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Interactions of food proteins with plant phenolics – modulation of structural, techno- and bio-functional properties of proteins

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List of Abbreviations

$ABTS^+$	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AMD	Age related macular degeneration
ANS	1-anilino-8-napthalensulfonate
BZ 1	BioZate 1
CA	Coffea arabica
CD	Circular Dichroism
CQA	Chlorogenic acid
CR	Coffea robusta
DM	Dry matter
DPPH	2,2 Diphenyl-1-picrylhydrazyl
DSC	Differential Scanning Calorimetry
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
FB	Faba beans
GC	Green coffee beans
GMP	Glycomacropeptide
KDa	Kilodalton
LE	Lutein ester
M/Z	Mass/charge
MALDI-TOF-MS	Matrix Assisted Laser Desorption/Ionization –Time of Flight-Mass Spectrometry
MCT	Medium chain triglyceride
MW	Molecular weight
O/W	Oil-in-water emulsion
OPA	Orthophtaldialdehyde
PBS	Phosphate-buffered saline
PPO	Polyphenol oxidase
RP-HPLC	Reverse phase- High Performance liquid Chromatography
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
T_d	Denaturation temperature
TEAC	Trolox equivalent antioxidant capacity
TNBS	Trinitrobenzenesulfonic acid
UV	Ultraviolet
β-LG	β-lactoglobulin
ΔH	Enthalpy of denaturation

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Significance of this study

Study 1 documents:

- The differences in CQA content and polyphenol oxidase activity in different apples.
- Identification of phenolic compounds in apple juice.
- Effect of storage conditions on polyphenol oxidase activity.
- The characterization of the interactions of CQA towards model amino acids
- The characterization of the interactions of CQA towards model proteins.
- Influence of the interaction conditions.

Study 2 documents:

- The interactions of the individual phenolic compounds towards coffee proteins
- Compatibility of protein extraction
- Simplification of the analytical procedure for determining the modification of coffee proteins
- Influence of such reactions on protein structure
- Perspective of improving and enhancing the coffee protein quality
- The first available in-silico modeling of modified coffee proteins documenting the potential sites and types of modification
- Finally, a mode of application of such modified proteins to provide a better protection of bioactive easily oxidized lipophilic compounds such as Lutein esters

Study 3 documents:

- The interactions of the individual coffee phenolic compounds towards model whey proteins
- Different modes of protein modification
- Simplification of the analytical procedure for determining the modification
- Influence of such reactions on protein structure
- The in-silico modeling of modified beta-Lactoglobulin documenting the potential sites and types of modification
- The effect of such modifications on the functional properties of the whey protein
- Finally, a mode of application of such modified proteins to provide a better protection of bioactive easily oxidized lipophilic compounds such as Lutein esters

1. Introduction

1.1 Interactions of proteins with phenolic compounds

The interactions between proteins and the phenolic compounds, which exist in many fruits, vegetables and beverages (e.g. coffee), have received considerable scientific attention in recent years (Clifford 1999). Phenolic compounds as food components represent - with more than 6000 identified substances - the main group of secondary metabolites in plant foods. They are characterized by a wide range of specific structures and functions, but also as generally possessing an aromatic ring bearing one or more hydroxyl substituents (Robards *et al.* 1999, Parr and Bolwell 2000). Interest in these compounds is related to their dual role as substrates for oxidative browning reactions and as antioxidants, underlining their impact on organoleptic and nutritional qualities of fruits and vegetables; their role in plant growth and metabolism; and, more recently, their demonstrated physiological activity in humans (Rawel *et al.* 2007).

Hydroxycinnamates - a class of polyphenols having a C6-C3 skeleton - and their derivatives can interact with proteins in many ways: with food proteins during food processing; with storage and physiological active proteins in the plant; with food proteins or enzymes in the course of digestion in the gastrointestinal tract; with blood plasma proteins; and, finally, with proteins in target tissues of organs in the human body. The interactions between phenolic compounds and proteins can be classified in two subgroups: non-covalent interactions (reversible), and covalent interactions (in most cases, irreversible (Prigent 2005, Rawel and Rohn 2010). The protein structure (amino acid sequence and the resulting structural conformation), and also external conditions – such as pH, temperature and ionic strength - control the non-covalent binding of the hydroxycinnamates to proteins (Prigent *et al.* 2003, Rawel *et al.* 2005a, Rawel *et al.* 2006). Principally, five potential types of non-covalent interactions have been suggested: hydrogen bonds, electrostatic interactions, hydrophobic interactions, van der Waals interactions and π bonds irreversible. However, the hydrophobic interactions and hydrogen bonds are the major forces for the interaction between the phenolic compound and proteins. Hydrophobic interactions may take place between phenolic compounds and amino acids, such as: alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine, and glycine residues. Furthermore, the amino acids (lysine, arginine, histidine, asparagine, glutamine, serine, threonine, aspartic acid,

glutamic acid, tyrosine, cysteine, and tryptophan) can be bound with phenolic compounds by hydrogen bonds, which may occur between their nitrogen or oxygen and hydroxyl groups of phenolic compounds (Prigent 2005, Rawel and Rohn 2010).

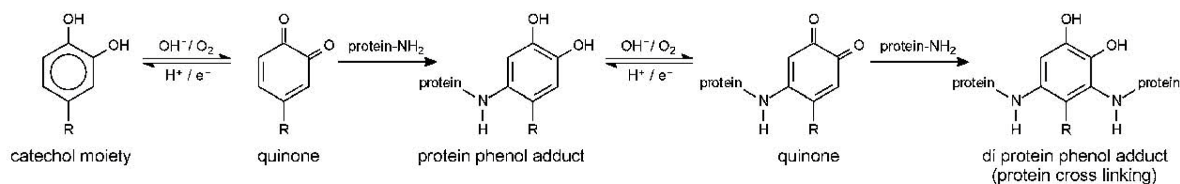


Figure 1-1 Proposed reaction of protein with hydroxycinnamates containing catechol structural elements
Adapted from (Rawel and Rohn 2010)

The phenolic compounds, e.g. chlorogenic acid, are not only susceptible to enzymatic oxidation but also oxidize easily non-enzymatically, in alkaline conditions. Both enzymatic and non-enzymatic oxidations occur in the presence of oxygen and produce quinone, a highly reactive substance, which normally further reacts with other quinone to produce a colored compound (dimer). Moreover, it can react covalently with amino acids incorporated in a peptide chain - for instance, lysine and cysteine - to produce the adducts (Felton *et al.* 1989, Rawel *et al.* 2000, Prigent *et al.* 2008, Schilling *et al.* 2008). Furthermore, the resulting reactive and redox active o-quinones are essential to the generation of electrophilic species capable of undergoing a nucleophilic addition to proteins (Figure 1-1) (Rawel and Rohn 2010). The application of a soft ionization technique in mass spectrometry, such as MALDI- or SELDI-TOF-MS, can be used to characterize the modified proteins (Prigent *et al.* 2003, Rawel *et al.* 2005c). Most of the literature sources deal with non-covalent interactions between phenolic compounds and proteins. In contrast, there is only a very limited data on the covalent bonds between these two reactants.

1.1.1 Effect of phenolic compounds on structural properties of proteins

The changes induced in structural properties of proteins after reacting with phenolic compounds were studied using many methods: DSC, CD, SDS-PAGE, MALDI-TOF-MS and ANS. DSC results reported that phenolic compounds improved the thermal stability of proteins to the denaturation, while CD data showed changes in secondary and tertiary structures by changes in % of α-Helix, β-strand, β-turn and unordered structure. Moreover, SDS-PAGE and MALDI-TOF results revealed the formation of high molecular fractions.

Finally, a decrease in the surface hydrophobicity of proteins was observed using the ANS method (Rawel *et al.* 2002b, Kroll *et al.* 2003, Rohn *et al.* 2005, Prigent *et al.* 2007, Aewsiri *et al.* 2009).

1.1.2 Effect of phenolic compounds on nutritional properties of proteins

The composition of amino acids, ratios of essential amino acids, susceptibility to hydrolysis during digestion, source, and the effects of processing are parameters which have an effect on the nutritional value of food proteins. The covalent and non-covalent binding of phenolic substances, in the presence and absence of polyphenol oxidase enzyme, to ε-amino groups on protein may cause deterioration in foods during processing and storage. Therefore, they lead to a loss in nutritional quality - a very important factor, especially in underdeveloped countries (Friedman 1997). The biological values and digestibility of proteins were decreased after the enzymatic and non-enzymatic reactions with different phenolic compounds (Horigome and Kandatsu 1968, Friedman *et al.* 1984, Petzke *et al.* 2005, Rohn *et al.* 2006). Moreover, the enzymatic and alkaline modifications of proteins with commercial and natural phenolic compounds decreased the available amino acids: lysine, cysteine, and tryptophan contents (Suryaprakash and Prakash 1995, Kroll *et al.* 2001, Rawel *et al.* 2002a, Rawel *et al.* 2002b, Kroll *et al.* 2003, Rawel *et al.* 2006, Aewsiri *et al.* 2009). Aside from lysine, cysteine and tryptophan, studies have also reported that methionine, histidine, tyrosine, and N-terminal proline of the protein molecule have been observed to be reaction partners (Rawel *et al.* 2002b, Kroll *et al.* 2003).

1.1.3 Effect of phenolic compounds on functional properties of proteins

Functional properties of proteins are those physicochemical properties which affect proteins' behavior in food systems during processing, preparation, consumption and storage, as well as contribute to the quality and sensory attributes of food systems. The most important functional properties of proteins in food applications are protein solubility, antioxidative capacity, foaming and emulsifying properties (Kinsella and Melachouris 1976). In recent studies, incubation of proteins with phenolic compounds at alkaline and enzymatic conditions caused a decrease in the solubility of proteins (Rawel *et al.* 2000, Rawel *et al.* 2002a, Kroll *et al.* 2003, Rohn *et al.* 2005, Prigent *et al.* 2007). On the other hand, the antioxidative capacity of

proteins was increased (Rohn *et al.* 2004, Almajano *et al.* 2007, Aewsiri *et al.* 2009). The interactions between phenolic compounds and proteins play a role in haze formation in beverages (Siebert 1999).

Improvement of network formation in gelatin gels, and giving them a greater mechanical strength and a higher thermal stability, can occur when proteins react covalently with phenolic compounds (Strauss and Gibson 2004). Emulsifying properties of proteins were changed after modification with phenolic compounds, where the stability of emulsions prepared using these proteins as emulsifiers was decreased, and the size of oil droplets was increased (Aewsiri *et al.* 2009). Finally, recent studies emphasize the potential utilization of such interactions as new emerging techniques – for example, the use of pigmentation to improve the color and hue of foodstuffs (e.g. wine, protein-enriched meals); or the application of hetero-cross-linking to improve the quality of bread, emulsions or foams in food processing (Rawel and Rohn 2010).

1.2 Polyphenol oxidase (PPO)

Polyphenol oxidases (PPOs) are copper - containing enzymes, which catalyze two different oxidative reactions (Figure 1-2): First, hydroxylation of monophenols to *o*-diphenols, by inserting oxygen in a position ortho- to an existing hydroxyl group in an aromatic ring (cresolase or monophenol monooxygenase activity EC 1.14.18.1). Secondly, oxidation of *o*-diphenol to the corresponding quinone (catecholase or diphenolase activity EC 1.10.3.1), which can react in further interactions, by polymerization with other phenolic compounds to form brown colored products, or by covalent reactions with nucleophilic molecules such as proteins (Figure 1-1) (Mazzafera and Robinson 2000, Prigent 2005, Mayer 2006, Ni Eidhin *et al.* 2006, Rawel and Rohn 2010). The distribution of PPO enzyme seems to be almost ubiquitous in animals, plants, fungi and bacteria. The plant PPO was detected in the chloroplast bound to thylakoid membranes (Martinez and Whitaker 1995). Apples, mushrooms, potatoes, bananas, peaches, and wines are rich sources for PPO enzyme (McEvily *et al.* 1992). The quality of fruits, vegetables and beverages, such as the appearance and organoleptic properties, is strongly associated with browning process during handling, processing and storage (McEvily *et al.* 1992).

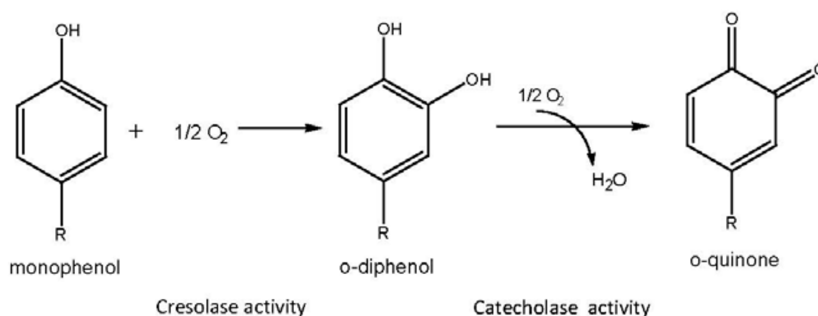


Figure 1-2 Reaction mechanism of PPO enzyme

Adapted from (Claus and Decker 2006)

Two mechanisms for food browning have been reported: enzymatic by PPO enzyme (as mentioned above); and non-enzymatic by the Maillard reaction that occurs during the heating of mixtures of amino acids and reducing sugars, in addition to the polymerization of oxidized phenolic compounds (Benjakul *et al.* 2005). In the last years, many efforts were made to find natural and nontoxic substances which can inhibit the polyphenol oxidase activity. Mushroom PPO activity was inhibited by various glycyl-dipeptides (e.g. GlyAsp, GlyGly, GlyHis and GlyLeu) (Girelli *et al.* 2004). The inhibition of PPO activity using proteins, peptides and amino acids can be explained by reacting with the *o*-quinones and chelating the essential copper at the active site of enzyme (Goetghebeur and Kermasha 1996, Gouzi *et al.* 2010).

SDS-PAGE for partially purified extracts of leaves and the endosperm of coffee beans showed two PPO bands with a molecular weight of approximately 45 and 64 kDa (Mazzafera and Robinson 2000, Goulart *et al.* 2003). The 64 kDa protein was a pre-protein of the 45 kDa protein, which was released after hydrolysis of a transit peptide by a plastid peptidase (Koussevitzky *et al.* 1998). Moreover, PPO isolated from Bramley's seedling apples had a recorded molecular weight of about 45 kDa (Benjakul *et al.* 2005, Ni Eidhin *et al.* 2006). The optimum pH for PPO enzyme isolated from different sources varied from 4.5 – 7.5, depending on the substrates used, such as 4-methylcatechol, chlorogenic acid, (+) – catechin and pyrogallol (Janovitz-Klapp *et al.* 1989, Richard-Forget *et al.* 1992, Zhou *et al.* 1993, Rocha and Morais 2001, Gouzi and Benmansour 2007). Furthermore, the optimum temperature observed in those reports ranged from 20°C to 30°C.

1.3 Characterization of green coffee beans

The coffee plant belongs to Rubiaceae family, *Coffea* genus, which comprises more than 70 other species. The two most commonly grown and consumed species are *Coffea arabica (CA)* and *Coffea robusta (CR)* (Ramalakshmi *et al*. 2008). *CA* accounts for 70-80% of the world's production, whereas *CR* represents about 20% (Kim and Lee 2010, Crozier *et al.* 2012). Green coffee (GC) beans are the seeds of bright red cherries of the coffee plant after drying and removing their outer casing (Tfouni *et al.* 2012). The chemical composition and quality of coffee beans are influenced by several parameters; for instance, the specific variety, species, the cultivated area and its weather, harvesting conditions, drying methods, and storage conditions (Schenker 2000, Silva *et al.* 2005). The content of crude protein in green *CA* and *CR* beans ranges from 8.5 to 13.8 %, after being corrected for the content of caffeine and trigonelline (Belitz *et al.* 2004, Casal *et al.* 2005, Rawel *et al.* 2005b). No significant differences in peptide content between five types from *CA* and four types from *CR* were found, while significant differences in peptide composition were observed. Molecular masses of the peptides in both coffees were between 4 kDa and 10 kDa (Ludwig *et al.* 2000).

Moreover, differences between green *CA* and *CR* in the concentration of free amino acid were observed; for *CA,* the concentration ranged from 2.7±0.1 to 4.8±0.3mg/kg; and, for *CR* from 3.5±0.2 to 6±0.3 mg/kg on DM (Arnold *et al.* 1994). Further, both coffees contained the same major and minor amino acids (Arnold *et al.* 1994, Casal *et al.* 2005), where the main amino acid was glutamic acid. Asparagine and aspartic acids were also observed in large amounts. In contrast, serine and alanine were present in smaller amounts. The storage of green *CA* under hermetic conditions for up to two years did not show significant changes, either in the total content of protein amino acids or in the concentration of most individual amino acids. Only the concentration of glutamine was significantly decreased (Selmar *et al.* 2008). Coffee deterioration can be monitored by endogenous coffee enzyme activities, esp. polyphenol oxidase, which decreases during coffee storage. The relationship between PPO activity and coffee quality is not well known. It appears to be possible that PPO may act as a proxy for other endogenous enzymes of coffee that preferentially modify lipids, proteins, carbohydrates or precursors of coffee flavor during storage, and that thereby affect final beverage quality (De Amorim and Silva 1968, Silva *et al.* 2005, Hodges and Farrell 2008).

Caffeine is an alkaloid with the substituted purine ring system. Coffee beans are one of the richest sources of caffeine. Consumer preference is given to *CA* than *CR* due to its organoleptic properties (Alonso-Salces *et al.* 2009, Crozier *et al.* 2012), where *CR* contains about 40 – 50% more caffeine than *CA* (Belachew 2003, Belitz *et al.* 2004, Casal *et al.* 2005, Hečimović *et al.* 2011). GC beans contain large amounts of chlorogenic (CQA), caffeic acids and other polyphenolic compounds, which are strong antioxidants in *in vitro* systems (Rice-Evans *et al.* 1996). So, in the last years, most of the literature has focused on the antioxidant capacity of coffee bean, which depends on the coffee species and varieties, e.g. *CR* beans yield greater antioxidant activity than *CA* beans. The antioxidant capacity of coffee beans can be explained by the presence of both natural phenolics and compounds formed during processing. Moreover, it has been argued that this could also be attributed to caffeine content (Hečimović *et al.* 2011, Vignoli *et al.* 2011).

1.3.1 Phenolic compounds in coffee beans

Total phenolics (as chlorogenic acid equivalents) of *CA* and *CR* present (4.8-5.7%) and (7.6%) on DM basis, respectively (Farah *et al.* 2005, Kim and Lee 2010).

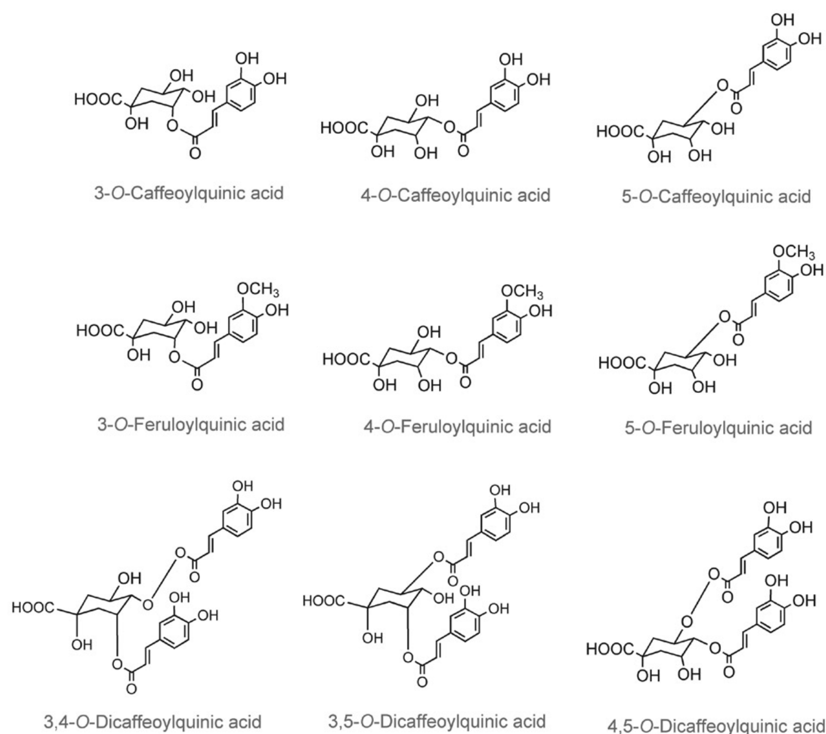


Figure 1-3 Major isomers of chlorogenic acid in green coffee beans

Adapted from (Stalmach *et al.* 2006)

Furthermore, many authors tend to report the total phenolics content as gallic acid or catechin equivalents. For example, Ramalakshmi *et al.* (2007) found that total polyphenols are less in defective coffee beans (3.0% to 4.1% - as gallic acid equivalents) when compared to graded beans (3.1% to 4.5%). Extensive literature exists on the composition of coffee phenolics, and more than 50 hydroxycinnamic acid derivatives have been identified recently (Jaiswal and Kuhnert 2010, Jaiswal and Kuhnert 2011). Coffee beans are one of the richest sources of caffeoylquinic acids (chlorogenic acids - CQA), an ester formed between caffeic and quinic acid. Where, besides CQA with three isomers (3-CQA, 4-CQA, and 5-CQA), dicaffeoylquinic acids (diCQA) with three isomers (3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) and feruloylquinic acids with three isomers (3-FQA, 4-FQA and 5-FQA) were found (Figure 1-3) (Campa *et al.* 2003, Farah *et al.* 2006, Stalmach *et al.* 2006, Koshiro 2007).

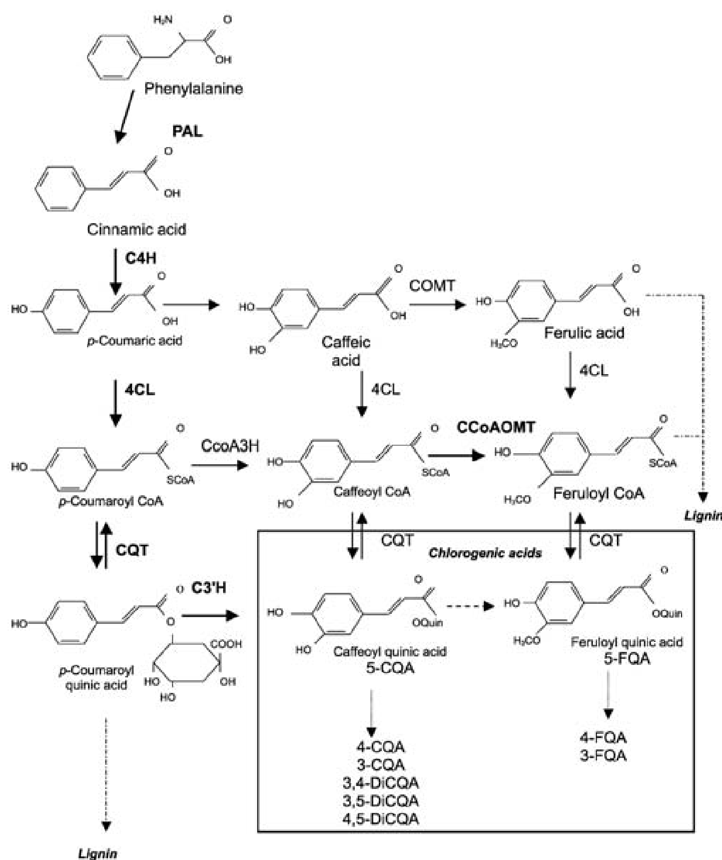


Figure 1-4 Different theoretical ways of chlorogenic acid synthesis in coffee trees

Where: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4 (hydroxy)cinnamoyl-CoA ligase; CQT, hydroxycinnamoyl- CoA:D-quinate hydroxycinnamoyltransferase; COMT, caffeic-acid/ 5-hydroxyferulic acid O-methyltransferase; CCoAOMT, caffeoyl- CoA O-methyltransferase; C30H, p-coumaroyl-shikimate/quinate 30- hydroxylase; DiCQA: dicaffeoyl quinic acid, adapted from (Campa *et al.* 2003)

Theoretical modes of CQA synthesis in coffee trees was shown in Figure 1-4. Various studies have shown that the CQA content of coffee beans is dependent on the species, varieties and processing conditions of the beans. For instance, differences were found in the total content of CQA, FQA and di-CQA between Guatemala and Brazil types representing *CA;* and the Uganda type representing *CR* (Trugo and Macrae 1984, Farah *et al.* 2005). Moreover, they reported that 5-CQA was always the main CQA derivative in all types.

1.3.2 Effects of various stages of roasting on the coffee beans

After roasting coffee beans at different degrees, the protein content was decreased. Only 13% of the roasted coffee protein was extracted into the brew coffee. This can be explained by the denaturation of protein; a reduced water solubility; and chemical reactions during roasting (Bekedam *et al.* 2006). A series of complex reactions is responsible for the flavor development during the roasting of coffee beans. Some of them may be attributed to Maillard and Strecker reactions, degradation of proteins, polysaccharides, trigonelline, and chlorogenic acids (De Maria *et al*. 1996, Jaiswal *et al.* 2012). Further, this process seems to be preceded by intensive interactions between the nitrogenous compounds and phenolic compounds (mainly CQAs) during the roasting process (Montavon *et al.* 2003b, Rawel *et al.* 2005b), and a reaction scheme can be postulated for the autoxidative mechanisms that occur during roasting in Figure 1-5.

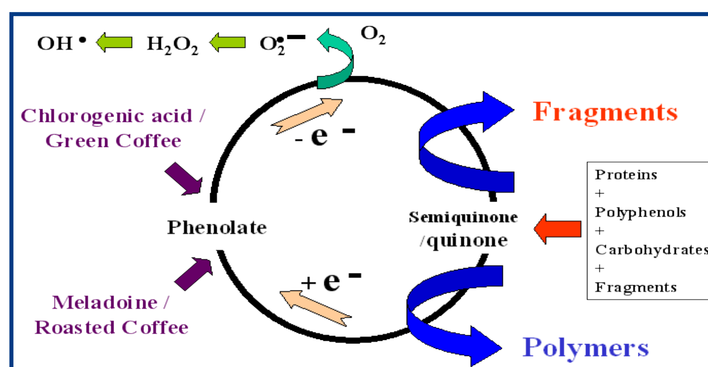


Figure 1-5 Proposed reaction scheme of autoxidative mechanisms in coffee

Adapted from (Montavon *et al.* 2003b)

The roasting process decreased the amino acid content in both *CA* and *CR* at different rates (Casal *et al.* 2005). This might be ascribed, not only to their inherent thermal stabilities, but also to the coffee matrix itself, since the same amino acids behaved differently in *CA* and *CR*.

Moreover, Hečimović *et al*. (2011) reported that coffee beans roasted at a light degree contained the highest caffeine content, which exhibited a decrease with a more intensive roasting compared to green coffee beans. The roasting conditions play an important role in the chlorogenic acid content in roasted coffee beans. For example, total chlorogenic acids were reduced to nearly 50% and almost to trace levels when green beans roasted at 230 °C for 12 min and at 250 °C for 21 min, respectively (Moon *et al.* 2009). Also, Farah *et al*. (2005) reported that after 5 min of roasting at 230 °C, the levels of 5-CQA had decreased substantially, while the levels of 3-CQA and 4-CQA had increased to twice their original values. Finally, coffee beans, which were roasted at different degrees - light, medium and dark - showed that the maximum total antioxidant activities were observed for light and medium roasting, although the CQA content of these samples decreased. Meanwhile, the lower antioxidant activity was observed for the dark coffee compared to the unroasted coffee (del Castillo *et al.* 2002, Somporn *et al.* 2011). In contrast, Daglia *et al.* (2000) reported that, the antioxidant capacity values were decreased slightly with light roast and increased with stronger roast.

1.4 Bioavailability, uptake and metabolism of chlorogenic acid (CQA)

The bioavailability of polyphenols depends on the chemical structure, type of compounds, food matrix, environmental factors, food processing, interaction with other compounds (bonds with proteins), extent of conjugation and individual colon microflora (Gonthier *et al.* 2003, D'Archivio *et al.* 2010, Shivashankara and Acharya 2010). Therefore, according to a few studies, the relative bioavailability of some polyphenols (hydroxycinnamates) were found to be in the following order: chlorogenic acid < rosmarinic acid < caffeic acid < ferulic acid < p-coumaric acid (Zhao and Moghadasian 2010). Moreover, bioavailability of CQA is affected by the addition of milk in coffee, because the milk protein can interact with polyphenols (Dupas *et al.* 2006, Duarte and Farah 2011). Normal human diets contain polyphenols and tannins, where the range from below 100 mg to higher than 2 g may be the daily intake (Clifford 2004). In contrast, the daily dietary intake of total CQA may vary substantially from roughly zero to perhaps close to 1g, and the coffee alone provides approximately 70% of the total (Clifford 2000, Olthof *et al.* 2001).

Previous studies reported that very little of the consumed dietary polyphenols reaches human plasma, where only some 5-10% is absorbed in the duodenum and over 95% of the ingested amount passes to the colon. After that, it may be metabolized by the gut's microflora, and the microbial metabolites absorbed and thus appear in the plasma as mammalian conjugates (Yang *et al.* 2001, Clifford 2004). CQA and caffeic acids absorb in the small intestine of humans and animals (Azuma *et al.* 2000, Olthof *et al.* 2001, Manach *et al.* 2004, Lafay *et al.* 2006a, Lafay *et al.* 2006b, Monteiro *et al.* 2007, Del Rio *et al.* 2010). Moreover, whereas caffeic acid – which is produced from hydrolysis of CQA by the action of esterases in colonic microflora – could be absorbed in the alimentary tract, CQA in its intact form could not be well absorbed in this part.

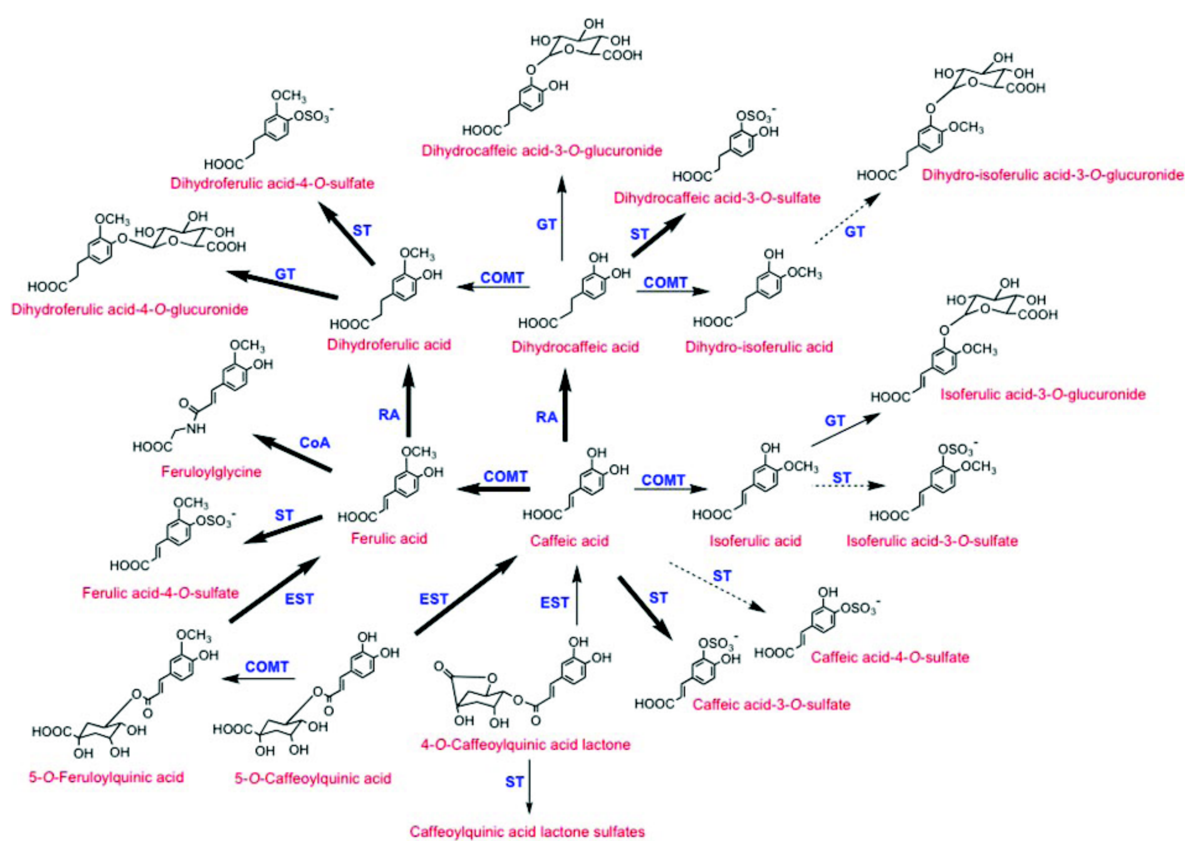


Figure 1-6 Proposed metabolism of chlorogenic acids following the ingestion of coffee by human volunteers

Where: 5-*O*-CQA and 5-*O*-FQA are illustrated structures but their respective 3- and 4-isomers would be metabolized in a similar manner and likewise with 4-*O*-CQAL and 3-*O*-CQAL. COMT, catechol-*O*-methyltransferase; EST, esterase; RA, reductase; GT, UDP-glucuronyltransferase; ST, sulfuryltransferase; Co-A, co-enzyme A. Bold arrows indicate major routes, dotted arrows minor pathways. Steps blocked in subjects with an ileostomy and hence occurring in the colon are indicated. Adapted from (Stalmach *et al.* 2009)

Azuma *et al.* (2000) proposed that the quinic acid moiety in the structure of CQA may be one of the reasons inhibiting CQA absorption in its intact form. Proposed metabolism of CQAs following the ingestion of coffee by healthy human volunteers is illustrated in Figure 1-6 (Stalmach *et al.* 2009, Stalmach *et al.* 2010). CQA and caffeic acids have been identified in rat plasma after the consumption of a diet supplemented with CQA (Azuma *et al.* 2000, Gonthier *et al.* 2003, Lafay *et al.* 2006a). Moreover, CQA derivatives, 3-CQA, 4-CQA, 5-CQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA, and 4,5 diCQA, were identified in human plasma after coffee consumption (Monteiro *et al.* 2007, Farah *et al.* 2008, Stalmach *et al.* 2009). However, the amount of 5-CQA alone was higher than other identified hydroxycinnamates in plasma.

