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Oxidation of emulsified oat proteins

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Tiivistelmä - Referat - Abstract

A review of the literature emphasised the significance of protein oxidation, which can lead to such modifications as the loss of essential amino acids and protein cross-links, and may bring about adverse effects on human nutrition and protein functionality. As the globulin fraction constitutes the predominant storage protein in oats, oxidation of oat globulin is in great need of attention and understanding.

Oat globulin-containing oil-in-water emulsions were used as a food model. Thus, the objectives of the present work include the oxidation of oat proteins as well as lipid oxidation in prepared food emulsions together with a commercial oat protein-containing cream, and the possible antioxidant activities of berry phenolics (i.e. ellagitannins) towards protein oxidation. Oat globulin was extracted with a cold isolation buffer preceded by the removal of water-soluble impurities. Oxidation took place in darkness by placing the emulsion and cream samples in an oven with continuous stirring and a constant temperature at 37 ^{o}C. Sampling for lipid and protein oxidation measurements was carried out at day 0, 3 6 and 9.

During the 9-day oxidation, no hexanal was detected in any oat protein-containing samples except for the ones without oat proteins, which were measured by headspace gas chromatography. The development of protein oxidation in prepared emulsions could not be revealed by the proposed loss of tryptophan fluorescence, as the tryptophan fluorescence actually increased and then decreased in the current study as opposed to continuous decrease as indicated in references, but carbonyl and dityrosine formation reflected the progression of protein oxidation. However, the same fluorescence techniques as in prepared emulsions ended up with contradictory fluorescence results in cream samples due to syneresis of oat creams during oxidation. In conclusion, fluorescence spectroscopy is a promising technique to investigate protein oxidation in food emulsions using carbonyl and dityrosine formation as oxidation markers.

Avainsanat - Nyckelord - Keywords

Oat protein, protein oxidation, fluorescence spectroscopy, lipid oxidation, ellagitannins

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PREFACE

The thesis work was carried out at Food Chemistry Division, Department of Food and Environmental Sciences, University of Helsinki.

First of all, I would like to convey my sincere gratitude to my supervisors Ph.D researcher Göker Gürbüz and Professor Marina Heinonen. I feel deeply indebted to you since my thesis work has confronted with many unexpected technique barriers, but you supported my idea, kept patient with me and helped me overcome those difficulties. During my thesis work, other group members also gave me warm encouragement and practical guidance, including Tuuli Koivumäki, Petri Kylli, Marjo Poutanen and Bei Wang.

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Finally, special thanks to my parents Chunyan Yang and Chuanbo Xu who give me endless support, encouragement and love, which motivates me to be brave in my life.

Helsinki, May 2013

Maonian Xu

LIST OF ABBREVIATIONS

3-OH-Kyn	3-Hydroxykynurenine
5-OH-Trp	5-hydroxy-tryptophan
A	Alanine
C	Cysteine
D	Aspartic acid
DiOia A/B	Diastereomers A and B of dioxindolylalanine
DNPH	2,4-dinitrophenylhydrazine
DTNB	2-nitrobenzoic acid
DTNP	5-nitropyridine
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
HNE	4-hydroxy-(2E)-nonenal
HS-GC	Headspace gas chromatography
I	Isoleucine
K	Lysine
Kyn	Kynurenine
KynA	Kynurenic acid
L	Leucine
LC-MS	Liquid chromatography- mass spectrometry
M	Methionine
N	Asparagine
NFK	N-formylkynurenine
NTB	2-nitro-5-thiobenzoate
NTSB	2-nitro-5-thiosulfobenzoate
Oia A/B	Diastereomers A and B of oxindolylalanine
P	Proline
PIC A/B	Diastereomers A and B of 3a-hydroxypyrroloindole-2-carboxylic acid
Q	Glutamine
R	Arginine

ROS	Reactive oxygen species
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

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1 INTRODUCTION

Protein oxidation in foods involves the reactions between food proteins and reactive oxygen species, such as peroxyl radicals from lipid oxidation and hydroxyl radicals from the Fenton reaction. From a molecular level, protein oxidation may lead to amino acid side chain modifications, protein cross-linking, protein backbone fragmentation and protein conformational changes (Lund and Baron 2010).

The significance of protein oxidation in the food industry is that it may have negative effects on human nutrition, which includes the oxidative degradation of essential amino acids and reduced protein digestibility, and food quality, which may impact on food texture such as decreased water-holding capacity of oxidized meat (Lund et al. 2011), whereas protein oxidation is also exploited to tailor new food structures by promoting protein cross-linking (Buchert et al. 2010). Due to the significance of protein oxidation, more research is needed to understand how food proteins are oxidized in a different food matrix and what effects protein oxidation may have on human nutrition and food quality.

Analysis of protein oxidation can be carried out by a variety of methods, such as chemical detection by certain reagents, fluorescence spectroscopy and liquid chromatography-mass spectrometry (LC-MS). Among those alternatives, fluorescence spectroscopy is a promising technique to investigate protein oxidation because of its non-destructive, rapid and highly sensitive nature (Lakowicz 2006). With this method, the extent of protein oxidation can be monitored by measuring fluorescent protein oxidation markers, such as the loss of intrinsic tryptophan fluorescence, carbonyl formation (Estévez et al. 2008) and dityrosine formation (Giulivi and Davies 1994).

Oats have the highest lipid content and a most even distribution of lipid (not centralized in the germ) among the major cereals, which brings about a more pronounced rancidity problem due to lipid oxidation (Webster 2002). Thus, it is considered that oat proteins may be more prone to oxidation, which can be initiated by the reactions with lipid oxidation products. However, oats are a valuable protein-rich cereal with the highest protein content and nutritionally balanced amino acid profile (Peterson 2011). Interestingly, the major storage protein in oats is saline-soluble globulin instead of alcohol-soluble prolamin (e.g. wheat and corn) and acid/alkaline soluble glutelin (i.e. rice) (McMullen 2000). Based on

the aforementioned uniqueness of oat lipid and protein, studies on oxidation of oat proteins, especially oat globulins, are therefore necessary.

In the current research, emulsions were used as a vehicle to study oxidation of oat proteins. The theoretical basis was that primary oxidation products of emulsified lipid react with oat proteins, leading to protein oxidation. Therefore, probing the oxidation of oat proteins in food emulsions and antioxidant attempts constituted the core of this Master's thesis. To this end, a series of coarse oil in water emulsions (10% oil, 0.1% protein) were prepared by first mixing lecithin (as an emulsifier), purified rapeseed oil and oat protein extract and then sonicating the mixture. Antioxidants, including ellagitannins and α-tocopherol, were added to emulsion samples to investigate their possible antioxidant activities towards protein oxidation. Oat proteins were isolation from wholegrain oat flour and ellagitannins from cloudberries (*Rubus chamaemorus*). Emulsions were incubated in darkness for 9 days in an oven with a constant temperature at 37 ^{o}C. Fluorescence spectroscopy was used to evaluate the progress of protein oxidation by measuring the loss of tryptophan fluorescence and the formation of carbonyls and dityrosine. Lipid oxidation was also investigated by determining hexanal formation by a static headspace gas chromatography system (HS-GC). As a reference, a commercial oat protein-containing cream (13% oil, 1% protein) was used to compare the oxidation process with prepared emulsions. Antioxidant trials against protein oxidation were also undertaken by adding ellagitannins and α-tocopherol.

The following review of the literature considers special attributes of oat proteins with a focus on the saline-soluble oat globulin fraction and the principles of protein oxidation.

2 LITERATURE REVIEW

2.1 Oat proteins

2.1.1 Protein content, Osborne fractions and amino acid profile of oats

Dehulled oat kernels (also called oat groat) possess the highest protein contents among the major cereals, varying from 12.4 to 24.5%, and the residual proteins in the removed hulls are negligible, being below 2% in the hull. The distribution of oat proteins in an oat groat is represented in Figure 1, where oat bran is the most protein-rich constituent followed by starchy endosperm and germ (McMullen 2000).

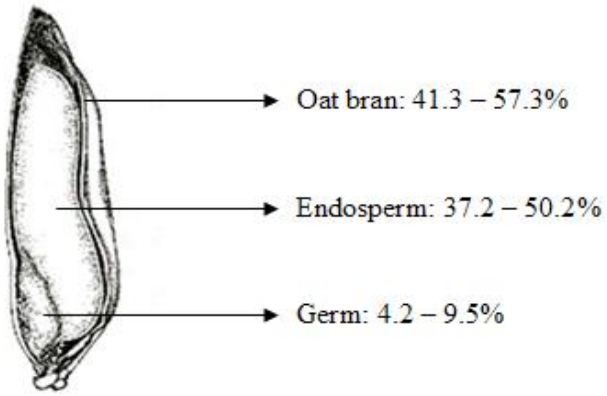


Figure 1. Protein distribution in an oat groat (*A. Sativa* L.) (Adjusted from Webster 2002).

The conventional protein separation technique of Osborne (1910) successively fractionates cereal protein into water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins and dilute alkaline or acid-soluble glutelins. The solubility and content of every fraction in oat protein is provided in Table 1. As seen, globulin is the most abundant fraction in oats accounting for 70% to 80% of total protein, followed by albumin, prolamin and glutelin. In contrast, all the other major cereals except for rice have prolamin as the dominant fraction (Webster 2002). However, an oat globulin content of 52% - 56% has also been reported (McMullen 2000). This discrepancy of globulin content mainly results from the variation between extraction methods. It is noteworthy that the commonly used salt solution extraction fails to recover all the globulin, leading to an underestimation of globulin content. This point of view is evidenced by the finding that the unextracted globulin is solubilized in sodium dodecyl sulfate-mercaptoethanol solutions and recovered from the glutelin fraction (Robert et al. 1983; Colyer and Luthe 1984).

Table 1. Osborne fractions of oat protein based on solubility (Welch 1995).

Osborne fraction	Solubility	Content (%)
Albumin	Water	15 – 20
Globulin	Dilute saline	70 – 80
Prolamin	Aqueous alcohol	5 – 10
Glutelin	Dilute alkali or acid	5 – 10

Oats have a superior amino acid profile to other cereals due to a nutritionally balanced essential amino acid content. Compared to other cereals, lysine and threonine are more abundant in oats at an averaged percentage of 4.2% and 3.3%, respectively. However, both of them are still below the FAO reference standards of 5.5% and 4% (Peterson 2010). Enrichment of such essential amino acids is, however, achieved by germination (Klose and Arendt 2012). Although oats are rich deposits of essential amino acids, non-essential amino acids like proline and glutamine contents are lower than other cereals (McMullen 2000). In terms of quantity, glutamic acid has the highest percentage (21.8%) in oat protein, followed by aspartic acid (8.9%), arginine (7%) and leucine (7%) (Draper 1973).

2.1.2 Oat globulin – the major Osborne fraction

Oat globulin is a general name representing a group of similar saline-soluble proteins, namely 3S, 7S, 12S and 18S globulins, among which the 12S globulin is the major fraction (90% of the total globulins) that is chiefly located in the endosperm (Burgess et al. 1983; Burgess and Miflin 1985). Studies on the quaternary structure of 12S globulin discovered that it is a protein hexamer containing six pairs of acidic and basic polypeptides or six subunits, and pairs are non-covalently linked to each other. Within each subunit, the acidic and the basic polypeptides are covalently linked by a disulfide bond (Shotwell et al. 1990). Features of the 12S globulin and its two constituent polypeptides are illustrated in Table 2.

Table 2. Molecular weights (MW) and isoelectric points (p*I*) of the 12S oat globulin and its two constituent polypeptides.

Protein	MW[1]	p*I*[2]
12S globulin	320,000 - 370,000	>5.5
Acidic polypeptide	35,000 - 40,000	5.5 - 6.5
Basic polypeptide	20,000 - 25,000	8 - 10

[1] MWs of proteins were obtained from Peterson (1978).
[2] p*I*s of proteins were obtained from Ma and Harwalkar (1984)

The basic polypeptides in the subunits are firmly folded in a core and surrounded by hydrophilic acidic polypeptides, and the latter are less folded but centered on proline residues (Shotwell et al. 1990). The amino acid sequence of a 12S oat globulin subunit is shown in Figure 2.

```
        10         20         30         40         50         60
MATTRFPSLL FYSCIFLLCN GSMAQLFGQS FTPWQSSRQG GLRGCKFDRL QAFEPLRQVR

        70         80         90        100        110        120
SQAGITEYFD EQNEQFRCAG VSVIRRVIEP QGLLLPQYHN APGLVYILQG RGFTGLTFPG

       130        140        150        160        170        180
CPATFQQQFQ QFDQARFAQG QSKSQNLKDE HQRVHHIKQG DVVALPAGIV HWCYNDGDAP

       190        200        210        220        230        240
IVAVYVFDVN NNANQLEPRQ KEFLLAGNNK REQQFGQNIF SGFSVQLLSE ALGISQQAAQ

       250        260        270        280        290        300
KIQSQNDQRG EIIRVSQGLQ FLKPFVSQQQ PVEHQAYQPI QSQQEQSTQY QVGQSPQYQE

       310        320        330        340        350        360
GQSTQYQSGQ SWDQSFNGLE ENFCSLEARQ NIENPKRADT YNPRAGRITH LNSKNFPTLN

       370        380        390        400        410        420
LVQMSATRVN LYQNAILSPY WNINAHSVMH MIQGRARVQV VNNHGQTVFN DILRRGQLLI

       430        440        450        460        470        480
IPQHYVVLKK AEREGCQYIS FKTTPNSMVS YIAGKTSILR ALPVDVLANA YRISRQESQN

       490        500        510
LKNNRGEEFG AFTPKFAQTG SQSYQDEGES SSTEKASE
```

Figure 2. The amino acid sequence of a 12S oat globulin subunit (Chang et al. 2011).

Similar to the secondary structures of other plant globulins, oat globulin has a small amount of α-helix (24%) but a large number of β-type structures (β-sheet 16%, β-turn/random coil 60%) without heating and pH adjustment (Ma et al. 2003). Light scattering measurements revealed the structure of the hexamer oat globulin as a cylinder-shaped molecule, built up by two trimeric rings with a height of about 8.5 nm, and the diameter of each ring is around 11.8 nm, as shown in Figure 3 (Zhao et al. 2004).

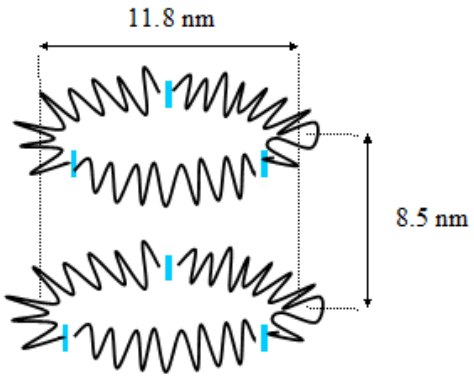


Figure 3. A proposed motif of an oat globulin hexamer according to Zhao et al. (2004).

The poor aqueous solubility of oat globulin proposes a major problem in the processing of liquid-based oat products. Loponen et al. (2007) pointed out that the solubilization of oat globulins requires a relatively high salt concentration or extreme pH conditions (Figure 4). Such low solubility can be explained by their unique amino acid sequence that glutamine-rich repeats of eight amino acids, having a neutral isoelectric point, are located near the C-terminus of the acidic polypeptide (Shotwell et al. 1988). Another unique attribute of oat globulins is that they have the highest thermal denaturation temperature among the major cereal storage proteins due to the rather compact quaternary structure of oat globulin (Marcone et al. 1998).

