, K. D., Bonanome, A., Coval, S. M., Binkoski, A. E., Hilpert, K. F., Griel, A. E. and Etherton, T. D. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am. J. Med. 113: 71-88.

Kylli, P., Nohynek, L., Puupponen-Pimiä, R., Westerlund-Wikström, B., McDougal, G., Stewart, D. and Heinonen, M. Unpublished results. Isolation, identification and bioactivities of rowanberry (*Sorbus aucuparia*) phenolics.

Ladokhin, A. S., Jayasinghe, S. and White, S. H. 2000. How to measure and analyze tryptophan fluorescence in membranes properly, and why bother? Anal. Biochem. 285: 235-245.

Lakowicz, J. R. 1999. Principles of fluorescence spectroscopy. Kluwer Academic: New York.

Leahy, M. M. and Warthese, J. J. 1983. The influence of Maillard browning and other factors on the stability of free tryptophan. J. Food Process. Preserv. 7: 25-39.

Lederer, M. O. 1996. Reactivity of lysine moieties toward gamma-hydroxy-alpha,beta-unsaturated epoxides: A model study on protein-lipid oxidation product interaction. J. Agric. Food Chem. 44: 2531-2537.

Lee, M. G. and Rogers, C. M. 1988. Degradation of tryptophan in aqueous solution. J. Paranter. Sci. Technol. 42: 20-22.

Lee, S. H., Oe, T. and Blair, I. A. 2002. 4,5-Epoxy-2(E)-decenal-Induced Formation of 1,N6-Etheno-2'-deoxyadenosine and 1,N2-Etheno-2'-deoxyguanosine Adducts. Chem. Res. Toxicol. 15: 300-304.

Levine, R. L., Williams, J. A., Stadtman, E. R. and Shacter, E. 1994. Carbonyl Assays for Determination of Oxidatively Modified Proteins. In: *Oxygen Radicals in Biological Systems, Pt C*. pp 346-357 Vol. 233.

Levine, R. L., Mosoni, L., Berlett, B. S. and Stadtman, E. R. 1996. Methionine residues as endogenous antioxidants in proteins. Proc. Natl. Acad. Sci. US. 93: 15036-15040.

Levine, R. L., Berlett, B. S., Moskovitz, J., Mosoni, L. and Stadtman, E. R. 1999. Methionine residues may protect proteins from critical oxidative damage. Mechanisms Ageing Developm. 107: 323-332.

Levine, R. L. and Stadtman, E. R. 2001. Oxidative modification of proteins during aging. Experim. Gerontol. 36: 1495-1502.

Levine, R. L. 2002. Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radic. Biol. Med. 32: 790-796.

Liu, Y. W., Han, C. H., Lee, M. H., Hsu, F. L. and Hou, W. C. 2003. Patatin, the tuber storage protein of potato (*Solanum tuberosum* L.), exhibits antioxidant activity in vitro. J. Agric. Food Chem. 51: 4389-4393.

Määtta-Riihinen, K. R., Kamal-Eldin, A., Mattila, P. H., Gonzalez-Paramas, A. M. and Torronen, A. R. 2004a. Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. J. Agric. Food Chem. 52: 4477-4486.

Määtta-Riihinen, K. R., Kamal-Eldin, A. and Torronen, A. R. 2004b. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (Family *rosaceae*). J. Agric. Food Chem. 52: 6178-6187.

Määttä-Riihinen, K. R., Kähkönen, M. P., Törrönen, A. R. and Heinonen, I. M. 2005. Catechins and procyanidins in berries of vaccinium species and their antioxidant activity. J. Agric. Food Chem. 53: 8485-8491.

Malencik, D. A., Sprouse, J. F., Swanson, C. A. and Anderson, S. R. 1996. Dityrosine: preparation, isolation, and analysis. Anal. Biochem. 242: 202-213.

Manderson, G. A., Hardman, M. J. and Creamer, L. K. 1999. Effect of heat treatment on bovine beta-lactoglobulin A, B, and C explored using thiol availability and fluorescence. J. Agric. Food Chem. 47: 3617-3627.

Mariutti, L. R. B., Orlien, V., Bragagnolo, N. and Skibsted, L. H. 2008. Effect of sage and garlic on lipid oxidation in high-pressure processed chicken meat. Eur. Food Res. Technol. 227: 337-344.