1.5 Health impacts of chlorogenic acid

The most common individual chlorogenic acid is 5-O -Caffeoylquinic acid (5-CQA). It exists as trans and cis isomers and during processing; trans isomers may be partially converted to cis (Mathew and Lakshminarayana 1969, Clifford 1999). Plant polyphenols have drawn increasing attention due to their antioxidant properties and their noticeable effects in the prevention of various oxidative stress - associated diseases. The action of phenolics as antioxidants is viewed as beneficial in both foods (e.g. gallates as a supplementary food ingredient) and the body, where they are oxidized to other food constituents or cellular components and tissues (Meyer *et al.* 1998, Robards *et al.* 1999, Miura *et al.* 2001, Rawel *et al.* 2007). Both chlorogenic and caffeic acids have been shown to be strongly antioxidant in *in vitro* systems, and there are no differences between caffeic acid and chlorogenic acid in their inhibitory effects on LDL oxidation (Rice-Evans *et al.* 1996, Olthof *et al.* 2001, Rodriguez de Sotillo and Hadley 2002, Ramalakshmi *et al.* 2007, Hoelzl *et al.* 2010).

CQA are known for their contribution to the final acidity, flavor, astringency and bitterness of the beverage. Furthermore, CQA and their lactones may have a potential bio-pharmacological importance to humans. The pharmacological activities of phenolic compound such as CQA have been associated with its antioxidant properties because they are thought to have positive effects on depression, alcoholism, chronic degenerative diseases, cardiovascular disease and cancer (Yang *et al.* 2001, Gonthier *et al.* 2003, Li and Chang 2005, Hoelzl *et al.* 2010, Zhao and Moghadasian 2010). CQA increased the concentration of copper, magnesium, sodium, and potassium in the plasma and improved glucose tolerance. Aside from this, CQA decreased

levels of cholesterol and triacylglycerols (in plasma, liver, adipose tissue and heart), in addition to significantly lowering insulin levels, enhancing lipid metabolism, and displaying an anti-obesity property (Rodriguez de Sotillo and Hadley 2002, Cho *et al.* 2010). Instant coffee enriched by 5-CQA affected the absorption and utilization of glucose from diet (Thom 2007). CQA showed competitive inhibition for glucose 6-phosphatase (Arion *et al.* 1997, Hemmerle *et al.* 1997). In addition, it effectively inhibited the growth of bacterial pathogens by binding it to the outer plasma membrane, disrupting the membrane, exhausting the intracellular potential, and releasing cytoplasm macromolecules, including nucleotide, which led to cell death (Lou *et al.* 2011). In another study, coffee extracts displayed antimicrobial activity (Almeida *et al.* 2006). Finally, many health benefits have been reported after the consumption of coffee, which has a high amount of CQA derivatives; for example, studies report a decrease in the relative risk of Alzheimer's disease, cardiovascular disease and diabetes type 2 (Lindsay *et al.* 2002, Salazar-Martinez *et al.* 2004, Ranheim and Halvorsen 2005).

1.6 Coffee storage proteins

Storage protein accounts for 45% of the total protein content of green coffee beans. The most abundant of these proteins are the legumin-like seed storage proteins of the 11S size class (Acuña *et al.* 1999, Rogers *et al.* 1999, Baú *et al.* 2001). The typical structure of an 11S storage protein consists of 3−6 monomers, which migrate into storage vacuoles (protein bodies) and generate by hydrophobic interactions the tri- and hexameric quaternary forms, with molecular weights of 150 − 400 kDa (Shutov and Vaintraub 1987). The rupture of the disulfide bonds in 11S monomers under reducing conditions releases the α (acidic) and β (basic) subunits (Fukushima 1991). *Coffea arabica (CA)* proteins, in the absence of a reducing agent, showed subunit with molecular weight of 55 kDa, and in the presence of reducing agent (2-mercaptoethanol) consisted of two polypeptides with molecular weights of 33 and 24 kDa (Acuña *et al.* 1999, Rogers *et al.* 1999).

Furthermore, under non-reducing conditions, *Coffea robusta (CR)* contained an abundant protein subunit at 58 kDa, producing two polypeptides under reducing conditions, with the α-component having ~32–38 kDa and the β-component ~20–22 kDa (Nunes and Coimbra 2002, Montavon *et al.* 2003a). More detailed studies of the coffee seed legumin suggest the presence

of different isoforms (Acuña *et al.* 1999, Rogers *et al.* 1999, Montavon *et al.* 2003a). Caffeoyl derivatives of amino acids have been isolated from green coffee (GC) beans. For example, caffeoyltryptophan in commercial green *CR* brands was identified (Morishita *et al.* 1987). The development of mass spectrometric methods has recently provided a more detailed biochemical characterization of the interactions of CQA and amino acids (Prigent *et al.* 2008, Schilling *et al.* 2008, Rawel and Rohn 2010). Despite the influential role that coffee proteins play in many chemical reactions during maturation, storage and roasting, little is known about it. A few studies have been carried out with *CA*, these especially with the water soluble fractions giving some indications of their importance in the coffee beverage quality (Amorim and Josephson 1975, Bade and Stegemann 1982, Arnold and Ludwig 1996, Baú *et al.* 2001).

1.7 Faba bean proteins

The faba plant belongs to the Fabaceae family, *vicia* genus. The faba bean (FB) is considered one of the main sources of cheap protein (27-34%), depending on genotypes, and energy in Africa, parts of Asia and Latin America (Haciseferogullari *et al.* 2003). Most of these proteins comprise globulins (79%), albumins (7%) and glutelins (6%) (Hossain and Mortuza 2006). The major seed storage proteins of FB are 7S and 11S globulins (Kimura *et al.* 2008). Moreover, the high lysine content encouraged the use of FB as a protein supplement for cereals in Saudi diets (El Fiel *et al.* 2002). The lowest solubility of dehulled FB protein isolate was found at a pH range between 4.0 and 6.0. In contrast, maximal solubility was obtained at pH values of 8.0 and 9.0 (Fernández-Quintela *et al.* 1997). Feeding rats on diets containing FB seeds, or the FB protein isolate, decreases significantly (LDL+VLDL) cholesterol in plasma, yet not in HDL-cholesterol; it also reduces the hepatic cholesterol and triacylglycerol (Macarulla *et al.* 2001). Furthermore, when the levels of anti-nutritional factors present are very low, dehulled FB meal may be used successfully as a substitute to soybeans as a source of amino acids for laying hens (Magoda and Gous 2011).

1.8 Whey proteins

Whey proteins (WP) are a by-product which is prepared from milk and cheese industries (Solak and Akin 2012). By the end of the first decade of the 21st century, they evolved to a variety of sophisticated and targeted food ingredients with a range of functional and

nutritional properties (Phillips and Williams 2011). Bovine whey proteins (WP) are one of the highest nutritious food supplements available for commercial use because of their high content in branched chain and essential amino acids (Bucci and Unlu 2000, Walzem *et al.* 2002).

WP present 20% of total milk protein and consist of several different protein fractions, including: β-lactoglobulin α-lactalbumin, immunoglobulin, glycomacropeptide (GMP), bovine serum albumin, lactoferrin and protease-peptones (Walzem *et al.* 2002, Doultani *et al.* 2003, Krissansen 2007, Solak and Akin 2012). Glycomacropeptide (GMP) results from action of chymosin on casein during the cheese making process and presents 10-15% of WP (Solak and Akin 2012). Moreover, BioZate1 (BZ1), which is prepared from the partially hydrolyzed whey proteins of milk, is a commercial food product of whey proteins containing bioactive peptides (Hui *et al.* 2007).

1.8.1 Functional properties of whey proteins (WP)

The functional properties of WP (e.g., gelation, emulsification, solubility, water binding, flavor binding, foaming, and viscosity development) are related to their efficacy in food production (Walzem *et al.* 2002). Consequently, WP are valuable food ingredients due to their ability to aggregate and provide structure to foods, their solubility over a wide pH range and other many functional properties (Kinsella 1984, Smithers *et al.* 1996, Rade-Kukic *et al.* 2011). Whey proteins are highly soluble over a wide pH range from 2.0 to 10.0; thus, they stabilize emulsions by creating interfacial films between hydrophilic and hydrophobic food components. Moreover, WP and its components could be used in the production of infant formulas, sports nutrition foods, meats, seafood, bakery, confections, snack foods, beverages and other food products, to improve their functional properties (Walzem *et al.* 2002, Veith and Reynolds 2004). For example, WP can be used in confections as a replacement for egg white, and in meat products as an extender (Varnam and Sutherland 1994).

1.8.2 Nutritional properties of whey proteins

Whey proteins can be used to fortify or enhance foods for several advantages compared to other proteins. For instance, WP contain the highest concentration of all the essential amino acids compared to any other natural food protein source (Walzem *et al.* 2002). Therefore, their

biological value is higher than other proteins (Shah 2000). WP have the highest amounts of branched-chain amino acids and they are a rich and balanced source of sulfur amino acids (methionine, cysteine). These amino acids provide a critical role as antioxidants (Shoveller *et al.* 2005, Pal *et al.* 2010). Hydrolysis of WP break down possible allergenic structures, so WP and their hydrolysates are used in infant formula, specialist enteral nutrition and in hypoallergenic infant formula (Phillips and Williams 2011). The high nutritional and functional value of β-LG - the main component in WP - is widely recognized and has made this protein an ingredient of choice in the formulation of modern foods and beverages (Chatterton *et al.* 2006, Kehoe *et al.* 2007).

1.8.3 Health benefits of whey proteins

WP contain three to four times more bioavailable cysteine than other proteins. Cysteine is a source of precursor for glutathione synthesis, an antioxidant and anticarcinogenic tripeptide produced by the liver for protection against intestinal tumors, in addition to its immune stimulatory properties (McIntosh *et al.* 1995, Solak and Akin 2012). WP play an important role in different applications: antioxidative, anticancer, anti-inflammatory, antihypertensive, antimicrobial and others (Krissansen 2007, Madureira *et al.* 2007). Moreover, WP play a role in the organization of the whole body protein metabolism (Bounous G *et al.* 1989, Walzem *et al.* 2002). In addition, they decrease the concentration of plasma and liver cholesterol as well as plasma triacylglycerols, in comparison to casein (Ritzel *et al.* 1979, Zhang and Beynen 1993, Pal *et al.* 2010). Later authors noticed that the supplementation with whey protein decreased both fasting insulin levels and homeostasis model assessment of insulin resistance scores compared to baseline. The inhibition of hepatic cholesterol synthesis by whey protein could explain the decrease in cholesterol concentration (Zhang and Beynen 1993).

Glycomacropeptide (GMP) has been reported to show antibacterial, antiviral, and immunomodulatory activities (Solak and Akin 2012). Also, foods fortified with GMP are used in feeding patients who suffer from phenylketonuria (PKU) disease (Brody 2000, Shah 2000, Walzem *et al.* 2002, Krissansen 2007). Furthermore, foods supplemented by GMP protein increased zinc absorption in infant rhesus monkeys (Kelleher *et al.* 2003). More recently, animal and clinical tests reported that the bioactive peptides (BZ1), which contain angiotensin converting enzyme (ACE) inhibitor peptides, appeared to help maintain a healthy blood

pressure - thus attracting more attention due to the importance of high blood pressure as a major risk factor in cardiovascular diseases (Hui *et al.* 2007).

1.8.4 β-Lactoglobulin (β-LG)

β-Lactoglobulin (β-LG), molecular weight (MW) 18362 form A and 18276 form B, is the major and most important protein in whey proteins (50 - 62% of WP), followed by α-Lactalbumin (25% of WP, MW 14000). It is found in the milk of cows and other ruminants, as well as in the milk of some non-ruminants such as pigs, horses and dolphins (McHugh and Krochta 1994, Walzem *et al.* 2002, Doultani *et al.* 2003, Krissansen 2007, Hernández-Ledesma *et al.* 2008, Phillips and Williams 2011). β-LG is defined as a retinol binding protein, which could play a role in the availability and absorption of vitamin A. The molecular weight of the β-LG monomer is composed of about 162 amino acids. It has five cysteine (Cys) units, four participate in two disulfide bridges (Cys 66-160 and Cys 106-119) and one is free (Cys 121) as shown in Figure 1-7. Cys 121 is buried between the β-sheet and the α-helix (Brownlow *et al.* 1997, Kontopidis *et al.* 2002). β-LG is one of the richest known food sources of the amino acids Leu, Ile and Val, where the β-LG molecule contains 22, 10 and 9 residues, respectively. Consequently, these amino acids giving β-LG some strongly hydrophobic regions, which are important for its functional properties (Phillips and Williams 2011).

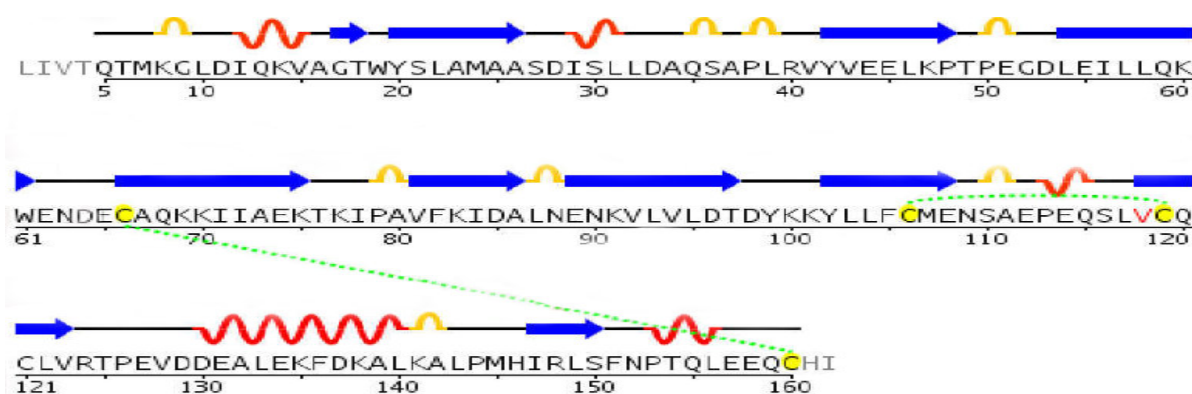


Figure 1-7 Amino acid sequence of β-lactoglobulin A

Where: β−turns, α- helices, β−strands) and disulphide bridges, adapted from (Kukic 2009)

The structure of β-LG contained α-helix (17-25%), β-sheet (37-40%), reverse turn (22-25%) and random coil (4-10%) (Creamer *et al.* 1983). The quaternary structure of β-LG depends on the medium pH, where at pH values between 5.2 and 7, it is stable as a dimer; with

a molecular weight of ca. 36,700 kDa, at pH values between 3.5 and 5.2 as an octamer; with a molecular weight of ca. 140,000 kDa, and at pH 3.0 and above 8.0, it is as a monomer with a molecular weight of ca. 18,000 kDa (de Wit 1989).

1.8.4.1 Digestibility and biological roles of β-LG

Despite the fact that β-LG properties have been studied using available biochemical technique, no specific biological function has been ascribed definitively to the protein. However, some functions have been reported. In the absence of direct crystallographic evidence, a preliminary modeling study revealed an internal cavity on the outer surface of protein, which can readily accommodate retinol and fatty acids, such as palmitate and stearate, in a manner similar to the related lipocalin, followed by transporting them (Sawyer *et al.* 1998, Sawyer and Kontopidis 2000). A study on the digestibility of native and heated-denatured β-LG *in vitro* and *in vivo* reported that, β-LG was not hydrolyzed by pepsin samples at pH (2-7) *in vitro*. On the other hand, heat-denatured β-LG was easily hydrolyzed. Further, native β-LG injected into the stomach of rats was not digested in the stomach in vivo, whereas heat-denatured β-LG was digested. However, both native β-LG and heat-denatured β-LG injected into the stomach were digested in the intestine in vivo (Kitabatake and Kinekawa 1998).
The low stability of β-LG against heat treatment is attributed to the low content of amino acid proline and the presence of cysteine, methionine and cystine residues. Its globular structure, however, is astonishingly stable against the acids and proteolytic enzymes present in the stomach (Papiz *et al.* 1986). Hernández-Ledesma *et al.* (2008) demonstrated that β-LG is an important source for biologically active peptides. Once these peptides are released by enzymatic proteolysis in vivo or in vitro, they play important roles in human health – for instance, antihypertensive, antioxidant and antimicrobial activities – in addition to their ability to decrease the body-cholesterol levels.

1.9 Lutein and lutein ester

Lutein and its isomer zeaxanthin are both dihydroxy carotenoids with the ionone ring systems being substituted at both the 3 and 3′ carbon atoms (Krinsky *et al.* 2003) (Figure 1-8). The molecular formula of lutein is $C_{40}H_{56}O_2$. Also, it has two secondary hydroxyl groups and

a polyene structure, beside ten methyl groups. From these data, lutein is classified as a xanthophyll or oxygenated carotenoid (Eichenberger and Grob 1965, Molnár *et al.* 2004).

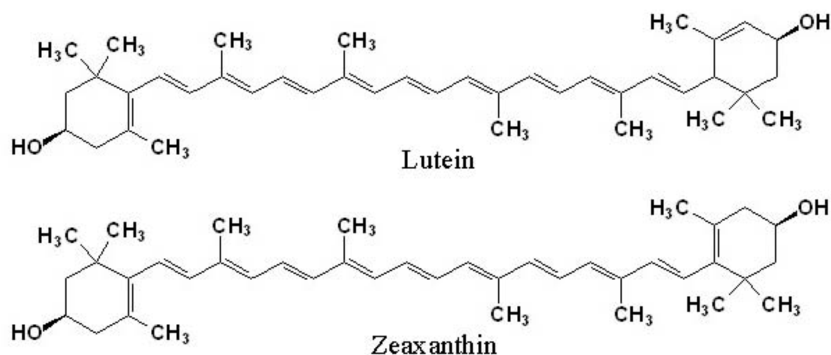


Figure 1-8 Chemical structure of lutein and its isomer zeaxanthin

Concentrated amounts of lutein and zeaxanthin are found in the retina of the eye - called macula lutea - in which they are responsible for central and high acuity vision (Landrum and Bone 2001, Alves-Rodrigues and Shao 2004). Many fruits and vegetables are sources for lutein and zeaxanthin; for example, maize has the highest content of lutein, while orange pepper has the highest amount of zeaxanthin. Additionally, substantial amounts of lutein and zeaxanthin (30– 50%) were also present in kiwi fruit, grapes, spinach, orange juice, zucchini and different kinds of squash. Moreover, they are also present in some animal products such as egg yolks, due to animals feeding on the plant products (Sommerburg *et al.* 1998). Lutein ester is an ester between lutein and various types of fatty acids such as lauric, myristic and palmitic acids. The economically most important source for lutein ester is the Tagetes species (Navarrete-Bolanos *et al.* 2005, Bhattacharyya *et al.* 2010).

1.9.1 Benefits of lutein

The high concentration of lutein in the macula lutea protects against age–related macular degeneration (AMD) (Lakshminarayana *et al.* 2008). AMD is a degenerative condition of the macula characterized by death or dysfunction of the photoreceptors (Carpentier *et al.* 2009). Epidemiologic studies suggested that xanthophylls can play a protective role in the eye and may help reduce the risk of AMD where, they indicated an inverse relationship between the lutein and zeaxanthin intake and both AMD and cataract (Ribaya-Mercado and Blumberg 2004, Bhattacharyya *et al.* 2010). In this case, the nutritional supplementation that contains lutein is highly recommended as a protective factor, where a lutein dose of 30 mg/day, in

addition to a zeaxanthin dose of 30 mg/day, raises serum concentrations and macular pigment density of these carotenoids in humans (Bone *et al.* 2003). The development of the two most common eye diseases in older people - cataract and macular degeneration - may be reduced using lutein and zeaxanthin (Mares-Perlman *et al.* 2002). Also, lutein has benefits beyond eye heath by decreasing the risk of atherosclerosis and thus preventing heart attack and stroke (Kritchevsky *et al.* 2000, Dwyer *et al.* 2001, Mares-Perlman *et al.* 2002). In addition, lutein inhibits specific isoforms of mammalian DNA polymerases and reduces inflammatory response (Horie *et al.* 2010). Moreover, it enhances skin health due to decreasing UV-induced damage (Stahl *et al.* 2000).

1.9.2 Bioavailability of lutein

Humans are not able to synthesize lutein, therefore it must be obtained through dietary lutein intake. Moreover, the bioavailability of lutein is very low, though large amounts of lutein are accumulated in the intestinal mucosa. This low bioavailability can be enhanced by interaction with food or food components. Furthermore, bile acids play an important role in the intestinal absorption of lutein, besides regulating the absorption of other lipophilic compounds such as cholesterol (Sato *et al.* 2011). Oleic acid or olive oil have also been reported as suitable fat sources to modulate the intestinal lutein absorption (Lakshminarayana *et al.* 2007). Moreover, the binding of lutein ester to proteins, in an emulsion model, may be addressed to improve its bioavailability (Ribeiro *et al.* 2006, Khalil *et al.* 2012)

1.9.3 Stability of lutein and lutein ester against UV light

Several studies on the influence of thermal treatment and light exposure on degradation and isomerization of lutein have been assessed. The UV-illumination and high temperature promoted lutein degradation, and the freezer temperature (- 20 °C) could satisfy the requirements for prevention of its degradation in solutions and in lutein - containing products (Shi and Chen 1997). Studies on the stability of lutein against UV light demonstrated that the most damaging wavelengths to lutein were identified in UV range of 200-400 nm and at 463 nm in a lutein-fortified model colloidal beverage (Kline *et al.* 2011). A recent study demonstrated that emulsified lutein ester with medium-chain triacylglycerols (MCT) oil exhibited the highest stability against a UV light (365 nm) compared to lutein ester in MCT oil extract without emulsification (Khalil *et al.* 2012).

2. Aim

The interactions of proteins with phenolic compounds have recently been gaining increasing interest. The phenolic compounds as food components represent the largest group of secondary metabolites in plant foods. Plant polyphenols have drawn increasing attention due to their antioxidant properties and their noticeable effects in the prevention of various oxidative stress - associated diseases. The phenolic compounds - e.g. chlorogenic acid (CQA) - are susceptible to oxidation by enzymes, especially polyphenol oxidase (PPO), and at alkaline conditions. Both enzymatic and non-enzymatic oxidations occur in the presence of oxygen and produce quinone, which normally further reacts with other quinone to produce colored products (dimers). Additionally, quinone is capable of undergoing a nucleophilic addition to proteins.

Green coffee beans are one of the richest sources of chlorogenic acids. Therefore, a green coffee extract would provide an eligible food relevant source for phenolic compounds for the modification of proteins. Lutein ester, labile lipophilic bioactive molecule, is sensitive to UV light. Moreover, humans are not able to synthesize lutein, so it has to be obtained through dietary lutein intake. Based on this background, the aims of the current study were as follows:

1- **Study 1**: Characterization of different apple varieties (one of the main sources of polyphenol oxidase [PPO]), in order to select the type with a high PPO activity and low phenolics content, and to use it in study 3. Moreover, it aims to describe the interactions of CQA with model amino components and proteins.

2- **Study 2**: Characterization of phenolic compounds in different coffee varieties. Despite the immense economic importance of coffee, only few studies have dealt with the biochemical and molecular properties of its seed storage proteins. Thus, this study aims to increase the knowledge of the composition and structure of green coffee bean proteins and to optimize the protein extraction, characterize the modifications by CQA, as well as to conceive the influence of the interactions with the phenolic compounds on structural and functional properties of protein. Finally, application of these proteins in a model multifunctional food system is envisaged.

3- **Study 3**: The interactions between phenolic compounds and milk whey proteins is an interesting research topic since these phenolic compounds are related to their dual role as substrates for oxidative browning reactions and as antioxidants. Therefore, the objectives

of this study were to modify (using enzymatic and alkaline conditions) the whey protein products (β-lactoglobulin, BioZate 1 and Glycomacropeptide) with an aqueous green coffee extract and commercial CQA, under the implementation of food relevant processing conditions; the characterization of the modified proteins with respect to the site and type of modification; and moreover to characterize the physico-chemical, structural and functional properties of such modified proteins. A final objective was to study the possibility for incorporation of these multifunctional ingredients into various food products and to enable consumers to benefit from the health - promoting effects of phytonutrients found in vegetables. For example, to combine high nutritional quality proteins, modified proteins, with the health - promoting substances (e.g. phenolic compounds) into a multifunctional ingredient that could be used to provide a better accessibility, protection and bioavailability of bioactive, easily oxidized lipophillic compounds (such as lutein ester).

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and instruments

Table 3-1 List of chemicals

Name	Company	Country
1-anilino-8-napthalensulfonate (ANS)	Sigma	Germany
2,2-diphenyl-1-picryl hydrazyl (DPPH)	Alfa Aesar	Germany
3,4- dicaffeoylquinic acid (3,4- di-CQA)	DEK GmbH	Germany
3,5- dicaffeoylquinic acid (3,5- di-CQA)	DEK GmbH	Germany
3-caffeoylquinic acid (3- CQA)	DEK GmbH	Germany
4- Methylcatechol, 95%	Sigma Aldrich	Germany
4,5- dicaffeoylquinic acid (4,5- di-CQA)	DEK GmbH	Germany
4-caffeoylquinic acid (4- CQA)	DEK GmbH	Germany
5,5`- Dithio-bis(2-Nitrobenzoic acid)	Sigma	USA
5-caffeoylquinic acid (5- CQA), 95%T	Sigma Aldrich	Germany
6-Hydroxy-2, 5, 7, 8-tetra-methylchroman-2 carboxylic acid	Sigma Aldrich	Germany
α-Cyano-4-hydroxy cinnamic acid	Bruker Daltonik	Germany
β-Mercaptoethanol	Roth	Germany
ABTS+	Sigma Aldrich	Germany
Acetic acid	Carl Roth	Germany
Acetonitrile	Sigma Aldrich	Germany
Acrylamid 2x	Serva	Germany
Ascorbic acid	Sigma	Germany
Boric acid	VEB Laborchemie Apolda	Germany
Caffeine	Carl Roth	Germany
Catechin	Extrasynthese	France
Chlorogenic acid hemihydrate	Sigma Aldrich	Germany
Citric acid	Roth	Germany
Citric acid monohydrate	Roth	Germany
Coomassie brilliant blue G 250	Serva	Germany
Copper sulphate	Fluka	Germany
Dihydroxyacetophenone (DHAP	Bruker Daltonik	Germany
Dipotassium dihydrogen phosphate	Serva	Germany
Disodium hydrogen phosphate dihydrate	Merck	Germany
Dithioerithritol	Raanal	Ungarn
Ethanol	Carl Roth	Germany
Folin- Ciocalteu- reagent	Sigma	Switzerland
Gallic acid monohydrate	Carl Roth	Germany

Table 3-1 List of chemicals (continued)

Glycerol	Roth	Germany
Glycin (99,5%)	Carl Roth	Germany
Hydrochloric acid	Carl Roth	Germany
Ferulic acid	Fluka	Germany
L-DOPA (L-3,4-dihydroxyphenylalanine)	Roth	Germany
L- Leucine	Serva	Germany
L-Cysteine hydrochloride monohydrate	Sigma Aldrich	Germany
L-Glutathione reduced	Fluka	Switzerland
L-Lysine monohydrochloride	Fluka	Switzerland
L-proline	Sigma	USA
Lutein extraction solvent	Bioanalyt	Germany
MCT-oil	Endima	Germany
Methanol	Roth	Germany
N-Acetyl-L- Cysteine	Sigma Aldrich	Germany
Nα-Boc-lysine	Sigma Aldrich	Germany
Tagetes concentrate oleoresin	BioExtract	India
O-phthaldialdehyde	Sigma	Germany
Peptide Calibration Standard	Bruker Daltonics	Germany
Pharmacia Protein mixture	GE Healthcare	UK
Poly(vinylpolypyrrolidone)	Sigma	Germany
Polyamide	Fluka	Germany
Potassium dihydrogen phosphate	Sigma	Germany
Protein Calibration Standard I	Bruker Daltonics	Germany
Sodium and potassium tartrate	Sigma	Germany
Sodium carbonate	Merek	Germany
Sodium carbonate water free	Carl Roth	Germany
Sodium chloride	Sigma	Germany
Sodium dodecyl sulphate	Serva	Germany
Sodium hydroxide	Roth	Germany
Sodium Tetraborate (borax)	Reachim	Ussr
Trichloroacetic acid	Carl Roth	Germany
Trifluoroacetic acid	New Jersey	USA
Trigonelline hydrochloride	Sigma Aldrich	Germany
Trinitrobenzenesulfonic acid	Sigma	Germany
Tris- Hydroxymethyl- aminomethan	Fluka	Switzerland
Trypsin working solution	Pierce Biotechnology	USA
Tryptic Digestion Kit	Pierce Biotechnology	USA
Tyrosinase Mushroom T3824-25KU	Sigma Aldrich	Germany
Urea	VEB Laborchemie Apolda	Germany

Table 3-2 List of instruments

Name	Model	Company	Country
Blender		IKA Labortechnik	Germany
Centrifuge	Heraeus megafuge 2.0R	Thermo scientific	Germany
Centrifuge	5415 R	Eppendorf	Germany
Eppendorf mixter	5432	Natheler, Hins	Germany
Eppendorf thermomixter	5355	Natheler, Hins	Germany
HPLC system	Gradient reversed-phase HPLC	Shimadzu	Japan
HPLC system	Gradient reversed-phase HPLC	JASCO	Japan
Jasco fluorescence detector	FP 920	JASCO	Germany
Kitchen machine	HR7775	Phillips	China
MALDI-TOF-MS	AUTOFLEX-III LRF200-CID	Bruker Daltonics	Germany
Mastersizer	MSS	Malvern	Germany
Microscope	BX50	Olympus optical Co	Japan
Micrplate reader	Fluostar Optima	BMG LABTECH	Germany
Scales	PT 120	Sartorius	Germany
Semi-micro Kjeldahl unit	Vapodest 30	Gerhardt	Germany
Spectrophotometer	Novaspec II	Pharmacia Biotech	England
Spectrophotometer	UVIKON 930	Kontron instrument	Switzerland
Ultra Centrifugal Mill	ZM 200	Retsch	Germay
Ultrasonic homogenizer	Sonopuls HD 2070	Bandelin electronic	Germany
UV lamp	VL- UV-6LM 965323	Vilber Lourmat	Germany
UV VIS spectrophotometer	SPEKOL	Carl - Zeiss	Germany

Table 3-3 List of HPLC parts

Instrument	Description	Manufacturer
Auto injector	SIL 10A	Shimadzu, Kyoto, Japan
Column	Prontosil 120-3-C18 ACE-EPS 150 x 4,6 mm; 3 µm	Bischoff chromatography, Leonberg Germany
Column	Perfectsil 300 – C 8 300 x 4,6 mm; 5 µm	Bischoff chromatography, Leonberg Germany
Column	Perfectsil C18 300 ODS 150 x 4,6 mm; 5 µm	MZ, Mainz, Germany
Column	C18 analytical Waters-Spherisorb SC-04, 125 x 4.0 mm, ODS2, 3.0 µm	Bischoff chromatography, Leonberg Germany
Column	Prontosil Sil Target ODS – 3HD,4.6 x 250 mm; 3.0 µm	MZ Analysentechnik und – geräte GmbH, Leonberg, Germany
Controller	SCL 10A VP	Shimadzu, Kyoto, Japan
Degasser	DGU 20A 5	Shimadzu, Kyoto, Japan
Detectors	DAD SPD M10AVP UV-Vis Detector SPD-10 AVP	Shimadzu Kyoto, Japan
Oven	CTO 10ASVP	Shimadzu Kyoto, Japan
Pumps	LC 10AD	Shimadzu Kyoto, Japan

3.1.2 Samples

Study 1: Five different varieties of fresh apples (Boskoop, Braeburn, Cox Orange, Golden Delicious and Jonagold) were purchased from a local supermarket (Berlin, Germany). Skimmed milk powder was also purchased (Sucofin, TSI GmbH, Zeven, Germany). Casein-Na was obtained from Carl Roth GmbH (Karlsruhe, Germany) and soy glycinin was prepared from defatted unheated soy flour (type 1, protein content ca. 52%, Sigma Chemicals Co., St. Louis, MO, USA) according to the method of Thanh and Shibasaki (1976).