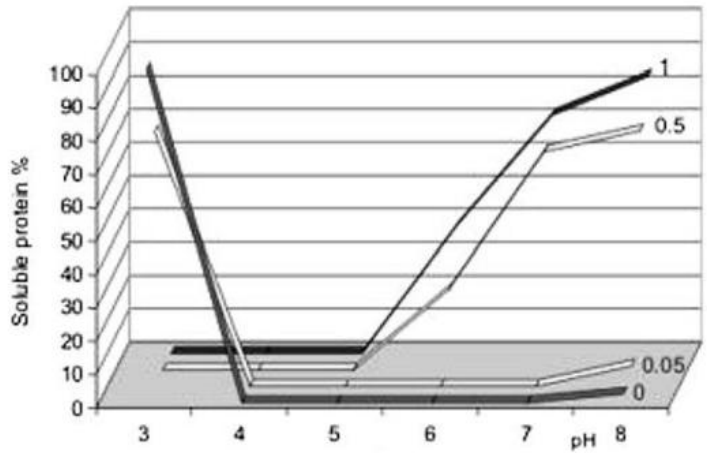


Figure 4. A diagram of oat globulin solubility dependent on pH and NaCl concentration (shown at the right end of the curves as mol/L) (Loponen et al. 2007).

2.2 Protein oxidation

Protein oxidation is the modification of protein by reactive oxygen species (ROS). Commonly encountered non-radical species involve hydrogen peroxide, lipid oxidation productions or even sugar oxidation products. ROS may attack amino acid side chains and/or protein backbones, leading to various amino acid side chain modifications, protein conformational changes, fragmentation and so forth (Lund and Baron 2010).

2.2.1 ROS—protein oxidation initiator

The oxidative modifications of food proteins by reactive oxygen species (ROS) is implicated in the loss of food quality (Lund et al. 2010). Examples of ROS in foods are hydroxyl radicals, singlet oxygen, peroxyl and alkoxyl radicles, which are formed from metal-catalyzed oxidation, irradiation and lipid oxidation, and they can be present as free

radical or non-radical species (Lee et al. 2004). In particular, hydroxyl radical (•OH) is an exceedingly reactive radical with the highest one-electron reduction potential among free radicals (Korycka-Dahl and Richardson 1978), and it can be generated through Fenton reaction, where metal cations participate in their lower valence (M^{n+}) (Haber and Weiss 1932). The reaction is shown below:

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + \bullet OH + OH^-.$$

For oil in water (o/w) food emulsions, protein oxidation may be initiated by lipid oxidation products, particularly peroxy radicals (ROO•) and alkoxyl radicals (RO•), through protein-lipid interactions (Viljanen et al. 2004). Those radicals can readily abstract hydrogen atoms from protein molecules (PH), producing protein radicals (P•). Then a series of reactions may happen. For example, protein cross-linking (P-P) can be formed by the reaction between two protein radicals (Buchert et al. 2010). The reactions are:

$$RO\bullet \,/\, ROO\bullet + PH = ROH$$
$$ROOH + P\bullet \rightarrow P\bullet + P\bullet = P\text{-}P$$

2.2.2 Amino acid side-chain modifications

Lund and Baron (2010) have given a comprehensive review about amino acid side chain modifications, which are classified into three groups including sulfur-containing, aromatic and aliphatic (without sulfur) amino acid side-chain modifications. In this section, special emphasis comes to the oxidation of tryptophan and protein carbonylation, as they are analyzed in the thesis and used as markers in protein oxidation.

The oxidation of tryptophan starts from pyrrole of the indole nucleus followed by the phenyl moiety when hydrogen peroxide and lipid oxidation products are used as oxidative agents (Simat and Steinhart 1999). The authors indicated the reason that the detected tryptophan (Trp) oxidation products are mainly derived from the oxidative modification of the pyrrole ring. The formation of Trp-derived products is shown in Figure 5. If the pyrrole ring is not cleaved during oxidation of tryptophan, the resulting compounds could be the diastereomers A and B of oxindolylalanine (Oia A/B), dioxindolylalanine (DiOia A/B) and 3-hydroxypyrroloindole-2-carboxylic acid (PIC A/B) (Salminen and Heinonen 2008). When tryptophan reacts with ROS such as singlet oxygen and ozone, the pyrrole ring is

broken and further degraded, producing N-formylkynurenine (NFK) and kynurenine (Kyn) (Ehrenshaft et al. 2009). Further degradation of Kyn generates kynurenic acid (KynA) and 3-hydroxykynurenine (3-OH-Kyn) (Simat and Steinhart 1999; Schwarcz 2004). Hydroxylation of the phenyl ring gives rise to 5-hydroxy-tryptophan (5-OH-Trp) (Salminen and Heinonen 2008).

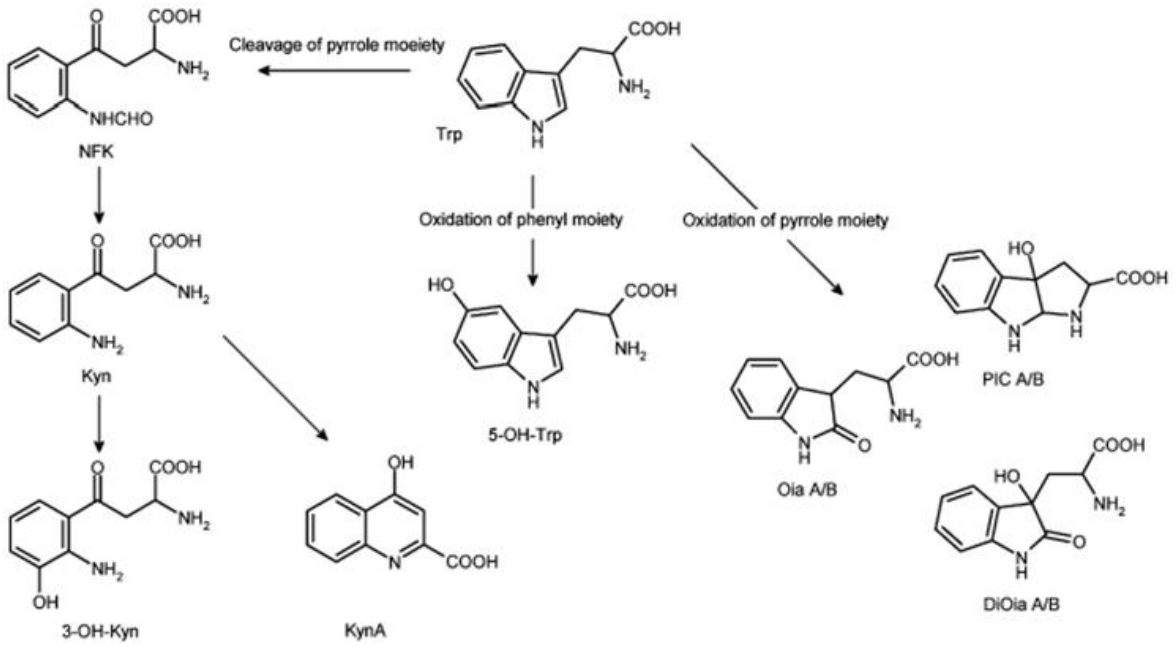


Figure 5. Oxidation of tryptophan (Trp: tryptophan; Oia A/B: diastereomers A and B of oxindolylalanine; DiOia A/B: diastereomers A and B of dioxindolylalanine; PIC A/B: diastereomers A and B of 3-hydroxypyrroloindole-2-carboxylic acid; NFK: N-formylkynurenine; Kyn: kynurenine; KynA: kynurenic acid; 3-OH-Kyn: 3-hydroxykynurenine; 5-OH-Trp: 5-hydroxy-tryptophan (Modified from Salminen and Heinonen 2008).

Estévez (2011) summarizes four mechanisms for protein carbonyl formation, namely direct side chain carbonylation, glycation, protein backbone cleavage and bonding of protein molecules to carbonyl compounds. Among the four alternatives, direct side chain carbonylation is regarded as the main pathway, while the others are likely to happen in a rather complex food matrix. In this pathway, a Fenton reaction is implicated with the direct side chain carbonylation which generates a hydroxyl radical (Figure 6), which abstracts a hydrogen atom from a protein molecule with a protein radical formed. After the deamination of the amino acid residue, a carbonyl group is produced (Xiong and Decker 1995). The susceptible amino acids to this pathway are lysine, threonine, arginine and proline (Stadtman and Levine 2003). Since proteins are able to chelate metals, hydroxyl radicals may have specific attacking sites on the target protein molecules (Stadtman and Levine 2003).

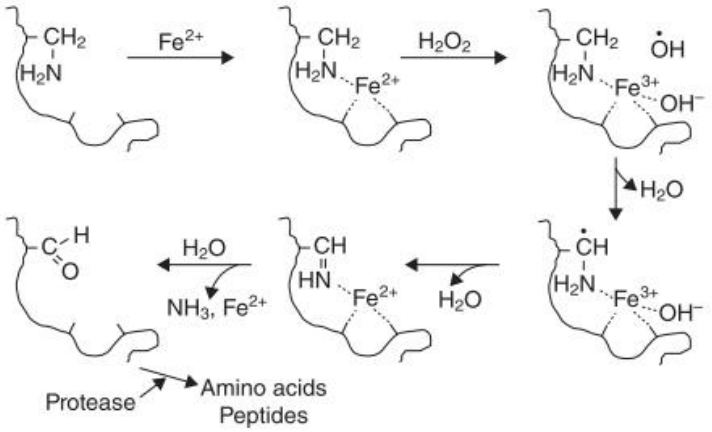


Figure 6. Metal-catalyzed protein carbonylation (Lund and Baron 2010).

In protein-stabilized oil in water emulsions as well as cells, protein carbonylation can result from the Michael addition reactions between lipid oxidation products, namely α, β-unsaturated aldehydes, and amino acid side chains of lysine, histidine, and cysteine (Viljanen et al. 2005; Stadtman 2006; Grimsrud et al. 2008). The mechanism of Michael addition-derived protein carbonylation is shown in Figure 7 by taking the example of reactions between 4-hydroxy-(2E)-nonenal (HNE) and lysyl residue on a protein molecule. Due to the electrophilic nature of the α-carbon, nucleophilic amino group of the lysyl residue directly adds to the α-carbon (Bruice 2011). Strictly speaking, protein carbonylation via this mechanism is not protein oxidation, but a direct addition of a carbonyl compound like HNE to the protein molecule (Lund and Baron 2010).

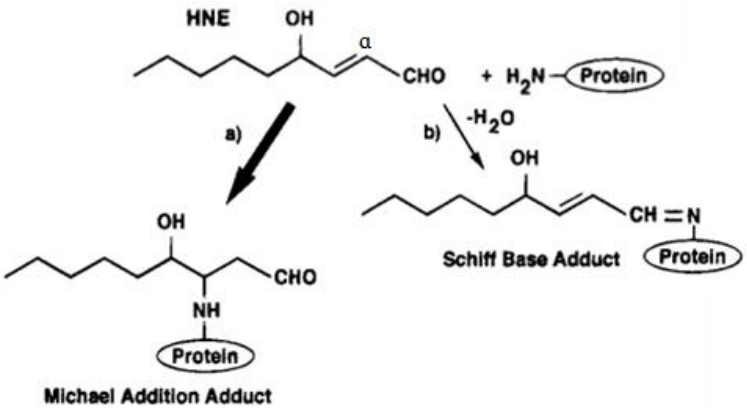


Figure 7. The reactions of HNE with the lysyl residue on a protein molecule via: a) Michael addition; b) Schiff base formation (Bruenner et al. 1995).

Bruenner et al. (1994) incubated β-lactoglobulin B with HNE, and they found that about 99% protein modifications are due to Michael addition. Together with Michael addition to the lysyl residue, HNE can also react with the lysyl residue forming an imine with a

nitrogen carbon double bond (Schiff base), but this is a minor reaction (Bruenner et al. 1995).

2.2.3 Protein cross-linking

Protein cross-links are of great importance to tailor food structure and influence protein functionality (Buchert et al. 2010). Among a variety of protein cross-links, disulfide bonds and dityrosine are most frequently encountered ones, and they are essential in cereal products, especially breads.

Disulfide bonds

Disulfide bonds can be formed by oxidizing thiol groups (-SH) as shown in Figure 8. The sulfur-hydrogen bond (S-H) in the thiol group is relatively weak, and thus the thiol group is more acidic than water. It is the most reactive amino acid residue among the 20 naturally occurring amino acids. A thiol group is readily deprotonated by oxidizing agents such as hydroperoxides, and two deprotonated thiol groups react to form a disulfide bond (Whitford 2005; Vollhardt and Schore 2011). The formation of disulfide bonds does not require the adjacence of two amino acid residue, and both intra- and inter-molecular disulfide bonds can be formed (Bruice 2011).

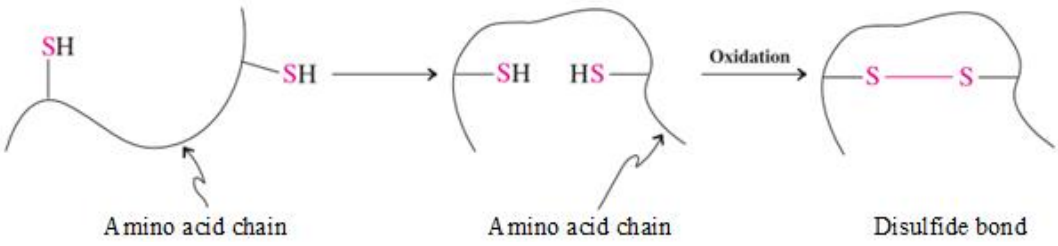


Figure 8. Formation of a disulfide bond (Vollhardt and Schore 2011).

Dityrosine cross-link

Giulivi et al. (2003) suggested that dityrosine be used as a specific marker for protein oxidation because it is the only stable oxidation product resistant to high pH, oxygen and enzymatic hydrolysis. The mechanism of dityrosine formation was elucidated by Giulivi and Davies (1994), which involves a three-step reaction: the generation of a tyrosyl radical, radical isomerization followed by diradical reaction and enolization (Figure 9). Dalsgaard

et al. (2011) argued that the generation of dityrosine prefers the proximity of two tyrosine residues.

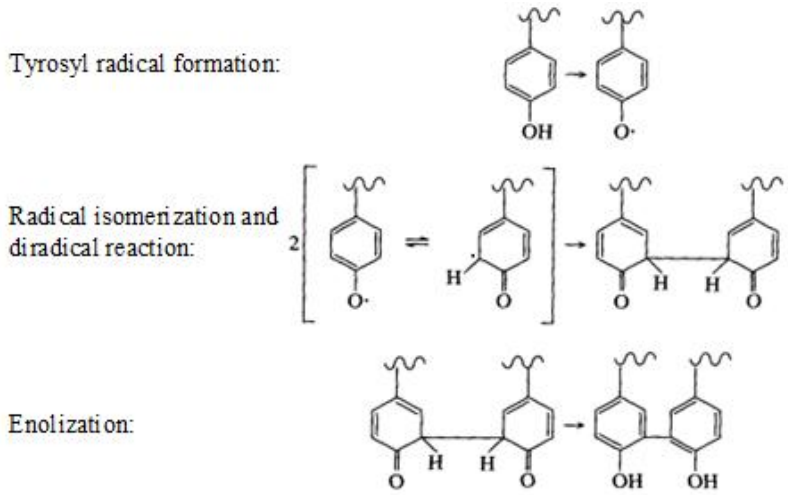


Figure 9. The mechanism of dityrosine formation (Giulivi and Davies 1994).