Marsh, D. and Pali, T. 2004. The protein-lipid interface: perspectives from magnetic resonance and crystal structures. Biochim. Biophys. Acta-Biomembranes. 1666: 118-141.

Matthaus, B. 2002. Antioxidant activity of extracts obtained from residues of different oilseeds. J. Agric. Food Chem. 50: 3444-3452.

McCarthy, T. L., Kerry, J. P., Kerry, J. F., Lynch, P. B. and Buckley, D. J. 2001. Assessment of the antioxidant potential of natural food and plant extracts in fresh and previously frozen pork patties. Meat Sci. 57: 177-184.

McClements, D. J. and Decker, E. A. 2000. Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. J. Food Sci. 65: 1270-1282.

Megli, F. M., Russo, L. and Sabatini, K. 2005. Oxidized phospholipids induce phase separation in lipid vesicles. FEBS Letters. 579: 4577-4584.

Meltretter, J., Seeber, S., Humeny, A., Becker, C. M. and Pischetsrieder, M. 2007. Site-specific formation of maillard, oxidation, and condensation products from whey proteins during reaction with lactose. J. Agric. Food Chem. 55: 6096-6103.

Meltretter, J., Becker, C. M. and Pischetsrieder, M. 2008a. Identification and site-specific relative quantification of beta-lactoglobulin modifications in heated milk and dairy products. J. Agric. Food Chem. 56: 5165-5171.

Meltretter, J. and Pischetsrieder, M. 2008b. Application of mass spectrometry for the detection of glycation and oxidation products in milk proteins. Maillard Reaction: Recent Adv. Food Biomed. Sci. 1126: 134-140.

Mercier, Y., Gatellier, P., Viau, M., Remignon, H. and Renerre, M. 1998. Effect of dietary fat and vitamin E on colour stability and on lipid and protein oxidation in Turkey meat during storage. Meat Sci. 48: 301-318.

Meynier, A., Rampon, V., Dalgalarrondo, M. and Genot, C. 2004. Hexanal and *t*-2-hexenal form covalent bonds with whey proteins and sodium caseinate in aqueous solution. Int. Dairy J. 14: 681-690.

Milde, J., Elstner, E. F. and Grassmann, J. 2004. Synergistic inhibition of low-density lipoprotein oxidation by rutin, gamma-terpinene-1 and ascorbic acid. Phytomed. 11: 105-113.

Milde, J., Elstner, E. F. and Grabmann, J. 2007. Synergistic effects of phenolics and carotenoids on human low-density lipoprotein oxidation. Molec. Nutr. Food Res. 51: 956-961.

Milder, I. E. J., Arts, I. C. W., van de Putte, B., Venema, D. P. and Hollman, P. C. H. 2005. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br. J. Nutr. 93: 393-402.

Mithen, R. F., Dekker, M., Verkerk, R., Rabot, S. and Johnson, I. T. 2000. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. J. Sci. Food Agric. 80: 967-984.

Moll, T. S., Harms, A. C. and Elfarra, A. A. 2000. A comprehensive structural analysis of hemoglobin adducts formed after *in vitro* exposure of erythrocytes to butadiene monoxide. Chem. Res. Toxicol. 13: 1103-1113.

Moller, J. K. S. and Skibsted, L. H. 2006. Myoglobins - The link between discoloration and lipid oxidation in muscle and meat. Quimica Nova. 29: 1270-1278.

Morales, F. J., Acar, O. C., Serpen, A., Arribas-Lorenzo, G. and Gokmen, V. 2007. Degradation of free tryptophan in a cookie model system and its application in commercial samples. J. Agric. Food Chem. 55: 6793-6797.

Moroni, F. 1999. Tryptophan metabolism and brain function: focus on kynurenine and other indole metabolites. Eur. J. Pharmacol. 375: 87-100.

Mueller, L. A., Goodman, C. D., Silady, R. A. and Walbot, V. 2000. AN9, a petunia glutathione S-transferase required for anthocyanin sequestration, is a flavonoid-binding protein. Plant Physiol. 123: 1561-1570.

Mullen, W., McGinn, J., Lean, M. E. J., MacLean, M. R., Gardner, P., Duthie, G. G., Yokota, T. and Crozier, A. 2002. Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. J. Agric. Food Chem. 50: 5191-5196.