Study 2: Coffee beans; different varieties of green coffee beans from five different countries: Brazil (B), Guatemala (G) and Columbia (K) representing *Coffea arabica* (*CA*), Uganda (U) and Indonesia (I) representing *Coffea canephora (robusta)* (*CR*) were employed. Coffee beans from Columbia were also roasted and provided by the kind courtesy of Deutsche Extraktkaffee GmbH (DEK GmbH, Berlin, Germany). All other samples were commercially obtained (Rohkaffee-Company, Berlin, Germany). Faba beans (FB); two varieties *Vicia faba var.* Giza 3 (Gi3) and *Vicia faba var.* Sakha 3 (S3) were obtained from Agriculture Research Center, Institute of Field Crops, Egypt.

Study 3: Milk whey proteins (WP); milk whey protein and its products, β-lactoglobulin (β-LG - Biopure, lot JE 002-8-415), BioZate 1 (BZ1) and Glycomacropeptide (GMP) powders were purchased from Davisco Foods International, Inc. (Le Sueur, MN, USA). β -LG powder contained 98% of protein, as determined by Kjeldahl analysis (N x 6.38); 0.1% fat, 1.9% ash and 4.7% moisture, as specified by the supplier. BZ1 powder contained 95.2% of protein, as determined by Kjeldahl analysis (N x 6.38); 0.5% fat, 4.9% ash and 4.5% moisture, as specified by the supplier and the degree of hydrolysis was 4.6% and GMP powder contained 83.7% of protein, as determined by Kjeldahl analysis (N x 6.47); 0.8% fat, 6.5% ash and 6% moisture, as specified by the supplier.

3.2 Methods

3.2.1 Determination of polyphenol oxidase (PPO) activity in apple juice

3.2.1.1 Extraction of PPO from apples

The fresh apples (Boskoop, Braeburn Cox Orange, Golden Delicious and Jonagold) and Braeburn apples, which were kept at different temperatures (room temperature, + 4, - 20 and – 80 °C) one day before extraction, were washed and cut into quarters. The core was removed,

and the apple quarters were pressed, on ice, in fruit - juicing equipment (Phillips, HR7775, China). Subsequently, the juices were filtered through a cloth sieve and stored at -20 °C until needed.

3.2.1.2 Polyphenol oxidase (PPO) activity

PPO activity was determined according to Schilling *et al.* (2008). The reaction mixture was made up of 1.5ml reaction buffer [0.5 mmol/L sodium dodecyl sulfate in McIlvaine buffer (pH 6.5) consisting of 30% 0.1 mol/L citric acid and 70% 0.2M disodium phosphate], 0.2ml of L-proline in reaction buffer and 0.1ml of extract. The reaction was started by adding 0.2ml of 25mM 4-methylcatechol dissolved in the reaction buffer. The formation of the pink proline-catechol adduct was recorded at 525nm (ε=1550 L mol^{-1} cm^{-1}) every 3 s for 6 min at room temperature. PPO activity was calculated from the slope within the initial linear range of the absorbance-time curve. For blank correction, the slopes of a sample blank (0.1ml water instead of juice) and a reagent blank (0.2ml reaction buffer instead of 25 mM 4-methylcatechol) were subtracted. The enzyme activity was expressed in U/L apple juice.

3.2.2 Effect of substrate substitution of PPO on its activity

For this purpose, 4 – methylcatechol was substituted with caffeic, chlorogenic and ferulic acids and L-DOPA (L-3, 4-dihydroxyphenylalanine). Furthermore, L-tyrosine instead of L-proline was used. The change in PPO activity was monitored by method (3.2.1.2) at a wavelength of 525 nm in case of using L-proline, while 475 nm was used with L-tyrosine.

3.2.3 Identification of phenolic compounds in apple juice by HPLC-MS

Chromatography was carried out on a Shimadzu HPLC system (Kyoto, Japan) using a Prontosil 120-3-C18 ACE-EPS column (Bischoff Analysentechnik und – geräte GmbH, Leonberg, Germany; 150 x 4, 6 mm; 3 µm) at 40°C, connected to a C18 pre-column containing the same material, with a flow rate of 1 ml/min and a wavelength UV-Vis detection from 200 to 700 nm. The eluents were A (2 % acetic acid) and B (methanol). The gradient was applied under the following conditions: 20 % eluent B, 3 min; 20–35 % eluent B, 17 min; 35–68 % eluent B, 17 min; 68 % eluent B, 3 min; 68–20 % eluent B, 3 min; 20 % eluent B, 12 min. Run time was 55 min and the injection volume was 5-20 µl. Negative and positive ion mass spectra of the samples were recorded in the m/z range of 200–1200Da, at a scan speed of

2000 amu /sec (event time 0.7 sec). Nitrogen was used as the drying gas at a flow rate of 1.5 L/min. The nebulizer temperature was set at 230°C and a potential of 3 KV for negative ionization and 4.5 KV for positive ionization were used. Concentrations of 10-100 µg/ml of chlorogenic acid were used for calibration. The content of chlorogenic acid was expressed as µg/ml juice.

3.2.4 Characterization of interactions of chlorogenic acid (CQA) with model amino components (Lysine and Boc-Lysine)

A 112 mM solution of the tert-butyloxycarbonyl-L-lysine (N_{α}-Boc-lysine) or lysine and a 28 mM solution of CQA were prepared in 0.1M potassium phosphate buffer (PBS) pH 7 and mixed at the ratio 1:1 (v/v) in the presence of (15 U/ml) fungal tyrosinase. The samples were stirred for 24 h at 40°C, and afterwards stored at - 80 °C until analysis. Moreover, the same reaction was also achieved using different amounts of enzyme (20, 60,120 and 240 U/ml), pHs (4 and 9) and incubation temperatures (25 and 60 °C). The control was prepared in the same way but without enzyme. The reaction was monitored at different time intervals (1, 2, 4, 6 and 24h) by applying the following methods:

3.2.4.1 The change in CQA content

The change in free CQA content, isomerization and adducts formation were studied by HPLC-MS, as mentioned above in 3.2.3.

3.2.4.2 The change in antioxidative capacity by TEAC assay

Trolox equivalent antioxidant capacity assay (TEAC) for samples was determined as described in Re *et al.* (1999) with a few modifications, where the $ABTS^{+\bullet}$ cation was produced by reacting of (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) $ABTS^{+}$ solution (700 µM) with 245 µM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{+\bullet}$ solution was diluted (1:10) with 5 mM phosphate buffer (PBS) pH 7.2-7.4. Dilutions of the samples were also prepared using the same buffer. 150 µL of diluted $ABTS^{+}$ solution was added to 10 µL of each sample solution and 6 min after initial mixing; the absorbance was measured at 730 nm using a fluorescence microplate reader (Fluostar Optima, BMG LABTECH, Jena, Germany) with OPTIMA Software Version 2.1. TEAC values were obtained by comparing the absorption

obtained from tested samples with those obtained from trolox (6-Hydroxy-2, 5, 7, 8-tetra-methylchroman-2-carboxylic acid) which served as standard with concentrations (0.125-2.5 mM). The results are expressed as mM Trolox equivalents (TE).

3.2.4.3 The change in free amino groups using o-phthaldialdehyde (OPA) method

The method using o-phthaldialdehyde (OPA) was applied for the quantitative determination of free α- and ϵ-amino groups (Roth 1971). OPA in the presence of reduced thiol groups reacts with the primary amino groups found in terminal amino acid and the ϵ-amino groups to form 1-alkylthio-2-alkylisoindoles (Simons and Johnson 1976, Simons Jr and Johnson 1978) that absorbs strongly at 340 nm (Svedas *et al.* 1980). The approach used in this study was based on Goodno *et al.*(1981) with some modifications, N-acetyl-L-cysteine (NAC) was used as a thiol component instead of mercaptoethanol. Briefly, the determination of free amino groups was carried out by mixing sample solutions with OPA reagent (0.3 % NAC (w/v) in a borate buffer pH 9.3, 20 % SDS (w/v) and 3.4 % OPA in methanol (w/v)). After 30 min of incubation at room temperature, the absorbance reading was made at 340 nm using a fluorescence microplate reader (Fluostar Optima, BMG LABTECH, Jena, Germany) with OPTIMA Software Version 2.1. Calibration was attained using L-Leucine in a concentrations range 0.25 – 6.00 mM. Blanks were prepared in the same order, only the OPA was replaced with methanol.

3.2.5 Characterization of green and roasted coffee and faba beans

3.2.5.1 Samples preparation

Coffee Columbia was roasted in Gene Café - Coffee Bean Roaster CBR-101 at different conditions as shown in Table 3-4. Roasted and unroasted coffee and faba beans were milled (particle size ≤ 0.4 mm) using an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany), equipped with a 24- tooth rotor and a ring sieve with a 0.4 mm mesh. The milling was conducted at a speed of 15000 rpm. Roasted samples were treated two times with hexane, then the samples were transferred to Petri dishes and dried in the desiccators. All of the powdered samples were freezed at - 20 ˚C until analysis.

Table 3-4 Parameters of roasting process of coffee bean (Columbia)

Degree of roasting	Time [min]	Temperature [˚C]
Light	12	230
Medium	14	240
City	17	250
French	21	250
Italian	23	250

3.2.5.2 Determination of protein content

The total crude protein content (using 6.25 as factor) was determined using a semi-micro Kjeldahl unit (Vapodest 30, Gerhardt, Bonn, Germany).

3.2.5.3 Determination of free amino acids content

3.2.5.3.1 Extraction of free amino acids

50 mg of each ground beans were extracted using 1.5 ml of 60% methanol, at 4°C overnight. The extracts were centrifuged at 10800 rpm for 10 min at 4 °C, the clear supernatant was carefully transferred to a fresh tube, and the solid part was extracted again with the same manner but for only 10 seconds.

3.2.5.3.2 HPLC analysis

The amino acids content of the extracts was determined as described previously (Roth 1971, Simons and Johnson 1976, Simons Jr and Johnson 1978, Svedas *et al.* 1980). 25 µl extract was mixed with 200µl of 10 mM borate buffer pH 9, in a HPLC vial. After that, 25 µl of OPA reagent (100 mg of Ortho-Phthalaldehyde and 10 µl of 3-Mercaptopropionic acid in 10 ml acetonitrile) was added. 10 µl mixture was immediately measured by a Shimadzu HPLC system (Kyoto, Japan) using a Prontosil Sil Target ODS – 3HD; 4.6 x 250 mm; 3.0 µm column (MZ Analysentechnik und – geräte GmbH, Leonberg, Germany) at 40°C, connected to a Prontosil Sil Target ODS – 3HD; 4.6 x 250 mm; 3.0 µm guard cartridge with a flow rate of 0.8 ml/min, with a UV-Vis detection at 330 nm and emission at 445 nm. The eluents were A (0.14 sodium acetate buffer, pH 6.4) and B (acetonitrile /water (60/40, v/v). The gradient applied is presented in Table 3-5.

Table 3-5 Gradient of eluents for determination of free amino acid by HPLC

Time [min]	0	4	12	30	40	41.5	44	45	47	48	60
Eluent A%	100	92	90	75	60	40	40	0	0	100	100
Eluent B%	0	8	10	25	40	60	60	100	100	0	0

Concentrations of 2-10 ng of amino acids (Ala, Arg, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Thre, Tyr, Val) /10 µl were used for calibration. The content of each amino acid was expressed as mg /100 g sample.

3.2.5.4 Determination of polyphenol oxidase (PPO) activity

3.2.5.4.1 Extraction of PPO from green coffee beans

For extraction of polyphenol oxidase (PPO), frozen samples were thawed until approximately 4°C was reached. The extraction procedure described by Baur *et al*. (2004) was applied. Approximately 600 mg of the sample and 600 mg of polyvinylpolypyrrolidone (PVPP) were weighed and mixed with 10 ml of chilled McIlvaine buffer pH 6.5 - consisting of 30% of 0.1 M citric acid and 70% of 0.2 M disodium phosphate - under continuous stirring for 1 h at 4 °C. Subsequently, the homogenate was centrifuged to separate solid residues from the extract. The homogenate was transferred into 2 ml tubes and centrifuged at 9300 g for 10 min at 4 °C. The clear supernatant was kept in an ice bath until PPO activity assay was performed. All samples were extracted in triplicate and the analysis performed in duplicate.

3.2.5.4.2 PPO activity

PPO activity for coffee extracts was determined as explained above in 3.2.1.2**.** The enzyme activity was calculated as U/100 g green coffee bean powder based on dry matter.

3.2.5.5 Characterization of phenolic compounds and caffeine content

3.2.5.5.1 Extraction of phenolic compounds

10 mg samples of each ground beans were extracted with 1 ml of methanol-acetic acid solvent (80% methanol, 19% distilled water, 1% acetic acid v/v), at room temperature for 30 min. The extracts were centrifuged at 9300 x g for 10 min at 4 °C, and the clear supernatant was carefully transferred to a fresh tube and stored at –20°C until needed.

3.2.5.5.2 Determination of total phenolic compounds content

Total phenolics content of the extracts was estimated using the Folin – Ciocalteu procedure as described above (Singleton and Rossi 1965, Kahkonen *et al.* 1999, Singleton *et al.* 1999, Ikawa *et al.* 2003), with a few modifications. Shortly, 0.1 ml of each extract was mixed with

the Folin – Ciocalteu reagent (0.5 ml) in test tubes and held at room temperature for 5 min. Then 0.4 ml of 7.5% sodium carbonate solution was added. The tubes were mixed and allowed to stand for 30 min at room temperature. The absorbance of the solutions was measured at 765 nm by a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, England). The quantification was performed by an external calibration with diluted standards using chlorogenic, gallic acids and catechin (10-80 µg/ml). The content was expressed as chlorogenic, gallic acid and catechin equivalents in milligrams per 100 gram dry material.

3.2.5.5.3 Identification of phenolic compounds and caffeine by HPLC – MS

HPLC conditions for identification of phenolic compounds and caffeine are described above in 3.2.3. Caffeine was measured at 280 nm. Concentrations of 10-100 µg/ml of chlorogenic acid and caffeine were used for calibration. The content of chlorogenic acid equivalents was expressed as mg/g dry material and caffeine equivalents as mg/100 g dry material.

3.2.5.6 Determination of antioxidative capacity by TEAC assay

The antioxidative capacity for extracts was determined by TEAC assay as described above in 3.2.4.2**.** The results are expressed as mM Trolox equivalents (TE)/100 g dry matter.

3.2.6 Modification of proteins by chlorogenic acid

3.2.6.1 Extraction of coffee and faba storage proteins

Green coffee (GC) proteins and faba bean (FB) proteins (reference proteins) were extracted by using different solvents and additives as described previously (Thanh and Shibasaki 1976, Kim and Kinsella 1986) with some modifications: 2g frozen GC flour and 1 g polyamide or polyvinylpolypyrrolidone (PVPP), applied as additives, were placed in a 50 ml centrifuge tube, except FB powder, where the extraction without these additives was performed. Then 20 ml of different solvents (0.04% ascorbic acid, 0.04% acetylcysteine in distilled water and 0.04% ascorbic acid in 0.03 M Tris-HCl pH 8 buffer) were added. All samples were shaken at room temperature for 3 hours. Extracts were centrifuged at 4000 x g for 20 min, then the clear supernatants were carefully removed, followed by dialysis against distilled water for 24 h at 4 °C and freeze dried. Proteins were stored at –20 °C until analysis.

3.2.6.2 Modification of β-Lactoglobulin (β-LG) protein with CQA

B-Lactoglobulin was modified under alkaline and enzymatic (PPO) conditions with two different CQA sources, as follows:

3.2.6.2.1 Modification of β-LG protein with green coffee beans extract.

3g ground green coffee (*CR*, Uganda) was extracted for 15 min with 90 ml boiling distilled water. The extract was allowed to cool to room temperature (25°C), centrifuged at 4000 x g for 10 min at 4 °C, and the supernatant was filled up to 250 ml. For enzymatic modification, an aliquot (25 ml) was added to 0.25g β-LG and mixed with 3 and 6 ml of apple juice (as a source for the PPO enzyme), the resulting pH was 4.7. For the alkaline modification, the pH of the extracted coffee phenolics was adjusted to pH 9, without any addition of apple juice. A blank control constituted of the same amount of β-LG (0.25 g) dispersed in distilled water. After 24 h reaction time, under continuous stirring at room temperature (25°C) with free exposure to air, the samples were dialyzed for 18–24 h against distilled water at room temperature, and, finally, lyophilized and saved at -20 °C until analysis.

3.2.6.2.2 Modification of β-LG protein with commercial CQA

60 mg of 5-CQA was dissolved in 100 ml distilled water. The enzymatic modification, with 3ml apple juice, and alkaline modifications were performed as mentioned in 3.2.6.2.1. The modification was done 3 times, to confirm the reproducibility of the modification procedure.

3.2.6.3 Modification of BioZate1 (BZ1) and Glycomacropeptide (GMP) with CQA

BZ1 and GMP proteins were only modified with green coffee beans extract at alkaline and enzymatic (using 3 ml apple juice) conditions as explained in 3.2.6.2.1. The modification was performed 3 times, to confirm the reproducibility of the modification procedure.

3.2.7 Chemical properties of modified proteins

3.2.7.1 Determination of the change in free CQA

Samples were removed at the beginning and after 24h reaction to determine the amount of free CQA by HPLC as described in 3.2.3.

3.2.7.2 Determination of covalent bound CQA by proteins

In order to calculate the amount of CQA bound to the proteins, 4 mg protein was dissolved in 1 ml of 8 M Urea solution followed by precipitation of protein with 20 % trichloroacetic acid (TCA). After this treatment, the precipitate was re-dissolved in 1 ml of 8M urea. Reverse phase HPLC (RP-HPLC) was conducted with a Shimadzu 10A system (Kyoto, Japan) with a Perfectsil column (C18 300 ODS 150 × 4,6 mm; 5 µm) at 37°C. The eluents were 0.1 % trifluoroacetic acid (v/v) (A) and acetonitrile (B). The gradient was applied under the following conditions: 10-18 % B, 22 min; 18–80% B, 8 min; 80 % B, 3 min; 80-10% B, 2 min; 10 % B, 7 min. The run time was 42 min. 50 µl were injected with a flow rate of 0.6 ml/min. Detection and external calibration using (10–1000 µg/ml) 5-CQA was conducted at 325 nm.

3.2.7.3 Determination of mole mass by electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, T= 14-18 % gel) according to Laemmli (1970) was applied for the molecular weight determination of the proteins. The band intensity was estimated using densitometer scanning with Quantity one software, version 4.5.2 (Bio-Rad, Universal Hood II, Bio-Rad Laboratories, Segrate, Milan, Italy). The **s**ample buffer applied was 0.05 M Tris-HCl buffer pH 6.8 containing 4 g sodium dodecyl sulfate, 12 g glycerol, 5 g 2-mercaptoethanol and 0.01 g Coomassie Brilliant blue R 250. The sample treatment consisted of denaturing the proteins (1mg/ml) by heating at 95°C for 3 min prior to analysis. Low molecular weight calibration kit for SDS electrophoresis (GE Healthcare Europe GmbH, Freiburg, Germany) was applied.

3.2.7.4 Matrix Assisted Laser Desorption/Ionization –Time of Flight-Mass Spectrometry (MALDI-TOF-MS) analysis

Experiments were performed by dissolving 1 mg of the protein sample in 1 ml 0.1% trifluoroacetic acid/acetonitrile (50%, v/v) containing 2 mg 1,4-dithioerythrit (DTT). Of this solution, 0.5 µl was brought on the target and covered with 0.5 µl of 2,5-Dihydroxy actetophenone (DHAP) as matrix (7.6 mg of 2,5-DHAP was dissolved in 375 µl ethanol and 125µl of a solution containing 18 mg/ml Diammonium hydrogen citrate [DAC] dissolved in water). After crystallization of the sample by air-drying, measurements were carried out on AUTOFLEX-III LRF200-CID, equipped with Smartbeam-Laser 200 (Bruker Daltonik GmbH,

Bremen, Germany). The instrument was internally calibrated using the signals of the positive $[M+H]^+$ mono isotopic ions of a protein calibration standard 1 (Bruker Daltonik GmbH, Bremen, Germany).

3.2.7.5 Determination of tryptophan fluorescence

Tryptophan fluorescence was determined as described in Jackman R and Yada R (1989). Two procedures were used to determine the amount of total and exposed (surface) tryptophan. Samples were dissolved in 5 mM PBS buffer pH 7.2 or 8M urea, for surface and total tryptophan determination, respectively. The sample containing 1 mg protein/ml was excited at 290 nm (slit 18 nm) and emission recorded over the wavelength range of 300 to 900 nm (slit 40 nm) using a Jasco fluorescence detector FP 920 (Gross-Umstadt, Germany; Tokyo, Japan). The peak area under the emission curve from 300 to 480 nm was used to calculate the tryptophan content. The calibration was done by applying 4 – 20 nM Tryptophan.

3.2.7.6 Determination of free amino groups by TNBS method

The changes in the content of free amino groups according to (Adler-Nissen 1976) were analyzed using trinitrobenzenesulfonic acid (TNBS). TNBS binds to the nucleophilic nitrogen of the primary amino groups found in terminal amino acids and the ε-amino groups and forms an adduct with yellow colour. Protein solution (mg/ml 1%SDS) was incubated with TNBS at pH 9 for 60 min and 40°C. After that, 1M HCL was immediately added to stop the reaction. The absorbance of the formed adduct was measured at 340 nm by a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, England). The calibration was performed using isoleucine in a concentration range of 20 – 100 nM. Blank was prepared in the same manner, with the sample being substituted with % SDS solution.

3.2.7.7 Determination of free thiol groups by DTNB method

The procedure using Ellman's reagent [5,5'-dithio-bis (2-nitrobenzoic acid); DTNB], was applied to determine the thiol (sulfhydryl) group (-SH) content according to Hofmann *et al.* (1978). The principle of the method is that colorless DTNB is converted to yellow 5-Mercapto-2-nitrobenzoic acid in the presence of thiol compounds, which has an absorption maximum at 412 nm. 2.5 mg proteins were dissolved in 1 ml of 200 mM Tris-HCL buffer containing 1% SDS at pH 8.0. A volume of 10 µL calibration or protein solutions were

transferred in 96-well-microtiter plate and the absorbance (A1) at 400 nm was measured using a Fluorescence microplate reader (Fluostar Optima, BMG LABTECH, Jena, Germany) with OPTIMA Software Version 2.1. Following that 150 µl of 0.0025 M DTNB in ethanol/buffer per well was added to these samples and the mixtures were left for 20 min at room temperature. Then, the absorbance (A2) at 400 nm was measured again. The corrected absorbance was calculated from (A2 - A1). Concentration of thiol groups in sample solutions was calculated by calibration with acetylcysteine in a concentration range of 6.7 – 333.5 µM.

3.2.8 Functional properties of modified proteins

3.2.8.1 Protein solubility

Modified and unmodified proteins (0.1% w/v) were dissolved in 0.05 M sodium phosphate buffer at different pHs from 2 to 10. The samples were centrifuged (10 min, 9300 x g and 4°C) and the protein concentrations of the supernatants were measured at 595 nm by spectrophotometer (a Novaspec II, Pharmacia Biotech, Cambridge, England) using a modified Lowry method (Lowry *et al.* 1951). The values obtained were compared with the solubility of the samples in 1% sodium dodecyl sulphate (SDS) solution, which was set to 100% solubility.

3.2.8.2 Determination of antioxidative capacity by TEAC assay

Trolox equivalent antioxidant capacity assay (TEAC) for proteins was determined as described in (3.2.4.2), with few modifications. The proteins were dissolved in 5 mM PBS buffer pH 7.2-7.4 and the dilutions were also prepared in the same buffer. 750 µL of diluted $ABTS^+$ solution was added to 50 µL of each sample solution and 6 min after initial mixing, the absorbance was measured at 730 nm using a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, England). The results are expressed as nmol TE/ mg protein.

3.2.8.3 Determination of antioxidative capacity by DPPH assay

Antioxidative capacity of proteins was determined by DPPH assay as described by Binsan *et al.* (2008) with a slight modification. 0.15 mM of 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol was added to 0.1% protein solution (in 5 mM PBS buffer pH 7.2) in ratio of (1:1, v/v). The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using

spectrophotometer (UVIKON 930, Kontron instrument, Switzerland). The blank was prepared in the same manner, except that 5 mM PBS buffer pH 7.2 was used instead of the sample. The calibration curve was prepared using trolox in the range of 12.5–100 µM. The activity was expressed as nmol TE/mg protein.

3.2.8.4 Determination of Surface hydrophobicity by ANS method

Surface hydrophobicity of the samples was determined using hydrophobic fluorescence reagent, 1-anilino-8-napthalensulfonate (ANS) as described in Hayakawa and Nakai (1985), with a slight modification. The applied protein concentrations were 0.125 – 0.5 mg/ml 5 mM PBS buffer (pH 7.2-7.4). 2 ml from each concentration was mixed with 20 µl of 4 mM ANS (in the same buffer). Fluorescence was recorded with (JASCO fluorescence detector FP 920, Japan) using excitation at 390 nm (slit 18 nm) and emission between 390 - 900 nm (slit 40 nm). The initial slope (So) of the fluorescence intensity versus soluble protein concentration was used as an index of the protein surface hydrophobicity.

3.2.8.5 Differential Scanning Calorimetry (DSC)

DSC of 15 % protein solutions in ml 5 mM PBS buffer pH 7.2 was performed using a SEIKO 120 DSC analyzer (equipped with thermal analysis system SSC5200, Seiko Instruments Inc., Japan) as outlined in Rawel *et al.* (2002b) with a few modifications. 40 µl sample solutions were analyzed in air tight 70 µl aluminum containers. Aluminum oxide in PBS buffer served as a reference. Thermal curves were established at a heating rate of 3°C min^{-1} over a temperature range of 20-105 °C. The thermal parameters T_d (denaturation temperature, temperature at maximum heat flux), ΔH (enthalpy of denaturation) and onset temperatures (T_{onset}) were determined from all thermal curves.

3.2.8.6 Circular Dichroism (CD) analysis

Circular Dichroism (CD) has been used extensively to give useful information about protein structure, the extent and rate of structural changes and ligand binding. In the protein design field, CD is used to assess the structure and stability of the designed protein fragments. CD depends on the differential absorption of left and right circularly polarized radiation by chromophores which either possess intrinsic chirality or are placed in chiral environments. Proteins possess a number of chromophores which can give rise to CD signals. In the far UV

region (240-180 nm), which corresponds to peptide bond absorption, the CD spectrum can be analysed to give the content of regular secondary structural features such as α-helix and β-sheet. The CD spectrum in the near UV region (320-260 nm) reflects the environments of the aromatic amino acid side chains and thus gives information about the tertiary structure of the protein (Kelly and Price 2000). Far-ultraviolet absorption (UV)-CD of the samples were recorded in the range of 190-260 nm in 5 mM PBS buffer pH 7.2 at a protein concentration of (0.2 g/L) using a Jasco J 710 spectropolarimeter. A quartz cylindrical cell having a 1 mm path length was used for the measurements. The parameters were set as follows: step resolution, 1 nm; speed, 50 nm /min; bandwidth, 1 nm; response, 4 s; and sensitivity, 200 mdeg. The mean ellipticity was determined using a mean residue molecular weight, calculated from the amino acid sequence of the corresponding proteins (SWISSPROT Protein bank, Data bank source). The CD spectra were analyzed by a curve-fitting software CDPro using CONTIN/LL, SELCON, and CDSSTR methods to obtain the secondary structural contents of the proteins. The estimation was performed using a 48 protein reference set (Sreerama *et al.* 2000, Sreerama and Woody 2000). CD spectra in near-UV (250-320 nm) were measured in 5 mM PBS buffer at a protein concentration of (0.2 g/L) to monitor the changes in tertiary structure. The parameters were set as follows: path length, 5 mm; step resolution, 1 nm; speed, 50 nm /min; bandwidth, 1 nm; response, 4 s; and sensitivity, 200 mdeg. Blank was PBS buffer without protein.

3.2.9 Emulsification properties of modified proteins

3.2.9.1 Preparation of emulsion

Emulsions with 10 % MCT (medium chain triglyceride) oil, containing oleoresin (lutein ester) 4 mg/ml, in 5 mM PBS buffer pH 7.24 were prepared by mixing the MCT oil and protein solution (0.2%) at 500 rpm for 10 min to form pre-emulsion. After that, the emulsion was homogenized by using an ultrasonic homogenizer (Sonopuls HD 2070 with titanium probe, Bandelin electronic GmbH & Co. KG, Berlin, Germany). The sonication time was 5 min in an ice bath at an energy input of 70% of the maximum power. Emulsions without any lutein ester were also prepared in the same manner.

3.2.9.2 Emulsion stability

The creaming stability of the emulsion was measured under centrifugal force. After homogenization, 1 ml emulsion was placed in a UV-cuvette and centrifuged at (1500-300) × g. After every 5 min centrifugation, the absorbance of emulsions at 500 nm was recorded by a UV VIS spectrophotometer (SPEKOL, Carl Zeiss, Germany).

3.2.9.3 Oil droplets size of emulsion

Oil droplets size and their distribution in the emulsion was evaluated by laser diffraction using Mastersizer S (Malvern instruments GmbH, Herrenberg, Germany), where laser diffraction is based on the principle that particles passing through a laser beam will scatter light at an angle that is directly related to their size (large particles scatter at low angles and small particles scatter at high angles). Emulsion samples were diluted in distilled water until the intensity of the laser beam decreased by ~14 % (obscuration). The average size of oil droplets and their size distribution was calculated by the equipment software according to Mie's theory. The size of particle for 10th, 50th (as the mass median diameter), and 90th percentile of the diameter were measured.

3.2.9.4 Microscopic imaging of emulsion

The distribution of particles in emulsions were examined and photographed using a microscope (normal light conditions, model BX50, Olympus optical Co Ltd, Tokyo, Japan) equipped with a Color view 12 CCD video camera (SIS, Münster, Germany) with magnification power of 40X. Images were processed using analysis TM 3.0 software (SIS).

3.2.10 Stability of emulsified lutein ester against UV light

3.2.10.1 Extraction of lutein ester

The previously prepared emulsions were exposed to ultra violet in 2 ml glass vials for 0, 24, 48 and 72 hours at 365 nm using UV lamp (VL-6LM 6W-365 nm tube, power 12 W, Angewandte Gentechnologie System GmbH, Heidelberg, Germany) at room temperature. Lutein ester was extracted from emulsion by adding 1000 µl of a specially developed hexane containing extraction solvent (BioAnalyt GmbH, Teltow, Germany) to 150 µl of emulsion, mixing for 10 seconds with further shaking for 10 min followed finally by centrifugation, for

5 min at 9300 × g at 4°C. The supernatant was transferred into a new 2 ml tube. The extraction step was repeated once more, and following that the volume was filled up to 2 ml with extraction solution for injection in the HPLC.

3.2.10.2 HPLC analysis

Lutein ester content was determined using a JASCO HPLC system (JASCO, Japan). The separation was carried out with a C18 analytical Waters-Spherisorb column SC-04 125×4.0 mm, ODS2, 3.0 µm (Bischoff Chromatography, Leonberg, Germany). The column temperature was kept at 30 °C. The binary mobile phase consisted of acetonitrile – methanol (9:1, v/v) (A) and ethyl acetate (B). Elution was carried out with a gradient program: 20 to100% B from 0-15 min and 100 to 20% B from 15-30 min; the flow rate was kept at 0.6 ml/min with an injection volume of 20 µl. The run time was 30 min and lutein ester was measured at 450 nm.