2.2.4 Protein backbone fragmentation and conformational rearrangements

A mechanism of protein backbone oxidation is proposed by Lund and Baron (2010). One hydrogen atom is abstracted from the α-carbon of an aliphatic amino acid generating an α-carbon-centered protein radical, which further reacts with oxygen and generate protein peroxyl radicals that undergo backbone fragmentation. Protein cross-linking can be formed by dimerization of two α-carbon-centered protein radicals. They also suggest that conformational rearrangements of protein molecules can be achieved by radical transfer from one polypeptide to another, but the exact mechanism remain unclear.

2.2.5 Analysis of protein oxidation

Common measurements in protein oxidation are illustrated in this section, including tryptophan fluorescence, analysis of protein carbonyls and protein cross-links (Estévez et al. 2008; Armenteros et al. 2009; Koivumaki et al. 2012). Special emphasis is given to the fluorescent properties of the intact molecules (i.e. tryptophan) and protein oxidation products (i.e. carbonyls and dityrosine), because in the current research, all protein oxidation measurements are conducted by fluorescence spectroscopy.

<u>Analysis of tryptophan oxidation</u>

Oxidative decomposition of tryptophan is regarded to be the primary stage during protein oxidation in oil-in-water emulsions (Estévez et al. 2008). It can be monitored by the loss of tryptophan fluorescence as well as the gaining of fluorescence from its oxidation products, such as N-formylkynurenine. The emission wavelength of tryptophan is in the range of 330-370 nm upon excitation at 280 nm. The loss of tryptophan fluorescence may result from the oxidative degradation of tryptophan and the formation of its radicals. Additionally, the shift of its maximum emission wavelength is indicative of the relative position of the tryptophan residues in the protein (Ladokhin et al. 2000). As a typical tryptophan oxidation product, N-formylkynurenine can be detected by fluorescence spectroscopy, which emits at 400 nm when excited at 330 nm (Dalsgaard et al. 2007).

Analysis of protein carbonyls

Protein carbonyl formation is believed to occur later than the loss of tryptophan fluorescence (Estévez et al. 2008). There are three well-recognized methods for the analysis of protein carbonyls in foods: fluorescence spectroscopy, the DNPH (2,4-dinitrophenylhydrazine) assay and LC-MS.

Carbonyl compounds can be detected by fluorescence spectrometry. It is reported that the carbonyls, which are produced from the reaction between lipid oxidation products and protein amino groups, are conjugated fluorophores with the emission wavelength of about 420 nm when excited at 350 nm (Viljanen et al. 2004).

Carbonyl compounds can react with DNPH to form a hydrazone (Figure 10) that can be detected by a spectrophotometer at 370 nm (Johnson 1953). The problem with this method is that DNPH does not only react with carbonyl groups, but also lipid oxidation products which may lead to an overestimation, if the protein material is not isolated prior to analysis. In addition, DNPH is practically added in excess in order to ensure a comprehensive derivatization of all carbonyls. However, the left DNPH has similar absorption to hydrazone and thus interfere the result, so the unbound DNPH is usually washed out before the quantification of carbonyl groups (Lund and Baron 2010).

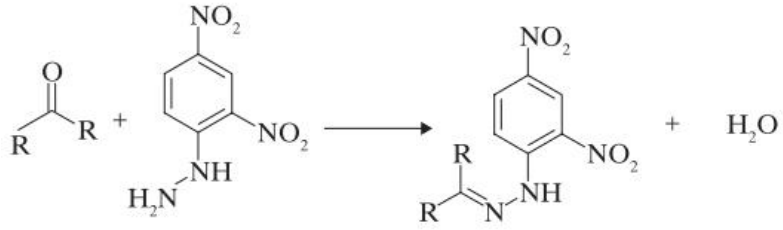


Figure 10. Reaction of DNPH with a carbonyl compound (Lund and Baron 2010).

However, the aforementioned two methods for carbonyl detection share the same limitation that they aim to measure the total carbonyl compounds instead of the carbonyl products produced during protein oxidation or even during the oxidation of specific amino acids. If the natural carbonyls in non-oxidized protein samples are taken into account, the results is rather an overestimation. To this end, a specific analytical method has been developed to detect the carbonyls from the oxidation of arginine and lysine in meat, which is based on LC-MS (Estévez et al. 2009).

Analysis of protein thiol groups

The analysis of protein thiol groups can be performed by derivatization with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), which is called Ellman's reagent. The reaction produces a coloured thiolate ion complex (Figure 11), which can be detected by a spectrophotometric measurement at 412 nm with an extinction coefficient of 13600 $mol^{-1}cm^{-1}$(Ellman 1959). Notably, the reaction is stringently pH-dependent. The effects of interfering chromophores are reduced by the use of 2,2'-dithiobis (5-nitropyridine) (DTNP) (Winterbourn 1990).

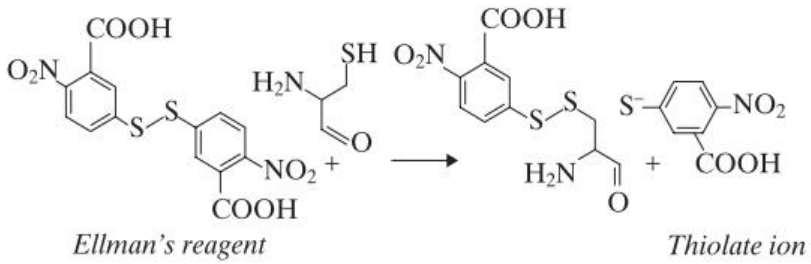


Figure 11. Detection of protein thiol groups by the DTNB assay (Lund and Baron 2010).

Analysis of disulfide bonds

Protein cross-links are formed through the covalent bonds between intramolecular or intermolecular amino acids. Apart from a large variety of enzyme-catalyzed protein cross-

links, disulfide bonds and dityrosine formation are the most characteristic ones. Disulfide bonds can be quantified through the reaction of 2-nitro-5-thiosulfobenzoate (NTSB) with disulfide bonds in the presence of excess sodium sulfite, which measures the reaction product 2-nitro-5-thiobenzoate (NTB) in darkness (Damodaran 1985). It is noteworthy that free thiol groups also participate in the reaction and contribute to the results, and thus they should be quantified earlier. Besides chemical reagents, Lutz et al. (2011) developed a liquid chromatography-tandem mass spectrometry technique to detect the disulfide bonds in wheat gluten.

Analysis of dityrosine

The fluorescent property of dityrosine facilitates its analysis due to its intense fluorescence emission around the 400-420 nm range and excites within either 315 nm (alkaline solution) or 284 nm (acidic solutions) absorption bands (Giulivi and Davies 1994; Malencik and Anderson 2003). Therefore, the formation of dityrosine is usually detected by a fluorometer. However, accurate quantitation of dityrosine is still hindered by the lack of commercially available standard compounds. By a peroxidase-catalyzed tyrosine cross-linking method (Huggins et al. 1996), dityrosine is synthesized and generally used as standard compounds. Later studies adopted this approach to obtain dityrosine standard and quantified dityrosine formation by various methods, such as gas chromatography or liquid chromatography coupled with mass spectrometry (Wells-Knecht et al. 1993; Hanft and Koehler 2005), and liquid chromatography coupled with a fluoremeter (Rodriguez-Mateos et al. 2006). Practically, the latter is widely used with simplification in data handling.

2.3 Investigations on oxidation of oat proteins

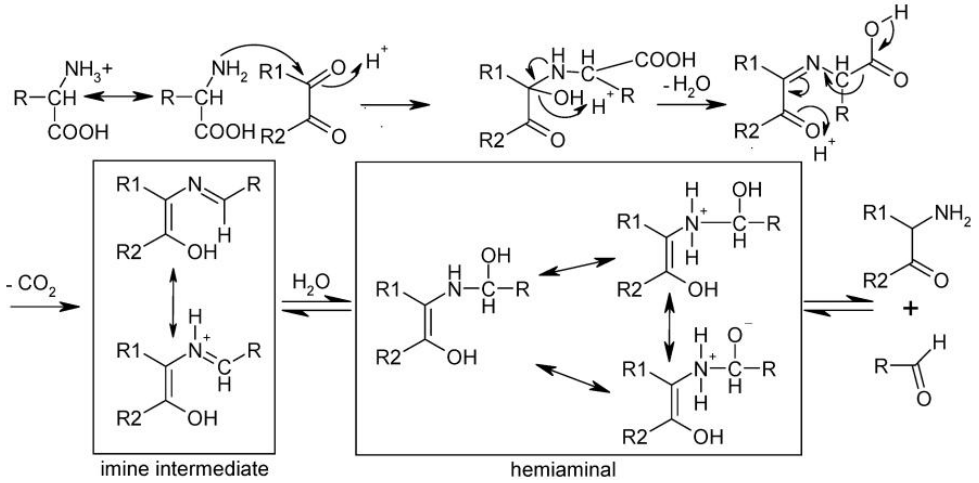
2.3.1 Thermal aggregation of oat globulin

Thermal aggregation of protein relates the application of protein as gelling agents. Most globular proteins are readily aggregated when heated. As in the case of oat globulin, it was found that the hexamer initially dissociates into subunits and then they associate into aggregates. In the formation of soluble aggregates, disulfide bonds play an important role, because when free sulfhydryl groups are blocked, formation of soluble aggregates is hindered (Ma and Harwalkar 1987). The heat-induced buffer-insoluble aggregates of oat globulin were studied by Raman spectrometry. The peak intensity of the disulfide bond

increases, while the tyrosine doublet band intensity decreases, suggesting that the tyrosine residues be buried or act as hydrogen bond donors, but the exact change of tyrosine residues remained unknown. Interestingly, the peak intensity of tryptophan is increased, suggesting that the tryptophan residues are buried upon heating (Ma et al. 2003).

2.3.2 Strecker degradation of α-amino acids during aging of oat beer

The level of oxidative indicators, 2-methyl-butanal and 3-methyl-butanal, slightly increased during aging of oat beer, but this level is considerably lower than those in barley beer due to the higher content of antioxidants in oat beer (Klose et al. 2011). The formation of such aldehydes involves a Strecker degradation mechanism which converts □-amino acids into aldehydes (Alexander and Radwan 1952), as shown in Figure 12.



R-	Amino acid	Aldehyde
$-CHCH_2CH_3$ (CH3 branch)	Isoleucine	2-methyl-butanal
$-CH_2CHCH_3$ (CH3 branch)	Leucine	3-methyl-butanal

Figure 12. Strecker degradation and aldehyde formation during beer aging (Schutter et al. 2008).

During brewing, α-amino acids in sweet wort react with α-dicarbonyl compounds that originate from Maillard reaction during wort boiling. This reaction takes place by a nucleophilic addition -elimination manner: first, the nucleophilic nitrogen atom on the amino group adds to the carbonyl carbon; second, the hydroxyl group on the carbonyl carbon gets protonated and a water molecule is eliminated, producing a imine intermediate with a nitrogen-carbon double bond (Bruice 2011). After the subsequent formation of

hemiaminal intermediate, aldehydes like 2-methyl-butanal and 3-methyl-butanal are released (Schutter et al. 2008). When isoleucine participates as an original reactant, 2-methyl-butanal is expected, while 3-methyl-butanal could be generated if leucine joins the reactions (Estévez et al. 2010).

2.3.3 Oat protein oxidation in bread-making

The limitations of oat flour to make bread are that oat flour lack gluten proteins, and that it has high content of dietary fiber (Renzetti et al. 2010). To this end, various oxidative enzymes, such as glucose oxidase, tyrosinase and laccase, have been used to strengthen the dough by promoting the intermolecular cross-linking of oat proteins through dityrosine, isodityrosine (Figure 13) or disulfide bonds (Renzetti et al. 2010; Flander et al. 2011). Among the potent enzymes, glucose oxidase is able to catalyze the oxidation of glucose and produce hydrogen peroxide, while tyrosinase and laccase specifically catalyze the cross-linking of biopolymers via their phenolic moieties (Buchert et al. 2010).

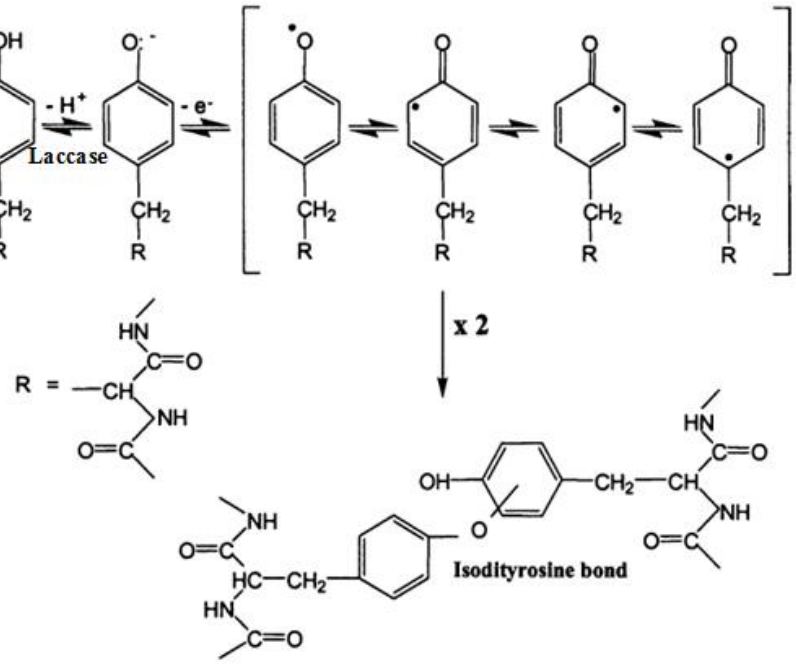


Figure 13. Laccase-catalyzed isodityrosine bond formation (Mattinen et al. 2005).

However, the research by Renzetti et al. (2010) shows that the application of glucose oxidase and laccase in oat bread-making leads to considerable protein aggregations, further causing undesirable texture due to the lack of soluble proteins to sustain the gas bubbles during bread-making. Instead, proteases are shown to be potent enzymes in oat bread-making, which hydrolyze oat proteins, produce a sufficient amount of soluble proteins, and

unfold the protein structure. Thus good textural quality is achieved, but severe protein oxidation is supposed to happen, in terms of the unfolding of oat proteins to expose more residues and observed protein polymerization. Another study suggests tyrosinase together with xylanase is beneficial to produce gluten-free oat bread with desirable texture (Flander et al. 2011). Apart from enzyme-catalyzed crosslinking to improve protein network, a non-enzymatic technique called "hydrostatic pressure" has recently come into use (Huttner et al. 2009). In this study, oat flour is mixed with water with a ratio of 1: 0.95 to produce a oat batter, which is then subject to hydrostatic pressure treatment. When the pressures are higher than 300 MPa, which are high enough to promote the deprotonation of thiol groups, disulfide bonds are formed.

2.3.4 Oxidative degradation of essential amino acids in oat flake processing

Total methionine and tryptophan contents in oat flakes were determined after processing, which were compared to those prior to processing (Horvatic and Vedrina-Dragojevic 2000). Results showed that the decrease of methionine after processing was about 13% and tryptophan about 10%, but the loss of both amino acids was the lowest among the tested cereal flakes including rye, wheat, oat and barley. In addition, lipid oxidation products were also measured during processing, which were significantly correlated to the oxidative degradation of the aforementioned two amino acids. Relevant studies also suggest that the high susceptibility of the amino acids to oxidative degradation is associated with lipid oxidation products, especially lipid peroxides (Cuq et al. 1983; Strange 1984).