Mullen, W., Yokota, T., Lean, M. E. J. and Crozier, A. 2003. Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruits by LC-MS^n. Phytochem. 64: 617-624.

Murphy, A., Kerry, J. P., Buckley, J. and Gray, I. 1998. The antioxidative properties of rosemary oleoresin and inhibition of off-flavours in precooked roast beef slices. J. Sci. Food Agric. 77: 235-243.

Nagakawa, M., Kato, S., Kataoka, S. and Hino, T. 1979. 3a-hydroperoxypyrroloindole from tryptophan - isolation and transformation to formylkynurenine. J. Am. Chem. Soc. 101: 3136-3137.

Nagakawa, M., Kato, S., Kataoka, S., Kodato, S., Watanebe, H., Okajima, H., Hino, T. and Witkop, B. 1981. Dye-sensitized photooxygentation of tryptophan: 3a-hydroperoxypyrroloindole as a labile precursor of formylkynurenine. Chem. Pharm. Bull. 29.

Naveena, B. M., Sen, A. R., Kingsly, R. P., Singh, D. B. and Kondaiah, N. 2008. Antioxidant activity of pomegranate rind powder extract in cooked chicken patties. Int. J. Food Sci. Technol. 43: 1807-1812.

Nuchi, C. D., McClements, D. J. and Decker, E. A. 2001. Impact of Tween 20 hydroperoxides and iron on the oxidation of methyl linoleate and salmon oil dispersions. J. Agric. Food Chem. 49: 4912-4916.

Nurmi, T., Voutilainen, S., Nyyssönen, K., Adlercreutz, H. and J.-T., S. 2003a. J. Chromatogr. B. 797: 101-110.

Nurmi, T., Voutilainen, S., Nyyssonen, K., Adlercreutz, H. and Salonen, J. T. 2003b. Liquid chromatography method for plant and mammalian lignans in human urine. J. Chromatogr. B - Anal. Technol. Biomed. Life Sci. 798: 101-110.

O'Keefe, S. F., Wilson, L. A., Resurreccion, A. P. and Murphy, P. A. 1991. Determination of the binding of hexanal to soy glycinin and beta-conglycinin in an aqueous model system using a headspace technique. J. Agric. Food Chem. 39: 1022-1028.

Okuno, E., Nakamura, M. and Schwarcz, R. 1991. Two kynurenine aminotransferases in human brain. Brain Res. 542: 307-312.

Oliver, C. N., Ahn, B.-W., Moerman, E. J., Goldstein, S. and Stadtman, E. R. 1987. Age-related changes in oxidized proteins. J. Biol. Chem. 262 5488-5491.

Ostergren, A., Annas, A., Skog, K., Lindquist, N. G. and Brittebo, E. B. 2004. Long-term retention of neurotoxic beta-carbolines in brain neuromelanin. J. Neural Transmission. 111: 141-157.

Paiva-Martins, F., Santos, V., Mangericao, H. and Gordon, M. H. 2006. Effects of copper on the antioxidant activity of olive polyphenols in bulk oil and oil-in-water emulsions. J. Agric. Food Chem. 54: 3738-3743.

Papadopoulou, A., Green, R. J. and Frazier, R. A. 2005. Interaction of flavonoids with bovine serum albumin: A fluorescence quenching study. J. Agric. Food Chem. 53: 158-163.

Papavergou, E. and Herraiz, T. 2003. Identification and occurrence of 1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid: the main beta-carboline alkaloid in smoked foods. Food Res. Int. 36: 843-848.

Parker, N. R., Jamie, J. F., Davies, M. J. and Truscott, R. J. W. 2004. Protein-bound kynurenine is a photosensitizer of oxidative damage. Free Radical Biol. Med. 37: 1479-1489.

Partridge, M. A. K., Jiang, Y., Skerritt, J. H. and Schaich, K. M. 2003. Immunochemical and electrophoretic analysis of the modification of wheat proteins in extruded flour products. Cereal Chem. 80: 791-798.

Peña-Ramos, E. A. and Xiong, Y. L. 2003. Whey and soy protein hydrolysates inhibit lipid oxidation in cooked pork patties. Meat Sci. 64: 259-263.