3.2.11 Identification of the sites and types of modification in proteins by MALDI-TOF-MS

3.2.11.1 Digestion of coffee storage proteins

Exemplary, the powdered coffee meals (50 mg) or the partly purified and extracted storage proteins (2mg) from *coffea arabica* (Brazil) and *Coffea robusta* (Indonesia) were dissolved in 1 ml SDS-PAGE sample buffer and 10 µl were separated by electrophoresis. SDS-PAGE was preformed with 14 % T gel as described in (3.2.7.3). The proteins were stained with a colloidal Coomassie brilliant blue G 250 solution. Proteins bands of interest were excised and exposed to a series of treatments: destaining buffers, reduction, and alkylation reagents. The treatments were conducted according to producer instructions as described in “In-Gel Tryptic Digestion Kit” (Pierce Biotechnology, Inc., Rockford, USA). The digestion by trypsin working solution was conducted at 30°C for 16 hours (overnight). The proteolytic digests were dissolved in 25 µl of digestion buffer (25 mM ammonium bicarbonate). A saturated matrix solution (α-Cyano-4-hydroxy cinnamic acid, HCCA) was prepared in 30:70 (v/v) acetonitrile: 0.1% TFA in water. Pre-mix equal volumes of sample solution and matrix solution were analyzed. A total 0.5 µl of this mixture was applied onto a steel target for mass spectrometry-analysis and it was left at room temperature to dry before analysis.

3.2.11.2 Digestion of modified β-LG proteins

Protein (1 mg) was dissolved in 1 ml of 50 mM ammonium bicarbonate (pH 7.5 to 8.5) buffer. To 20 µl of this solution, 77.5 µl of 50 mM ammonium bicarbonate buffer and 2.5 µl trypsin working solution were added. The digestion was conducted at 37°C for 24 hours and stopped by adding 5 µl of 10% trifluoroacetic acid (v/v). A saturated matrix solution HCCA was prepared. For MS-Analysis, 0.5 µl of the sample solution was applied onto a steel target then covered by 0.5 µl matrix and it was left at room temperature to dry before analysis.

3.2.11.3 MALDI-TOF-MS analysis

The analysis was performed on AUTOFLEX-III LRF200-CID, equipped with Smartbeam-Laser 200 (Bruker Daltonik GmbH, Bremen, Germany) in the reflector-mode operation; the acceleration voltage was 20 kV, and the effective flight path was 200 cm. The instrument was internally calibrated using the signals of the positive $[M+H]^+$ mono isotopic ions of a peptide calibration standard II (Bruker Daltonik GmbH, Bremen, Germany). The data analysis was performed using the software packet: Bruker Daltonics FlexAnalysis (Vers. 3,3; Bruker Daltonik GmbH, Bremen, Germany). Sequence database search using the m/z values of the digested peptides was performed with Bruker Daltonics BioTools (Vers. 3,2; Bruker Daltonik GmbH, Bremen, Germany) combined with the MASCOT search program (Matrix Science Ltd., London) (Perkins *et al.* 1999). Following protein data bases were applied: SwissProt 2011_06 database (http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-age+top;http://www.uniprot.org/) and Plants_EST (http://www.plantgdb.org/search/). Depending on the database, individual protein scores defined as significant were applicable ($p < 0.05$). On basis of the sequence information available, further analysis of the data was performed using the software "Sequence editor" (Vers. 3,2; Bruker Daltonik GmbH, Bremen, Germany). Following parameters were considered during the search: Fixed modifications such as reductive disulfide cleavage and carbamidomethylation, i.e., reaction with iodoacetamide; Mass tolerance MS (the peptide mass error) of 100 ppm; and the number of missed cleavages (or partials accounting for tolerated internal missed cleavage sites in the matching peptides). The latter option was according to the best results obtained (generally 2 partials were applied). Sequence coverage of at least 20 % of the digested proteins was set as an internal quality parameter. Analysis of optional modifications by CQA was based on the covalent reaction of lysine and

thiol amino acid side groups with 5-CQA (Schilling *et al.* 2008), and those theoretically possible. The details are described in (Ali *et al.* 2012).

3.2.12 Molecular modeling experiments

Molecular docking and energy minimization experiments were performed on a Workstation with Dual Xenon Quad Cores using the following molecular modeling software: Molecular Operating Environment (MOE), 2010.10 (MOE), Gaussian 09w (Frisch 2009) and Molegro 5.0 (Thomsen and Christensen 2006). Both programs MOE 2010.10 and Yasara 12.01(Krieger *et al.* 2002, Krieger *et al.* 2009) were used for homology modeling of the 11S globulin.

3.2.12.1 Homology Modeling

Templates applied for the modeling of the 11S globulin were X-ray structures deposited in the Protein Data Bank (PDB) under the PDB codes 3FZ3-B-D, 2D5F-B, 3C3V-A (monomer) and 3KSC, 2E9Q, 1UD1 (trimer). The structures were prepared for homology modeling by adding hydrogen atoms and partial charges to the peptides using the Protonate3D application of MOE2010.10. In Yasara, the homology modeling macro in the standard configuration was used for the modeling. The consensus models were then finally built with MOE and Yasara by applying the homology macro. This consensus model was refined with a molecular dynamics (MD) simulation. The stereochemistry quality aspects of the resulting model were checked using MOE. For the *in-silico* experiments the protein structure from bovine β-lactoglobulin (unlganded form, PDB code:3NPO; http://www.rcsb.org/pdb/explore/explore.do?structureId= 3npo) was applied. Complimentary PDB code 3NQ3 was also considered.

3.2.12.2 Ligand Structure Optimization

The initial structures of different CQAs were built using ChemOffice Suite 2010 and transferred to a MOE database. Molecular mechanical energies of the different conformers of each ligand were minimized until a root mean-square deviation (rmsd) of 0.01 kcal/mol was reached. Energy minimization was performed using the MMFF94 force field option (Halgren 1996) with the restriction to preserve original chirality of the molecules. The resulting structures were finally optimized with Gaussian09W A02 (Level of Theory HF/6-31).

3.2.12.3 Docking

The docking experiments were performed with MOE and Molegro. These two different docking environments were used to compare the results for validity. With respect to the position of a ligand to a reactive amino acid, rotations of the whole molecule as well as rotations around single bonds were allowed with the restriction that the original configuration would still be preserved. In Molegro, a search space around the reactive amino acid was defined as a sphere having a 15Å diameter. In the program MOE, a pharmacore (aromatic phenyl reactant) was defined in a vicinity of the reactive amino acid side chain.

3.2.13 Statistical analysis

All experiments were conducted at least three times. All data are expressed as means of their standard deviations. The results were analyzed using SPSS statistical software (SPSS, version 18) where applicable. Values of $P < 0.05$ were considered statistically significant.

4. Results and Discussion

4.1 Study 1: Preliminary characterization of interactions of chlorogenic acid (CQA) with amino acids and proteins

4.1.1 Characterization of different apple juices

Several varieties of apples (Boskoop, Braeburn, Cox Orange, Golden Delicious and Jonagold) were tested for their polyphenol oxidase activity and chlorogenic acid (CQA) content to determine the variety which has the highest activity and lower CQA content and thus suitable for using in the following analyses (study 3).

4.1.1.1 Polyphenol oxidase (PPO) activity in different apple varieties

PPOs are responsible for the enzymatic browning reaction that causes deterioration in fruits or vegetables. The ability of polyphenol oxidases to act on phenolic compounds makes them highly useful biocatalysts for various biotechnological applications. The PPO apple activity was calculated from the slope of the linear range of 4-MC-proline-adduct absorbance formed during the reaction between phenolic compounds**,** 4-Methylcatechol (4-MC), and proline in presence of apple juice versus time curve using the Lambert Beer's law**.** Figure 4-1**A** shows an example for the linear of the absorbance of formed adduct at 525 nm versus time reaction curve for the juice extracted from Braeburn apple.

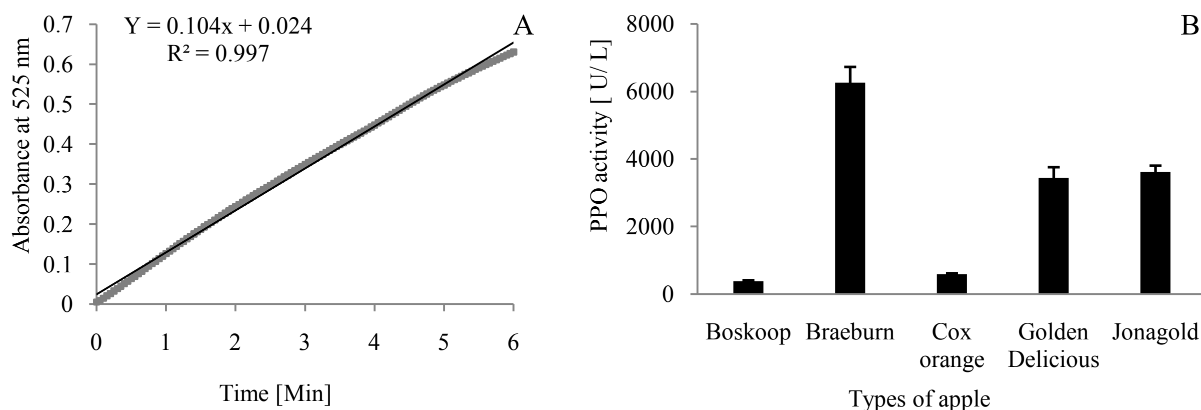


Figure 4-1 Linear PPO activity diagram for Braeburn variety apple (A) and PPO activity (U/L juice) for different varieties of apple (B)

Where: PPO, Polyphenol oxidase; 1 U corresponds to 1µmol/min, which is equal to U/60 µkat

The PPO activities for five apple varieties are outlined in Figure 4-1B. The differences in the investigated varieties are substantial. The Braeburn variety showed the highest activity with

6262.4 ± 462.6 U/L juice followed by 3612.9±186.1 and 3440.9 ± 315.4 U/L for Jonagold and Golden Delicious varieties, respectively. In contrast, the Boskoop and Cox orange varieties had the lower enzyme activity, where they had about 94 and 91% lower activities (respectively) compared to Braeburn. One possible reason for the difference between studied varieties in PPO enzyme activities is the ascorbic acid content. Ascorbic acid exists naturally in apples and acts as an antioxidant. Thus, it has an inhibiting effect on polyphenol oxidases (McEvily *et al.* 1992). The amount of ascorbic acid varies according to apple varieties and harvest conditions. Another reason is the pH of the apples (Rocha and Morais 2001). Additionally, these values may be due to the differences in genotype, locations, postharvest treatment, maturation and storage conditions of apple varieties.

4.1.1.2 Identification of phenolic compounds in Braeburn apple variety by HPLC-MS

Exemplary HPLC chromatogram of apple juice (Braeburn) is shown in Figure 4-2. The HPLC−MS analysis data for braeburn variety showed many compounds at 275 and 325 nm, where two hydroxycinnamates, 5-O-caffeoylquinic acid and 4-O-p-coumaroylquinic acid, peak 1 (Rt = 10.6 min and the mass spectral data of $[M-H]^-$ = m/z 353) and peak 2 (Rt =12.95 min and the mass spectral data $[M-H]^-$ = m/z 337), respectively were detected.

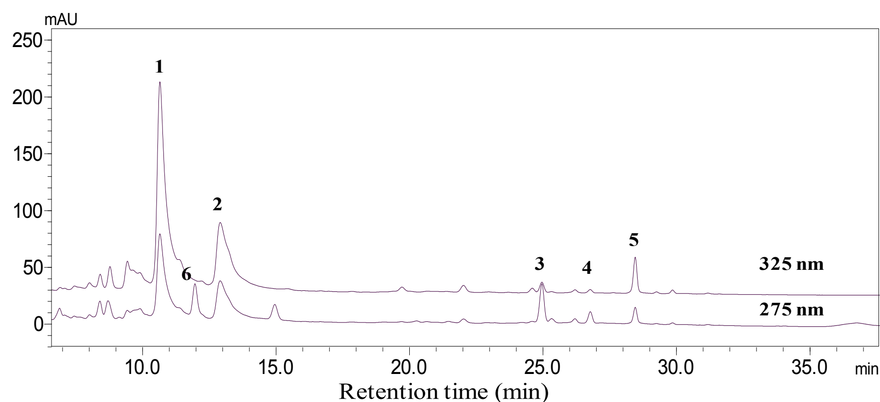


Figure 4-2 UV chromatograms of the phenolics from apple juice (Braeburn variety)

Furthermore, phloretin-2′- O-(2″-O-xylosyl) glucoside, peak 3 (Rt = 25 min and the mass $[M-H]^-$ at m/z 567) was detected. One quercetin glycoside, quercetin-rhamnose-hexose-rhamnose, may be allocated at peak 4 (Rt = 26.8 min, mass $[M-H]^-$ = m/z 754) and unknown component, peak 5 (Rt = 28.5 min, mass $[M-H]^-$ = m/z 975) were also detected. Finally, (+) - catechin, peak 6 (Rt = 12.05 min, mass $[M-H]^-$ = m/z 289) was found. All compounds were detected at

λmax = 275 and 325 nm except catechin was only detected at λmax = 275 nm. These results are in agreement with those published elsewhere (Bernillon *et al.* 2004, Marks *et al.* 2007).

4.1.1.3 Chlorogenic acid (CQA) content in different apple varieties

CQA is known to be susceptible to degradation by PPO enzyme, thus the browning of apples is due to enzymatic oxidation of phenolic compounds. Therefore, the susceptibility of apples to browning and differences in enzyme activity are related to the varietal differences (Rocha *et al.* 1998). HPLC chromatogram of apple juice (Braeburn) showed that 5- CQA (peak 1) presents the main component as compared to the other constituents (Figure 4-2A). These results are in line with previous studies (Eisele and Drake 2005, Marks *et al.* 2007, Markowski.J *et al.* 2009). The comparison between five different varieties of apples illustrated clear differences in their CQA content (Figure 4-3).

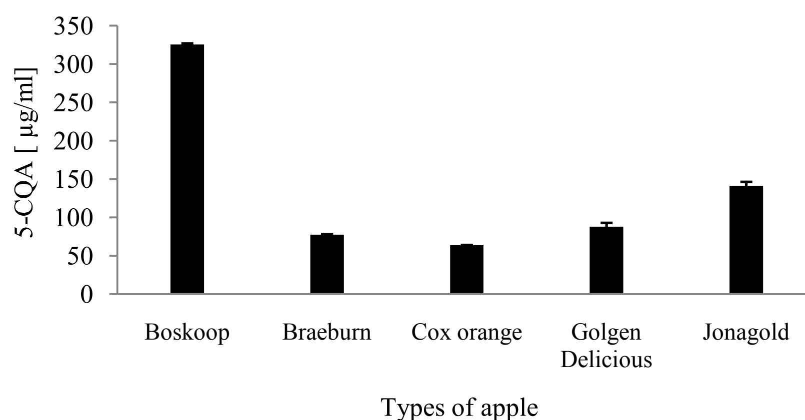


Figure 4-3 The content of chlorogenic acid present in different apple varieties

As can be seen, Boskoop variety had the highest CQA amount with 325.3±1.3 µg/ml juice, followed by Jonagold, 141.5±4.6 µg/ml. By contrast, Cox orange contained the lowest content with 63.6±0.2 µg/ml apple juice. These differences may be due to the differences in genotype, locations, postharvest treatment, maturation and storage conditions of apple varieties. In conclusion, the previous results illustrated that the Braeburn variety exposed the highest PPO activity and the lower CQA content, thus it was selected for further work (study 3).

4.1.2 Effect of storage temperature on PPO activity

The effects of the Braeburn apples stored one day before juice extraction at different temperatures - room temperature, 4°C, -20°C and -80°C - and storage of the juices at -20 °C

for two weeks on PPO activity, are illustrated in Figure 4-4. The results exhibited that there are significant differences at $P < 0.05$ between the various storage conditions, where the highest PPO activity was found for the apples which were previously frozen at -20 °C with value 15208.6 U/L apple juice, closely followed by apples, which were frozen at -80 °C, 12163.4 U/L. On the other hand, the lowest PPO activities were found in apples, which were previously stored at room temperature and in the refrigerator at + 4 °C, with values 1780.7 and 5209.5 U/L, respectively. The high activities of previously frozen apple might be caused by the destruction of cell membranes by additional ice crystals. Thus, the membrane-bound polyphenols oxidase during juice extraction can be removed better from the apple matrix.

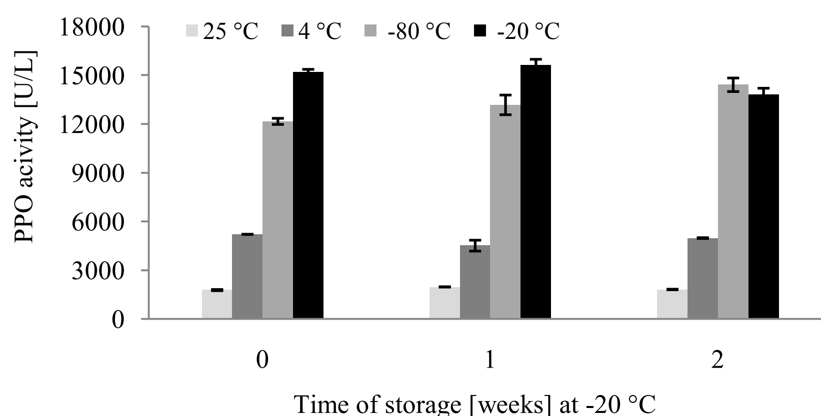


Figure 4-4 Influence of storage at – 20 °C on PPO activity

Where: 0 = apple samples stored at different temperature and 1/2 = effect of storage of the juices at -20°C

Further, the low activity at room temperature and + 4 °C may be due to presence of quinones and their endogenous phenolic precursors in crude extracts of the apples, which may react with the enzyme, resulting in activity losses (Rocha *et al.* 1998). The decision on the best storage condition for extracted juices was made on the basis of the PPO activity. Thus, all juices were directly saved, after pressing, at - 20 °C for two weeks. The results in Figure 4-4 demonstrated that there are slight changes in PPO activity for all juices after two weeks storage. Also, the juices of apples which were previously stored at -20 and – 80°C are still having very high PPO activity even after two weeks storage. These results seem to be in the line with Gouzi and Benmansour (2007), who reported that the PPO activity was relatively stable after 44 days storage at – 15 °C.

4.1.3 Effect of substrate substitution on PPO activity

According to the literature, 4- methylcatechol (4-MC) and L- Proline were used in the determination of PPO activity. The present study examined the effect of other substances on PPO activity. PPO activities after using ferulic, chlorogenic and caffeic acids and L-3,4-dihydroxyphenylalanine (L- DOPA) instead of 4-MC and by the substitution of L-proline by L-tyrosine are presented in Table 4-1. All substrates except ferulic acid were appropriate to react with the PPO. This result reinforces the use of ferulic acid as PPO inhibitor where, 0 % PPO activity was detected toward the ferulic acid. These results are in line with Ni Eidhin et al. (2006). From the apparent values, chlorogenic acid - followed by caffeic acid in the presence of L- proline - showed the best substrates for Braeburn apple PPO, where the values were 63.66 ± 1.22 and 33.55 ± 1.22 nkat / ml apple juice, respectively. On the other hand, the opposite was observed when L - tyrosine was used instead of L- proline. The PPO activity in case of combination with L-proline was higher than with L-tyrosine, especially with chlorogenic acid, where a three fold increased activity of PPO was noted. These results are in accordance with those previously reported by other authors (Rocha and Morais 2001, Ni Eidhin *et al.* 2006). They reported that tyrosine is not a good substrate for apple PPO activity.

Table 4-1 Effect of the substrate substitution on PPO activity

Substrates	PPO activity [nkat/ml]	
	L-Proline	L-Tyrosine
Caffeic acid	33.55±1.22	23.51±2.63
Chlorogenic acid	63.66±1.22	20.22±3.04
Ferulic acid	0.00±0.00	-
L- DOPA [10mM]	1.72±0.00	17.63±0.61

Table 4-1 shows that the PPO activity in case of the combination of L-DOPA with L-tyrosine was higher than with L-proline, where the PPO activity with L-tyrosine was 17.62 nkat / ml while with L-proline it was only 1.72 nkat / ml, which corresponds to only about one-tenth. This observation may be explained that the increase in the PPO activity was due to L-tyrosine, where, since 0.1 M L-tyrosine was used and the PPO has a tyrosinase activity, the L-tyrosine can be oxidized to L-DOPA (Ali *et al.* 2007, Surwase *et al.* 2012).

4.1.4 Preliminary characterization of interactions of CQA with model amino acids

The CQA is susceptible to both enzymatic and non-enzymatic oxidation in the presence of oxygen. In both cases the subsequent oxidation of these to reactive and redox active o-quinones appear to be necessary to generate electrophilic species capable of undergoing a nucleophilic addition to proteins (Rawel and Rohn 2010). Principally, two types of interactions have been reported – non-covalent and enzymatic (under the action of PPO) / non-enzymatic covalent modification of the protein side chains (Rawel and Rohn 2010). A covalent interaction of proteins preferably takes place at the ε-amino group of lysine and thiol groups of cysteine with a prior conversion to quinone. In previous studies, the covalent interactions between chlorogenic acid (5-CQA) quinone and amino acids derivatives in presence of tyrosinase were studied by HPLC-MS (Namiki *et al.* 2001, Yabuta *et al.* 2001, Prigent *et al.* 2008, Schilling *et al.* 2008). With a view to understanding the covalent interactions between CQA and amino acid side chains, the incubation of lysine and N-BOC-lysine at 40°C for 24 h with 5-CQA in the presence of tyrosinase in a simple model system was applied as described in 3.2.4). This reaction can be modulated through the loss of CQA and the amount of free amino groups and the change in the antioxidative capacity. The effect of both enzymatically and non - enzymatically (control) reactions of 5-CQA with lysine on composition of CQA content are given in Figure 4-5.

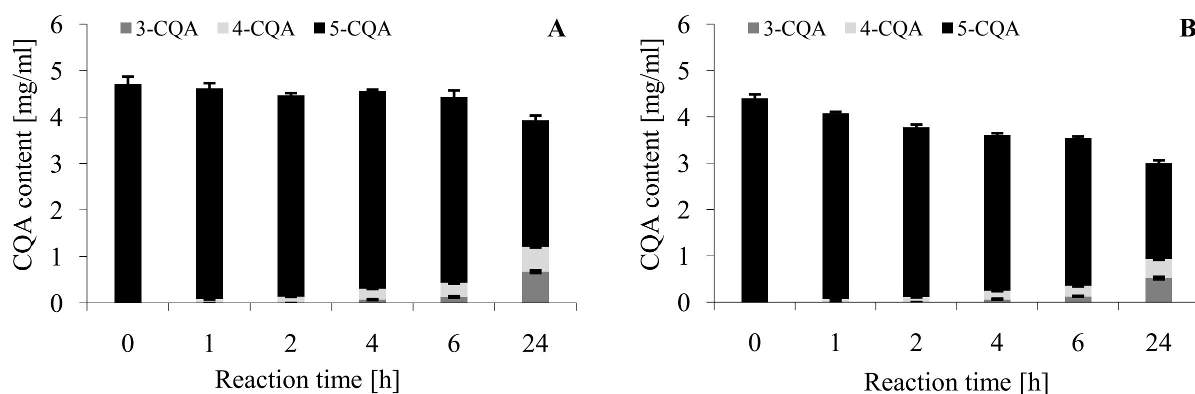


Figure 4-5 Effect of reaction of 5-CQA with lysine on the composition of CQA (mg/ml) depending on reaction time

Where: A) = Control experiment without tyrosinase and B) = Sample with tyrosinase

The results showed that the reaction initially leads to the formation of 3-CQA and 4-CQA isomers in both reactions; in parallel there was a decrease in the amount of 5-CQA, however

the loss in enzymatically modulated reaction (at pH 7) was higher than in the control reaction. The loss in 5-CQA content after 24 h reaction was 53.2 and 42.6% for enzymatically and control conditions, respectively. Further, the change in free amino groups (FAG) was monitored by OPA method and the data are shown in Figure 4-7A. As can be observed, the amount of free amino groups was decreased depending on the reaction time, where % of decrease in FAG was 5.4 and 7.6% after 1 and 24h reaction, respectively. The decrease in FAG content can be explained by the oxidation giving rise to the formation of a dimer which subsequently forms an adduct with lysine to finally result in a benzacridine derivative - as reported by Namiki *et al.* (2001) and Yabuta *et al.* (2001) and confirmed by Schilling *et al.* (2008) - with the aid of HPLC coupled with ESI-MS^n. Accordingly, based on these observations, a tentative list of possible adduct structures was derived (Figure 4-6). In addition, the interaction between 5-CQA and amino acid lysine showed only a slight effect on the antioxidative capacity as exemplarily documented depending on the reaction time (Figure 4-7B).

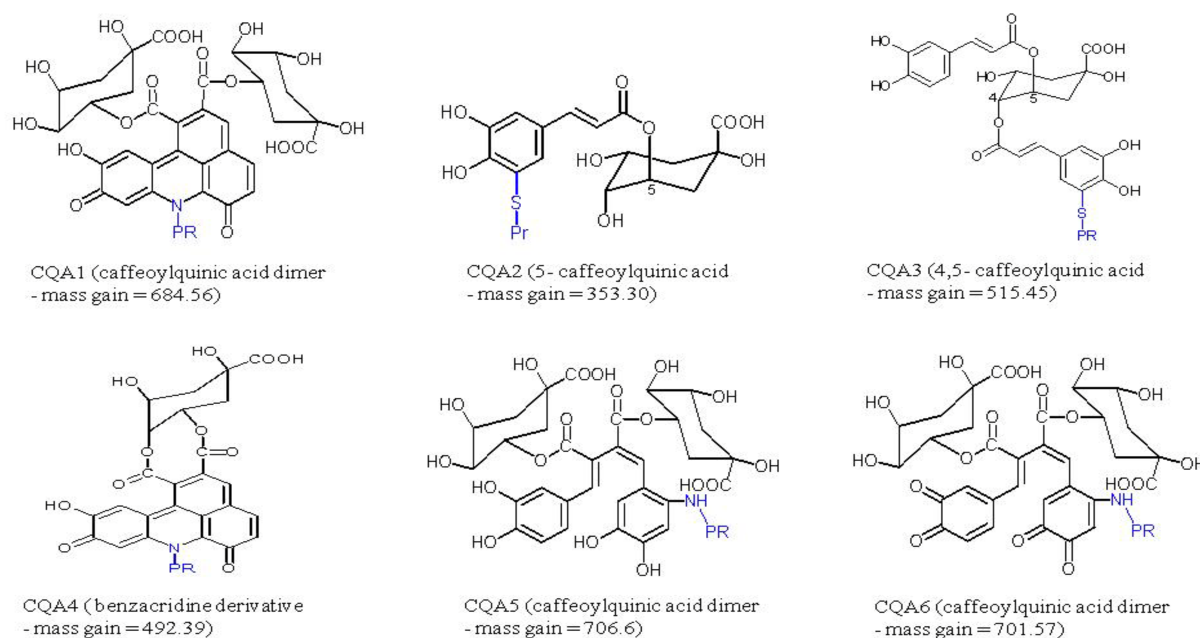


Figure 4-6 Proposed modifications of CQA derivatives with thiol groups of cysteine and lysine amino side chains

* Pr-N- / Pr-S- Protein molecule involved in the adduct formation with CQA derivatives

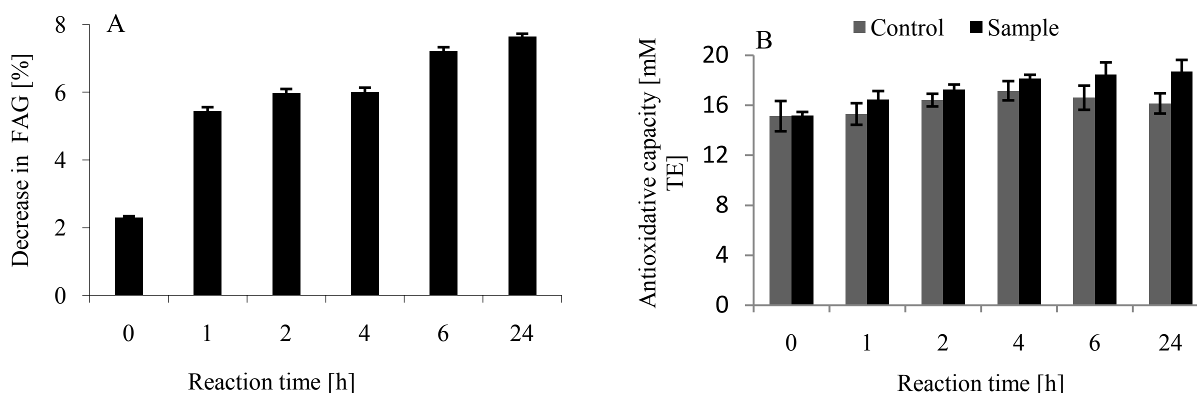


Figure 4-7 Effect of reaction of 5-CQA with lysine on the amount of free amino groups (FAG) (A) and change in the antioxidative capacity (B) depending on reaction time

Finally, identification of adducts formed by HPLC-MS could be also used to characterize the interaction between 5-CQA and lysine and N-BOC-lysine. The HPLC-MS data showed a large number of intermediary substances of low intensities (Figure 4-8), where among others, following few can be proposed: a $[M+H]^-$ ion at m/z 705 - corresponding to a CQA dimer (Figure 4-9A). This m/z value was detected as peaks of high intensity at many retention times. A $[M+H]^+$ ion at m/z 533 and Rt= 34.3 min- corresponding to a CQA dimer minus a quinic acid moiety and $[M-H]^-$ ion at m/z 514, corresponding to Di-CQA were also detected (Appendix, Figure 7-1).

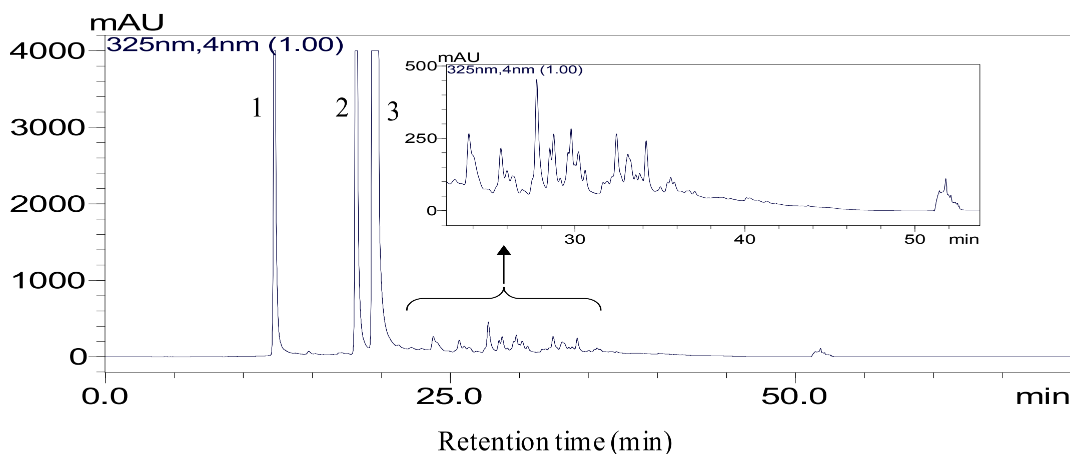


Figure 4-8 Exemplary HPLC chromatograms for chlorogenic acid isomers and covalent interaction products of chlorogenic acid and N-BOC-lysine at (325 nm)

Where: 1, 3-O-caffeoylquinic acid; 2, 4-O-caffeoylquinic acid; 3, 5-O-caffeoylquinic acid and reaction conditions: pH 7, Incubation temperature = 40°C, Tyrosinase = 20U/ml

Furthermore, a $[M\text{-}H]^+$ ion at m/z 826 and Rt = 40.13 min - corresponding to a benzacridine derivative with lysine was recorded (Appendix, Figure 7-1). Finally, a $[M\text{-}H]^+$ ion at m/z 947 and Rt= 33.06 min - corresponding to an adduct of a CQA dimer with N-BOC-lysine (Figure 4-9B). These results, and those supplied by other research teams (Namiki *et al.* 2001, Yabuta *et al.* 2001, Prigent *et al.* 2008, Schilling *et al.* 2008) seem to be in agreement. Furthermore, this reaction was also done between 5-CQA and amino acid lysine at different pH, incubation temperature and the amount of tyrosinase applied and our results document that this reaction can be modulated through the loss of CQA and the amount of free amino groups (Appendix, Figure 7-2 - Figure 7-6). The results demonstrated that, in enzymatically modulated reaction, the amount of decrease in CQA content was related with the amount of enzyme, where the values were 3.27 and 1.86 mg/ml when the amount of enzyme increased from 20 to 240 U/ml, respectively. On the other hand, no change was found in control reaction (Appendix, Figure 7-2).