2.3.5 Possible protein oxidation pathways in an oat-based beverage

In oat-based beverages like Oatly milk (Oatly AB, Landskrona, Sweden), protein oxidation is supposed to first occur in the continuous phase. Zhang et al. (2007) monitored the nutritional changes of an oat-based beverage from production to a long-term storage (64 weeks). Fatty acid profile was almost unchanged, and only a 9% loss of linolenic acid was observed in the presence of iron for one year. Efficient inhibition of lipid oxidation wa due to natural content of vitamin E in the lipid and the phenolic compounds in oats as potent antioxidants. Because of the limited lipid oxidation, oat proteins located in the emulsion interface are supposed to be slightly oxidized. However, the continuous phase in the research contained elevated level of dissolved oxygen, although it remained in low concentration of 0.71 mg/L after 64 weeks and the rate was gradually reduced. Based on

the observations, the authors raised the idea that a balance between oxygen consumption and oxygen uptake was slowly developed. Thus it is rational to infer that the compounds in the continuous phase, including soluble oat proteins, scavenge the oxygen and undergo oxidation. The protective role of soluble protein in continuous phase towards lipid oxidation is observed, where proteins oxidize before lipid oxidation (Salminen et al. 2010).

3 EXPERIMENTAL RESEARCH

3.1 Aim

The current study aimed to investigate the oxidation of oat proteins (especially saline-soluble oat globulins) as well as lipid oxidation using rapeseed oil in water emulsions as a vehicle, and the possible inhibition of oat protein oxidation by special phenolic compounds (i.e. ellagitannins). The latter was an attempt to mimic a real food: oat milk with cloudberry as a flavour.

3.2 Materials and methods

3.2.1 Materials

Wholegrain oat flour (Provena, Raisio, Finland), oat protein-containing cream (Oatly AB, Landskrona, Sweden), rapeseed oil (Bunge Finland Oy, Raisio, Finland) and cloudberry (*Rubus chamaemorus*) were purchased from local stores and marketplace. Whole grain oat flour is produced from pure oat and labeled as gluten-free with a 14% protein. Oat protein-containing cream contains 1% protein and 13% fat. It also contains rapeseed oil and palm oil, rapeseed lecithin as an emulsifier and both of xanthan gum and sea salts as stabilizers. Bovine serum albumin (BSA) and Amberlite XAD-7 nonionic polymeric adsorbent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex LH-20 adsorbent was provided by Pharmacia (Uppsala, Sweden). Sodium hydroxide (NaOH) in solid pellets, sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$), disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$), sodium chloride (NaCl), sodium tartrate dihydrate ($C_4H_4Na_2O_6 \cdot H_2O$), copper sulfate ($CuSO_4$), formic acid (HCOOH), sodium carbonate (Na_2CO_3), Folin-Ciocalteu's phenol reagent and aluminium oxide (Al_2O_3) powder for column chromatography were bought from Merck (Darmstadt, Germany). All solvents were of HPLC grade, among which heptane was supplied by J.T Baker (Deventer, Holland), acetonitrile was got from Sigma (Steinheim, Germany), hexanal was from Merck (Munich, Germany), and methanol and acetone were obtained from Rathburn Chemicals (Walkerburn, Scotland). Water was purified by a Milli-Q system (Millipore Corp., Bedford, MA, USA). Emultop (partially hydrolyzed soybean lecithin) was received as a gift from Cargill (Hamburg, Germany).

3.2.2 Isolation of ellagitannins

The isolation of ellagitannins refers to the work by Kähkönen et al. (2012), as outlined in Figure 14. Lyophilized berries were first ground into powder by a blender before solvent extraction. An accelerated solvent extractor was used to extract using 70% aqueous acetone in order to obtain a raw extract. Then the raw extract was filtered and purified by two consecutive reversed-phase column chromatography (i.e. Amberlite XAD-7 and Sephadex LH20) to remove polar impurities such as organic acids, sugars and flavonoids. Hence, an ellagitannin-rich extract was obtained. Ellagitannin dimers and trimers were further separated by a preparative HPLC system. The dimeric ellagitannin fraction, sanguiin H-6, was collected and used as a potential antioxidant in the present research.

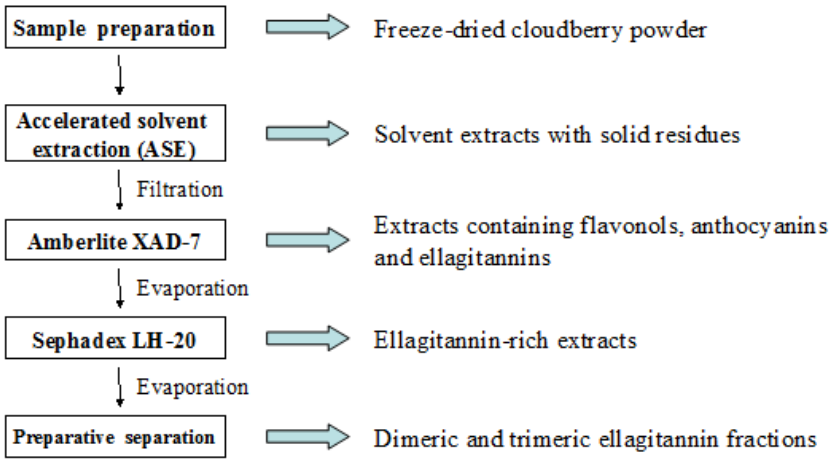


Figure 14. An outline of isolation of ellagitannins (Kähkönen et al. 2012).

Quantitation of sanguiin H-6 in methanol was made using a UV-spectrophotometer (Perkin-Elmer, Buckinghamshire, England). The concentration was determined by Beer-Lambert Law: $A = \varepsilon dc$, where A is absorbance, d is the path length in centimeter (d = 1cm), c is the molar concentration (mol/L), and ε is the molar extinction coefficient of sanguiin H-6 in methanol (ε_{260nm} = 72070 $M^{-1}com^{-1}$) (Gasperotti et al. 2010). By taking into account of the molar mass of sanguiin H-6 as 1870.1540 g/mol (Gasperotti et al. 2010), the concentration of sanguiin H-6 in methanol was calculated as 254.3 mg/L.

3.2.3 Extraction of oat protein

Oat globulins were extracted from wholegrain oat flour according to the procedure used by Chang et al. (2011) with modifications (Figure 15). A sample (8 g) of wholegrain oat flour

was mixed with MQ-water (40 mL). The mixture was stirred until the oat flour was fully hydrated, and then the slurry was allowed to rest in an ice bath for 30 min. After centrifugation (4000 rpm, 20 min, 4°C), the supernatant was discarded and the residue was washed with 40 mL Milli-Q-water twice. Then the residue was mixed with 15 mL cold isolation buffer (10 mM sodium phosphate, 0.5 M sodium chloride, pH 7.2). Before the final centrifugation, the mixture was homogenized for 30 s and let rest for 30 min. The supernatant was filtered and collected as oat protein extract, which was stored in a 50 mL Sarstedt tube at 4°C before the preparation of oil-in-water emulsions.

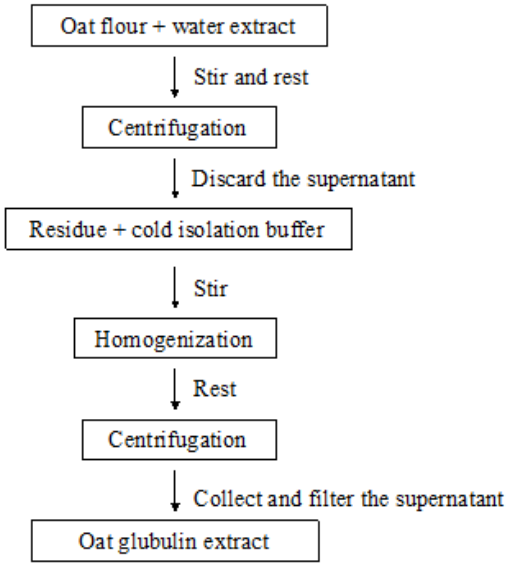


Figure 15. The procedure for oat globulin extraction.

The protein concentration of the oat extract was measured by the Lowry method (Lowry et al. 1951) using BSA as a standard. Quantitation of proteins required an alkaline Cu-reagent solution, which was prepared by mixing three solutions: 4% Na-tartrate solution, 2% $CuSO_4$ solution and 10% Na_2CO_3 in 0.5 M NaOH solution. Folin-Ciocalteu's phenol reagent (5 mL) was diluted with 50 mL Milli-Q water. Those two solutions were prepared right before use. BSA standard solutions were prepared with four concentrations: 0.05, 0.10, 0.20 and 0.40 mg/mL. An aliquot of oat protein extract (1 mL) was pipetted into a test tube and diluted with 9 mL Milli-Q water, and then the diluted oat protein solution was mixed. Diluted protein solution was pipetted into two replicate test tubes, and each contained 1 mL. Each of the standard solutions (1 mL) was pipetted into test tubes, while a reagent blank test tube contained 1 mL Milli-Q water, so there were in total 7 test tubes: 4 for standard solutions, two replicate protein solutions and 1 blank. Freshly made alkaline Cu-reagent solution (1 mL) was added to each tube. Then the tubes were mixed and let

stand for 10 min at room temperature. Afterwards, 3 mL of diluted Folin-Ciocalteu's phenol reagent solution was added into each tube. The tubes were also mixed and incubated in a 50^{o}C water bath for 10 min. When the tubes cooled down to room temperature, their absorbances were measured by a UV/Vis-spectrophotometer (Perkin-Elmer, Buckinghamshire, England) at 540 nm. The reagent blank was used for autozero of the spectrophotometer. The protein concentration in the extract was 14.5 mg/mL.

3.2.4 Purification of rapeseed oil

Rapeseed oil (100 g) was purified using open column chromatography (diameter 29 mm, length 510 mm) packed with 180 g Al_2O_3 powder, in order to remove polar pro-oxidants and antioxidants (Lampi et al. 1999). The Al_2O_3 powders were heated (100^{o}C for 16 h and 200^{o}C for 8 h) before use. Heptane was used as mobile phase and the separation was accelerated by a suction system at room temperature. Purified rapeseed oil dissolved in heptane was preserved in a glass bottle covered with aluminium foil, and it is stored in a freezer at -20^{o}C before use. The rapeseed oil concentration was determined by pipetting 5 mL purified oil solution into 2 replicate beakers with known weights. Beakers with oil solutions were put under nitrogen gas to evaporate heptane. Added weights were purified oil. Oil concentration was calculated as 0.306 g/mL according to the equation below:

$$\text{Oil concentration (g/mL)} = \frac{\text{Weight of beaker and oil (g)} - \text{Weight of empty beaker (g)}}{5\,\text{mL}}$$

Before the preparation of emulsions, purified rapeseed oil was checked if it was free of tocopherols with a HPLC method (Haila and Heinonen 1994). The HPLC system was composed of a Waters 515 pump (Waters Corporation, Milford, MA, USA), an autosampler, a silica guard column (4×3.0 mm, Phenomenex), an Inertsil 5 SI column (300×3.9 mm, 5 μm particle size, Varian Inc., Pal Alto, CA, USA) and a Waters 2475 scanning fluorescence detector. This system was controlled by the Empower 2 program. Before injection, purified rapeseed oil in heptane solution (50 mg/10 mL) was filtered (Acrodisc GHP, 0.45 Pm, 13 mm) directly into a HPLC vial. Oil samples were analyzed in duplicate (25 μl injections) with a palm oil extract used as a standard to identify tocopherols. Analytes were isocratically eluted within 20 min using a mobile phase containing 3 % dioxane and 97% heptane mixture with a flow rate of 2 mL/min. The excitation wavelength (λ_{ex}) of the fluorescence detector was 292 nm and the emission

wavelength (λ_{em}) was 325 nm. Column temperature was kept at 30°C and the autosampler tray was cooled to 4°C. Results showed that the purified oil was free of tocopherols.

3.2.5 Emulsion preparation

A series of oil-in-water emulsions (10% w/w) were prepared in oxidation studies. This series consisted of five groups of emulsions including oil control group (OC) containing 10% oil, protein control group (PC) containing 0.1% oat proteins or 1 mg oat proteins per 1 mL emulsion, tocopherol group with α-tocopherol concentration at 50 µg/g oil (PT 50), ellagitannin groups with ellagitannin concentrations at 25 µg/g oil and 50 µg/g oil (PE 25 and PE 50). The information of each group in the emulsion series is shown in Table 3.

Table 3. Emulsion group in the emulsion series.

Group	Concentration of oat protein (mg/mL emulsion)	Concentration of the antioxidant (µg/g oil)	Oil content (%)
OC	0	0	10
PC	1 (0.1%)	0	10
PT50	1 (0.1%)	50 (α-tocopherol)	10
PE25	1 (0.1%)	25 (Ellagitannin)	10
PE50	1 (0.1%)	50 (Ellagitannin)	10

Figure 16 shows the emulsion preparation procedure for the PC group. Lecithin powders were sonicated within Milli-Q-water for 3 min to obtain a 4% emulsifier solution. Purified rapeseed oil in heptane solution was evaporated under nitrogen gas to remove heptane, which was mixed with emulsifier solution and oat protein extract (in cold isolation buffer containing10 mM sodium phosphate, 0.5 M sodium chloride with a pH of 7.2) to obtain a mixture with 10% oil, 0.1% oat protein. The mixture was immersed in an ice bath and sonicated for 2 min to prepare a 10% oil-in-water emulsion with 0.1% oat protein. An aliquot (4 mL) of the emulsion was pipetted into each of four replicate emulsion vials. The OC group was prepared by replacing the oat protein extract with the same volume of cold isolation buffer.

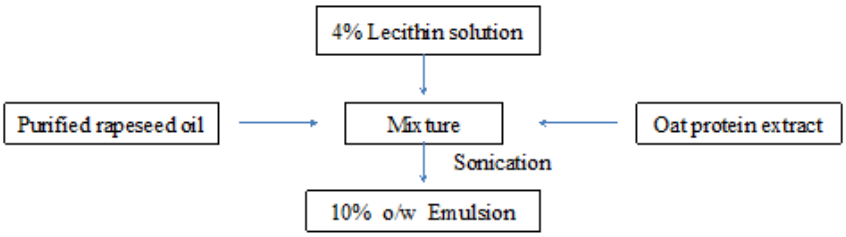


Figure 16. Emulsion preparation procedure for protein control group.

As a reference sample, an oat protein-containing cream (1% protein, 13% oil) was used to prepare the cream series, consisting of such groups as cream control (CC), tocopherol group (CT50) and two ellagitannin groups (CE25 and CE50). Cream was weighed (4 g) into each of four replicate emulsion vials to prepare a group. The composition of cream series is shown in Table 4.

Table 4. Cream group in the cream series.

Group	Concentration of oat protein (mg/mL emulsion)[1)]	Concentration of the antioxidant (μg/g oil)	Oil content (%)[1)]
CC	10 (1%)	0	13
CT50	10 (1%)	50 (α-tocopherol)	13
CE25	10 (1%)	25 (Ellagitannin)	13
CE50	10 (1%)	50 (Ellagitannin)	13

[1)]Protein and oil contents of the reference cream product was from product labeling.