Peterson, J., Dwyer, J., Bhagwat, S., Haytowitz, D., Holden, J., Eldridge, A. L., Beecher, G. and Aladesanmi, J. 2005. Major flavonoids in dry tea. J. Food Compos. Anal. 18: 487-501.

Pietraforte, D., Turco, L., Azzini, E. and Minetti, M. 2002. On-line EPR study of free radicals induced by peroxidase/H_2O_2 in human low-density lipoprotein. Biochim. Biophys. Acta-Molecul. Cell Biol. Lipids. 1583: 176-184.

Pripp, A. H., Vreeker, R. and van Duynhoven, J. 2005. Binding of olive oil phenolics to food proteins. J. Sci. Food Agric. 85: 354-362.

Puupponen-Pimia, R., Nohynek, L., Alakomi, H. L. and Oksman-Caldentey, K. M. 2005. Bioactive berry compounds - novel tools against human pathogens. Appl. Microbiol. Biotechnol. 67: 8-18.

Rababah, T., Hettiarachchy, N., Horax, R., Eswaranandam, S., Mauromoustakos, A., Dickson, J. and Niebuhr, S. 2004. Effect of electron beam irradiation and storage at 5 °C on thiobarbituric acid reactive substances and carbonyl contents in chicken breast meat infused with antioxidants and selected plant extracts. J. Agric. Food Chem. 52: 8236-8241.

Rampon, V., Lethuaut, L., Mouhous-Riou, N. and Genot, C. 2001. Interface characterization and aging of bovine serum albumin stabilized oil-in-water emulsions as revealed by front-surface fluorescence. J. Agric. Food Chem. 49: 4046-4051.

Rawel, H. M., Kroll, J. and Rohn, S. 2001. Reactions of phenolic substances with lysozyme - physicochemical characterisation and proteolytic digestion of the derivatives. Food Chem. 72: 59-71.

Refsgaard, H. H. F., Tsai, L. and Stadtman, E. R. 2000. Modifications of proteins by polyunsaturated fatty acid peroxidation products. Proc. Natl. Acad. Sci. US. 97: 611-616.

Reinbold, J., Rychlik, M., Asam, S., Wieser, H. and Koehler, P. 2008. Concentrations of total glutathione and cysteine in wheat flour as affected by sulfur deficiency and correlation to quality parameters. J. Agric. Food Chem. 56: 6844-6850.

Requena, J. R., Chao, C. C., Levine, R. L. and Stadtman, E. R. 2001. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. Proc. Natl. Acad. Sci. US. 98: 69-74.

Retsky, K. L., Chen, K., Zeind, J. and Frei, B. 1999. Inhibition of copper-induced LDL oxidation by vitamin C is associated with decreased copper-binding to LDL and 2-oxo-histidine formation. Free Radic. Biol. Med. 26: 90-98.

Rey, A. I., Hopia, A., Kivikari, R. and Kahkonen, M. 2005. Use of natural food/plant extracts: cloudberry (*Rubus Chamaemorus*), beetroot (*Beta Vulgaris "Vulgaris"*) or willow herb (*Epilobium angustifolium*) to reduce lipid oxidation of cooked pork patties. Lwt-Food Sci. Technol. 38: 363-370.

Rice-Evans, C. and Burdon, R. 1993. Free-radical lipid interactions and their pathological consequences. Prog. Lipid Res. 32: 71-110.

Rice-Evans, C. A., Miller, N. J. and Paganga, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20: 933-956.

Riihimäki, L. H., Vainio, M. J., Heikura, J. M. S., Valkonen, K. H., Virtanen, V. T. and Vuorela, P. M. 2008. Binding of phenolic compounds and their derivatives to bovine and reindeer beta-lactoglobulin. J. Agric. Food Chem. 56: 7721-7729.

Ronsein, G. E., Oliveira, M. C. B., Miyamoto, S., Medeiros, M. H. G. and Di Mascio, P. 2008. Tryptophan oxidation by singlet molecular oxygen [$O_2(^1\Delta_g)$]: Mechanistic studies using ^{18}O-labeled hydroperoxides, mass spectrometry, and light emission measurements. Chem. Res. Toxicol. 21: 1271-1283.

Roskar, R., Vivoda, M. and Kmetec, V. 2008. Use of isothermal microcalorimetry for prediction of oxidative stability of several amino acids. J. Thermal Anal. Calorim. 92: 791-794.