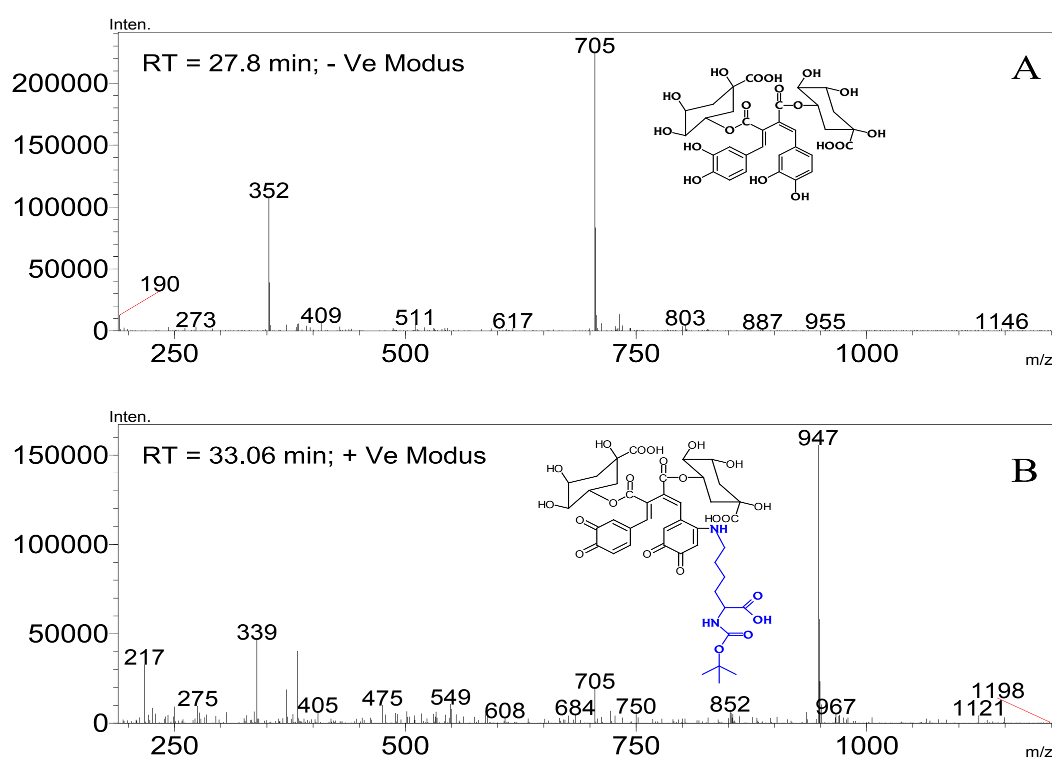


Figure 4-9 Mass spectra of the reaction products between amino components and CQA

Where: A) = CQA dimer and B) = N-BOC-lysine -CQA adduct

Moreover, the amount of FAG was also decreased depending on amount of enzyme, where % of decrease in FAG was 7.6 % at 20U enzyme/ml then increased significantly till 38.5% at 240 U/ml (Appendix, Figure 7-3). Maximum loss of CQA occurs at pH 9 (mainly due to non-enzymatic oxidation processes) and at pH 7 and 25°C (due to enzymatic conversion) (Appendix, Figure 7-4 - Figure 7-6). Moreover, the optimal conditions for the conversion as assessed by both the loss of CQA and free amino groups of lysine can be given at pH 7 and 25°C, the conversion increasing with incubation time and depending also on the amount of tyrosinase present. In conclusion, these results gave us an indication of the most effective conditions necessary for the reaction between CQA and proteins (study 2 and 3).

4.1.5 Preliminary characterization of interactions between CQA and proteins

In order to illustrate the interactions of CQAs with proteins in the next studies, experiments were conducted with different reference food proteins by following the quenching of intrinsic tryptophan fluorescence of proteins by ligand binding (Rawel *et al.* 2006). The interaction of 5-CQA with model proteins (milk whey β-lactoglobulin, casein-Na, soy glycinin and skimmed spray dried milk) was monitored. The results (Figure 4-10 A) document the concentration-dependent effect of the binding of 5-CQA to these proteins, which seems to be relatively independent of the protein applied. The binding depends on the protein concentration applied as illustrated using β- lactoglobulin as a model system (Figure 4-10 B). At the concentration of 40 µM 5-CQA, a 50% decrease in tryptophan fluorescence was obtained at a CQA: protein ratio of 1:7 (14.4:100 mg/L). Therefore, maintaining this ratio; the binding was further studied for different absolute concentrations of both reactants (Figure 4-10 C). These results show that a certain minimum absolute concentration of both ligands is necessary to provoke the binding. Thereafter, with higher absolute concentrations of both ligands present, and maintaining a CQA: protein ratio of 1:7, a saturation of the binding sites may result. Finally, each of the major hydroxycinnamic acid derivatives was fractionated from green coffee beans (Columbia) and their binding to β-lactoglobulin was studied at a constant concentration of 40 µM, directly after addition of the ligand and after incubation at room temperature for 24h (Figure 4-10 D). It can be noted that the di-CQA are more reactive than other CQA isomers, with 3,5 di-CQA being the most eligible reaction partner. A further incubation for 24 h (Figure 4-10 D) results in a continued binding of CQA to β-lactoglobulins.

Such interaction may have a character of covalent modification in proteins as described previously (Prigent *et al.* 2003, Rawel and Rohn 2010). A slight shift for feruloylquinic acids (FQA) and for protein alone was also observed, which may putatively be attributed to structural changes, involving thiol-disulfide interexchange.

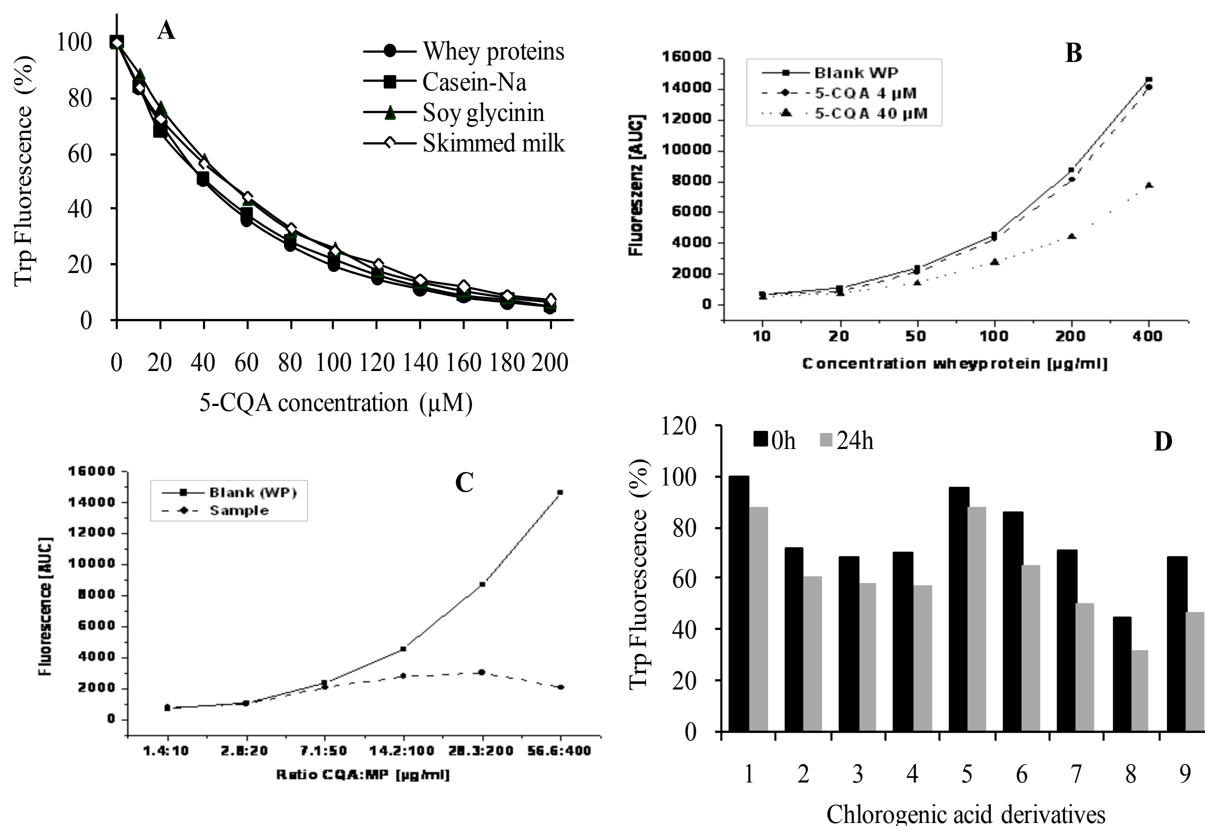


Figure 4-10 Binding of 5-CQA as determined by the quenching of intrinsic tryptophan fluorescence

Where: a) with different protein containing model systems, b) depending on amount of protein added with 5-CQA concentration remaining constant, c) depending on different 5-CQA/whey protein concentration ratios and d) with different CQA derivatives at a constant concentration of 40 µM directly after addition of the ligand and after incubation at room temperature for 24h; 1, whey protein control; 2, 3-CQA; 3, 4-CQA; 4, 5-CQA; 5, FQA; 6, CQL; 7, 3-4 di-CQA; 8, 3-5 di-CQA; 9, 4-5 di-CQA; CQA, Caffeoyl-Quinic Acid; FQA, Feruloyl-Quinic Acid; CQL, Caffeoyl-Quinic Acid lactone and di-CQA, di Caffeoyl-Quinic Acid

4.2 Study 2: Characterization and application of coffee storage proteins in a model multifunctional food

It was demonstrated in study 1 that, chlorogenic acid (CQA) can react covalently with amino acid side chains and proteins under enzymatic and alkaline conditions. These reactions effect the physicochemical and digestion properties of proteins, which are important in food industries (Rawel *et al.* 2001a, Prigent *et al.* 2008), as well as the nutritional value of proteins by the reaction with the essential amino acids and inhibition of protein enzymes (Kroll *et al.* 2003, Prigent *et al.* 2008, Schilling *et al.* 2008). Therefore, the aim of this study was to understand these interactions and their effect on the properties of coffee storage proteins extracted from different types of coffee beans including *Coffea arabica (CA)* and *coffea robusta (CR)* species, which have not only protein and a high CQA content but also polyphenol oxidase enzyme. The data obtained was compared to the faba bean protein as control. The main protein (storage protein) from the faba bean is similar to the coffee bean protein, both belonging to 11S class proteins, thus providing a sound basis for such a comparison (Rawel *et al.* 2005b, Ali *et al.* 2012).

4.2.1 General characterization of coffee and faba beans

The general characterization data of coffee and faba beans are provided in Table 4-2. Total protein content is obtained by using factor 6.25, consequently *CA* and *CR* species contained total protein content in the range 10.3 ± 0.1 – 13.00 ± 2.5 % on DM basis. These values are in line with those previously reported (Belitz *et al.* 2004, Rawel *et al.* 2005b, Souci *et al.* 2008).
A high protein content was found in ground FB, 25.8 ± 0.4 and 28 ± 0.7% for FB Sakha3 (S3) and Giza 3 (Gi3), respectively. The values are lower than values obtained previously (Lattanzio *et al.* 1983, Duc 1997, Haciseferogullari *et al.* 2003, Alghamdi 2009). In contrast, they are in agreement with values obtained by Musallam *et al.* (2004). Also, results in Table 4-2 show that there are no significant differences among *CR* and *CA* in protein content at ($p < 0.05$). On the other hand, significant differences between FB and two *Coffea* species were found. Caffeine is a major alkaloid in green coffee (GC) beans. The results documented in Table 4-2 show significant differences between *CR* and *CA* beans in caffeine content (at $p < 0.05$), where *CR* contains about two fold more content than *CA*. The values of caffeine in both *Coffea* species ranged between 0.9 and 1.6 ± 0.1 g/100g DM. These values are similar to those

reported (Martín *et al.* 1998, Belachew 2003, Franca *et al.* 2005, Rawel *et al.* 2005b, Ramalakshmi *et al.* 2007). On the other hand, the two faba beans contained no caffeine. Total phenolic compounds in the acidified methanol extracts as determined by the Folin-Ciocalteu and HPLC methods using 5-CQA as standard are furnished in Table 4-2.

Table 4-2 General characterization of coffee and faba beans based on dry matter

Parameters	*Coffea arabica*		*Coffea robusta*		Faba beans	
	Brazil	Guatemala	Uganda	Indonesia	Giza 3	Sakha 3
Protein-N [g/100g]	11.9±0.1	10.3±0.1	13.0±2.5	11.2±0.1	28±0.7	25.8±0.4
Caffeine [g/100g]	0.9±0.0	0.8±0.0	1.5±0.0	1.6±0.1	Traces	Traces
Total phenolics (Folin) [g/100g]	4.1±0.1	3.6±0.2	7.8±0.1	6.9±0.5	0.6±0.1	0.4±0
Total phenolics (HPLC) [g/100g]	4.3±0.0	4.0±0.1	7.8±0.2	7.0±0.1	Traces	Traces
Antioxidative capacity [mM/100g]	11.4±0.5	10.2±0.3	18.0±0.7	18.5±0.2	4.9±0.3	5±0.5
PPO activity [U/100 g]	291.8±39.2	159.9±5.9	104.7±0.9	107.3±17.0	Nd	Nd

Nd = not determined

The results demonstrate that there are slight differences in phenolics content between the two applied methods. Total phenolic compounds by the Folin-Ciocalteu method (reported as CQA equivalents) were 3.6 - 5.5% (*CA*) and 6.9 - 7.8% (*CR*), 0.6% FB-Gi3 and 0.4 % FB-S3 on a DM basis compared to 4 - 4.3 %, 7- 7.8%, and traces by the HPLC method, respectively. These results are comparable to those recently reported by Kim and Lee (2010), where 4.8% as CQA equivalents were found. Partially also agreeing to those reported for *CA* (5.4-5.7%) and *CR* (7.6%) on DM basis (Farah *et al.* 2005).

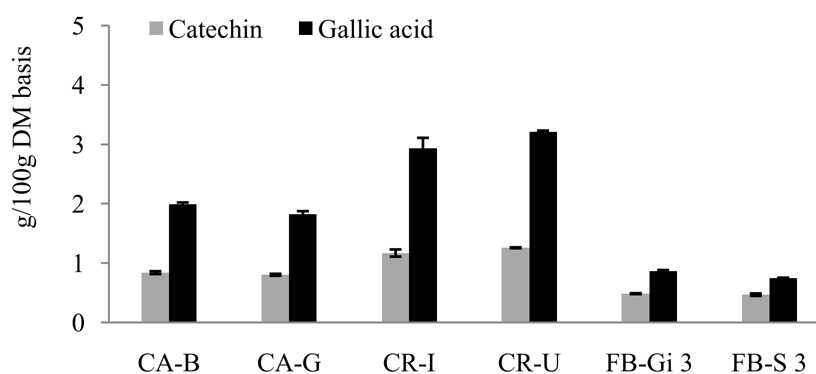


Figure 4-11 Total phenolic compounds as gallic acid and catechin equivalents (g/100g DM) for green coffee and faba beans

Where: CA, *Coffea arabica*, CR, *Coffea robusta* from: B, Brazil; G, Guatemala; I, Indonesia; U, Uganda; FB, faba beans; Gi 3, Giza 3, and S 3, Sakha 3

Furthermore, many authors tend to report the total phenol contents as gallic acid or catechin equivalents. Generally lower values are obtained when these standards are used for calibration (Figure 4-11). There were significant differences in total phenolic content expressed as chlorogenic, gallic acid and catechin equivalents. These differences may be due to the differences in genotype, locations and postharvest treatment or the quality of the beans being assessed.

The antioxidative capacity of coffee beans is related to the presence of polyphenols, as discussed above. The TEAC assay was used to determine the total antioxidative capacity of the coffee and faba bean extracts and the results are given in Table 4-2. The TEAC assay is based on an electron transfer to an $ABTS^{\bullet +}$ radical. Consequently, differences in the determined values are to be expected and are recorded. Significant differences were found among the species of *Coffea* and FB extracts by the $ABTS^{\bullet +}$, where the antioxidative capacity (Trolox equivalents) ranged from 10.2 to 11.4 (*CA*) and 18 to 18.5 (*CR*) mM/100g DM compared to 4.9 and 5 mM/100 g DM for FB-Gi3 and FB-S3, respectively. These results are higher than those reported by Gómez-Ruiz *et al.* (2008). They found that the value of antioxidant activity for green coffee from *CA* was 6.15 ± 0.62 µM according to $ABTS^{\bullet +}$. These differences may not only be due to the content of phenolic compounds as mentioned above but also due to conditions of agricultural practices, maturation, postharvest treatment and storage. The high antioxidative capacity of coffee methanol extract is due to the presence of phenolic compounds including chlorogenic acid and its isomers, which make them more suitable as a source of natural antioxidants; their utility compared to faba beans can be explored in the context of the food industry.

Polyphenol oxidase (PPO) is a copper containing enzyme responsible for hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-diquinones. Proline was used as substrate to determine PPO activity in the different coffee beans and data are outlined in Table 4-2. The results showed that both *CA* varieties have higher activities resulting in significant differences to *CR* ($p < 0.05$). Therefore, the highest PPO activity was found in coffee from Brazil, 291.8 ± 39.2 U/100g DM followed by coffee from Guatemala, 159.9 ± 5.9 U/100g DM.

4.2.2 Characterization of the individual phenolic compounds from coffee and faba beans by HPLC-MS

Phenolic acids exist widely in nature as combinations of free acids, esters or ethers. The largest amounts of CQA in plants were found in green coffee beans (Farah *et al.* 2005). The exemplary chromatograms at 325 nm and the identification parameters used to analyze the main phenolic compounds in green coffee are shown in Figure 4-12 and Table 4-3, respectively. Caffeoylquinic acids (CQA), with three esters (3-, 4- and 5-CQA); di-caffeoylquinic acids (diCQA), with three isomers (3,4- 3,5- and 4,5-diCQA) and feruloylquinic acids (FQA), with two isomers (3- and 4-FQA) were identified in coffea species by HPLC-MS.

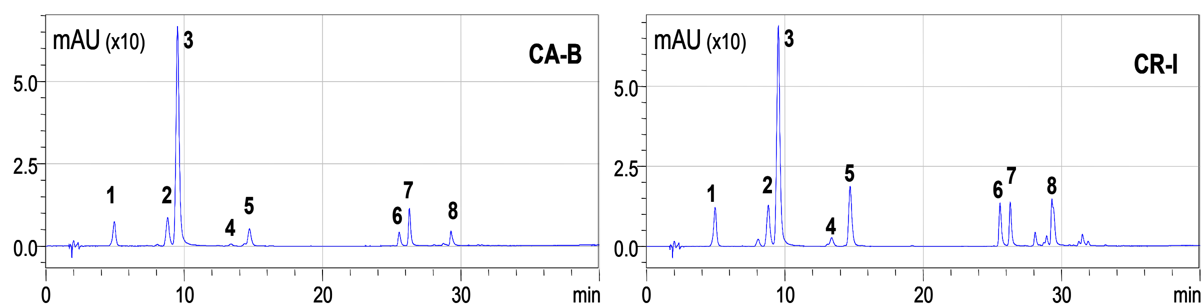


Figure 4-12 Exemplary HPLC chromatograms for phenolics in green coffee beans

Table 4-3 Identification parameters applied for phenolic compounds in green coffee bean

Peak	1	2	3	4	5	6	7	8
m/z*	353	353	353	367	367	515	515	515
UV/Vis (nm)	280/325	280/325	280/325	280/325	280/325	325	325	325
Identity	3-CQA	4-CQA	5-CQA	FQA 1	FQA 2	3,4-di-CQA	3,5-di-CQA	4,5-di-CQA

* affirmed by HPLC-MS, Where: CA, Coffea arabica; CR, Coffea robusta from: B, Brazil; I, Indonesia CQA, Caffeoyl-Quinic Acid; FQA, Feruloyl-Quinic Acid and di-CQA, di Caffeoyl-Quinic Acid)

These results are in the line with previous studies (Farah *et al.* 2006, Farah *et al.* 2008, Alonso-Salces *et al.* 2009, Ramalakshmi *et al.* 2011). The comparison between *CA* and *CR* beans shows typical phenol profiles to be present in each case with clear differences in their amounts as depicted in Table 4-4. As can be seen, 5-Caffeoylquinic acid (5-CQA) was the major phenolic compound in both *CA* and *CR*. Moreover, the percent of 5-CQA in *CA* was significantly higher than *CR*, where 5-CQA accounts for ca. 57 and ca. 35-37% of *CA and CR*, respectively. Moreover, significant differences, in the amounts of CQA, FQA and Di-CQA, at

($p < 0.05$) were observed for both species. These results are in line with previous studies (Farah *et al.* 2005, Farah *et al.* 2006, Alonso-Salces *et al.* 2009, Ramalakshmi *et al.* 2011).

Table 4-4 The content of CQA and its isomers (mg/g) on DM basis in green coffee beans

Samples	*Coffea arabica*				*Coffea robusta*			
	Brazil		Guatemala		Uganda		Indonesia	
Phenolics	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3-CQA	3.05	0.23	2.80	0.14	4.45	0.21	5.17	0.79
4-CQA	3.78	0.01	3.36	0.07	5.50	0.08	5.01	0.43
5-CQA	24.17	1.73	22.80	0.68	29.54	0.73	24.50	1.61
FQA	1.11	0.01	1.14	0.01	1.57	0.04	1.8	0.07
FQA	2.75	0.16	2.58	0.03	6.65	0.08	7.15	0.61
3,4-di-CQA	1.97	0.05	1.87	0.07	4.33	0.14	4.33	0.14
3,5-di-CQA	3.65	0.09	3.13	0.20	4.02	0.25	4.17	0.23
4,5-di-CQA	2.04	0.05	2.35	0.09	5.93	0.1	5.96	0.33
Unknown	-	-	-	-	16.18	1.23	11.59	2.29
Total	42.53	-	40.03	-	78.18	-	69.68	-
% 5- CQA	56.8	-	57	-	37.8	-	35.2	-

Where: CQA, Caffeoyl-Quinic Acid; FQA, Feruloyl-Quinic Acid and di-CQA, di Caffeoyl-Quinic Acid)

4.2.3 Characterization of the free amino acids content in green coffee and faba beans

Depending on the types of coffee and faba beans, the variations in the levels of the different amino acids are illustrated in exemplary chromatograms for coffee and faba beans in (Appendix, Figure 7-7) and Table 4-5.The results showed that there are clear differences between *CA and CR* where, total amounts of free amino acids were 215.5 and 273.7 for *CA* (Brazil and Guatemala) while *CR* (Uganda and Indonesia) contained 159.6 and 158.3 mg /100 g of ground beans, respectively. These differences may also be due to the differences in genotype, locations, postharvest treatment, maturation and storage.

The main amino acid in the two *coffea* species was Glu. This result is in the line with different citations (Arnold and Ludwig 1996, Bytof *et al.* 2005, Casal *et al.* 2005). The amino acids with the highest levels were Ser, Asp, Asn and Ala. These results partially agree with some studies (Shimizu and Mazzafera 2000, Muller and Hofmann 2005, Dias *et al.* 2012)**.** Moreover, slight differences between the types of *coffea* were found. On the other hand, the main amino acids in FB were Arg, Glu, Asp and Asn. The results do not completely agree with those published elsewhere, their content as already mentioned depending on various factors (Alghamdi 2009). Finally, there are significant differences between *coffea* species and

faba varieties in the total amino acids content (Table 4-5).

Table 4-5 Free amino acids determined in the coffee and faba beans (mg/100g powder on DM basis)

Samples	*Coffea arabica*				*Coffea robusta*				*Faba*			
	Brazil		Guatemala		Uganda		Indonesia		Giza 3		Sakha 3	
Amino acids	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Asp	30.9	2.5	39.1	3.7	12.2	0.9	13.6	0.1	43.9	5.4	35.4	7.2
Glu	78.9	6.7	82.4	11.9	37.9	0.2	36.8	1.8	79.2	10.0	79.4	18.2
Asn	17.6	1.6	23.8	0.1	11.8	1.2	13.9	0.5	14.3	1.0	10.3	1.7
Ser	14.5	0.9	29.8	1.7	16.4	0.4	17.2	0.8	6.8	0.1	6.8	0.3
Gln	5.8	0.3	8.1	1.5	4.3	0.3	4.7	0.1	4.1	0.1	3.7	0.3
Gly	5.3	0.2	10.0	3.6	10.0	0.7	8.8	0.0	11.0	0.2	10.8	1.0
Thr	7.5	0.3	10.2	3.3	7.1	0.4	6.3	0.0	9.7	0.2	10.8	0.9
Arg	6.5	0.5	10.1	2.1	9.7	0.8	10.4	0.4	280.0	11.1	114.8	15.2
Ala	11.3	1.0	15.8	0.1	10.0	1.2	8.8	0.3	10.3	0.8	8.0	1.5
Tyr	3.6	0.2	5.5	0.7	5.7	0.6	6.2	0.3	5.8	0.5	4.0	0.6
Val	5.7	0.2	8.3	1.7	6.0	0.3	6.1	0.2	5.4	0.2	5.2	0.6
Ile	5.8	0.3	8.4	0.9	7.4	0.6	6.1	0.1	4.2	0.1	3.7	0.3
Phe	10.2	1.4	9.9	0.2	9.1	1.3	7.7	0.3	8.8	0.6	8.6	1.9
Leu	4.1	0.4	4.3	0.1	5.1	0.3	4.4	0.1	3.9	0.2	3.5	0.7
Lys	7.8	0.1	8.1	1.1	7.0	0.0	7.3	0.0	9.3	0.3	9.7	0.5
Total	215.6		273.7		159.6		158.3		496.8		314.6	

4.2.4 Effects of various stages of roasting on the coffee beans

4.2.4.1 Effect on protein

During the coffee roasting process, many compounds may be changed in its content and others newly formed. The effect of the roasting degree on proteins in *CA* (Columbia) was determined by investigating the changes in the protein profile of coffee changes during roasting.

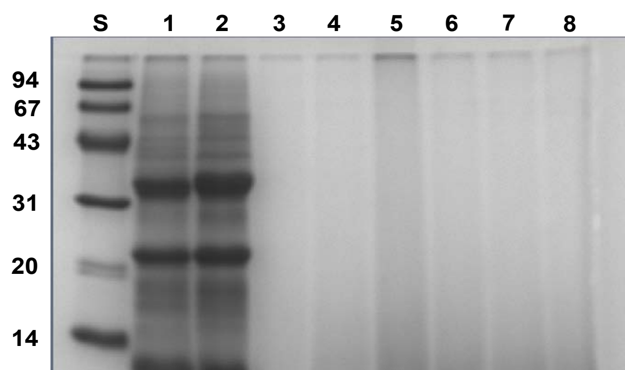


Figure 4-13 SDS-PAGE for roasted coffee from Columbia at different roasting degrees

Where: S, Standard proteins; 2, Unroasted; 3, Light roasted; 4, Medium roasted; 5, City roasted; 5, French roasted and 7, Italian roasted coffee

Figure 4-13 shows SDS-PAGE for coffee Columbia at different roasting degrees, as described in methods section. In lane 2, green coffee, two bands which refer to α and β- chains with molecular weight ~ 34 and 21 KDa respectively were detected. After that, no detectable protein bands after roasting, lanes 3-8, were found. These results can be explained by polymerization and fragmentation reactions during the roasting as well as the integration of protein into melanoidins during Maillard reaction (Montavon *et al.* 2003b).

4.2.4.2 Effect on caffeine content

Regarding the effect of the roasting degree on caffeine content, it is worth noting that there is little agreement in the literature. According to the roasting degree, there was slight change in caffeine content as illustrated in Figure 4-14. As can be observed, caffeine content was slightly decreased at the first roasting option, where the caffeine content in green coffee bean (unroasted) was 1.35 g /100g powder coffee which decreased to 1.10 and 1.27 g/100g in light and medium roast, respectively. Nevertheless, a slight increase in caffeine content at light and medium roast conditions was reported (Hečimović *et al.* 2011, Tfouni *et al.* 2012). This difference can be ascribed to the difference in roasting conditions, for example time and temperature. On the other hand, our results are in accordance with the ones previously reported by Casal *et al.* (2005). They reported that a slight decrease in caffeine content was recorded after roasting both *C. robusta* and *C. arabica* at 230 and 240 °C for 15 min. Figure 4-14 also shows that, there was a slight increase in caffeine content in city, French and Italian roast and these results again differ from Tfouni *et al.* (2012), who found that the caffeine content was decreased at stronger roast degrees.

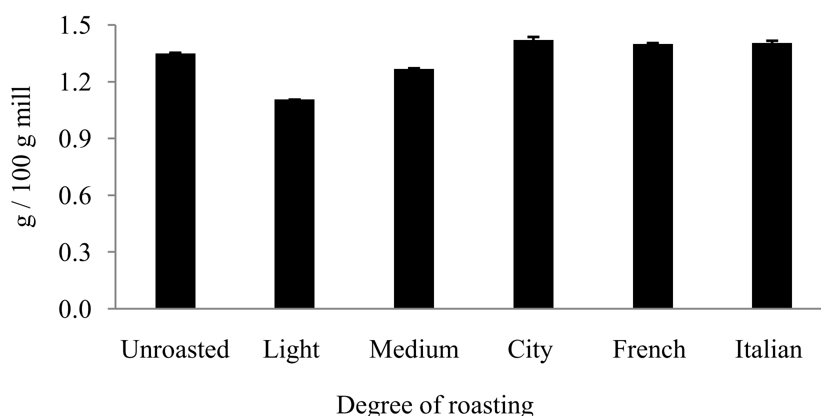


Figure 4-14 Effect of roasting conditions on caffeine content (µg/mg milled coffee from Columbia)

4.2.4.3 Effect on phenolics content

Coffee beans from Columbia were roasted under different conditions as described in the methods section. An exemplary chromatogram and the identification parameters used to analyze the main phenolic compounds in roasted coffee are given in the Appendix, Figure 7-8 and Table 7-1, respectively. HPLC data demonstrate that, during roasting, new derivatives such as chlorogenic acid lactones (CQL) and *p*-coumaric acid (p-CA) are formed or released. Seven CQL have been identified as a consequence of roasting: 3-CQL, 4-CQL, 3-coumaroylquinic-1,5-lactone, 4 coumaroylquinic-1,5-lactone, 3-feruloylquinic-1,5-lactone, 4-feruloylquinic-1,5-lactone, and 3,4-dicaffeoylquinic-1,5-lactone, where 3-CQL and 4-CQL represent the most abundant lactones in *CA* (Farah *et al.* 2005). The loss of CQAs during the roasting process of coffee was observed and the data are shown in Table 4-6. The results show that 5-CQA is the major phenolic constituent in both green and roasted coffees. The 5-CQA level drastically decreased from 48.27 to 0.99 mg/g in green and Italian roasted coffee respectively, while the levels of 3-CQA, 4-CQA and FQA1 had increased to twice their original values at the beginning of the roast process, as under conditions of light roast.

Table 4-6 Effect of various stages of roasting on CQA and its isomers (mg/g milled coffee from Columbia)

Phenolics	Roasting degree					
	Unroasted	Light	Medium	City	French	Italian
3-CQA	4.66±0.07	7.59±0.08	4.01±0.06	1.39±0.01	0.97±0.01	0.96±0.01
4-CQA	5.85±0.26	8.55±0.10	5.11±0.07	1.74±0.02	1.08±0.02	1.01±0.00
5-CQA	48.27±0.44	18.31±0.35	9.10±0.24	1.89±0.05	1.06±0.02	0.99±0.01
Total CQA	58.78±0.77	34.45±0.52	18.21±0.26	5.01±0.05	3.11±0.05	2.96±0.02
FQA 1	1.52±0.14	3.16±0.00	1.54±0.03	1.34±0.00	1.03±0.01	1.01±0.01
FQA 2	5.49±0.12	2.93±0.13	1.94±0.04	1.17±0.02	1.10±0.01	0.95±0.00
Total FQA	7.01±0.26	5.80±0.37	3.48±0.01	2.51±0.02	2.14±0.01	1.97±0.02
3,4- Di-CQA	2.71±0.19	1.88±0.09	1.26±0.01	Nd	Nd	Nd
3,5- Di-CQA	5.88±0.18	1.83±0.14	1.26±0.01	Nd	Nd	Nd
4,5- Di-CQA	3.20±0.14	2.09±0.14	1.34±0.05	Nd	Nd	Nd
Total Di-CQA	11.79±0.51	5.80±0.37	3.86±0.03	Nd	Nd	Nd
Total	77.58±1.55	46.05±1.26	25.55±0.22	7.52±0.03	5.22±0.05	4.93±0.00

Where: CQA, Caffeoyl-Quinic Acid; FQA, Feruloyl-Quinic Acid, di-CQA, di Caffeoyl-Quinic Acid and Nd, not detected

It has been suggested that roasting causes isomerization of 5-CQA prior to the formation of the lactones, thus partly explaining this observation. A breakage of the carbon-carbon bonds

of CQA, resulting in isomerization and degradation was caused by the high temperature of the roasting process (Farah *et al.* 2005). In addition, it is possible that partial hydrolysis of Di-CQA to monoester derivatives occurs in addition to isomerization. The amount of loss in total CQAs depends on the roasting conditions applied (Bicchi *et al.* 1995, Belitz *et al.* 2004, Sacchetti *et al.* 2009, Somporn *et al.* 2011, Tfouni *et al.* 2012), where the % of loss in total CQAs increased from ca. 40 % in light roast to 93 % in Italian roast. The loss in CQA content may be also due to the series of complex reactions responsible for the flavor development during the roasting of coffee beans. Some of them may be allocated to Maillard and Strecker reactions, degradation of proteins, polysaccharides, trigonelline and chlorogenic acids, and the oxidation of CQA to oxidized form (quinone which can react with protein during the roasting) (De Maria *et al.* 1996, Montavon *et al.* 2003b, Rawel *et al.* 2005b). Finally, the decrease in total di-CQA can be summed to be in the range of 50-100%, whereas the total FQA was degraded to about 72 %.