The preparation of emulsion and cream groups with antioxidant compounds (Figure 17) followed the same protocol: addition of antioxidant solutions, solvent evaporation and addition of emulsions. First, α-tocopherol in ethanol stock solution (384.0 μg/mL) was prepared. Second, an aliquot of α-tocopherol stock solutions were pipetted into emulsion vials before adding emulsions, and evaporated to dryness by nitrogen gas. Then emulsions (4 mL) with 0.1% oat proteins were added into the vials to reach a final α-tocopherol content of 50 μg /g oil and stirred, which was the PT50 group. PE 25 and PE 50 groups were then prepared by pipetting ellagitannin stock solutions (254.3 mg/L) into empty emulsion vials which were evaporated, added with emulsions and stirred, to reach the final concentrations of 25 and 50 μg/g oil, respectively. Similarly, cream groups with α-tocopherol (CT50) and ellagitannins (CE25 and CE50) were also prepared.

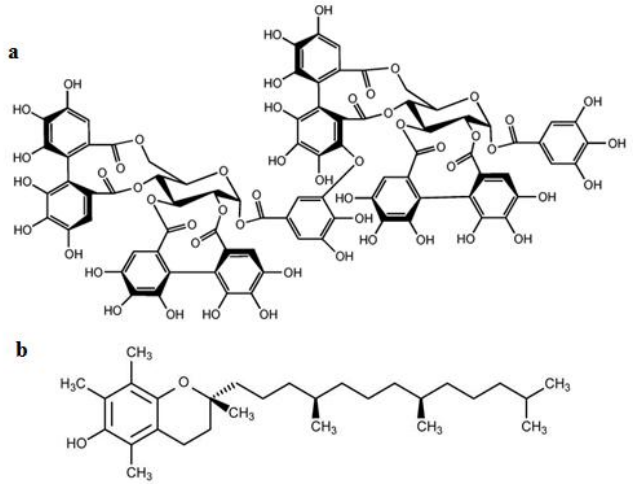


Figure 17. Structures of antioxidant compounds used in the current study: dimeric ellagitannin sanguiin H6 (a), and α-tocopherol (b).

Oxidation was carried out in darkness by placing sealed emulsion vials on the magnetic tray inside an oven with a steady temperature at 37°C. During oxidation, emulsions and creams were constantly stirred by magnet stirrers.

3.2.6 Oxidation measurements

Oxidation studies were carried out by measuring hexanal formation and fluorescence intensities of protein oxidation markers including tryptophan, carbonyls and dityrosine. Sampling for oxidation studies was carried out on day 0, 3, 6 and 9 from each replicate emulsion and cream vial, as shown in Figure 18.

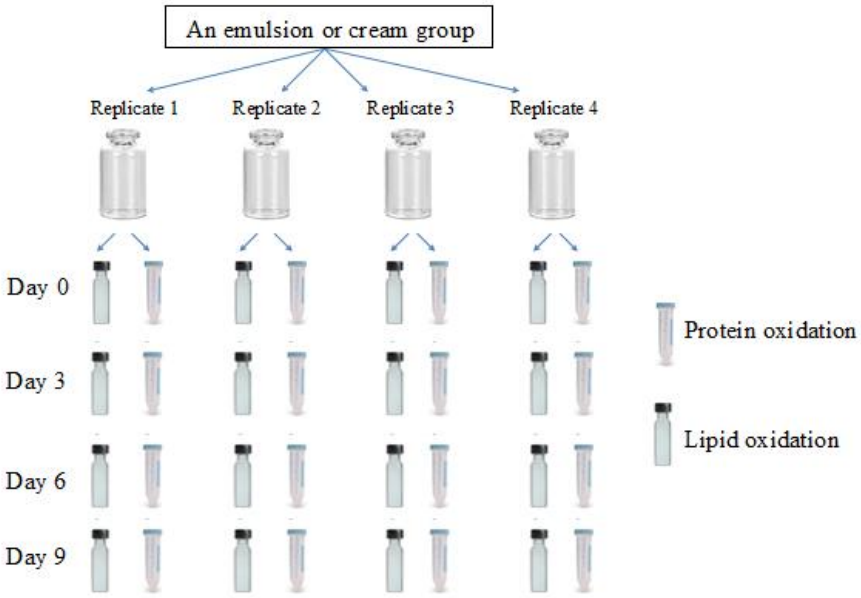


Figure 18. Oxidation measurements of an emulsion or cream group.

Lipid oxidation was studied by measuring hexanal formation using a HS-GC technique during the oxidation of emulsions and creams (Frankel et al. 1994). The system was composed of an Autosystem XL gas chromatograph, an HS40XL headspace sampler, a flame ionization detector, and a NB-54 column for separation. The parameters for GC analysis were set as: sample temperature 80°C, needle temperature 100°C, transfer temperature 100°C, GC cycle time 10 min, thermostatting time 18 minutes, pressurization time 2 min, injection time 0.1 min, withdrawal time 0.2 min and vial venting off. For each sample, an aliquot of emulsion or cream samples (250 μL) was pipetted into a headspace vial, which was sealed with a PTFE-coated septa and an aluminium cap. A hexanal solution was used as the standard for the determination of emerged hexanal in samples.

The oxidation of oat proteins was evaluated by measuring three oxidation markers including the loss of tryptophan fluorescence, formation of carbonyls and formation of dityrosine (Heinonen et al. 1998; Estévez et al. 2008; Koivumaki et al. 2012), using fluorescence spectroscopy (LS55 Perkin Elmer luminescence spectrometer, USA). The spectrometer was controlled by a FL WINLAB software. On each oxidation day, an aliquot of emulsion or cream samples (250 μL) was dissolved in phosphate buffer (750 μL, pH 7.2) and diluted. For emulsion samples, dilution factors for the measurements of tryptophan fluorescence, carbonyls and dityrosine were 1:250, 1:500 and 1:500, whereas for cream samples they were 1:2000, 1:1000 and 1:2000, respectively. Diluted samples for measurements were dispended in a 4 mL quartz spectrofluorometer cell. Emission spectra of tryptophan were recorded from 300 nm to 400 nm upon excitation at 283 nm. Emission spectra of carbonyls were recorded from 400 nm to 500 nm when the excitation was at 350 nm, and dityrosine was measured by recording their emission spectra from 400 nm to 480 nm with the excitation wavelength set at 315 nm. The intensities of the highest peaks were recorded as the results of protein fluorescence measurements. In all of the measurements, excitation and emission slit widths were set at 10 nm and data was collected at 500 nm per minute. Change of fluorescence intensities of one of the three markers during oxidation (ΔC) was calculated as: $\Delta C = C_t - C_0$, where C_t is the fluorescence intensity of one marker at day t (t = 3, 6 or 9), and C_0 is the initial fluorescence of the marker at day 0 in a sample.

Differences between changes of fluorescence intensities among emulsion or cream groups were tested by one-way analysis of variance using Minitab Statistical Software (Addison-Wesley, Reading, MA). The significance level was $P < 0.05$.

3.3 Results

3.3.1 Lipid oxidation in emulsion and cream samples

Hexanal, a secondary lipid oxidation product, was detected by HS-GC using a hexanal standard solution. Figure 20 shows the GC chromatogram of volatile compounds in an oxidized emulsion sample. Hexanal was identified by a hexanal standard with a retention time at about 7.26 min. The generation of hexanal in cream and emulsion samples was recorded. Hexanal formation was plotted against the time of oxidation.

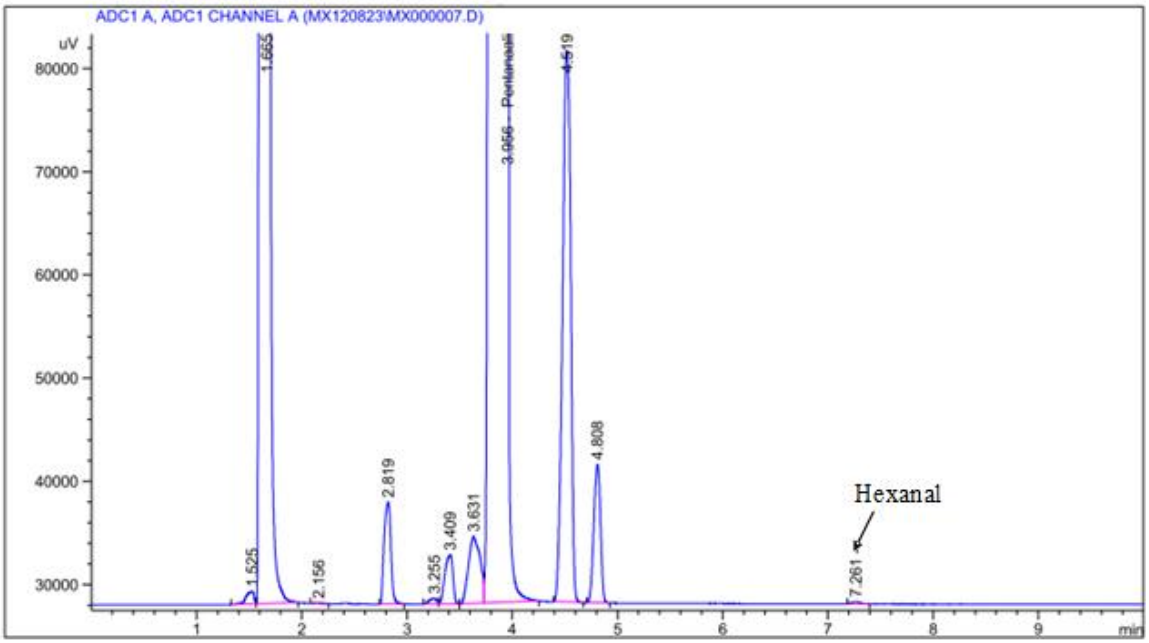


Figure 19. A HS-GC chromatogram of an emulsion sample from the oil control (OC) group on day 9.

Apart from the emulsions in the oil control group, no hexanal formation was detected in the other emulsion or cream samples. Figure 20 shows the elevated hexanal formation in lipid control emulsions. Formation of hexanal was measured on the third day, followed by a steady increase during the rest of the oxidation days. On day 9, all oat protein containing-emulsions were broken down by adding salt (0.1 g NaCl/mL emulsion) to achieve phase separation, and samples were taken from the upper layer for headspace GC analysis owing to the high affinity of hexanal to lipid phase (Druaux and Voilley 1997). No headspace hexanal was detected in the emulsions from the PC, PE25 and PT50 groups, but the ones from the PE50 group appeared to have hexanal formation.

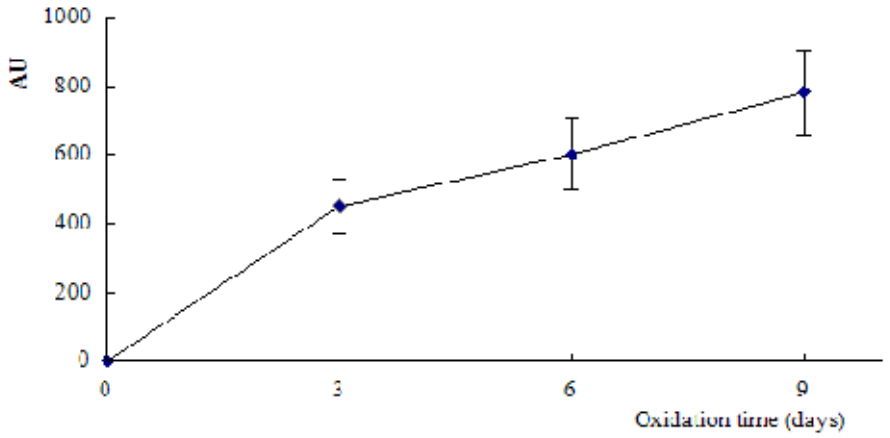


Figure 20. Increase of hexanal (arbitrary units, AU) during oxidation of emulsions in oil control (OC) group.

3.3.2 Protein oxidation in prepared emulsions

Tryptophan fluorescence

All of the emulsion groups displayed an increase of tryptophan fluorescence intensities compared with those on day 0 during oxidation, except for the PT50 group on day 3 which had a decrease of tryptophan fluorescence (Figure 21). Although there was a decrease of tryptophan fluorescence in the PT50 group on day 3, tryptophan fluorescence increased on day 6 and day 9. The increase of tryptophan fluorescence in the PC group climbed to the highest level on day 6, and then it dropped on day 9, when it however still emitted the highest tryptophan fluorescence. On day 9, the PE25 group showed lowest tryptophan fluorescence, followed by the PT50, PE50 and PC groups.

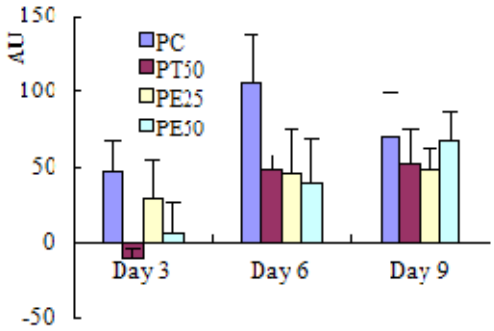


Figure 21. Changes of natural tryptophan fluorescence intensities (arbitrary units, AU) during oxidation of samples in the emulsion series.

The particular mode of tryptophan fluorescence evolution in the PC group can also be visualized in a more informative way, as shown in Figure 23. The peak around 345 nm designated tryptophan residues, and a slight red shift of tryptophan fluorescence was observed from 344.5 nm on day 6 to 345.1 nm on day 9. However, in the antioxidant studies, both of the PT 50 and PE 50 groups displayed a blue shift from 345.4 nm to 344.5 nm and from 344.7 nm to 343.4 nm, respectively, whereas PE 25 group had a relatively steady emission wavelength stabilized around 344.5 nm (spectra not shown here).