Ruel, G., Pomerleau, S., Couture, P., Lamarche, B. and Couillard, C. 2005. Changes in plasma antioxidant capacity and oxidized low-density lipoprotein levels in men after short-term cranberry juice consumption. Metabol. Clin. Exp. 54: 856-861.

Ryynänen, M., Lampi, A. M., Salo-Väänänen, P., Ollilainen, V. and Piironen, V. 2004. A small-scale sample preparation method with HPLC analysis for determination of tocopherols and tocotrienols in cereals. J. Food Comp. Anal. 17: 749-765.

Salminen, H., Estevez, M., Kivikari, R. and Heinonen, M. 2006. Inhibition of protein and lipid oxidation by rapeseed, camelina and soy meal in cooked pork meat patties. Eur. Food Res. Technol. 223: 461-468.

Sano, A. and Nakamura, H. 1998. Chemiluminescence detection of thiols by high-performance liquid chromatography using *o*-phthalaldehyde and *N*-(4-aminobutyl)-*N*-ethylisoluminol as precolumn derivatization reagents. Anal. Sci. 14: 731-735.

Schaich, K. M. and Karel, M. 1976. Free-radical reactions of peroxidizing lipids with amino-acids and proteins - ESR Study. Lipids. 11: 392-400.

Schaich, K. M. and Rebello, C. A. 1999. Extrusion chemistry of wheat flour proteins: I. Free radical formation. Cereal Chem. 76: 748-755.

Schaich, K. M. 2008. Co-oxidation of proteins by oxidizing lipids. In: *Lipid Oxidation Pathways*. Kamal-Eldin, A.,Min, D. B., Eds., pp 181-272 AOCS Press, Urbana, IL, Vol. 2.

Schieberle, P. 1996. Odour-active compounds in moderately roasted sesame. Food Chem. 55: 145-152.

Schrocksnadel, K., Widner, B., Bergant, A., Neurauter, G., Schennach, H., Schrocksnadel, H. and Fuchs, D. 2003. Longitudinal study of tryptophan degradation during and after pregnancy. Life Sci. 72: 785-793.

Schwarcz, R. 2004. The kynurenine pathway of tryptophan degradation as a drug target. Curr. Opinion Pharmacol. 4: 12-17.

Seyoum, A., Asres, K. and El-Fiky, F. K. 2006. Structure-radical scavenging activity relationships of flavonoids. Phytochem. 67: 2058-2070.

Siebert, K. J., Carrasco, A. and Lynn, P. Y. 1996a. Formation of protein-polyphenol haze in beverages. Journal of Agricultural and Food Chemistry. 44: 1997-2005.

Siebert, K. J., Troukhanova, N. V. and Lynn, P. Y. 1996b. Nature of polyphenol-protein interactions. J. Agric. Food Chem. 44: 80-85.

Siebert, K. J. 1999. Effects of protein-polyphenol interactions on beverage haze, stabilization, and analysis. J. Agric. Food Chem. 47: 353-362.

Simat, T., Meyer, K. and Steinhart, H. 1994. Synthesis and analysis of oxidation and carbonyl condensation compounds of tryptophan. J. Chromatogr. A. 661: 93-99.

Simat, T., van Wickern, B., Eulitz, K. and Steinhart, H. 1996. Contaminants in biotechnologically manufactured -tryptophan. J. Chromatogr. B: Biomed. Sci. Appl. 685: 41-51.

Simat, T. J. and Steinhart, H. 1998. Oxidation of free tryptophan and tryptophan residues in peptides and proteins. J. Agric. Food Chem. 46: 490-498.

Singleton, V. L. and Rossi, J. A. 1965. Calorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16: 144-158.

Siriwardhana, N. and Jeon, Y. J. 2004. Antioxidative effect of cactus pear fruit (*Opuntia ficus-indica*) extract on lipid peroxidation inhibition in oils and emulsion model systems. Eur. Food Res. Technol. 219: 369-376.

Smith, S. A., Pestka, J. J., Gray, J. I. and Smith, D. M. 1999. Production and specificity of polyclonal antibodies to hexanal-lysine adducts. J. Agric. Food Chem. 47: 1389-1395.

Soares, S., Mateus, N. and De Freitas, V. 2007. Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary alpha-amylase (HSA) by fluorescence quenching. J. Agric. Food Chem. 55: 6726-6735.