4.2.4.4 Effect on free amino acids content

Recent work clearly shows that free amino acids and peptides are necessary for the generation of coffee aroma during the Maillard reactions. The free amino acid amounts in green and roasted coffee Columbia are listed in Table 4-7.

Table 4-7 Effect of roasting process on free amino acids content (mg/100g milled coffee Columbia beans)

Amino acids	Roasting degree					
	Unroasted	Light	Medium	City	French	Italian
Asp	11.9	11.1	10.9	10.8	11.1	11.1
Glu	24.9	14.4	12.1	9.0	9.0	14.4
Asn	9.5	4.9	4.9	4.8	4.9	4.9
Ser	7.0	2.9	2.4	1.6	3.7	2.9
Gln	4.7	2.9	2.9	2.9	2.9	2.9
Thr	4.9	2.4	2.3	2.4	2.8	2.4
Arg	3.7	1.1	0.9	0.9	1.0	1.1
Ala	8.5	4.4	4.3	4.3	4.6	4.4
Tyr	6.5	5.3	5.4	5.4	5.3	5.3
Val	13.4	6.7	6.6	6.7	6.7	6.7
Met	2.9	2.5	2.8	2.6	2.5	2.5
Ile	7.2	6.3	6.2	6.2	6.4	6.3
Phe	6.7	4.4	4.4	4.4	4.4	4.4
Leu	5.7	4.3	4.4	4.6	4.8	4.3
Lys	13.1	12.4	12.0	12.6	13.6	12.4
Total	130.7	85.9	82.5	79.2	83.6	85.9

Standard deviations of all data were less than 5%

Total free amino acids are higher in green coffee than roasted coffee and these results are in accordance with Casal *et al.* (2005), where the values were 130.7 and 85.9 mg /100g, respectively. Furthermore, the results showed that Glu acid is the major free amino acid in both green and roasted coffees. Also, depending on the inherent thermal stabilities of amino acids, all amino compounds analyzed decreased with roasting at different rates. For example, the level of Ser decreased considerably in all the samples followed by Arg, Val, Thr, Glu and Gln. The explanation for this decrease is the free amino acids have free amino groups available for participation in the Maillard reaction during the roasting process and are involved in the formation of both the flavor and the color of the coffee.

4.2.4.5 Effect on antioxidative capacity

During the process of roasting, the amount of detectable phenolic compounds was decreased. Therefore, the antioxidative potential changes depending on the parameters applied during roasting. Data in Figure 4-15 illustrate the antioxidative capacity for the extracts which were obtained from roasted coffee Columbia using acidic methanol extract. The antioxidative capacity value was 34.07 mM TE/ 100 g mill for green coffee. When considered in terms of the degree of roasting, the total antioxidative capacity increased slightly at light till city roasting. Afterwards it slightly decreased with stronger roasting, 31.33 mM TE/ 100 g mill.

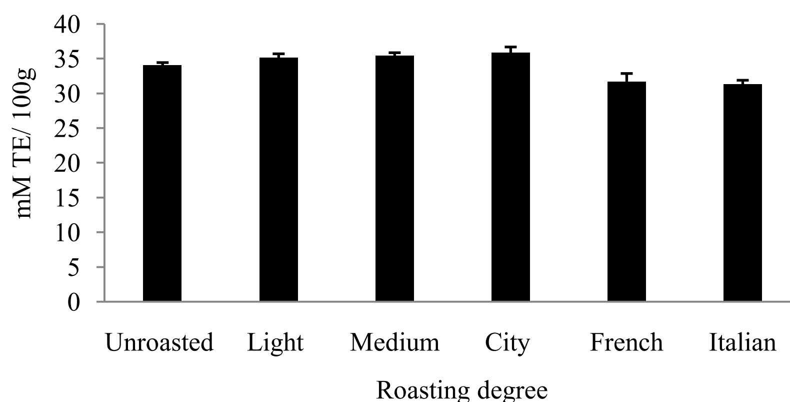


Figure 4-15 Effect of roasting conditions on antioxidative capacity (mM TE/100 g mill coffee Columbia)

The results are in the line with del Castillo *et al.* (2002) and Sacchetti *et al.* (2009). The changes in the antioxidative capacity of coffee upon roasting are related to the degradation of chlorogenic acids (del Castillo *et al.* 2002, Nebesny and Budryn 2003). Many of the volatile substances, such as furans and pyrroles, w hich are formed by pyrolysis upon coffee roasting,

also showed antioxidative efficacy (Fuster *et al.* 2000). The HPLC analysis was also applied to generate fractions from green and roasted coffee beans. The samples thus obtained were used to obtain the antioxidative potentials of the individual CQA. The comparison of the data from TEAC assay for commercially available standards, HPLC fractions and chemically synthesized CQA is depicted in Figure 4-16. The HPLC fractions generally showed higher values. This may result from the co-elution of substances underlying the individual peaks. The antioxidative capacity for 5-CQA (0.9-1.4 nmol TE/nmol) agrees with those described (1.15 nmol TE/nmol CQA) elsewhere (Gómez-Ruiz *et al.* 2007, Gómez-Ruiz *et al.* 2008). The highest TEAC values were obtained for di-CQA, where 4,5 di-CQA appears to be the most active component.

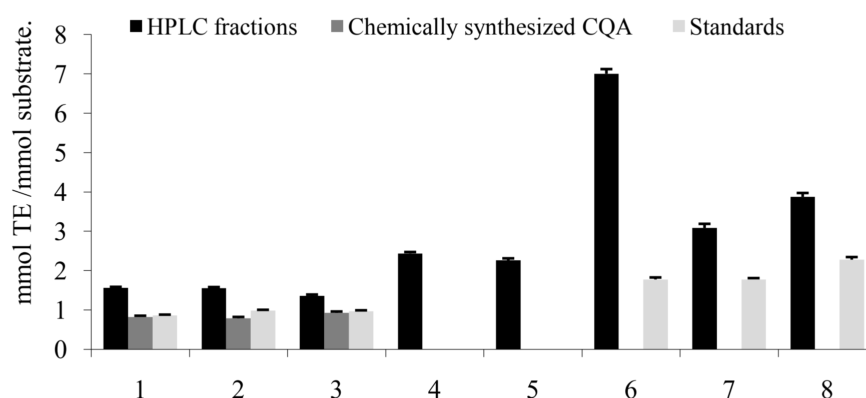


Figure 4-16 Comparison of the antioxidative capacity as measured by TEAC assay for commercially available standards, chemically synthesized isomers and fractions isolated by HPLC from green and roasted *Coffea arabica* from Columbia

Where: 1, 3-CQA; 2, 4-CQA; 3, 5-CQA; 4, FQA; 5, CQL; 6, 3-4 di-CQA; 7, 3-5 di-CQA; 8, 4-5 di-CQA; CQA, Caffeoyl-Quinic Acid; FQA, Feruloyl-Quinic Acid; CQL, Caffeoyl-Quinic Acid lactone and di-CQA, di Caffeoyl-Quinic Acid

Thus, knowing the individual TEAC values of the analyzed CQA fractions, it was possible to estimate their individual contributions to the total determined antioxidative capacity (Appendix, Table 7-2). This compilation of data delivered the following observations: 1) The sum of individual contributions for the green beans was generally higher than that of the total activity measured in the extract, the major contributor being 5-CQA with ca. 48-51% for *C. arabica* and 32-37% for *C. robusta*. The results therefore also show that the phenolic CQA compounds are the main contributors and the synergic effect of all CQA components present together as in the extract of green coffee may decrease the total TEAC capacity measured.

2) In roast coffee, it was found that a substantial part (ca. 80%) of the activity is allocated to an “unknown” fraction, the total activity measured in the extract being higher than the sum of the individual contributions (5-CQA with ca. 4% allotment). Further, “only” the extracted components could be analyzed, other potential insoluble contributors are not included. Therefore, as a result it can be assumed that a unique complex combination of enzymatic and non-enzymatic browning processes during roasting may lead to distinct antioxidative operating browning products, their characteristics as yet unknown. A substantial contribution to these unknown fractions may also originate from the nitrogenous fraction of the coffee, which needs to be investigated further.

4.2.5 Coloration of the extracted coffee and faba proteins

The extraction of coffee and faba bean proteins was conducted with different combinations as described in method section, with the color of proteins thus obtained being intensely affected (Figure 4-17). The color ranged from light yellow/cream white to dark green compared to the color of extracted faba protein (Appendix, Figure 7-9). It has been shown that the principle structure of green and yellow pigments resulting from the reaction of CQA with an amino compound under aeration in alkali was a semiquinone type radical compound of the trihydroxy benzacridine derivative (Namiki *et al.* 2001). On the other hand, brown colored pigments generally result from enzymatic (polyphenols oxidase - modulated) oxidation of CQA and their reaction with proteins. A treatment of acetylcysteine and ascorbic acid in presence of polyamide delivered dark brown products, suggesting an extensive oxidation of the phenolic compounds in the extracted proteins. While in the case of Tris-HCL pH 8 in presence of polyamide, a dark green color was formed (Figure 4-17), suggesting the reaction of a dimer of oxidized phenolics compounds with primary amino groups (Namiki *et al.* 2001, Yabuta *et al.* 2001).

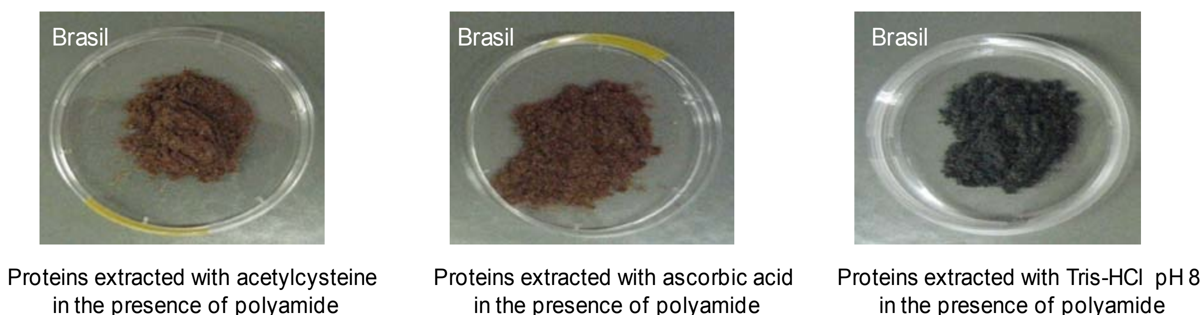
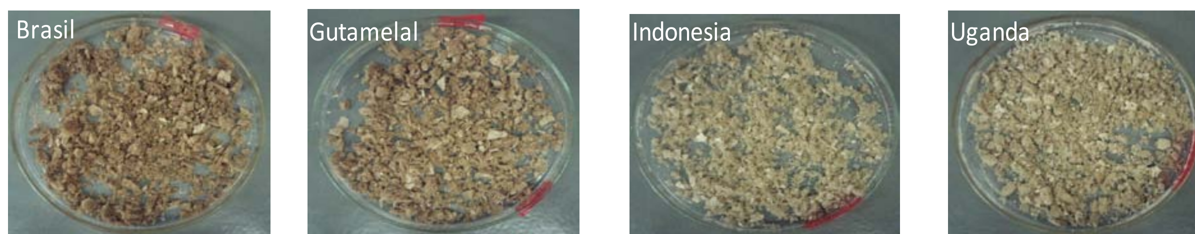


Figure 4-17 Coloration of proteins extracted from green coffee beans with different solvent compositions



Proteins extracted with Ascorbic acid in the presence of (PVPP)

Figure 4-18 Coloration of proteins extracted from different green coffee beans with optimized conditions

Furthermore, PVPP was used instead of polyamide and according to the results obtained; the lightest cream-colored products were obtained by combining ascorbic acid with PVPP instead of polyamide (Figure 4-18). Both these components seem to be necessary to prevent further reaction of the proteins with CQA during the extraction, where PVPP is known to bind phenolic compounds and the presence of ascorbic acid helps in preventing the oxidation of CQA as well as in inhibiting the indigenous polyphenol oxidase activity (Janovitz-Klapp *et al.* 1990). The proteins obtained from *CR* beans were a shade lighter than those from *CA* (Figure 4-18). These results seem also to depend primarily on the amount of polyphenol oxidase activity in the beans (Table 4-2).

4.2.6 Structural properties of coffee and faba proteins

The first step towards the characterization of green coffee and faba proteins is to optimize the protein extraction using various solvents/additives with the least possible modification of the proteins. Therefore, in the next step the structural properties of the coffee bean proteins are compared to that of faba bean proteins.

4.2.6.1 Surface hydrophobicity

To illustrate that non-covalent/covalent interactions between CQA derivatives and their oxidized products to occurs upon the surface of the coffee protein molecules, the extracted coffee proteins were tested with respect to the nature of their surface following the binding of a hydrophobic probe, 8-anilino-1-naphthalenesulfonic acid (ANS). ANS, known to bind to hydrophobic pockets on the protein surface, is a much-utilized fluorescent 'hydrophobic probe' for examining the non-polar character of proteins and membranes. Probe ANS is an environmentally sensitive fluorophore. It is essentially non-fluorescent in an aqueous solution and becomes distinctly fluorescent in a polar environment. The quantum yield of ANS

fluorescence intensity increases significantly after binding to the hydrophobic portions of proteins, and for this reason it is also often used to study conformational changes in a given protein by probing its hydrophobic binding sites. The enhancement of fluorescence due to the binding of ANS to extracted coffee proteins was determined and compared to model 11S proteins obtained from FB (Figure 4-19). The data showed that the coffee proteins were not capable of binding any ANS, thus exhibiting a very hydrophilic protein surface. The faba bean proteins in comparison exhibited the customary high binding of ANS.

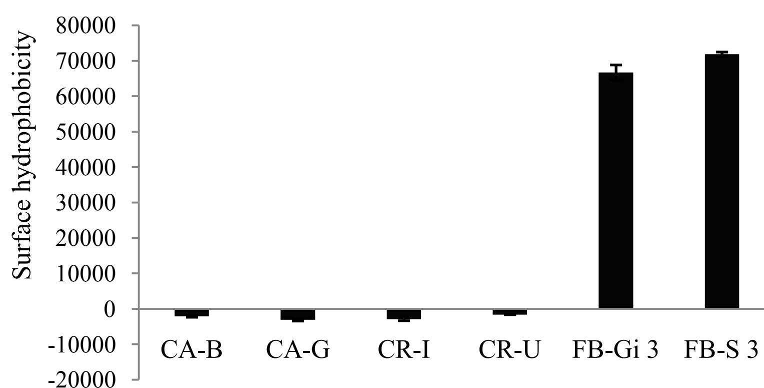


Figure 4-19 Surface hydrophobicity of extracted coffee and faba proteins

Where: CA, Coffea arabica; CR, Coffea robusta; Coffee beans from: B, Brazil, G, Guatemala, I, Indonesia, U, Uganda; and FB, faba beans from: Gi 3, Giza 3 and S 3, Sakha 3

Recent work shows that phenolic compounds appear to decrease the surface hydrophobicity of proteins (Rawel *et al.* 2002a, Aewsiri *et al.* 2009). The decrease in surface hydrophobicity for modified proteins was possibly caused by the increase in the number of hydroxyl and carboxyl groups of phenolic compounds attached. Further, the covalent attachment of the phenolic compound to proteins also causes the blocking of the hydrophilic groups like amino and thiol groups (Kroll *et al.* 2003, Aewsiri *et al.* 2009).Therefore, it still remains unclear if this surface blanketing of the coffee proteins (micelle) via non-covalent binding of CQA derivatives is a result of the protein extraction or their typical status in the beans.

4.2.6.2 Circular Dichroism (CD) spectroscopy

The circular dichroism analysis confirms the dominating β-sheet conformation in the secondary structure, which is a further characteristic molecular feature strongly influencing the functional properties of 11S globulins. These results are in agreement with Rawel *et al.*

(2005b). Both near-UV and far-UV CD-spectra and the assignment of the calculated structural fractions are shown in (Figure 4-20 and Table 4-8).

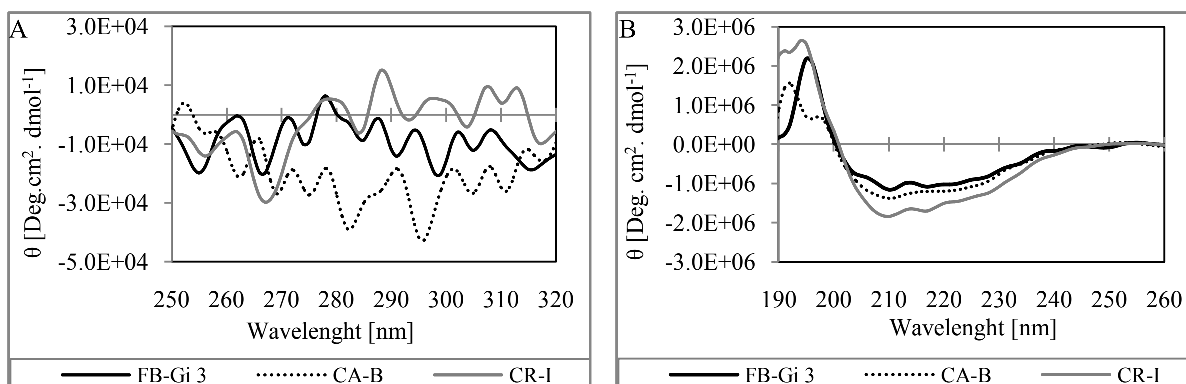


Figure 4-20 CD spectra of the coffee and faba proteins, near-UV (A) and Far-UV (B)

Where: CA, *Coffea arabica*; CR, Coffea robusta; B, Brazil, I, Indonesia; FB, faba beans, Gi 3, Giza 3 and θ; molar ellipticity

Table 4-8 The content of secondary structure elements in coffee and faba beans proteins as determined by far-UV CD spectrometry

Properties	*Coffea arabica*		*Coffea robusta*		*Faba*
	Brazil	Guatemala	Uganda	Indonesia	Giza 3
α-Helix (%)	5.0	4.9	4.1	7.7	3.3
β-Sheet (%)	38.4	28.1	38.8	35.8	34
β-Turn (%)	19.5	18.6	20.2	20.3	19.3
Unordered (%)	37.1	48.4	36.8	36.2	43.4

Far UV CD-spectra data indicate that there are clear differences between types of *CA* in % of β-sheet and unordered, where the coffee Brazil protein contained, 38.4% β-sheet and 37.1% unordered while, coffee Guatemala contained 28.1% β-sheet and 48.4% unordered elements. In contrast, data illustrated that the differences between types of *CR* were noticed in % of α-helix and β-sheet, where the coffee Uganda and Indonesia proteins contained 4.1% and 7.7 α-Helix and 38.8 and 35.8 % β-sheet, respectively. The values of β-turn and unordered are in accordance with Rawel *et al.* (2005b), whereas the different values in % of α-helix and β-sheet were recorded. The differences could be explained by different experimental conditions, and the application of different methods for the collection and evaluation of CD data. In addition, the preparation of proteins (extraction conditions) might have also influenced the data produced. The coffee data were compared with the protein extracted from faba beans (as control), where an increase in both % of α-Helix and β-sheet was found. Thus conformational

analysis based on CD data suggests that the faba protein contains α-helix 3.3%, β-sheet 34%, β-turn 19.3%, and unordered 43.4% (Table 4-8).

The increase in both % of α-helix and β-sheet values may be related to the interaction between CQA and coffee protein during the extraction. There is an agreement among these data and a previous study, which reported that the interaction between phenolic compounds (Apigenin, kaempferol and quercetin) with soy glycinin (also an 11S protein), produced an increase in α-helix and β-strand (Rawel *et al.* 2002a), while interaction of chlorogenic acid with human serum albumin and bovine serum albumin was shown to cause a decrease in α-helix structure with other structures unaltered (Muralidhara and Prakash 1995, Rawel *et al.* 2002b).

4.2.6.3 Covalent bound CQA

A RP-HPLC method was used to estimate the amount of CQA covalently bound to the extracted proteins. For this purpose the proteins were dissolved in 8 M urea and precipitated to separate the non-covalently bound CQA from the protein-CQA adduct followed by subsequent chromatography. The liberated CQA and the remaining coffee protein adduct showed similar UV-Vis scans, both absorbing at the same wavelength (325 nm), but were eluted at different retention times (Figure 4-21) (Rawel *et al.* 2002a, Rawel *et al.* 2005b). The more hydrophobic coffee protein elutes at the same retention time for faba protein under the HPLC conditions applied at 280 nm. The protein extracted from faba beans, since it was not modified with CQA did not show any relevant absorption at 325 nm (Data are not shown). Therefore, using an external 5-CQA calibration curve, this property was utilized to estimate the amount of CQA attached covalently in the coffee protein adduct (Figure 4-22).

Although this approach does not give the exact amount of CQA bound due to the unknown nature and absorbance behavior of the covalently bound CQA moiety / reactions products to proteins, the method still permits a rough evaluation of the extent of modification and provides a valuable tool in assessing the quality of the extracted proteins. The results showed that although the extracted coffee proteins were exhaustively dialyzed against distilled water, some CQA still remains non-covalently bound to the coffee proteins and is released only after the treatment with urea, precipitation of proteins, re-dissolving and consequent chromatographic separation. Therefore, the liberated CQA amounted to 1.55 – 3.25 nmol/mg protein (Figure 4-22), the value being slightly higher for the proteins extracted from *CR*

(samples 6 and 7). On the other hand the amount of CQA estimated to be firmly involved in the adduct formation with the proteins was in the range of 5.56 – 10.05 nmol/mg protein, the amount being significantly (two times) higher for the proteins extracted from *CA* compared to *CR* (Figure 4-22, samples 4-7).

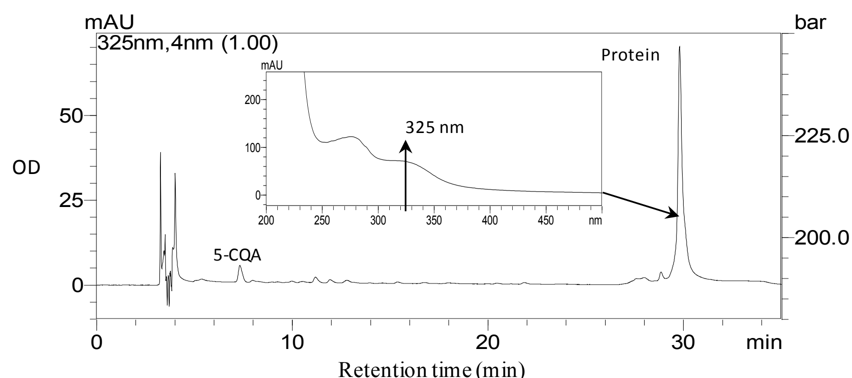


Figure 4-21 RP-HPLC for the extracted coffee Brazil proteins and their corresponding UV–vis spectra

Moreover no significant differences were found between types of each species. The values are higher than the values reported by Rawel *et al.* (2005b). These differences may be due to the differences in types, maturation degree and extraction methods. The data implies that approximately 0.3 - 0.6 µmol CQA pro µmol of the subunit of the coffee 11s protein (based on molecular weight of 60 kDa) are involved in adduct formation. These results again reflect the role of the polyphenol oxidase activity in the beans for the adduct formation with coffee proteins, the amount of available CQA playing only a secondary role. The corresponding PPO activity and total CQA determined for the two *coffea* species are provided in Table 4-2. Interestingly, the proteins extracted with Tris-HCL pH 8 in the presence of polyamide gave the same amount of CQA bound, suggesting approximately the same degree of modification (Figure 4-22, samples 3 and 4). A stable free radical is initially assumed to have formed, which in the presence of an amino compound led to the formation of a benzacridine derivative involving a trihydroxy structure (Namiki *et al.* 2001).The reduced form of the benzacridine derivative involving a trihydroxy structure derivative was found to be yellow, being very reactive with oxygen yielding semiquinone and quinone type of products with characteristic green colors (Namiki *et al.* 2001, Yabuta *et al.* 2001). The previous studies on covalent interactions of chlorogenic acid with lysozyme, whey, and soy proteins as well as with enzymes like α-amylase and trypsin document the formation of such yellow/green pigments

under autoxidative conditions, the color being dependent on pH conditions during the derivatization (Rawel and Rohn 2010).

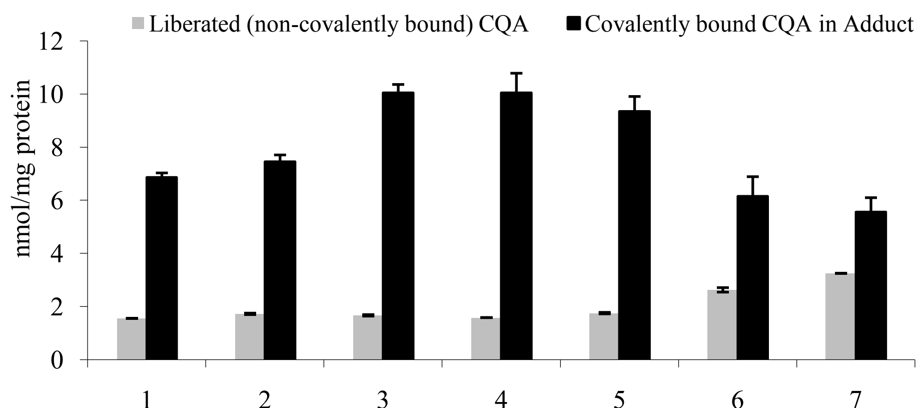


Figure 4-22 Amount of CQA equivalents (nmol/mg) of the coffee proteins extracted with different additives and from different coffee types

Where: 1, proteins extracted from coffee Brazil with acetylcysteine in the presence of polyamide; 2, proteins extracted from coffee Brazil with ascorbic acid in the presence of polyamide; 3, proteins extracted from coffee Brazil with Tris-HCl at pH 8 in the presence of polyamide; 4, proteins extracted from coffee Brazil with ascorbic acid in the presence of PVPP; 5, proteins extracted from coffee Guatemala with ascorbic acid in the presence of PVPP; 6, proteins extracted from coffee Indonesia with ascorbic acid in the presence of PVPP; and 7, proteins extracted from coffee Indonesia with ascorbic acid in the presence of PVPP.

The use of acetylcysteine and ascorbic acid in the presence of polyamide showed lower amounts of CQA covalently bound (Figure 4-22, samples 1 and 2) compared to sample 3, but also delivered dark brown products, suggesting an extensive oxidation of the phenolics contained in the extracted proteins.

4.2.6.4 Electrophoresis data

SDS-PAGE separation of extracted coffee proteins was conducted under reducing and non-reducing conditions. The typical structure of an 11s coffee storage protein with molecular weights of 300-400 kDa can be separated under non-reducing conditions, SDS or urea, to give 6 subunit pairs with molecular weights of 55-60 kDa, each pair being connected by a disulphide link. The rupture of these disulphide bonds under reducing conditions releases the α (acidic) and β (basic) subunits - two polypeptides with molecular weights around 33 and 24 kDa, respectively (Fukushima 1991, Shewry 1995, Fukshima 2001). As discussed in the methods, the extractions of coffee and faba bean proteins were conducted with different additives/solvents and the differences in quality of protein under reducing and unreducing

conditions are shown in Figure 4-23. The lane 1 and 6 showed that the best conditions for protein extraction were ascorbic acid/ polyamide and ascorbic acid in Tri-HCL at pH 8 for coffee Brazil and faba Ciza 3 beans, respectively. Otherwise, lanes 3 illustrated that in case of using Tris-HCl pH 8 in the presence of polyamide, the protein seems to be more modified with phenolics compounds because of the alkali solution being suitable for this reaction.

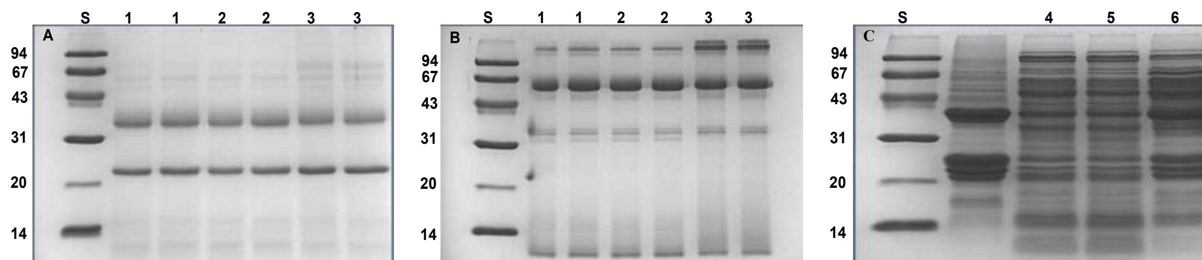


Figure 4-23 SDS-PAGE under reducing (A) and non-reducing (B) conditions of protein extracted from Brazilian green coffee beans and under reducing (C) conditions of proteins extracted from faba beans Giza 3

Where: S, Standard; 1, protein extracted with ascorbic acid in the presence of polyamide; 2, proteins extracted with acetylcysteine in the presence of polyamide and 3, protein extracted with Tris-HCl pH 8 in the presence of polyamide; 4, protein extracted with ascorbic acid; 5, protein extracted with acetylcysteine and 6, protein extracted with Tris-HCl pH 8

Furthermore, PVPP was used instead of polyamide (Figure 4-24A). The results demonstrated that using PVPP improved the quality of the protein obtained compared to those extracted using polyamide, where the results showed that the best conditions were using of PVPP in the presence of the antioxidant ascorbic acid (lane 2). These results supported the results in Figure 4-17 and Figure 4-18. Both these components seem to be necessary to prevent further reaction of the proteins with CQA during the extraction, where PVPP and polyamide are known to bind phenolic compounds and the presence of ascorbic acid helps in preventing the oxidation of CQA, as well as in inhibiting the indigenous polyphenol oxidase activity, because it is among those types of inhibitors, that principally affect the active site for the phenolic substrate. In this context, the aromatic carboxylic acids of the benzoic and cinnamic series have been widely studied (Janovitz-Klapp *et al.* 1990).

Small differences in the molecular weight distribution between different extracted proteins were also observed under reducing conditions. Therefore, when the coffee proteins were reduced with 2-mercaptoethanol, bands at lower apparent molecular masses were found (33.9 ± 0.3 and 24 ± 0.2 kDa) (Figure 4-24B). These results are in line with previous studies (Acuña *et al.* 1999, Rogers *et al.* 1999), where the reported molecular mass of the two subunits of

coffee protein were 33 and 24 kDa. Moreover, protein bands with an apparent molecular mass of 57 ± 3 kDa were predominant in samples of *CA* and *CR* extracted with a buffer that did not contain the reducing agent (Figure 4-24C). These results are in agreement with previous studies (Acuña *et al.* 1999, Baú *et al.* 2001).