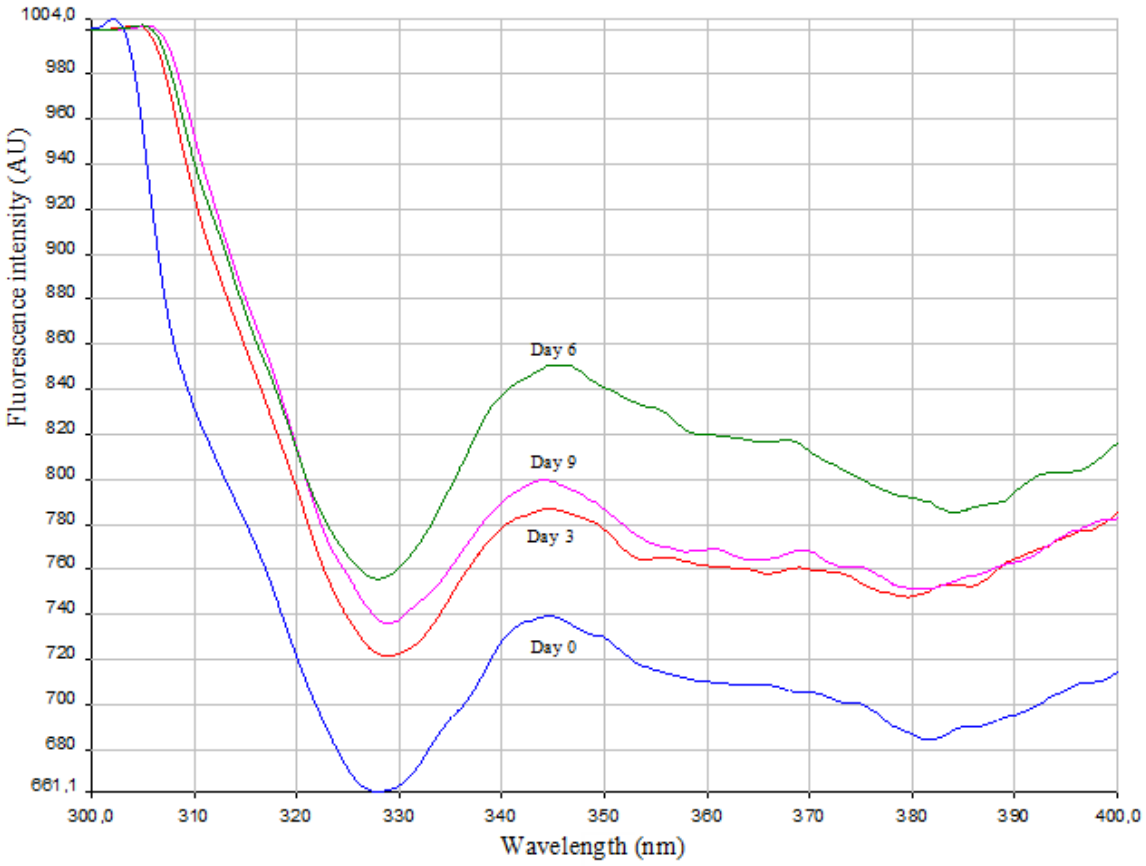


Figure 22. Fluorescence spectrum of tryptophan (λ excitation = 283 nm) in emulsions from the protein control (PC) group during oxidation for 9 days.

Carbonyl formation

Carbonyls were formed in every emulsion group (Figure 23). The PC group dominated the level of carbonyl formation among the emulsion groups. The PT50 group had the lowest carbonyl fluorescence on day 3 and day 6, but on day 9 it had higher increaser of carbonyl fluorescence than the PE25 and PE50 groups. On the final day, the PE25 group exhibited the lowest increase of carbonyl fluorescence.

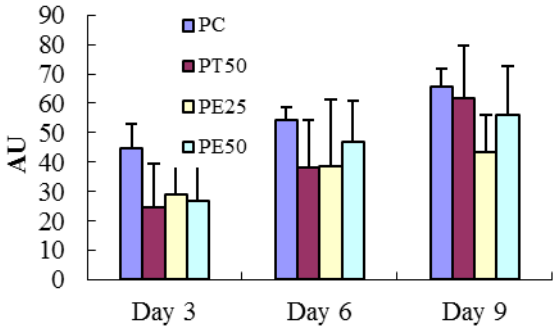


Figure 23. Changes of carbonyl formation (arbitrary units, AU) during oxidation of emulsions in the emulsion series.

Figure 24 shows the increase of carbonyl formation in the PC group. Carbonyls were identified by the small peaks around 420 nm. During the 9-day oxidation, about 68% of the total carbonyl fluorescence increase was due to the first 3-day oxidation, and the rest 32% was caused by the subsequent 6-day incubation.

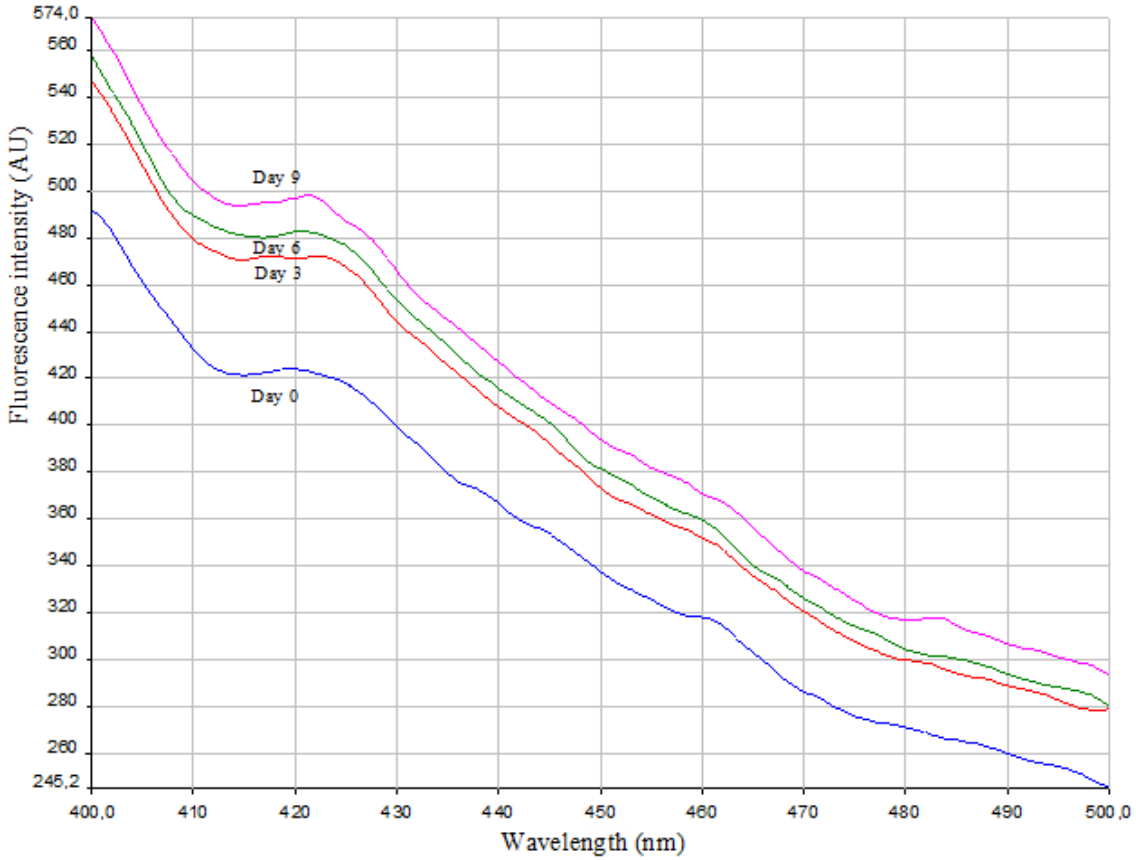


Figure 24. Fluorescence spectrum of carbonyls (λ excitation = 350 nm) in emulsions from PC group during oxidation for 9 days.

Dityrosine formation

Dityrosine fluorescence increased in all the emulsion groups (Figure 25). On day 3, the PE25 group showed the highest dityrosine formation, but it turned out to be the one with lowest increase of dityrosine fluorescence on day 9, which was however not significantly different ($P < 0.05$) from the increases of dityrosine fluorescence in other emulsion groups on day 9. A higher ellagitannins load at 50 µg/g oil did not inhibit dityrosine formation but the PE50 group eventually contained the highest dityrosine among the emulsion groups. Interestingly, PT50 group also presented to be the one with lowest dityrosine fluorescence initially on day 3 and day 9. Similar to the case of carbonyl formation, the PE50 group also accumulated a high content of dityrosine comparable to the contents of dityrosine in the other emulsion groups.

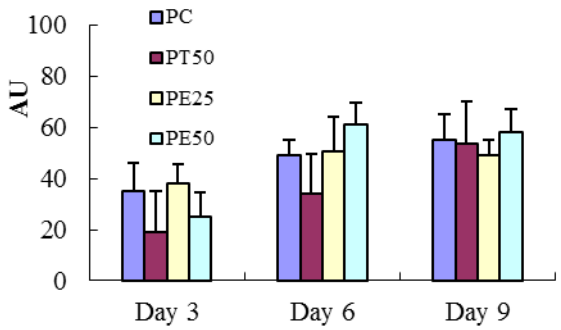


Figure 25. Changes of dityrosine formation (arbitrary units, AU) during oxidation of emulsion samples in the emulsion series.

Dityrosine formation in the PC group could be seen in Figure 26. The peaks around 427 nm represented the dityrosine fluorescence. Corresponding to the tendency in carbonyl formation, the first 3-day oxidation produced 64% of the total dityrosine and the rest was generated after the third day. Single peak were characteristic of the analyte without redundant or interfering signals in contrast with the spectra of tryptophan fluorescence (300 to 310 nm) and carbonyl fluorescence (400 to 415 nm).

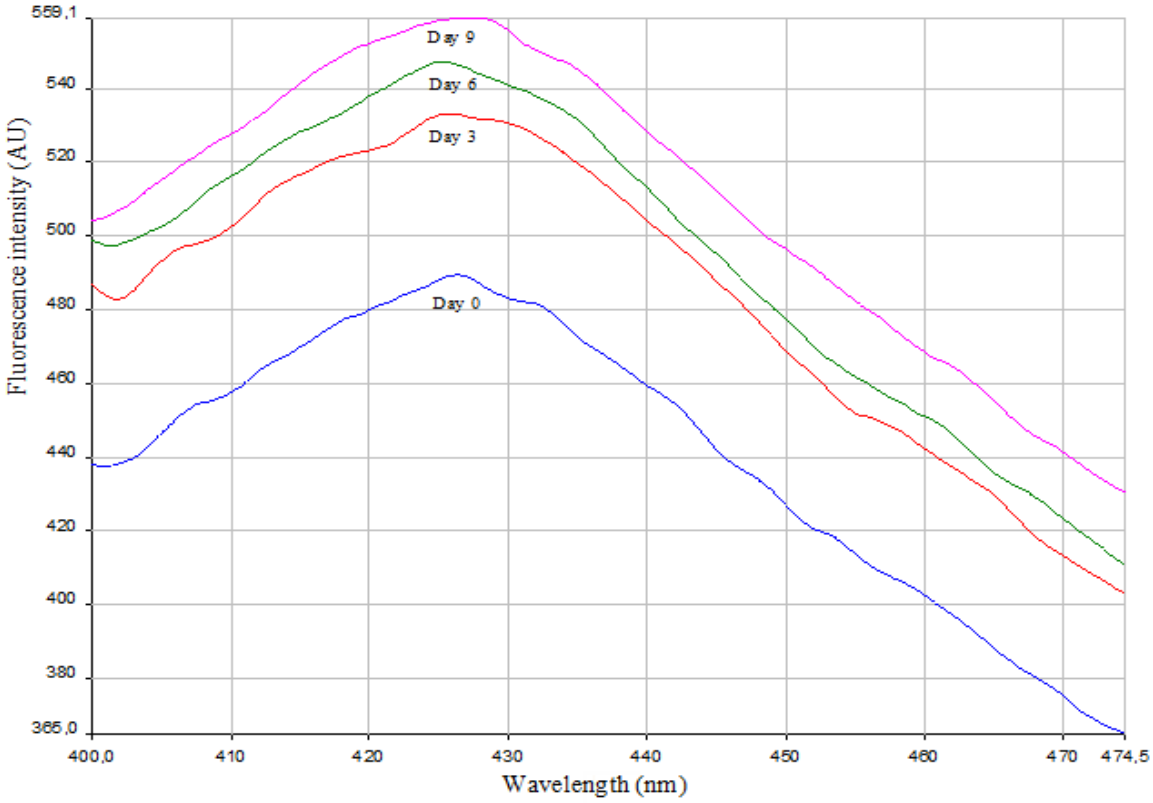


Figure 26. Fluorescence spectrum of dityrosine (λ excitation = 315 nm) in emulsion samples in the protein control (PC) group during oxidation for 9 days.

3.3.4 Protein oxidation in cream samples

Tryptophan fluorescence

Although the CT50 and CE25 groups had an increase of tryptophan fluorescence on day 3, they showed a decrease of tryptophan fluorescence on day 9 (Figure 27). The CC and CE50 groups had a decrease of tryptophan fluorescence, with the former more pronounced.

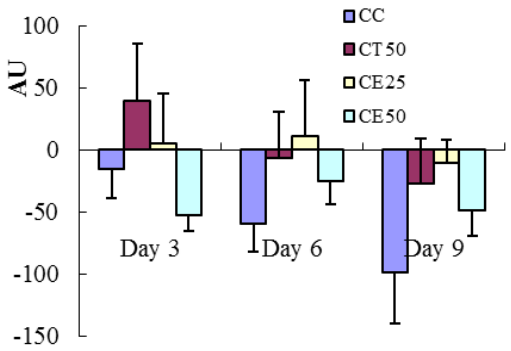


Figure 27. Changes of natural tryptophan fluorescence (arbitrary units, AU) during a 9-day oxidation of creams in the cream series.

The tryptophan fluorescence in the CC group was shown in Figure 28. Intense fluorescence peaks around 345 nm indicated tryptophan residues on protein molecules. The 9-day oxidation of cream samples resulted in an obvious loss of tryptophan fluorescence. Additionally, a red shift of tryptophan fluorescence was found from 345.7 nm on day 0 to 347.1 nm on day 9.

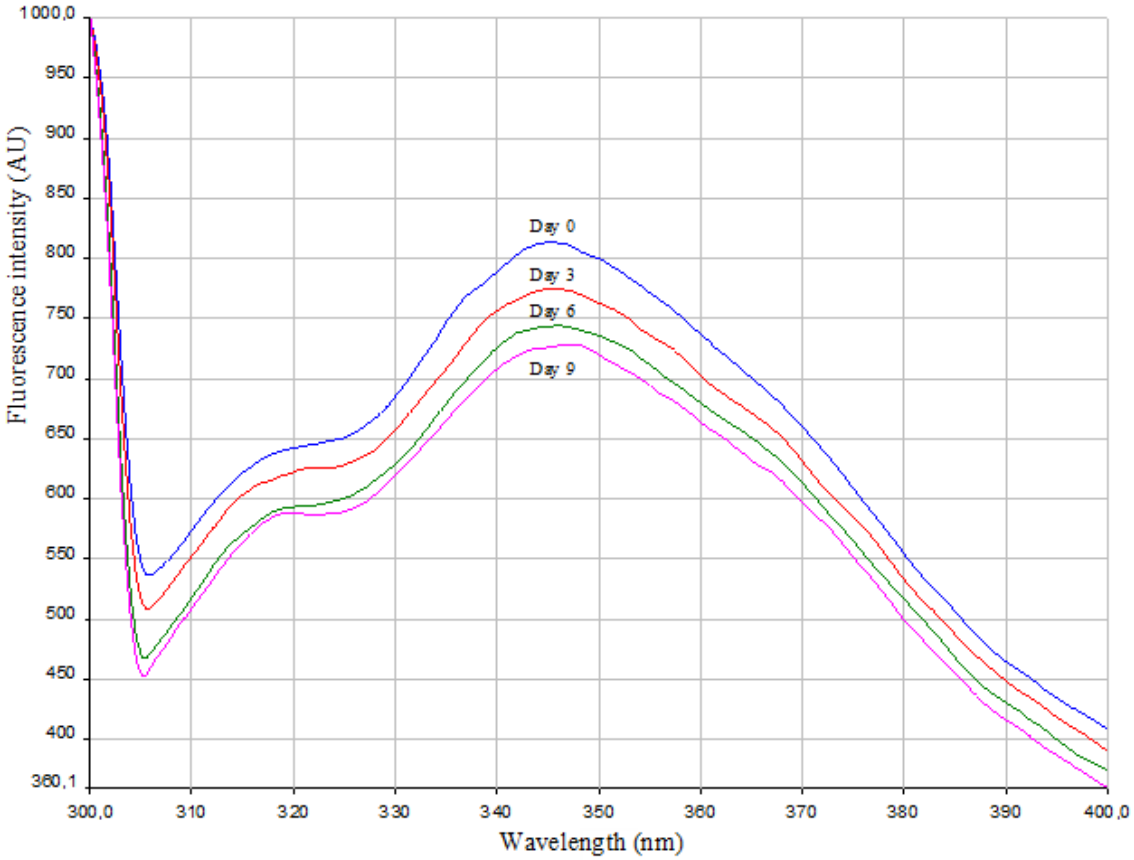


Figure 28. Fluorescence spectrum of tryptophan (λ excitation = 283 nm) in cream samples in the cream control (CC) group during oxidation for 9 days.