Stadtman, E. R. 2006. Protein oxidation and aging. Free Radical Res. 40: 1250-1258.

Stadtman, E. R. and Levine, R. L. 2003. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids. 25: 207-218.

Steinhart, H., Vollmar, M. and Sailer, C. 1993. Pro- and antioxidative effect of ascorbic acid on L-tryptophan in the system iron(3+)/ascorbic acid/oxygen. J. Agric. Food Chem. 41: 2275-2277.

Sultana, B. and Anwar, F. 2008. Flavonols (kaempferol, quercetin, myricetin) contents of selected fruits, vegetables and medicinal plants. Food Chem. 108: 879-884.

Sun, J., Chu, Y.-F., Wu, X. and Liu, R. H. 2002. Antioxidant and Antiproliferative Activities of Common Fruits. J. Agric. Food Chem. 50: 7449-7454.

Sysak, P. K., Foote, C. S. and Ching, T.-Y. 1977. Chemistry of singlet oxygen - XXV. Photooxygenation of methionine. Photochem. Photobiol. 27: 565-569.

Szuchman-Sapir, A. J., Pattison, D. I., Ellis, N. A., Hawkins, C. L., Davies, M. J. and Witting, P. K. 2008. Hypochlorous acid oxidizes methionine and tryptophan residues in myoglobin. Free Radic. Biol. Med. 45: 789-798.

Tang, S. Z., Kerry, J. P., Sheehan, D., Buckley, D. J. and Morrissey, P. A. 2001. Antioxidative effect of dietary tea catechins on lipid oxidation of long-term frozen stored chicken meat. Meat Sci. 57: 331-336.

Tang, S. Z., Kerry, J. P., Sheehan, D. and Buckley, D. J. 2002. Antioxidative mechanisms of tea catechins in chicken meat systems. Food Chem. 76: 45-51.

Tashiro, Y., Okitani, A., Utsunomiya, N., Kaneko, S. and Kato, H. 1985. Changes in lysozyme due to interaction with vaporized hexanal. Agric. Biol. Chem. 49: 1739-1747.

Teshima, N., Nobuta, T. and Sakai, T. 2008. Simultaneous spectrophotometric determination of ascorbic acid and glutathione by kinetic-based flow injection analysis. Bunseki Kagaku. 57: 327-333.

Tomita, M., Irie, M. and Ukita, T. 1969. Sensitized photooxidation of histidine and its derivatives. Products and mechanism of the reaction. Biochem. 8: 5149-5160.

Traldi, P., Favretto, D., Seraglia, R. and Lapolla, A. 1997. Mass spectrometry in the study of non-enzymatic glyco-oxidation of proteins. Rapid Comm. Mass Spectrom. 11: 673-678.

Tsai, P. J. and She, C. H. 2006. Significance of phenol-protein interactions in modifying the antioxidant capacity of peas. J. Agric. Food Chem. 54: 8491-8494.

Tsuchiya, K., Akai, K., Tokumura, A., Abe, S., Tamaki, T., Takiguchi, Y. and Fukuzawa, K. 2005. Oxygen radicals photo-induced by ferric nitrilotriacetate complex. Biochim. Biophysic. Acta - General Subjects. 1725: 111-119.

Tuberoso, C. I. G., Kowalczyk, A., Sarritzu, E. and Cabras, P. 2007. Determination of antioxidant compounds and antioxidant activity in commercial oilseeds for food use. Food Chem. 103: 1494-1501.

Uchida, K. and Kawakishi, S. 1986. Selective oxidation of imidazole ring in histidine residues by the ascorbic acid-copper ion system. Biochem. Biophysic. Res. Comm. 138: 659-665.

Uchida, K. and Stadtman, E. R. 1993. Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase - a possible involvement of intramolecular and intermolecular cross-linking reaction. J. Biol. Chem. 268: 6388-6393.

Uchida, K. and Kawakishi, S. 1994. Identification of oxidized histidine generated at the active site of Cu,Zn-superoxide dismutase exposed to H_2O_2. Selective generation of 2-oxo-histidine at the histidine 118. J Biol. Chem. 269: 2405-2410.

Uchida, K. 2003. Histidine and lysine as targets of oxidative modification. Amino Acids. 25: 249-257.