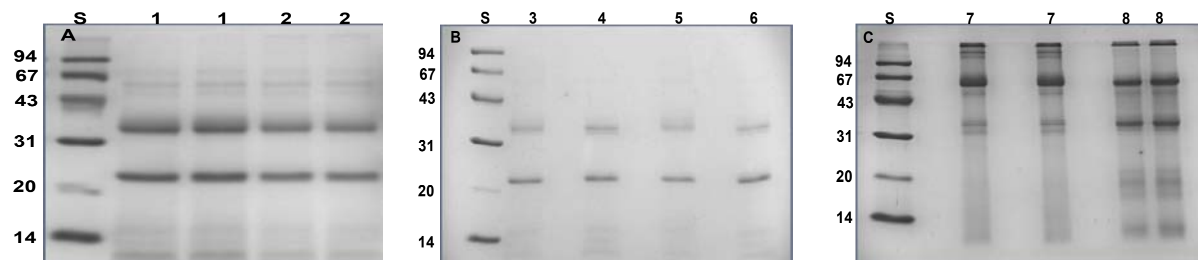


Figure 4-24 SDS-PAGE under reducing (A and B) and non-reducing (C) conditions of proteins extracted from different green coffee beans

Where: S, Standard; 1, protein extracted from coffee with ascorbic acid in the presence of polyamide; 2, protein extracted from coffee with ascorbic acid in the presence of PVPP; 3, protein extracted from coffee Brazil with ascorbic acid in the presence of PVPP; 4, protein extracted from coffee Guatemala with ascorbic acid in the presence of PVPP; 5, protein extracted from coffee Indonesia with ascorbic acid in the presence of PVPP; 6, protein extracted from coffee Uganda with ascorbic acid in the presence of PVPP; 7, protein extracted from coffee Brazil with ascorbic acid in the presence of PVPP and 8, protein extracted from coffee Indonesia with ascorbic acid in the presence of PVPP

Further, marked differences in the electrophoretic patterns under non-reducing conditions were also noted between the samples of *CA* and *CR*. Proteins of *CA* were more aggregated and delivered high-molecular fractions, where as the extracts of *CR* showed a higher quantity of low molecular weight fractions, with part of the α-chain being liberated during the application of extraction conditions. On the basis of the coloration of the extracted proteins, and while considering the presence of high molecular fractions, it may be possible to determine the quality of the extracted proteins.

4.2.6.5 Identification of the sites and types of modification in the coffee proteins

In-gel digestion combined with MALDI-TOF-MS provided tentative allocation of the modification types and sites in the protein. To obtain some more information on the types and sites of adduct formation in the coffee proteins, the coffee proteins were directly extracted from the coffee meal with an SDS-PAGE sample buffer containing 0.05 M Tris-HCl pH 6.8, 4 % SDS and 5% 2-Mercaptoethanol and, subsequently, separated by electrophoresis. The bands of interest (α- and β- polypeptide chains of the coffee 11S proteins) were excised, treated as described in methods section, and finally digested by trypsin.

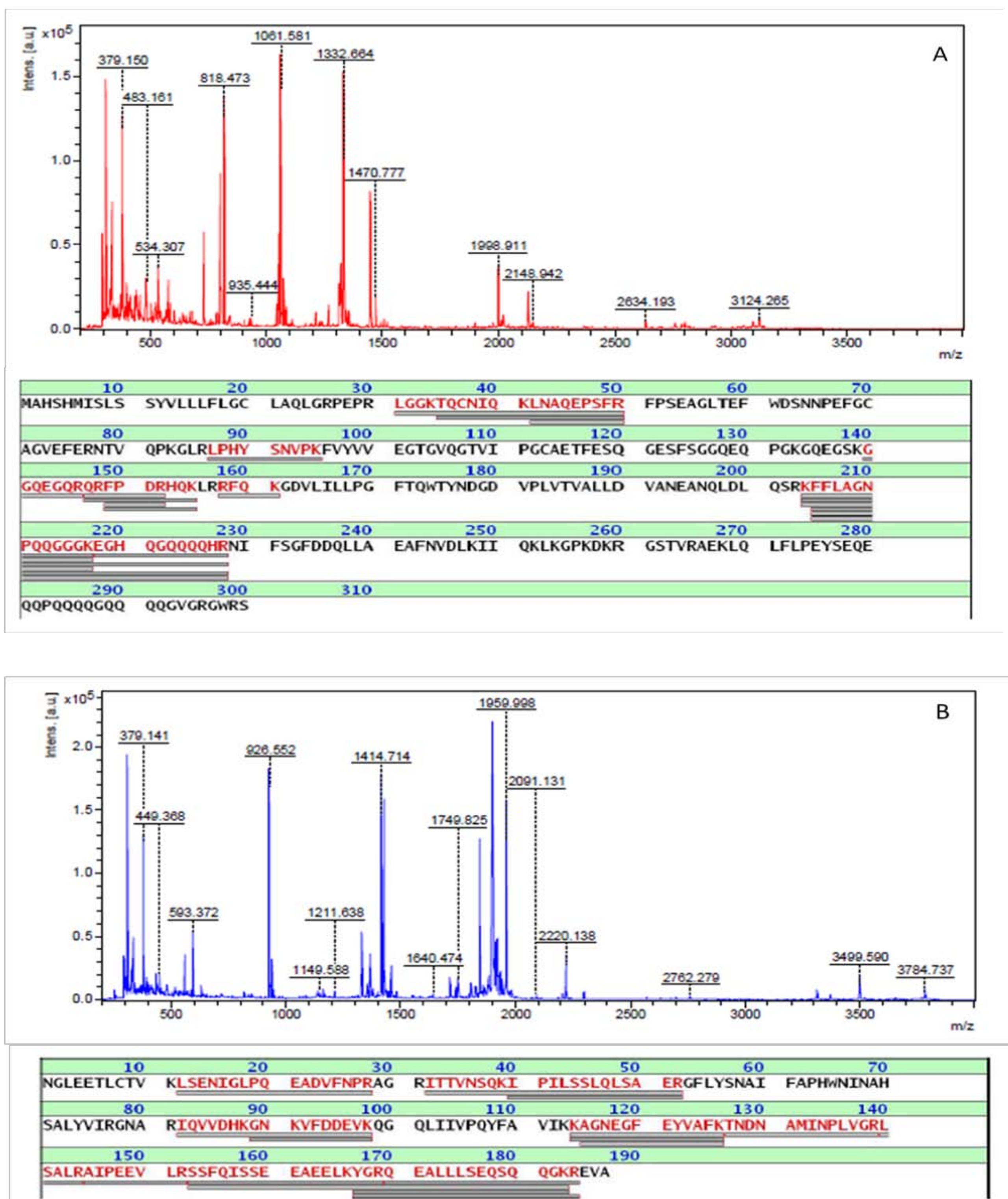


Figure 4-25 MALDI-TOF-MS analysis of the in-gel tryptic digested α-polypeptide chains (A) and β-polypeptide chains (B) from coffee beans from Brazil (*C. arabica*)

The α and β peptides of *CA* from Brazil thus produced were analyzed with MALDI-TOF-MS to produce a peptide mass fingerprint (Figure 4-25), which were then submitted to a Mascot

MS search in the databank Plants_EST. On basis of the preliminary identification (data are not shown) and comparison of the derived sequence to the available complete primary structure for coffee 11S storage proteins in the protein knowledgebase (UniProtKB; http:// www.uniprot.org/), the following candidate was found to be the best match: P93079 (P93079_COFAR). Therefore, further analysis with regard to the identification of the type and site of modification in the protein with CQA was performed applying this P93079 complete model sequence belonging to the 11S seed storage protein (globulin) family.

Table 4-9 Proposed the sites and types of modification in coffee 11S storage proteins

Proteins		Modification types*	Modification sites	Peptides
Coffea arabica	α-polypeptide chains	CQA 6 CQA 6 CQA 6 CQA 4	K34 K41 K204 K217	31-50 31-51 204-217 204-217
	β-polypeptide chains	CQA 4 CQA 3	K91 K114	89-98 114-126
Coffea robusta	α-polypeptide chains	CQA 2	K155	153-158
	β-polypeptide chains	none	none	-

* Modification types were shown in Figure 4-6

The tentative classification of the modification type and sites is given in Table 4-9 (on the basis of the proposed different types of modifications; see Figure 4-6 and Ali *et al.* 2012). It appears that the storage proteins from *CA* beans are more intensively involved in covalent interactions with CQA compared to *CR*, agreeing with data on amounts of CQA bound (samples 4 and 5 in Figure 4-22). Further, as shown in Table 4-9, the CQA modification of coffee storage protein also proceeds with a preferred reaction at the α chain, where many sites, K34, K41, K204 and K217 were modified, whereas the β chain of the 11S proteins remains more or less intact.

4.2.6.6 Tentative modeling of the modification

To understand the CQA modification of the coffee storage protein, it is necessary to consider the structure of 11S storage proteins in more detail. The 11S storage proteins of legumes are built up of polymorphic subunits encoded by multigene families (Schwenke 2001, Rawel *et al.* 2005b). They form, however, regular quaternary structures, hexameric in the case of legumin-

like proteins, whose association-dissociation behaviour and three-dimensional and surface structure as well as the conformational stability are very important for understanding the functionality of these proteins. It is the non-specific hydrophobic interaction of the 50–60 kDa monomers that allows them to assemble in a legumin-like quaternary structure. The two disulfide-bridged polypeptide chains of these subunits fulfill different functions in stabilizing the globular structure (Schwenke 2001, Rawel *et al.* 2005b).

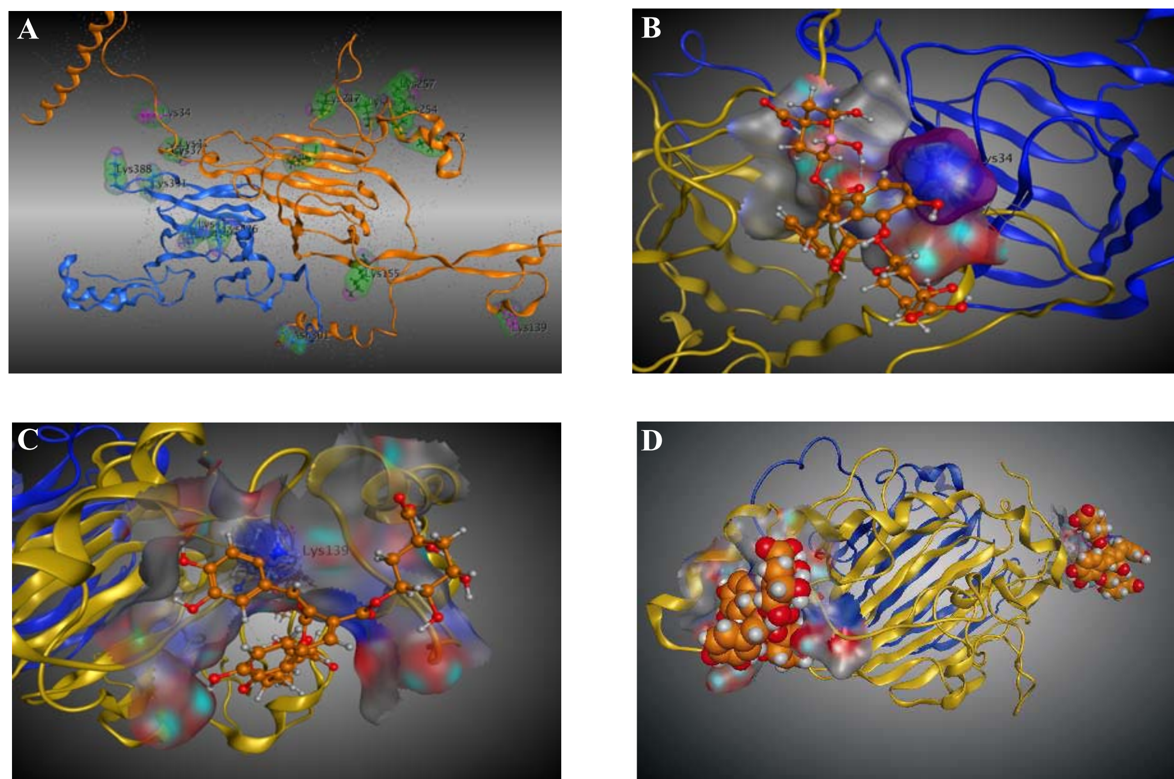


Figure 4-26 Tentative modeling of the modification of 11S coffee protein

Where: (A) The possible modification sites in the 11S coffee protein; (B) modeling of CQA modification at the lysine site 34; (C) modelling of CQA modification at the lysine site 139 and (D) Positions of the modifications in the monomer of 11S coffee protein

The alignment of 11S storage proteins from different sources and the S-S bridge connecting the α chain to the corresponding β chain has been reported (Ali *et al.* 2012). However, the most important fact is that the strong hydrophilic C-terminal region of the α chain is located at the surface of the protein molecule and protects the structural domains from the solvent. It is therefore of high importance not only for the solubility and interfacial properties of 11S globulins but also seems to be the preferred reaction site for chlorogenic acid (Schwenke 2001, Rawel *et al.* 2005b). The limited tryptic hydrolysis of 11S globulins results in the

splitting of the surface-exposed regions of α chains, while the β chains located in the inner part of the protein molecules remain intact, (Schwenke 2001).

Thus, on basis of these theoretical and practical observations, and considering the data determined in this study, the first available in-silico modeling of modified coffee proteins is reported, documenting the occurrence of covalent reactions between hydroxycinnamic acid derivatives and coffee storage proteins. The modeling is the next step to obtain more information on the type and site of adduct formation in the coffee proteins. The possible modification sites in the 11S coffee protein were shown in Figure 4-26A. The accessibility of two reaction sites (Lys 34 and Lys 139) was simulated by molecular modelling to underline the possibility of these modifications. Thereafter, the pharmacore in the vicinity of Lys 34 and Lys 139 in the 11S coffee protein was defined. On the basis of this definition, the modification of the sites was conducted, such that the docking distance of under 4Ǻ for different poses could be achieved (Ali *et al.* 2012). The results of these simulations confirm the accessibility of the two reaction sites Lys 34 and Lys 139 and are illustrated in panels B and C of Figure 4-26, respectively. The modeling of the two modification sites in the monomer of the coffee 11S protein is given in Figure 4-26D. Simulations showing the exposure of the charged molecular surface of the protein based on the molecular electrostatic potential, and the surface accessibility of the trimer of 11S coffee protein with the corresponding modifications have been reported (Ali *et al.* 2012).

4.2.7 Functional properties of coffee and faba proteins

4.2.7.1 Solubility of proteins

Solubility is an important requisite for a functional ingredient protein and is critically necessary for products such as beverages. The solubility, as a function of pH, of coffee Brazil (*CA*), coffee Indonesia (*CR*) and faba Sahka3 proteins was studied, and the results are shown in Figure 4-27. The solubility of coffee proteins at pH 2 and 3 was lower than FB protein (as control), where it was 43.51±2 and 33.19±1% for coffee brazil, 43.86±1.1 and 44.26±0.5 % for coffee Indonesia, compared to 72.15± 0.7 and 53.53±1.4% for faba protein, respectively. These results refer to that coffee proteins are more acidic. Our results are in the line with Rawel *et al.* (2005b). Data also show that there are slight differences between *CA* and *CR.* Moreover, significant differences between coffee and faba proteins were found at pH from 4

to 5.5, where the solubility ranged from 55.83 to 78.91 and from 26.17 to 38.98% for coffee and faba proteins, respectively. These differences may be caused by the reaction between CQA and the coffee protein during the extraction or harvesting process.

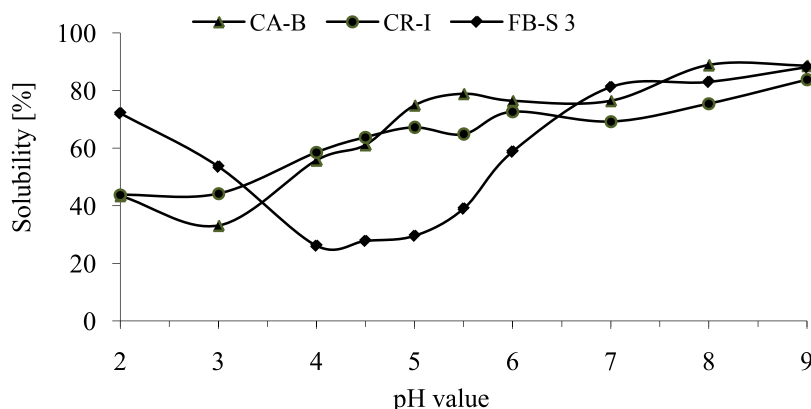


Figure 4-27 Solubility of coffee and faba proteins as a function of pH

Where: CA, *Coffea arabica*; CR, *Coffea robusta*; Coffee beans from: B, Brazil, and I, Indonesia, faba beans from: S 3, Sakha 3

4.2.7.2 Antioxidative capacity of the extracted coffee proteins

The antioxidative capacity of the extracted coffee proteins (mM TE/g) was also determined by TEAC assay, and the data are shown in Figure 4-28. The protein extraction regime influences the antioxidative capacity considerably, with the treatment of acetylcysteine and ascorbic acid in the presence of polyamide leading to the highest activity documented 3.14 and 2.97 mM TE/g protein, respectively. Considering the dark pigmentation of these protein products (Figure 4-17) and the observed low amount of CQA bound (Figure 4-22), it can be proposed that these samples are likely to contain products resulting from progressed PPO modulated oxidation of CQA. Moreover, the results show that proteins extracted with Tris-HCL pH 8 in the presence of polyamide were also darkly pigmented, and are thus also likely to contain similar potentially antioxidative operating reaction products. It seems that during the extraction, a series of CQA derivatives and their oxidized products, which have antioxidant potential, become non-covalently attached to surface of the coffee protein molecules (Rawel and Rohn 2010). Furthermore, proteins which were extracted in the presence of PVPP instead of polyamide showed low antioxidative capacity and no significant differences at ($p < 0.05$) between *CR* and *CA* were observed, where the values ranged from 1.50 to 1.67 mM TE/g protein (Figure 4-28, samples 4-7).

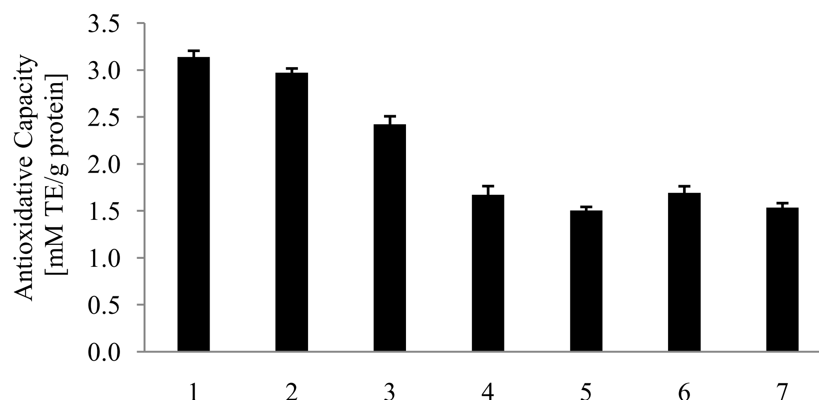


Figure 4-28 Antioxidative capacity of the proteins extracted from different coffee types with different additives

Where: 1, proteins extracted from coffee Brazil with acetylcysteine in the presence of polyamide; 2, proteins extracted from coffee Brazil with ascorbic acid in the presence of polyamide; 3, proteins extracted from coffee Brazil with Tris-HCl at pH 8 in the presence of polyamide; 4, proteins extracted from coffee Brazil with ascorbic acid in the presence of PVPP; 5, proteins extracted from coffee Guatemala with ascorbic acid in the presence of PVPP; 6, proteins extracted from coffee Indonesia with ascorbic acid in the presence of PVPP; and 7, proteins extracted from coffee Indonesia with ascorbic acid in the presence of PVPP.

4.2.7.3 Emulsification properties

4.2.7.3.1 Oil droplets size

Figure 4-29 presents data for the mean particle size of oil droplets in the emulsions (MCT oil in water), which were prepared using three different proteins, two coffee proteins (from Brazil and Indonesia) and one faba protein (Sakha 3) as emulsifiers. The droplet size was measured almost immediately after the emulsification.

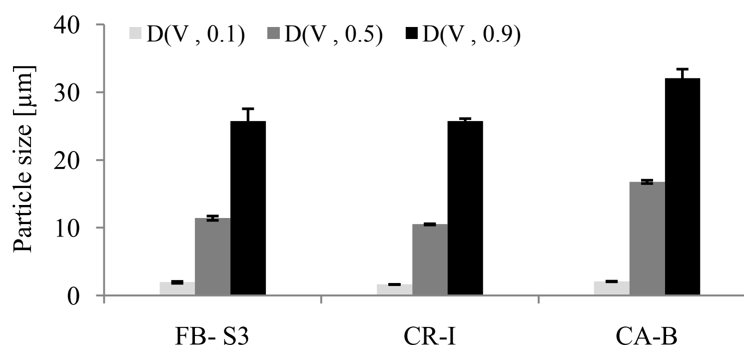


Figure 4-29 Mean particle size of freshly prepared MCT oil-PBS buffer emulsions made with coffee and faba proteins

Where: CA, *Coffea arabica*; CR, *Coffea robusta*; B, Brazil; I, Indonesia; FB, faba beans and S 3, Sakha 3

As documented by the characterization of emulsion with regard to the 10th percentile of particles size D (v, 0.1), no significant differences between the emulsions were detected. On the other hand, the 50th and 90th percentile of the particle size D (v, 0.5) and D (v, 0.9), showed that a considerable increase in size distribution could be observed for coffee protein CA-B, whereas CR-I and faba proteins exhibited more or less similar behavior (Figure 4-29). The large size of the emulsion droplets prepared using CA-B may be related to the concentration and solubility of proteins used. Aewsiri *et al.* (2009) mentioned that when the high concentrations of cuttlefish skin gelatin either with or without modification were used as emulsifiers, the droplet size of the emulsion was decreased distinctly. Also, in a recent study, the droplet size of the emulsion stabilized by whey protein isolate or sodium caseinate was decreased when the concentration of the protein increased (Cornacchia and Roos 2011). These emulsions were also studied by the microscopic imaging of the particles which did not show an optimal distribution of the oil droplets, with subsequent observation of their aggregation (Appendix, Figure 7-10). Comparatively larger droplets for CA-B and faba Sakha 3 proteins seems also to effect the stability of the emulsion as documented in Figure 4-30.

4.2.7.3.2 Stability of emulsion

The creaming stability of emulsions, prepared by two different types of coffee proteins and one faba protein as control, was measured as the emulsion turbidity at 500 nm wavelength, and the data are presented in Figure 4-30. The emulsions were centrifuged to provoke the formation of the serum layer. The results revealed that, during the first 25 min both the Indonesia and Brazil protein emulsions were stable compared to the faba Sakha 3 protein emulsion. After this period, the stability of the emulsion employing coffee Brazil decreased. Coffee Indonesia protein- stabilized emulsion was relatively stable as compared to both coffee Brazil and Sakha 3, and did not show any creaming till after 40 min centrifugation. Moreover, emulsion stabilized using coffee Indonesia protein showed the lowest decrease in stability after 65 min centrifugation, of 17.3% compared to 52.6 and 65.6% for emulsions stabilized using coffee Brazil and faba Sakha 3 proteins, respectively. Therefore, the amount of creaming formation increased as a function of centrifugation time for all emulsions. An instant in-situ creaming, as most likely caused by flocculation and an instant oiling-off occurs in all emulsions suggesting that weak viscoelastic protein films were formed around oil droplets.

The decrease in the stability of emulsion when emulsified with coffee proteins may be related to covalent bound CQA amount, as mentioned above in Figure 4-22, which caused the decreases in surface hydrophobicity of the coffee protein. Aewsiri *et al.* (2009) reported that the modification of cuttlefish skin gelatin with oxidized caffeic acid, ferulic acid and tannic acids also showed a decrease in surface hydrophobicity.

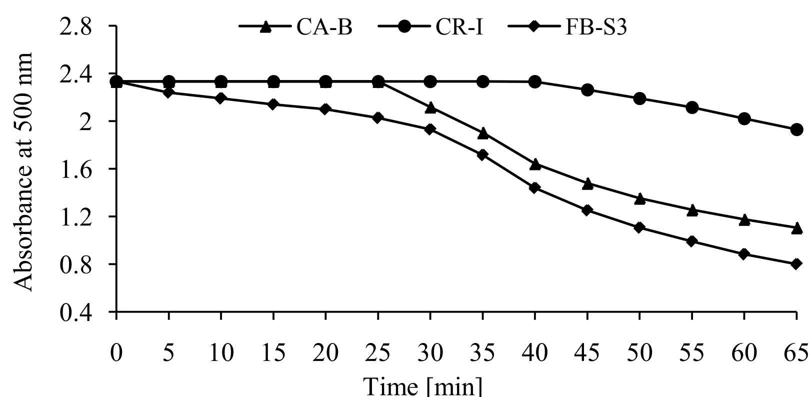


Figure 4-30 Stability of emulsions prepared using coffee and faba proteins

Where; CA, *Coffea arabica*; CR, *Coffea robusta*; B, Brazil; I, Indonesia; FB, faba beans and S 3, Sakha 3

Therefore this decreases the ability of gelatin to localize at the oil - water interface. In addition, the tendency to the aggregation of cuttlefish skin gelatin molecules was also related to the modification of protein with oxidized phenolic compounds. Finally, an insufficient amount of proteins at the oil - water interface is one of the most important reasons for unstable emulsions, where enough of a soluble protein amount is needed to cover the oil droplet completely, thereby resulting in a more stable emulsified system (Aewsiri *et al.* 2009).

4.2.7.3.3 Stability of emulsified lutein ester

Stability of labile lipophilic bioactive molecules (e.g. lutein ester) in food model systems is a very important aspect for the food industries. Lutein has to be obtained through dietary lutein intake because humans are not able to synthesize it. Furthermore, the bioavailability of lutein is very low, although large amounts of lutein are accumulated in the intestinal mucosa. This low bioavailability can be enhanced by interaction with food or food components (Khalil *et al.* 2012). In model emulsions, lutein ester was emulsified by coffee Indonesia and Brazil and faba proteins, and the percent of the residual lutein ester (LE), after exposure of these emulsions for 0, 24, 48 and 72 hours to UV light at 365 nm, is outlined in Figure 4-31. The

calculations showed that the protein emulsified lutein esters in MCT oil exhibited a higher stability against UV light compared to lutein esters in MCT oil without emulsification, where after 72 hours storage no LE was detected in un-emulsified sample. In addition, coffee Indonesia protein emulsified oil exhibited a high % of lutein ester remaining after 72 h of UV exposure followed by both emulsions emulsified by coffee Brazil and faba proteins, where the values were 47.5, 19 and 17.8 %, respectively. The differences in the amounts of LE remaining are most likely related to the degree of modification of proteins with CQA and the flocculation of droplets/aggregation of emulsion during the storage, whereby the introduced antioxidative capacity of the proteins seems to play an interesting role (Figure 4-28).

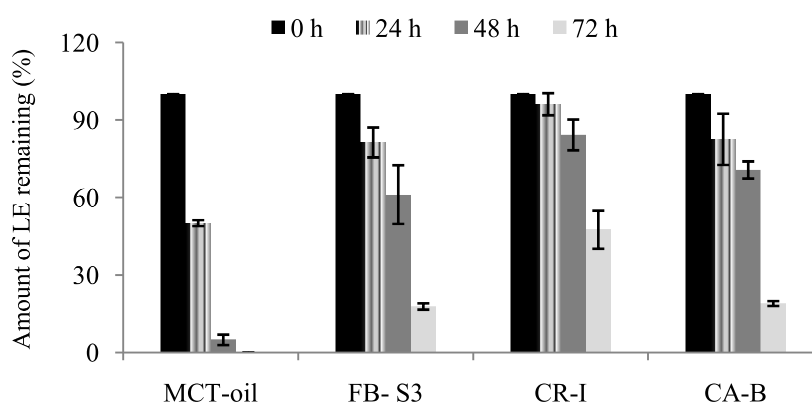


Figure 4-31 The change in the UV-stability of lutein ester in protein emulsions as affected by time of UV exposure

Where: CA, *Coffea arabica*; CR, *Coffea robusta*; B, Brazil; I, Indonesia; FB, faba beans and S 3, Sakha 3

4.3 Study 3: Effect of modification of milk whey protein products by CQA on its chemical, structural and functional properties

Recent studies reporting on the composition of coffee phenolics have documented more than 50 hydroxycinnamic acid derivatives being present (Jaiswal and Kuhnert 2010, Jaiswal and Kuhnert 2011). Depending on the amount of phenolic compound present, *Coffea robusta* (coffee from Uganda) was selected to modify milk whey protein products (WP), β-Lactoglobulin (β-LG), BioZate 1 (BZ1) and Glycomacropeptide (GMP) products. Distribution and information on the typical hydroxycinnamic acids derivatives found in the coffee Uganda is provided (Table 4-4). In the next step different commercially available apple samples were tested with respect to their PPO activity and 5-CQA content (the first study). The variety of braeburn, having a high PPO activity (6262 U/L juice) and low 5-CQA content in its juice (77 mg/L), was found to be suitable for the planned experiments. The modification of WP products was conducted with a hot water extract from selected green coffee powder and with commercial CQA solution as mentioned in methods section.

4.3.1 Effect of the modification with CQA on chemical properties of proteins

4.3.1.1 The change in free CQA isomers in solutions applied for the modifications

The change induced in the composition and content of CQA isomers at the beginning and after 24 h modification time was determined by HPLC and the data are shown in Table 4-10. The results showed that the 5-caffeoyl-quinic acid (5-CQA) is the major phenolic constituent contributing to 35% of the total phenolics determined. Under alkaline conditions, pH 9, 3- and 4- CQA isomers were increased after 24 h modification, in parallel 5- CQA was decreased under both enzymatic and alkaline modifications. However, the amount of decrease in 5-CQA in alkaline modification was higher than during enzymatic modification. These results could be clarified by isomerization of 5- CQA to both 3- and 4- CQA in alkaline solution. These data are in agreement with Prigent *et al.* (2008) and Schilling *et al.* (2008). Moreover, HPLC data also demonstrated that the amounts of all CQA isomers were decreased in both modification variations. In case of the enzymatic modification, the percent of decrease in CQA isomers was positively correlated with the amount of enzyme, where the % loss in total CQA, FQA and di-CQA was increased from 9.19, 4.40 and 41.38 to 22.94, 12.11 and 62.64%, respectively when the amount of apple juice increased from 3 ml to 6 ml.