Carbonyl formation

Carbonyls in all cream groups decreased during oxidation. Among them, the CC and CT50 groups demonstrated a greater decrease than the ellagitannin-containing groups. Finally, the CC group had the highest decrease of carbonyl fluorescence, followed by the CT50, CE25 and CE50 groups (Figure 29). The continuous loss of carbonyl fluorescence was illustrated in Figure 30. The peaks representing carbonyls were not apparent.

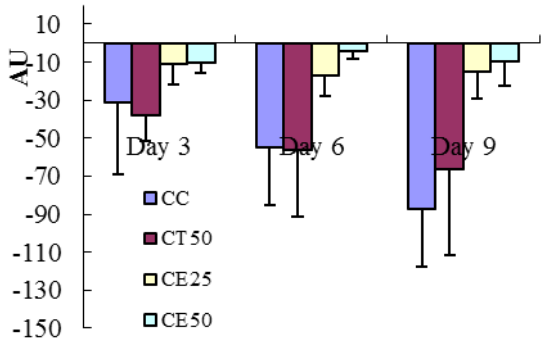


Figure 29. Carbonyl formation (arbitrary units, AU) during oxidation of creams in the cream series.

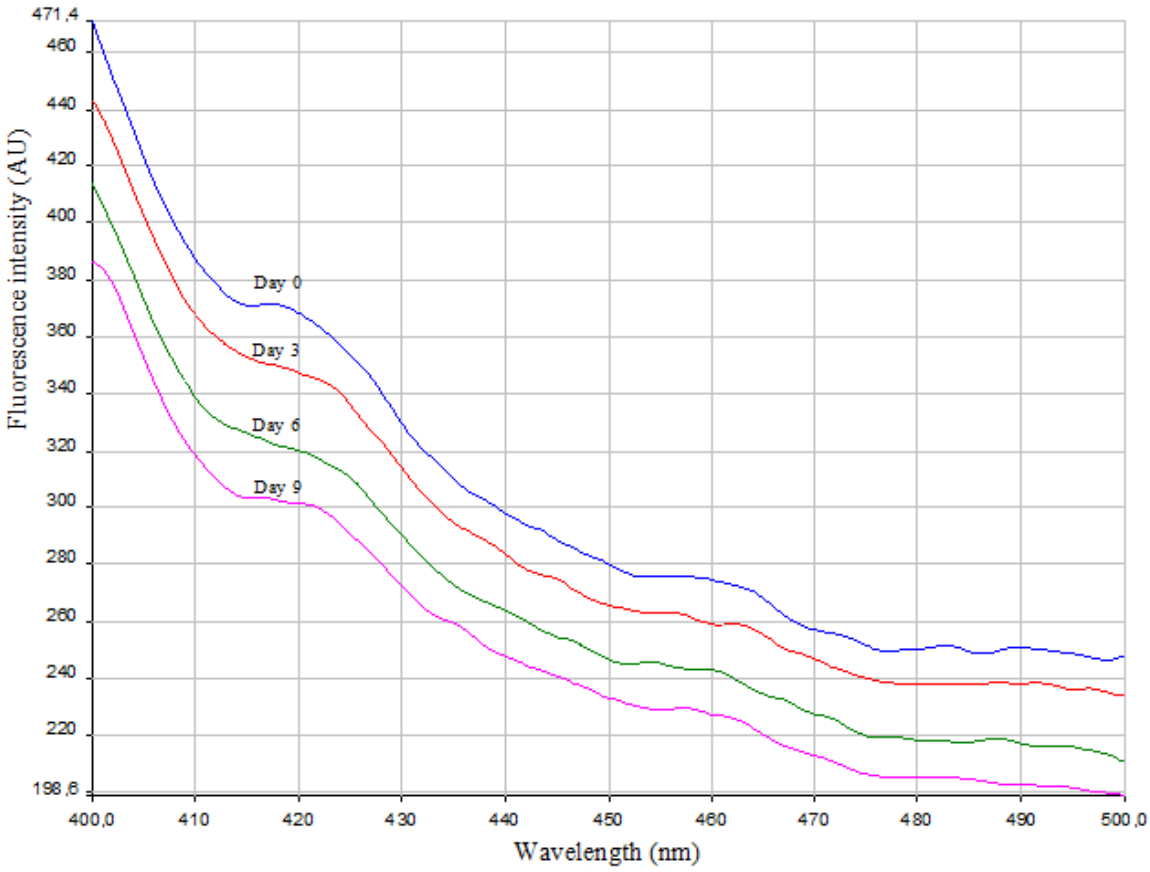


Figure 30. Fluorescence spectrum of carbonyls (λ excitation = 350 nm) in creams from the cream control (CC) group during a 9-day oxidation.

Dityrosine formation

Dityrosine fluorescence decreased almost in all the cream groups except for the CE25 groups on day 6 and day 9. Similarly, the highest decrease also occurred in the CC group, followed by the CT50 and CE50 groups (Figure 31). The decrease of dityrosine fluorescence in the CC group was shown in Figure 32.

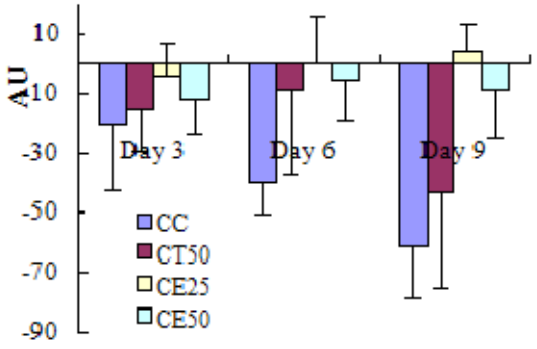


Figure 31. Dityrosine formation (arbitrary units, AU) during oxidation of creams in the cream series.

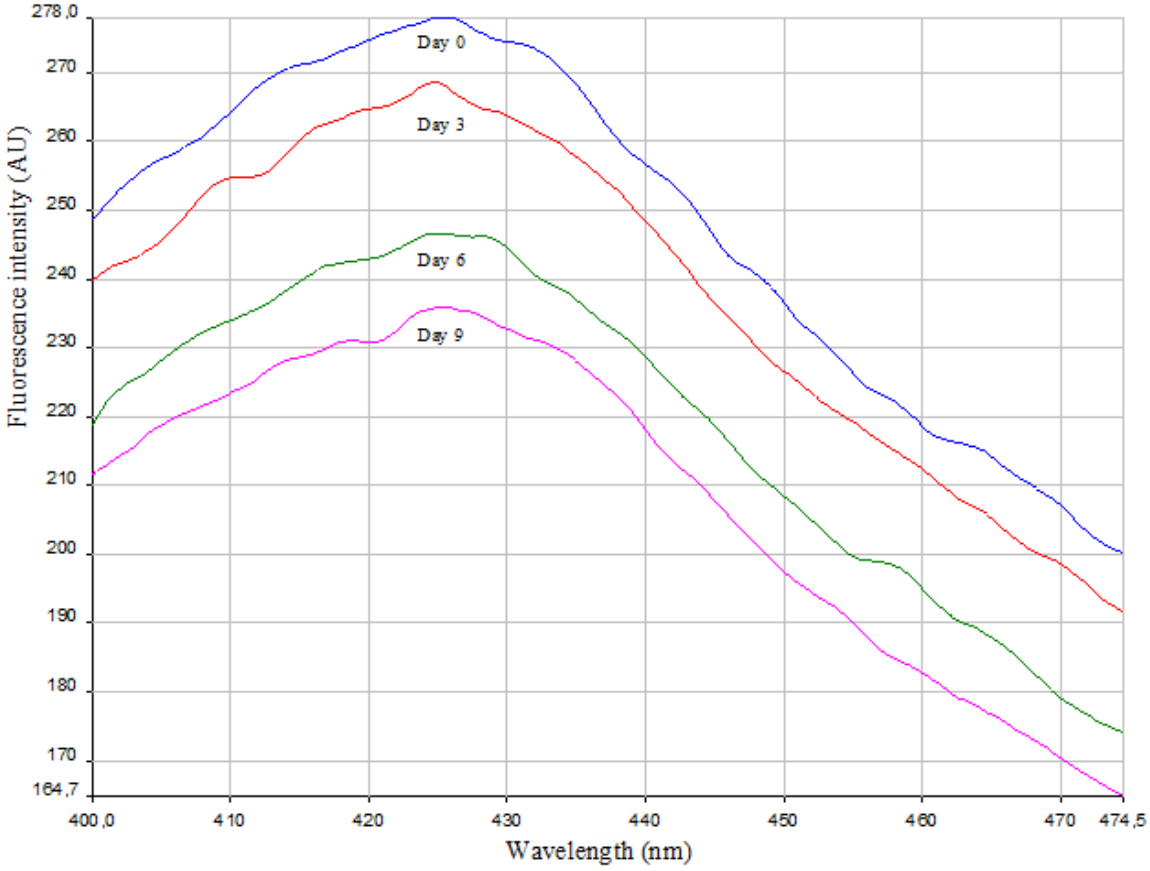


Figure 32. Fluorescence spectrum of dityrosine (λ excitation = 315 nm) in creams from the cream control (CC) group during oxidation for 9 days.

After 3-day oxidation, syneresis in cream vials from the CT 50 and CC groups was observed, and this phenomenon was more pronounced at day 6 and day 9. Syneresis also took place in the CE 25 and CE50 groups although it was not visible during oxidation, because solids in white color were found sticking onto the vial walls in all cream samples. The picture for visible syneresis in a cream vial from the CT50 group on day 3 was shown in Figure 33.

Figure 33. Syneresis in a cream vial from the CT50 group observed on day 3.

3.4 Discussion

3.4.1 Lipid oxidation in emulsions and creams

Hexanal has been conventionally used as an indicator of lipid oxidation in rapeseed oil as it is a major secondary lipid oxidation product (Frankel 1985). Elevated level of hexanal in the OC group indicated the progression of lipid oxidation. However, hexanal was not found in the counterpart emulsion groups supplemented with oat protein and antioxidants. This could mean that oat protein or together with ellagitannins or α-tocopherol might exert antioxidant activities towards lipid oxidation in the current study.

Food proteins are well-recognized antioxidants towards lipid oxidation through their reactions with free lipid radicals, primary and secondary lipid oxidation products like hexanal (Elias et al. 2008), but it can hardly explain the missing of hexanal in the emulsion series. It is known that aldehydes including hexanal are able to form covalent bonds with amine groups of protein molecules to produce Schiff bases (Figure 34), whereas α, β-unsaturated aldehydes can also react with amine groups to form Michael addition adducts (Viljanen et al. 2005). Both accounted for the covalent bonding mechanisms, leading to an increase of protein molecular weights and modifications of protein functions (Leaver et al. 1999). However, Schiff base formation is considered as a minor event for the missing of hexanal, as this reaction prefers a weak acidic environment (Bruice 2011) in comparison to the weak alkaline emulsion medium maintained by sodium phosphate buffer system in the current study.

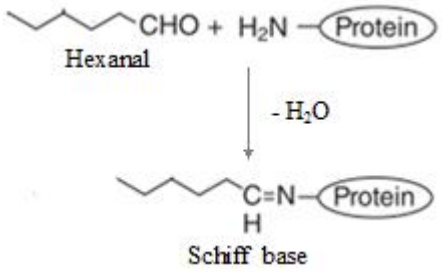


Figure 34. Covalent bonding of a protein molecule with a hexanal molecule by a Schiff base formation.

In addition to the unfavorable pH value, the availability of reactants is another issue of consideration. There is research evidence that unlike hexanal isomers such as *trans*-2-hexanal, hexanal itself only reacts with lysyl residues of proteins (Meynier et al. 2004; Meynier et al. 2005), but the availability of lysine in the prepared emulsion is big issue if

two factors were taken into account: first, the protein concentration in prepared emulsions (1 mg/mL or 0.1%) was much lower than that in the referred studies (30 mg/mL or 3%); second, referred studies use sodium caseinates which are more lysine-rich than oat globulins (Draper 1973; Jost et al. 1999). Therefore, there must be other mechanisms implicated with the missing of hexanal in the emulsion series.

Apart from covalent bonding of proteins with hexanal, proteins can also form reversible hydrophobic interactions with hexanal so that the volatility of hexanal is decreased (Druaux and Voilley 1997). The retention of hexanal by its hydrophobic binding with proteins is drastically increased with increasing pH from 6 to 9 (Well et al. 2003), so a relatively high retention of hexanal in the emulsions could be predicted, but it is still hard to know how much it contributed to the missing of hexanal in the oat protein-containing emulsions during oxidation. The hexanal discovered after the breakdown of emulsions in the PE50 group was probably trapped by the emulsion matrix through reversible hydrophobic interactions, thus it was released once the emulsion structure was disrupted by adding salts to emulsion samples.

Specifically, the unique antioxidant capacity of oat flour or alkaline extract of oat proteins were closely linked to an enzymatic oxidation of hexanal into water-soluble hexanoic acid and a physical protection of food lipids from oxidative agents by oat proteins. Lehto et al. (2003) suggested that wholegrain oat flour itself has an aldehyde dehydrogenase type activity that can oxidize hexanal into hexanoic acid. This phenomenon is estimated to be the major culprit decreasing 80% hexanal in the referred study, while irreversible covalent bonding and simple adsorption combine to account for the rest 20%. The current study also used wholegrain oat flour as a raw material, but it is still unknown if the enzyme with such an aldehyde dehydrogenase type activity would survive in the buffered system (sodium phosphate buffer, pH 7.2) and exert an oxidation activity converting hexanal into hexanoic acids, because Lehto and coworkers did not study the enzymatic activity in the buffer.

If the enzyme did exist in the emulsion samples with oat protein extract, it is possible to explain the reason why there was hexanal formation after breaking down the emulsions in the PE50 group by adding salts. It is well-documented that tannins can inhibit enzymatic reactions by the formation of tannin-protein complexes (Haslam and Lilley 1988). Compared with the emulsion media in the PE25, it is probable that fewer enzymes were left active to oxidize hexanal into water-soluble hexanoic acid in the PE50 group due to a

stronger inhibitory effect of more ellagitannins on the enzymatic reactions. This finding was in accordance with the fluorescence results that PE25 group contained less protein oxidation products (carbonyls and dityrosine) than PE50 group. However, Kähkönen et al. (2012) found that cloudberry ellagitannin isolate has a higher inhibitory effect on hexanal formation with increasing phenolic concentrations from 1.4 (63% inhibition) to 4.2 μg/mL (94% inhibition) using human low-density lipoprotein (LDL) dispersion as a model, which is contradictory to the current findings. The reason might be that in the current study, it was relatively difficult for ellagitannins to locate themselves into the oil/water interfaces to exert an antioxidant activity, since in the emulsion samples, oat proteins, lecithin and ellagitannins would compete with each other to reside in the interfaces.