Van Acker, S. A. B. E., Van Den Berg, D.-j., Tromp, M. N. J. L., Griffioen, D. H., Van Bennekom, W. P., Van Der Vijgh, W. J. F. and Bast, A. 1996. Structural aspects of antioxidant activity of flavonoids. Free Radical Biol. Med. 20: 331-342.

Vanhooren, A., Devreese, B., Vanhee, K., Van Beeumen, J. and Hanssens, I. 2002. Photoexcitation of tryptphan groups induces reduction of two disulfide bonds in goat alpha-lactalbumin. Biochem. 41: 11035-11043.

Vattem, D. A., Lin, Y. T., Ghaedian, R. and Shetty, K. 2005a. Cranberry synergies for dietary management of *Helicobacter pylori* infections. Process Biochem. 40: 1583-1592.

Vattem, D. A., Randhir, R. and Shetty, K. 2005b. Cranberry phenolics-mediated antioxidant enzyme response in oxidatively stressed porcine muscle. Process Biochem. 40: 2225-2238.

Vazquez, S., Parker, N. R., Sheil, M. and Truscott, R. J. W. 2004. Protein-bound kynurenine decreases with the progression of age-related nuclear cataract. Investig. Ophthalmol. Visual Sci. 45: 879-883.

Velasco, J., Dobarganes, M. C. and Marquez-Ruiz, G. 2004. Antioxidant activity of phenolic compounds in sunflower oil-in-water emulsions containing sodium caseinate and lactose. Eur. J. Lipid Sci. Technol. 106: 325-333.

Viljanen, K., Kivikari, R. and Heinonen, M. 2004a. Protein-lipid interactions during liposome oxidation with added anthocyanin and other phenolic compounds. J. Agric. Food Chem. 52: 1104-1111.

Viljanen, K., Kylli, P., Kivikari, R. and Heinonen, M. 2004b. Inhibition of protein and lipid oxidation in liposomes by berry phenolics. J. Agric. Food Chem. 52: 7419-7424.

Viljanen, K., Halmos, A. L., Sinclair, A. and Heinonen, M. 2005a. Effect of blackberry and raspberry juice on whey protein emulsion stability. Eur. Food Res. Technol. 221: 602-609.

Viljanen, K., Kylli, P., Hubbermann, E. M., Schwarz, K. and Heinonen, M. 2005b. Anthocyanin antioxidant activity and partition behavior in whey protein emulsion. J. Agric. Food Chem. 53: 2022-2027.

von Elbe, J. H. and Schwartz, S. T. 1996. Colorants. In: *Food Chemistry*. Fennema, O. R., Ed. Marcel Dekker, NY, USA.

Vuorela, S., Meyer, A. S. and Heinonen, M. 2003. Quantitative analysis of the main phenolics in rapeseed meal and oils processed differently using enzymatic hydrolysis and HPLC. Eur. Food Res. Technol. 217: 517-523.

Vuorela, S., Meyer, A. S. and Heinonen, M. 2004. Impact of isolation method on the antioxidant activity of rapeseed meal phenolics. J. Agric. Food Chem. 52: 8202-8207.

Vuorela, S., Kreander, K., Karonen, M., Nieminen, R., Hamalainen, M., Galkin, A., Laitinen, L., Salminen, J. P., Moilanen, E., Pihlaja, K., Vuorela, H., Vuorela, P. and Heinonen, M. 2005a. Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health-related effects. J. Agric. Food Chem. 53: 5922-5931.

Vuorela, S., Salminen, H., Makela, M., Kivikari, R., Karonen, M. and Heinonen, M. 2005b. Effect of plant phenolics on protein and lipid oxidation in cooked pork meat patties. J. Agric. Food Chem. 53: 8492-8497.

Wang, S. Y., Feng, R., Bowman, L., Penhallegon, R., Ding, M. and Lu, Y. 2005. Antioxidant activity in lingonberries (*Vaccinium vitis-idaea* L.) and its inhibitory effect on activator protein-1, nuclear factor-kappaB, and mitogen-activated protein kinases activation. J. Agric. Food Chem. 53: 3156-3166.

Waseem, A., Yaqoob, M. and Nabi, A. 2008. Flow-injection determination of cysteine in pharmaceuticals based on luminol-persulphate chemiluminescence detection. Luminescence. 23: 144-149.