Lehtinen and Laakso (2000) suggested that oat protein-rich fraction may provide a physical encapsulation of free fatty acids by restricting their contact with oxidative. An earlier research by the same authors supported that suggestion that oat protein could provide a physical protection to lipid from oxidation, and they highlighted that high antioxidant capacity of the oat extract was very pronounced in an alkaline condition (pH 8 - 10) but it collapsed upon a pH drop to 6 (Lehtinen and Laakso 1998). Being pH- sensitive, the antioxidant capacity of the extract is very likely to be attributed to oat globulin, because its solubility is much higher in the pH range from 8 to 10 (>80%) (Figure 4) but collapses when pH drops to 6, which is in agreement with the composition analysis of the extract.

No hexanal formation in oat creams could be explained by high protein content and hydrocolloid formation. There were more proteins in creams (1%) acting as antioxidants towards lipid oxidation, than those in prepared emulsions (0.1%). Xanthan gum is used as a thickener in creams to create viscosity, which reduces the release of hexanal by viscosity and molecular interactions (Yven et al.1998). The high viscosity in cream samples might resist the diffusion of oxygen into creams to react with lipid, and it might also resist the diffusion of hexanal from dispersed lipid phase to continuous water phase, and then to gas phase (de Roos 2003). Instead, Bylaite et al. (2005) argue that hydrophobic interaction between xanthan gum and hexanal is the major cause for a reduced release of hexanal other than viscosity. The proposed aldehyde dehydrogenase type activity is much less likely to happen, since the production of cream product includes an ultra-high temperature treatment which destroys the enzyme. The inactivation temperature of the enzyme is about 85^{o}C (Lehto et al. 2003).

The absence of hexanal in emulsion and cream samples could not give rise to the conclusion that there was no lipid oxidation taking place in the samples. The reason is that no attempts to measure the primary lipid oxidation products (e.g. conjugated dienes) have been taken. Thus, if there was the formation of primary lipid oxidation products, lipid oxidation must have occurred even though no hexanal was formed. In addition, the claim that oat proteins as well as phenolics are efficient antioxidants towards lipid oxidation could not be launched.

3.4.2 Protein oxidation in the emulsion series

Tryptophan fluorescence

Tryptophan is the main contributor to protein fluorescence, because it absorbs the least energy for excitation and has the highest extinction coefficient. Fluorescence spectra of tryptophan are quite useful to understand solvent polarity and protein folding (Lakowicz 2006). Loss of tryptophan fluorescence has been conventionally used as an indicator of protein oxidation (Heinonen et al. 1998; Viljanen et al. 2005; Estévez et al. 2008) due to the oxidative modification of the indole ring of the tryptophan residue. In contrast, the PC group did not display a continuous loss of tryptophan fluorescence during oxidation, but the tryptophan fluorescence actually increased from day 3 to day 6 during oxidation. The increase of tryptophan fluorescence in the PC group from day 3 to day 9 was probably caused by the unfolding of oat globulin. When oat globulin is heated, protein molecules gradually unfold themselves, and more tryptophan residues are exposed to the solvent environment, leading to the increase of tryptophan fluorescence (Ma and Harwalkar 1988). Therefore, oxidation of emulsions under 37^{o}C might slightly stimulate the unfolding of oat globulin compared to the oat globulin in protein extraction at about 4^{o}C, and thus more tryptophan residues were possibly exposed due to temperature increase, leading to an increase of tryptophan fluorescence.

Decrease of tryptophan fluorescence was not witnessed until day 9, and it was likely caused by quenching of tryptophan fluorescence due to continuous molecule unfolding (Yamagishi et al. 1981) or by other mechanisms, such as oxidative degradation (Gieβauf et al. 1995). Interestingly, the red shift of tryptophan fluorescence from 344.52 nm (day 6) to 345.10 nm (day 9) was found, which meant that slight protein denaturation happened (Kronman and Holmes 1971).

Due to interactions between antioxidant compounds and oat globulin, no decreases of tryptophan fluorescence have been observed in the PT50, PE25 and PE50 groups. This may also be explained by the shifts of the tryptophan fluorescence peak wavelengths except for the case in the PE25 group which showed a steady tryptophan fluorescence peak wavelength around 344.4 nm. The PT50 and PE50 group had a clear blue shift. Particularly, the PE50 group had a more pronounced blue shift from 344.7 nm to 343.4 nm, indicating a more severe shielding of the protein molecules (Ma and Harwalkar 1987) and a stronger interaction between ellagitannins and oat globulin. Due to the continuous shielding of oat globulin molecules, tryptophan residues were also less exposed to the aqueous phase, leading to an increase of tryptophan fluorescence during oxidation.

According to the results in the present study, loss of tryptophan fluorescence was not recommended as a protein oxidation marker when one is to study the oxidation of heat-coagulable proteins like oat globulin, because conformational changes in such proteins are more pronounced and contributed to the changes of tryptophan fluorescence. Proteins commonly used in oxidizable food models, such as β-lactoglobulin, ovalbumin, BSA and collagen, coagulate or form gels after protein denaturation when they are heated in water (Aguilera and Rademacher 2004), but oat globulin readily coagulates before denaturation (Ma and Harwalkar 1987). Therefore, changes of tryptophan fluorescence could not reveal the real oxidative modifications of oat globulin, and the loss of tryptophan fluorescence as a protein oxidation marker is only recommended for those proteins with a good conformational stability when they are heated.

Carbonyl formation

Carbonyl gain occurred in all of the groups in the emulsion series. A higher dose of ellagitannin in the PE50 group did not inhibit carbonyl formation but rather accelerated it compared with the PE25 group which exhibited an antioxidant activity of. Similarly, this type of antioxidant activity of ellagitannin towards carbonyl formation was also observed in a lactalbumin-lecithin liposome model, where a higher dose of ellagitannin at 4.2 μg/mL has a weaker antioxidant activity than a lower dose of 1.4 μg/mL (Viljanen et al. 2004). Since tannins can form strong interactions with proteins which could result in the formation of tannin-protein complexes, such type of antioxidant activity of ellagitannin towards protein oxidation might result from different binding patterns between ellagitannin and protein molecules (Li and Gu 2011).

One interesting finding was that α-tocopherol displayed a significant antioxidant activity during the oxidation from day 3 to day 6, but on day 9 no significant difference of carbonyl compounds was found between the PT50 and PC groups, indicating that α-tocopherol exerted an antioxidant activity in the early stage and then it decomposed. Similar founding is that α-tocopherol decomposes more quickly than its counterpart tocopherol analogues (Koski et al. 2002). Since the oxidation was carried out in the dark, lipid oxidation did not involve photo-oxidation, and α-tocopherol in the PE50 group could play a unique role as an effective radical scavenger to react with peroxyl radicles by donating hydrogen atoms (Buettner 1993), so a significant antioxidant activity during the oxidation from day 3 to day 6 could be found.

As addressed in literature review, another issue associated with carbonyl formation is the magnitude that protein oxidation-induced carbonyl formation accounted for the total carbonyl formation, as multiple pathways collaborate to produce carbonyl compounds in a complex food matrix such as food emulsions (Estévez 2011). In an *in vitro* study where HNE was incubated with β-lactoglobulin B, most of protein modifications were from Michael addition reactions (Bruenner et al. 1994), which are the major sources of protein carbonylation other than protein oxidation reactions. Therefore, a more specific method is needed to elucidate protein oxidation-induced protein carbonylation.

Dityrosine formation

Dityrosine accumulated in all of the groups in the emulsion series during oxidation, and its formation correlated well with carbonyl formation that the PE50 group contained more dityrosine and carbonyls than the PE25 group, which correlated well to the carbonyl formation in the emulsion series. Ellagitannin had a significant antioxidant activity towards dityrosine formation at the concentration of 25 μg/g oil. The antioxidant activity of α-tocopherol towards dityrosine formation also correlated well with that in the carbonyl formation: a more pronounced inhibitory effect at early oxidation stage.

It is noteworthy that the highest peak emission wavelengths for dityrosine identification in all emulsion and cream samples were around 427 nm, which were higher than most literature values ranging from 400-420 nm in alkaline solutions as illustrated in the literature review. This discrepancy might result from the difference of solvent environment. Since sodium chloride and phosphate in emulsions increased the polarity of

water molecules, those water molecules reoriented themselves around the excited-state fluorophores with dipoles. It reduced the energy of fluorophores and shifted the emission to higher wavelengths (Lakowicz 2006). In contrast, the highest peak emission wavelengths for tryptophan and carbonyl identification in the present study were not considerably affected by the relatively polar solvent environment.

However, accurate identification of dityrosine in the present model indeed needs a dityrosine standard or a more specific method than fluorescence spectroscopy. In addition to the discrepancy of the highest peak emission wavelengths of dityrosine between the observed and reference values, there are some lipid oxidation-derived compounds having similar fluorescence properties to dityrosine, which might also emit fluorescence around 427 nm. For example, the reaction between lysine and hexanal can result in fluorescent products which has maximum emission wavelength around 416 - 420 nm when excited at 327 - 370 nm (Veberg et al. 2006). In an oxidized lens protein study, dityrosine only accounts for 1% of the dityrosine-like fluorescence (Wells-Knecht et al. 1993). Thus an inference can be drawn that the dityrosine spectra in the present study could be the sum of spectra of a series of oxidation-induced fluorescent products (Liang 1999), but this point of view needs a more specific method to determine if other oxidation products apart from dityrosine emit the same fluorescence around 427 nm as dityrosine.

Compared with carbonyl and tryptophan fluorescence spectra, dityrosine fluorescence spectra displayed very intense peaks, meaning that dityrosine had a more specific fluorescence property and less interfered by other compounds. Thus dityrosine is suggested to be used as a protein oxidation marker when the protein oxidation of a complex food matrix is to be investigated.

3.4.3 Protein oxidation in the cream series

Tryptophan fluorescence decreased during oxidation in all cream groups, which seemed to lead to an inference that tryptophan was oxidatively degraded according to the literature (Estévez et al. 2008). However, this inference was not in agreement with the decrease of carbonyl and dityrosine contents, since theoretically both of them should increase or at least remain stable during the oxidation as opposed to the decrease of both.

As discussed in the section of protein oxidation in the emulsion series, changes of tryptophan fluorescence may result from other mechanisms than protein oxidation alone, such as conformational changes of protein molecules. Moreover, the decrease of tryptophan fluorescence in the cream series could also be caused by syneresis, which is the separation of liquid from solids (Mizrahi 2010). Xanthan gum, which is used as a stabilizer in the cream, is considered as the main contributor to syneresis during cream storage by inducing emulsion droplet flocculation (Krstonosic et al. 2009). As liquid was expelled, dispersed proteins aggregated and adhered to the vial wall. Therefore, a less concentrated cream resulted, and simultaneous decreasing of tryptophan, carbonyl and dityrosine fluorescence was observed. Protein oxidation studies on the cream series were thus hindered by cream syneresis.

Although the protein oxidation studies were not feasible by the cream model, other information from fluorescence spectra of tryptophan and carbonyls could also be obtained. For example, the fluorescence spectra of carbonyls in the CC group (Figure 29) showed unclear fluorescence peaks of carbonyls due to the fluorescence emitted by impurities from 400 to 420 nm. The impurities were supposed to be sodium salts when the spectra in Figure 29 were compared with the corresponding ones in the study by Estévez et al. (2008). The referred study used potassium phosphate instead of sodium phosphate buffer, and the resulting spectra have clear fluorescence peaks for carbonyls and no intense fluorescence from 400 to 420 nm. This argument could be also supported by the fluorescence spectra of sodium phosphate buffer (not shown here), which showed strong fluorescence from 400 to 420 nm. Therefore, two actions could be taken to decrease the interference of sodium salts on fluorescence spectra of carbonyls: dialysis of protein extract and replacement of sodium by potassium.

When the tryptophan fluorescence spectra of the emulsion series are compared with those of the cream series, it is obvious that the spectra from the cream series demonstrated much more intense and clear peak shapes than those from the emulsion series. This might be caused by the presence of phenolic compounds that have similar fluorescent properties to tryptophan. Phenolic compounds could be co-extracted with oat globulin (Ma et al. 2000), and the present work used whole-grain oat flour as a raw material which contains phenolic-rich oat bran (Webster 2002). However, during the production of oat protein-containing cream, oat bran has been removed and less interference from those phenolics is expected.

In both of the emulsion and cream series, dityrosine fluorescence spectra showed intense base peaks, regardless of the presence of sodium salts and possibly co-extracted phenolic compounds. Thus here it is emphasised that dityrosine or dityrosine-like oxidation compounds could be used as a better protein oxidation marker.

4 CONCLUSION

The current thesis work investigated lipid oxidation (e.g. hexanal formation) and protein oxidation in prepared oat protein-containing emulsion and cream samples, and inhibitory effects of ellagitannins and α-tocopherol on protein oxidation.

Hexanal was effectively inhibited by oat protein as no hexanal was detected in any oat protein-containing emulsion and cream during oxidation. Since no attempts to detect primary lipid oxidation products have been made, it is not certain if lipid oxidation took place in emulsion and cream samples. However, at least it is sure that lipid oxidation did occur in the emulsion samples with higher ellagitannin dose, because disruption of emulsion structure in those emulsion samples did release hexanal, which also indicated that hexanal could be trapped by the emulsion matrix. The greater protein content and high viscosity of cream samples could partly explain the fact that no hexanal was detected in those samples.

Fluorescence spectroscopy is a promising tool to evaluate protein oxidation in food emulsions by measuring protein oxidation makers, namely loss of tryptophan fluorescence, carbonyl formation and dityrosine formation. The proposed method to monitor the loss of tryptophan is not a suitable way to evaluate protein oxidation, because it not only results from oxidative degradations, but also conformational changes of protein molecules. Possibly, oat globulin slightly unfolded, leading to an increase of tryptophan fluorescence rather than a loss. Therefore, this marker is recommended for the protein oxidation investigations on the proteins with a high structural stability. However, both carbonyl and dityrosine formation reflected the progression of protein oxidation, and the latter is highly recommended. The relevance of carbonyl formation to protein oxidation is not certain since it could also be formed by non-oxidation pathways. Hence, it is highlighted here that the formation of dityrosine, or more precisely "dityrosine-like compounds", could be used as a better protein oxidation marker.

Antioxidant studies towards protein oxidation suggested that ellagitannins at a higher dose at 50 μg/g oil was less efficient than a lower concentration at 25 μg/g oil. α-Tocopherol preferentially exerted an antioxidant activity in the early stage of oxidation, and it was not as efficient as ellagitannins to inhibit protein oxidation.

Protein oxidation results from fluorescence spectra of the cream series could not reveal the oxidation extent in the cream series, due to syneresis of cream samples. The viscous nature and structural instability of the cream could hide protein oxidation products and lead to an underestimation, which was verified by the concurrent decreasing of fluorescence of all the protein oxidation markers. Therefore, it is emphasised here that when the samples are food hydrocolloids, structural stability of samples is the essential prerequisite if fluorescence spectroscopy shall be used.

As suggestions for further studies, several points should be noticed. First of all, dialysis of oat protein extract is recommended to improve emulsion stability and enhance protein content. In addition, oat proteins should also be further purified to remove impurities, such as phenolics or oil. As a minor objective, lipid oxidation studies should encompass the detection of primary lipid oxidation products. More specific protein oxidation techniques are still needed to shed light on the contribution of protein oxidation-derived carbonyl and dityrosine formation to their total formation.

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