Wernicke, C., Schott, Y., Enzensperger, C., Schulze, G., Lehmann, J. and Rommelspacher, H. 2007. Cytotoxicity of beta-carbolines in dopamine transporter expressing cells: Structure-activity relationships. Biochem. Pharmacol. 74: 1065-1077.

Widner, B., Ledochowski, M. and Fuchs, D. 2000. Interferon-gamma-induced tryptophan degradation: Neuropsychiatric and immunological consequences. Curr. Drug Metabol. 1: 193-204.

Widner, B., Laich, A., Sperner-Unterweger, B., Ledochowski, M. and Fuchs, D. 2002a. Neopterin production, tryptophan degradation, and mental depression - What is the link? Brain Behavior and Immunity. 16: 590-595.

Widner, B., Leblhuber, F. and Fuchs, D. 2002b. Increased neopterin production and tryptophan degradation in advanced Parkinson's disease. J. Neural Transmis. 109: 181-189.

Winterbourn, C. C. and Hampton, M. B. 2008. Thiol chemistry and specificity in redox signaling. Free Radic. Biol. Med. 45: 549-561.

Xiao, J., Suzuki, M., Jiang, X., Chen, X., Yamamoto, K., Ren, F. and Xu, M. 2008. Influence of B-ring hydroxylation on interactions of flavonols with bovine serum albumin. J. Agric. Food Chem. 56: 2350-2356.

Yamada, S., Funada, T., Shibata, N., Kobayashi, M., Kawai, Y., Tatsuda, E., Furuhata, A. and Uchida, K. 2004. Protein-bound 4-hydroxy-2-hexenal as a marker of oxidized *n*-3 polyunsaturated fatty acids. J. Lipid Res. 45: 626-634.

Yamaki, S., Kato, T. and Kikugawa, K. 1992. Characteristics of fluorescence formed by the reaction of proteins with unsaturated aldehydes, possible degradation products of lipid radicals. Chem. Pharm. Bull. 40: 2138-2142.

Yamakura, F. and Ikeda, K. 2006. Modification of tryptophan and tryptophan residues in proteins by reactive nitrogen species. Nitric Oxide. 14: 152-161.

Yeomans, V. C., Linseisen, J. and Wolfram, G. 2005. Interactive effects of polyphenols, tocopherol and ascorbic acid on the Cu^{2+}-mediated oxidative modification of human low density lipoproteins. Eur. J. Nutr. 44: 422-428.

Yuan, Q., Zhu, X. and Sayre, L. M. 2007. Chemical nature of stochastic generation of protein-based carbonyls: metal-catalyzed oxidation versus modification by products of lipid oxidation. Chem. Res. Toxicol. 20: 129-139.

Zamora, R. and Hidalgo, F. J. 1994. Modification of lysine amino groups by the lipid peroxidation product 4,5(*E*)-epoxy-2(*E*)-heptenal. Lipids. 24: 243-249.

Zamora, R. and Hidalgo, F. J. 1995. Linoleic-acid oxidation in the presence of amino-compounds produces pyrroles by carbonyl amine reactions. Biochim. Biophysic. Acta - Lipids Lipid Metab. 1258: 319-327.

Zamora, R., Alaiz, M. and Hidalgo, F. J. 1999a. Determination of ε-*N*-pyrrolylnorleucine in fresh food products. J. Agric. Food Chem. 47: 1942-1947.

Zamora, R., Alaiz, M. and Hidalgo, F. J. 1999b. Modification of histidine residues by 4,5-epoxy-2-alkenals. Chem. Res. Toxicol. 12: 654-660.

Zamora, R. and Hidalgo, F. J. 2001. Inhibition of proteolysis in oxidized lipid-damaged proteins. J. Agric. Food Chem. 49: 6006-6011.

Zamora, R., Navarro, J. L., Gallardo, E. and Hidalgo, F. J. 2006. Chemical conversion of alpha-amino acids into alpha-keto acids by 4,5-epoxy-2-decenal. J. Agric. Food Chem. 54: 6101-6105.

Zhao, B. and Hall, C. A. 2007. Antioxidant activity of raisin extracts in bulk oil, oil in water emulsion, and sunflower butter model systems. J. Am. Oil Chem. Soc. 84: 1137-1142.