Table 4-10 Content of CQA isomers (mg/g protein) in coffee Uganda extract at the beginning and the end of β-Lactoglobulin, Glycomacropeptide and BioZate1 modifications

	CQA isomers	1	2	3	4	5	6	7	8	9
Content of chlorogenic acid isomers at 0 h	3- CQA	6.2±0.1	5.8±0.2	8.8±0.1	N.d	6.7±0.3	9.1±0.1	7.3±0.2	9.5±0.0	7.3±0.2
	4- CQA	7.1±0.1	6.8±0.2	10.2±0.1	N.d	10.6±0.3	10.5±0.1	8.2±0.4	10.8±0.0	8.7±0.1
	5- CQA	46.7±0.2	44.3±1.3	40.4±0.2	56.2±0.9	52.0±2.3	49.4±0.0	51.6±1.4	47.5±0.3	51.1±0.5
	FQA	1.0±0.0	1.0±0.0	1.7±0.0	N.d	N.d	1.6±0.1	1.2±0.0	1.8±0.0	1.1±0.1
	FQA	8.2±0.2	7.9±0.1	7.4±0.1	N.d	N.d	9.0±0.0	9.5±0.0	9.0±0.0	8.8±0.7
	3,4-di-CQA	2.2±0.3	1.7±0.2	2.7±0.2	N.d	N.d	2.9±0.3	2.3±0.0	2.6±0.0	1.9±0.0
	3,5-di-CQA	3.1±0.1	2.4±0.1	3.6±0.2	N.d	N.d	4.6±0.0	3.4±0.2	4.8±0.1	3.2±0.0
	4,5-di-CQA	1.7±0.1	1.3±0.1	1.3±0.1	N.d	N.d	2.5±0.0	2.1±0.2	2.4±0.1	1.6±0.1
	Total-CQA	60.0±0.3	56.9±1.7	59.3±0.1	56.2±0.9	69.3±3.0	69.0±0.2	67.2±2.0	67.8±0.3	67.1±0.8
	Total-FQA	9.3±0.0	8.9±0.0	9.2±0.1	N.d	N.d	10.5±0.1	10.6±0.0	10.7±0.0	9.9±0.8
	Total-di-CQA	7.0±0.3	5.4±0.3	7.6±0.1	N.d	N.d	10.0±0.3	7.7±0.4	9.8±0.2	6.7±0.1
Content of chlorogenic acid isomers at 24h	3- CQA	5.7±0.1	4.7±0.0	13.0±0.1	N.d	13.3±0.5	13.4±0.1	6.8±0.0	9.8±0.1	6.2±0.2
	4- CQA	6.7±0.3	5.4±0.0	9.5±0.1	N.d	11.0±0.2	9.6±0.1	7.9±0.0	6.8±0.0	7.2±0.3
	5- CQA	42.2±0.7	33.8±0.6	19.1±0.7	36.2±0.1	29.6±0.2	17.8±0.4	49.0±1.2	9.5±0.2	45.8±2.1
	FQA	1.0±0.0	1.0±0.0	2.2±0.0	N.d	N.d	2.3±0.1	1.2±0.1	2.5±0.0	1.3±0.0
	FQA	7.9±0.1	6.9±0.1	3.7±0.1	N.d	N.d	4.0±0.0	9.2±0.4	2.8±0.1	9.0±0.1
	3,4-di-CQA	1.3±0.2	0.7±0.0	0.9±0.0	N.d	N.d	0.6±0.0	2.0±0.1	Nd*	2.2±0.0
	3,5-di-CQA	1.9±0.2	0.8±0.0	0.9±0.0	N.d	N.d	0.6±0.0	3.2±0.5	Nd	3.3±0.0
	4,5-di-CQA	0.9±0.0	0.5±0.0	0.4±0.0	N.d	N.d	0.3±0.0	1.5±0.3	Nd	1.4±0.0
	Total-CQA	54.5±1.1	43.9±0.7	41.6±0.9	36.2±0.1	53.9±0.8	40.8±0.6	63.6±1.2	26.1±0.3	59.2±2.6
	Total-FQA	8.9±0.0	7.8±0.0	5.9±0.2	N.d	N.d	6.3±0.1	10.5±0.5	5.3±0.1	10.2±0.1
	Total-di-CQA	4.1±0.1	2.0±0.1	2.1±0.1	N.d	N.d	1.5±0.1	6.8±1.0	0.0±0.0	6.9±0.1
% Change	5- CQA	9.76	23.68	52.73	35.58	43.04	63.96	5.17	79.95	10.49
	Total-CQA	9.14	22.94	29.95	35.58	22.16	40.9	5.28	61.49	11.76
	Total-FQA	4.4	12.11	35.99	N.d	N.d	39.86	1.54	50.45	-3.03
	Total-di-CQA	41.38	62.64	71.74	N.d	N.d	85.46	11.78	100	-3.21

Where: β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, β-LG + 3 ml juice + coffee extract; 2, β-LG + 6 ml juice + coffee extract; 3, β-LG + pH 9 + coffee extract; 4, β-LG + 3 ml juice + CQA solution; 5, β-LG + pH 9 + CQA solution; 6, GMP + pH 9 + coffee extract; 7, GMP + 3 ml juice + coffee extract; 8, BZ1+ pH 9 + coffee extract; 9, BZ1 + 3 ml juice + coffee extract; CQA, Caffeoyl-Quinic Acid; FQA, Feruloyl-Quinic Acid and di-CQA, di-Caffeoyl-Quinic Acid and N.d, not detected

Therefore, it can be observed that the most reactive hydroxycinnamic acid derivatives were the di-caffeoylquinic acids (di-CQA). Table 4-10 also shows the change in CQA isomers in the case of whey protein products (BZ1 and GMP). The results show that there is an increase in 3- CQA isomer in alkaline modification. The 5- CQA was decreased in both modifications; however the % of decrease in alkaline modification was higher than the enzymatic

modification, where the values were 63.96 and 5.17 % for GMP at alkaline and enzymatic modification respectively, whereas 79.95 and 10.49 % were found for BZ1. Moreover, all CQA isomers were decreased in the modified GMP. In the case of enzymatically modified BZ1 (sample 9), total FQA and di-CQA seem to be unchanged. On the other hand, total di-CQA in alkaline modified BZ1 (sample 8) was almost undetectable. One possible explanation for the decrease in CQA is the oxidation of CQA in alkaline solutions or in the presence of polyphenol oxidase enzyme, to the respective quinones (Rawel *et al.* 2002b).

4.3.1.2 The change in free amino groups (FAG) content

The trinitrobenzenesulfonic acid (TNBS) substrate, which binds to the nucleophilic nitrogen of the ε-amino side chains of lysine, was used to determine the change in amount of free amino groups of modified proteins. The interaction of CQA with the free amino groups of the β-LG, BZ1 and GMP products results in the consequent binding and blocking of this reaction site for TNBS (Kroll *et al.* 2003). This leads to a significant decrease in the content of the free amino groups in all modified proteins compared to controls (Figure 4-32). These results are in the line with previous studies (Rawel *et al.* 2001a, Rawel *et al.* 2001b, Rawel *et al.* 2002b, Rohn *et al.* 2006, Prigent *et al.* 2007, Aewsiri *et al.* 2009), who reported that the interaction between phenolic compounds and whey protein and other proteins caused a decrease in the amount of free amino groups.

SDS was used to destroy the non-covalent protein interactions, so we can suppose that the derivatization has occurred through covalent binding. 861.33 nmol free amino groups/mg unmodified β-LG is found. The results confirm that the application of green coffee extract in enzymatic modification showed a positive relationship between the amount of blocked free amino groups and the amount of apple juice added, where % of blocked amino groups was increased from 4.6 to 7% upon increasing the amount of apple juice from 3ml to 6ml. On the other hand, the application of coffee extract at pH 9 tends to block approximately 12.9 % of the free amino groups. With regard to the application of commercial CQA at enzymatic and alkaline modifications (sample 5 and 6), it leads to a significant decrease at $P < 0.05$ in free amino groups, 23.4 and 24.7%, respectively, compared to coffee extract (Figure 4-32A). The change in free amino group contents of BZ1 and GMP modified by both methods are shown in Figure 4-32B. The amount of free amino groups for unmodified BZ1 was 1094.3 nmol /mg.

This value is higher than native β-LG which had 861.33 nmol/mg: this difference may be ascribed to the hydrolysis process, which occurred during BZ1 preparation. Modified BZ1 under alkaline and enzymatic conditions showed a significant decrease in the amount of free amino groups compared to unmodified BZ1, while no significant differences between either of them were noticed. After 24h of alkaline and enzymatic modifications of BZ1, a 25.3 and 26.5 % decrease in amount of free amino groups respectively, was found. On the other hand, in case of GMP, the amount of free amino groups was increased after 24h of reaction. Significant differences at $P < 0.05$ between GMP modified at pH 9 and GMP modified by the PPO enzyme were found, where the percentage increase was 78.3 and 55.3% for alkaline and enzymatic modifications, respectively. This observation is remarkable, and the modification conditions appear to facilitate the hydrolysis of these proteins. No clear explanation can be given here at the moment and further experiments may facilitate the understanding of the observed phenomenon.

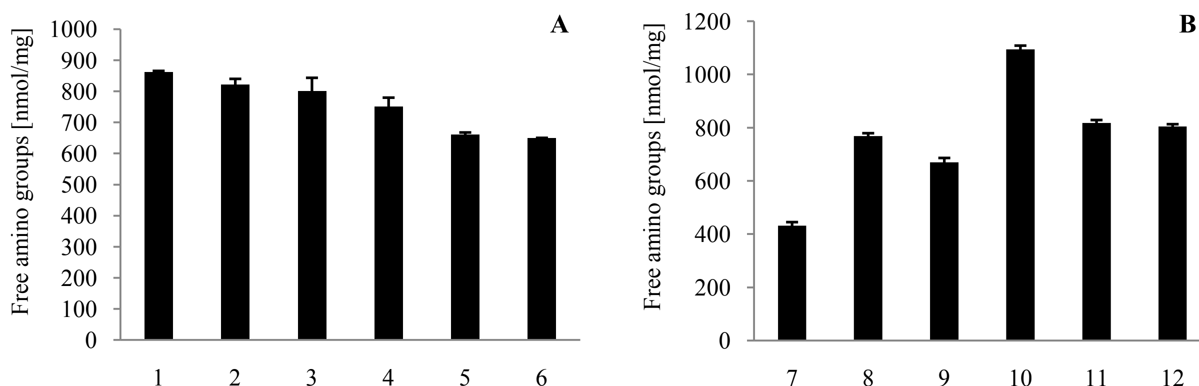


Figure 4-32 Free amino groups content of β-Lactoglobulin (A), Glycomacropeptide and BioZate1 (B) – chlorogenic acid derivatives (nmol/mg protein)

Where: β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution; 7, unmodified GMP; 8, GMP + pH 9 + coffee extract; 9, GMP + 3 ml juice + coffee extract; 10, unmodified BZ1; 11, BZ1+ pH 9 + coffee extract and 12, BZ1 + 3 ml juice + coffee extract

In conclusion, the decrease in the amount of free amino groups in the proteins studied was associated with the loss in CQA isomers as mentioned above. It may be due to the oxidation of phenolic substances in alkaline solutions or in the presence of polyphenol oxidase to respective quinones, which can react with free amino groups of proteins (Rawel *et al.* 2001a, Kroll *et al.* 2003, Aewsiri *et al.* 2009). The nutritional consequence of these oxidative

reactions of phenolic compounds in the food systems is limited availability of the essential amino acid lysine. These interactions do not only take place with amino acid lysine groups, but also with tryptophan, methionine, histidine, tyrosine and cysteine of the protein molecule (Rawel *et al.* 2002b, Kroll *et al.* 2003, Schilling *et al.* 2008).

4.3.1.3 The change in the thiol groups content

β-LG contains one reactive free thiol group (at Cys121) per molecule. In addition, it is stabilized by two disulphide bridges, one inside the molecule between Cys106 and Cys119 and the second is near the C-terminus between Cys66 and Cys160. A reducing agent (5, 5′-Dithio-bis-(2-nitrobenzoic acid) – DTNB) was used for the colorimetric determination of the content of thiol groups. The modification of β-LG with green coffee extract and commercial CQA under the alkaline and PPO modulated reactions, leads to a significant decrease in the number of thiol groups (Figure 4-33) compared to controls. These results are in accordance with one previously reported (Rawel *et al.* 2002a, Rohn *et al.* 2002, Rohn *et al.* 2005, Rawel and Rohn 2010, Rade-Kukic *et al.* 2011).

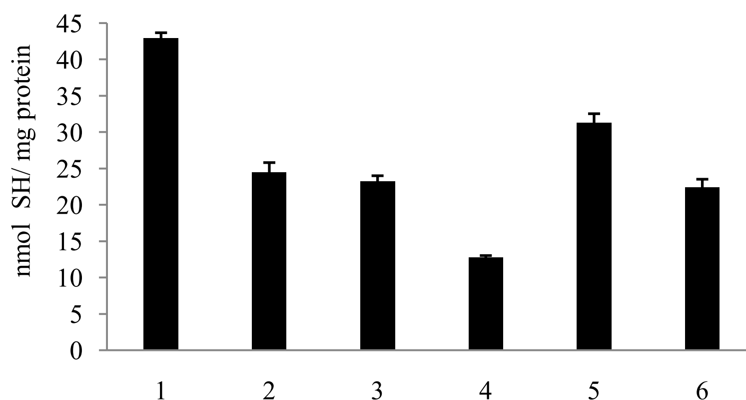


Figure 4-33 Thiol groups content of β-Lactoglobulin protein-chlorogenic acid derivatives (nmol/mg protein)

Where: β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution and 6, β-LG + pH 9 + CQA solution

Data showed that there are no significant differences at $P < 0.05$ between modified β-LG with 3 ml and 6 ml apple juice. However, significant differences between β-LG modified under alkaline and enzymatic conditions were found. Therefore, the highest decrease was observed in modified β-LG with coffee extract at pH 9 followed by modified β-LG with CQA

commercial at pH 9, where the % of loss was 70.23 and 47.83 %, respectively. Similar experiments during the derivatization of hydrolyzed whey protein (BZ1) and GMP were done but there was no change in thiol groups (data are not shown). The explanation of the decrease in the amount of thiol groups can be illustrated by two reaction mechanisms. The first one is the reaction of the thiol group with oxidized phenolic compounds (Rawel *et al.* 2004). Although the reaction was done in the presence of SDS and under reducing conditions, the thiol groups were not altered to disulfide bridges, but had reacted with the phenolic compounds (Rawel *et al.* 2003, Rawel *et al.* 2004). The second mechanism can be explained by the redox interaction in two steps. In the first step, the phenolic compounds interact with the disulfide bridges, the phenol is oxidized to the corresponding quinone and the disulfide bridges are reduced to free thiol groups, then, in the second step, a nucleophilic addition with each other may take place (Rawel *et al.* 2004, Prigent *et al.* 2008, Schilling *et al.* 2008). Such interactions between phenolics and the thiol groups were confirmed using a lysozyme protein, which does not have any free thiol groups (Rawel *et al.* 2003).

4.3.1.4 The change in the tryptophan content

A possibility of the reaction of oxidized phenolic compounds with other amino acids, e.g. tryptophan, has also been found (Rohn *et al.* 2005). The quenching of fluorescence intensity as an indicator of tryptophan changes was studied after activation at 290 nm and measurement of the emission between 300 and 900 nm. The effect of modification of whey protein products, β-LG, BZ1 and GMP dissolved in urea and PBS buffer pH 7.2 on the tryptophan content are outlined in Figure 4-34. As is known, urea destroys non-covalent protein interactions, so the results in Figure 4-34 showed that the amount of tryptophan in proteins measured in the presence of 8 M urea was higher than measured in the presence of a PBS buffer. The data demonstrated that both modification methods caused a decrease in tryptophan content. These results agree with Rawel *et al.* (2001a) and Rohn *et al.* (2005). Therefore, all values obtained for the modified proteins are significantly different from the control protein. It can also be noticed that in case of β-LG, the % of loss in tryptophan content was significantly increased from 71.52 to 88.2%, when the amount of apple juice increased from 3 ml to 6 ml. Moreover, the % of loss during enzymatic modification was higher compared to alkaline modification. β-LG modified by coffee extract and 6 ml apple juice showed the highest

decrease, while the β-LG at pH 9 modified by CQA solution showed the lowest decrease, 63.7%.

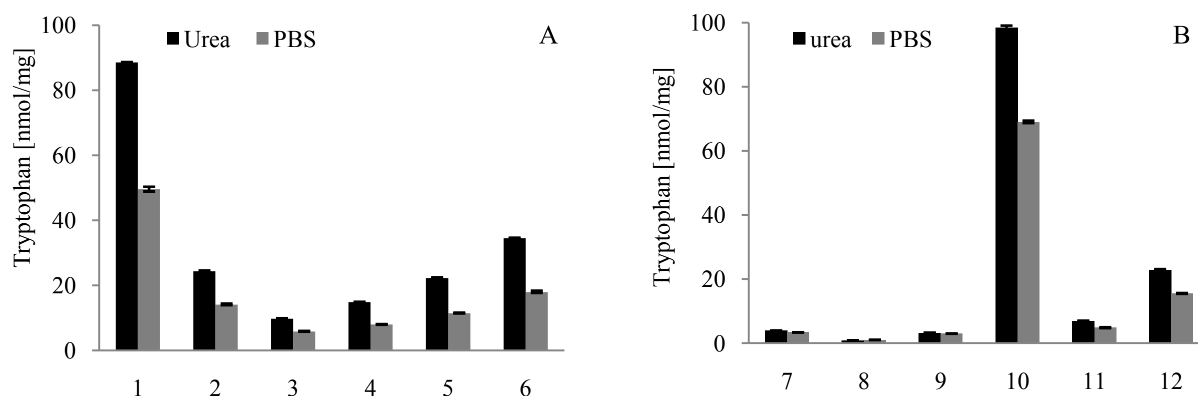


Figure 4-34 Tryptophan content of β-Lactoglobulin (A), Glycomacropeptide and BioZate1 (B) - chlorogenic acid derivatives (nmol/mg protein)

Where: β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution; 7, unmodified GMP; 8, GMP + pH 9 + coffee extract; 9, GMP + 3 ml juice + coffee extract; 10, unmodified BZ1; 11, BZ1+ pH 9 + coffee extract and 12, BZ1 + 3 ml juice + coffee extract

On the other hand, the opposite results were found in the case of BZ1 and GMP, where alkaline modification showed a higher decrease than enzymatic modification. Therefore, 69.8 and 93% of tryptophan fluorescence were quenched for GMP and BZ1 at alkaline conditions, while 11.6 and 77.5% were found at enzymatic condition, respectively. The amount of tryptophan in BZ1 control was higher than β-LG control, where the values were 98.5 and 88.8 nmol/mg proteins, respectively. This may be attributed to the hydrolysis process, which happened in BZ1 preparation. A previous study by Rawel *et al.* (2003) put forward the possibility that this decrease in tryptophan content attributable to a nucleophilic addition of the heterocyclic nitrogen of the tryptophan moiety to an oxidized species of a phenolic compound, although as yet such adducts have not been identified. In conclusion, learning more about the number of modified amino side chains may help us to show a partial indication of the extent of protein modification. Therefore, the amino acids blocked and the position of these acids in the β-LG structure will be discussed in the next steps.

4.3.2 Effect of CQA on structural properties of proteins

4.3.2.1 Changes in surface hydrophobicity

ANS, known to bind to hydrophobic pockets on the protein surface, is a much-utilized fluorescent for examining the non-polar character of proteins. It is also often used to study conformational changes in a given protein by probing its surface hydrophobic binding sites. The changes in the surface hydrophobicity, by the low initial slopes (S_o, hydrophobicity index) calculated from the corresponding fluorescence intensity vs. concentrations of β-LG, BZ1and GMP proteins compared to the controls, were studied, and the data are illustrated in Figure 4-35. In general, the modification of these products with coffee extract and commercial CQA leads to a significant decrease depending upon the mode of modification. These results were in line with Rawel *et al.* (2002a), who demonstrated that the modification of soy protein with chlorogenic, caffeic and gallic acids, and some flavonoids decreased the surface hydrophobicity of protein. Also, Aewsiri *et al.* (2009) reported that the surface hydrophobicity of cuttlefish skin gelatin decreased when modified with different oxidised phenolic compounds.

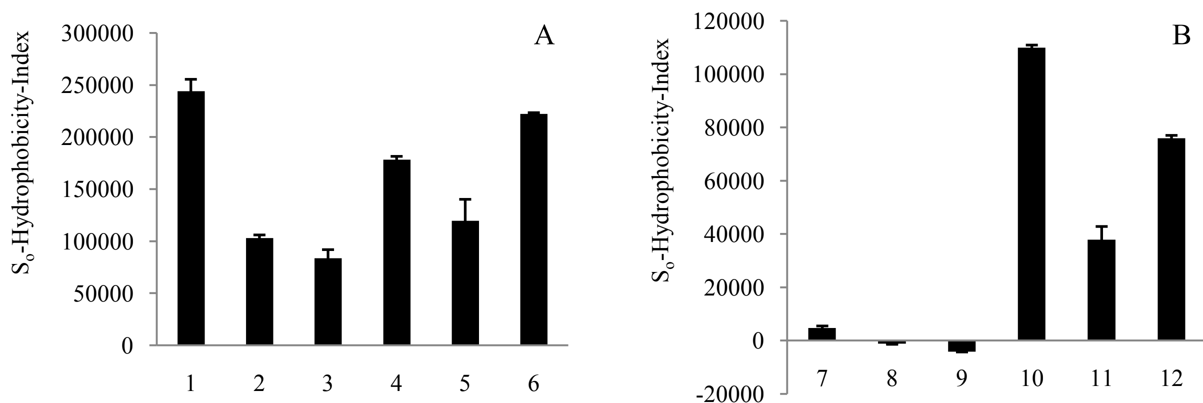


Figure 4-35 Surface hydrophobicity of β-Lactoglobulin (A), Glycomacropeptide and BioZate1 (B) - chlorogenic acid derivatives

Where: β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution; 7, unmodified GMP; 8, GMP + pH 9 + coffee extract; 9, GMP + 3 ml juice + coffee extract; 10, unmodified BZ1; 11, BZ1+ pH 9 + coffee extract and 12, BZ1 + 3 ml juice + coffee extract

The highest decrease in the surface hydrophobicity was recorded in modified β-LG with 6 ml apple juice (Figure 4-35A, sample 3) followed by samples 2 and 5, where the % decrease was

65.8, 57.8 and 51, respectively. Possible explanations for these decreases were given (Kroll *et al.* 2003, Aewsiri *et al.* 2009). They reported that the decrease in surface hydrophobicity for modified proteins was caused by the increase in the number of hydroxyl and carboxyl groups of phenolic compounds attached. This decrease could also be due to the covalent attachment of the phenolic compound to proteins causing the blocking of the hydrophilic groups like amino and thiol groups.

Results in Figure 4-35A also showed that β-LG modified with PPO enzyme from apple juice exhibits a more hydrophilic surface property than both alkaline and unmodified. This also substantiates the hypothesized oxidized state of the reaction products in modified β-LG at alkaline condition, the phenolic OH-groups of the CQA adducts with protein side chains not being available and the hydrophobic vicinity of tryptophan (W19 and W61) being involved in the CQA-protein interactions. Furthermore, the GMP hardly binds ANS. BZ1 samples modified at alkaline conditions exhibited more decrease in hydrophobicity than those modified with PPO enzyme.

4.3.2.2 Circular Dichroism (CD) spectroscopy

Circular Dichroism (CD) spectroscopy is a valuable method for examining the extent of structural changes in the proteins as a result of CQA binding. The secondary structure of modified β-LG, GMP and BZ1 proteins and their controls was estimated by a far-UV-CD analysis, while a near-UV analysis was used to evaluate the tertiary structure of these proteins.

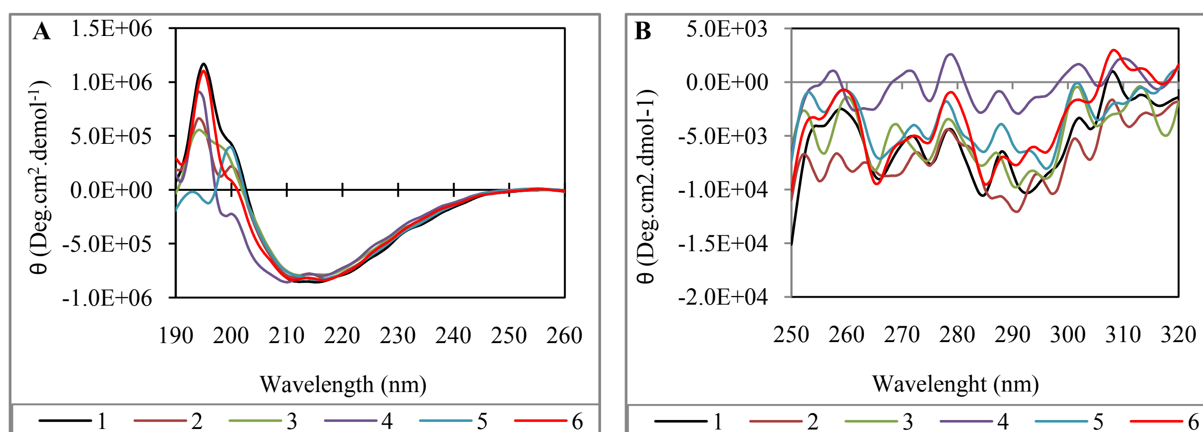


Figure 4-36 Far-UV (A) and near-UV (B) CD spectra of β-Lactoglobulin-chlorogenic acid derivatives

Where: β-LG, β-Lactoglobulin; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution and θ; molar ellipticity

The obtained β-LG proteins far-UV and near-UV CD spectra are shown in Figure 4-36, and the effects of modification methods on the normalized content of secondary structure parameters in proteins, as calculated by far-UV CD spectrometry using the CONTIN method, are shown in Table 4-11. According to CONTIN allocation, unmodified β-LG contained 20.5% α-helix, 39.9% β-sheet, 15.2% turn and 24.5% unordered structure elements. The protein conformational analysis based on CD data suggests that β-LG at pH 7.1 contains α-helical 16%, β-sheet 33.6%, β-turn 20.9%, and random coil 29.6% (Rade-Kukic *et al.* 2011), while for free β-LG, 12% α-helical, 58% β-sheet, 10% turn and 20% random coil were reported by Belatik *et al.* (2012). These differences could be explained by different experimental conditions, the application of different methods for collection, and the software used for the evaluation of CD data. Additionally, the preparation of whey proteins (e.g. the drying conditions) might have also influenced the measurement, as might the presence of ca. 7% of α-lactalbumin and ca. 4% of BSA in the first study.

Table 4-11 Effect of modification by CQA on the normalized content of secondary structure parameters in proteins, as calculated by far-UV CD spectrometry

Proteins	CD [%]				DSC		
	α-Helix	β-Strand	β-Turn	Unordered	T_{onset}	T_d (°C)	ΔH mJ/mg]
1	20.5	39.9	15.2	24.5	69.6	75	10.8
2	9.0	36.3	20.7	34.1	74.1	79.8	9.9
3	7.3	35.2	22.5	35	74.1	80.6	7.4
4	9.3	29.5	17.6	43.5	67.2	76.7	3.4
5	5.7	38.1	21.5	34.6	74.9	80.5	8.8
6	8.2	34.9	22.9	34	69.3	76.7	9

Where: all values are mean and the standard deviations are less than 3%; β-LG, β-Lactoglobulin; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution; $T_{onset,}$ onset temperature; T_d, denaturation temperature and ΔH, enthalpy of denaturation

The CQA modification leads to major changes in the secondary structures (Table 4-11), therefore to proteins becoming more unordered. It is also evident that the interaction of CQA with protein is accompanied by a corresponding loss of structured elements (α-helix and β-strand) and an increase in the amount allocated to the random coil factions. There is an agreement among those formerly reported (Rawel *et al.* 2002a), where an increase in α-helix and β-strand was reported after modification of soy glycinin with phenolic compounds, while the modification of human serum albumin and bovine serum albumin with chlorogenic acid

was shown to cause a decrease in α-helix structure (Muralidhara and Prakash 1995, Rawel *et al.* 2002b). Moreover, the highest increase in unordered structure, ca. 77.6%, compared to unmodified β-LG, was noticed in β-LG modified with coffee extract at alkaline conditions, whereas the highest decrease in α-helical element, ca. 72.2%, was found in β-LG modified with CQA solution at enzymatic conditions (Table 4-11, samples 4 and 5). Rade-Kukic *et al.* (2011) reported that bound allyl isothiocyanate with β-LG protein at pH 7.1 caused changes in both secondary and tertiary structures, where up to 2.4 and 2.5% increase in α-helix and unordered structures respectively, were found. Furthermore, after the alkaline modification of the coffee proteins in presence of chlorogenic acid, the content of the β-strand remained more less unchanged, with a slight decrease in the random coil being observed (Rawel *et al.* 2005a). Finally, dimer separation, untying and partial unfolding of the β-LG molecule, as a result of steric hindrance and hydrophobicity of the introduced molecule, could be sufficient to explain the changes in α-helix and unordered structures (Rade-Kukic *et al.* 2011). The influence of the modification on the tertiary structure of β-LG was also evaluated by means of a near-UV study. The CD of proteins in the near-UV (310–240 nm) derives from transitions in the prosthetic groups. The variation of CD in this region can be used to monitor changes in conformation and local environment. These spectra (Figure 4-36B) highlight the conformational changes induced by the reaction with CQA, affecting the tertiary structure and perturbing the initial conformation of the protein, the effect being stronger in alkaline modification. Therefore, the CD-analysis shows that the reaction of CQA with proteins induced micro-environmental and conformational changes.

4.3.2.3 Differential Scanning Calorimetry (DSC)

To study the thermo stability of the modified proteins, the DSC was applied. It is well known that denaturation processes in proteins commonly take place in two steps: the first one is reversible, corresponding to the unfolding process, and the second one is irreversible, leading to the degradation of the unfolded protein molecule (Cueto *et al.* 2003). The effects of alkaline and enzymatic modifications on DSC characteristics of β-LG with and without CQA are presented in Table 4-11. The DSC of unmodified β-LG showed one transition with a peak temperature (T_d) at 75°C and an enthalpy (ΔH) of 10.8 mJ /mg. Upon modification with CQA, there is a corresponding increase in the denaturation temperature (T_d) and a decrease in ΔH,

where the highest increase in T_d was found in β-LG modified with PPO enzyme compared to that which was modified at pH 9.

The denaturation temperature increased by ca. 4.8, 5.6 and 5.5 °C for β-LG modified with 3 and 6ml apple juice in coffee extract and 3 ml apple juice in CQA solution respectively, and the same increase amount, 1.7 °C was noticed in β-LG modified by both coffee extract and CQA solution at pH 9 compared to unmodified β-LG. Consequently, the higher denaturation temperature reflects a higher thermal stability of β-LG modified at enzymatic conditions, compared to those modified at alkaline conditions. These results are in agreement with Prigent *et al.* (2007) who noted that modification of *α*-lactalbumin, lysozyme and bovine serum albumin proteins with the oxidation products of CQA increased denaturation temperature between 1 to 5°C, while Rawel *et al.* (2002b) found the opposite, where a decrease in the T_d for bovine serum albumin protein after modification with CQA was found.

In contrast, this order differed for ΔH values, where the highest decrease in ΔH was noted in β-LG modified with coffee extract in alkaline modification (Table 4-11, sample 4), where 68.7% decrease was found compared to unmodified β-LG. Also a decrease in the ΔH for BSA–chlorogenic acid derivatives was noticed (Rawel *et al.* 2002b). Therefore, both T_d and ΔH parameters are being differently affected depending on reaction conditions. It is interesting to note that while the modified β-LG proteins are thermally more stable, they required less amount of energy to unfold than unmodified β-LG. These results suggest that modification may have partially unfolded certain regions of β-LG resulting in lowering the ΔH and conferred stability in other regions of the protein, resulting in higher T_d.

4.3.2.4 The amount of covalent bound CQA

Two types of interactions between phenolic compounds and proteins can be classified: covalent and non-covalent interactions (Rawel and Rohn 2010). The reactive and redox active *o*-quinones, which are generated from the hydroxycinnamic acid derivatives under the conditions applied, provide an electrophilic species capable of undergoing a nucleophilic addition to proteins, thereby covalently modifying the proteins. A rough evaluation of the extent of modification (covalent bound 5-CQA equivalents in nmol/mg protein, Figure 4-37) is possible by applying a RP-HPLC method, which also provides a valuable tool in assessing the quality of the modified proteins. 8 M urea was used to destroy the non-covalent bound

CQA. The results showed that both CQA and CQA- proteins adducts were absorbed at the same wavelength (325 nm), but were eluted at different retention times, while protein control did not show any relevant absorption at 325 nm. In the enzymatic modification, the amount of covalent CQA increased significantly from 18.27 to 23.03 nmol/mg protein, when the amount of apple juice increased from 3 ml to 6 ml (Figure 4-37A).

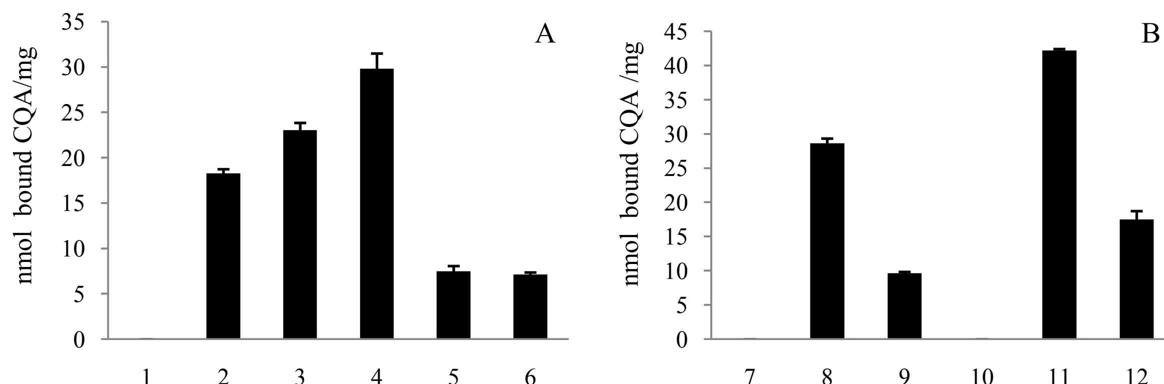


Figure 4-37 Amount of covalent bound CQA in β-Lactoglobulin (A), Glycomacropeptide and BioZate1 (B) - chlorogenic acid derivatives (nmol/mg protein)

Where: β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution; 7, unmodified GMP; 8, GMP + pH 9 + coffee extract; 9, GMP + 3 ml juice + coffee extract; 10, unmodified BZ1; 11, BZ1+ pH 9 + coffee extract and 12, BZ1 + 3 ml juice + coffee extract

Moreover, the results showed that the highest amount of covalent CQA was detected in the modified β-LG with coffee extract at pH 9, where the value was 29.81 nmol/mg protein. On the other hand, the protein modified with CQA solution at enzymatic and alkaline conditions showed the lowest values, 7.47 and 7.14 nmol/mg protein, respectively. Consequently, there are significant differences at $P < 0.05$ between proteins modified with coffee extract and CQA solution. An explanation for this observation can be attributed to the presence of Di-CQAs, which were present in the coffee extracts and were shown to be more reactive (Figure 4-10). Finally, in case of hydrolyzed whey protein BZ1 and GMP, the alkaline modification also showed the highest values compared to enzymatic modification, and significant differences between both alkaline and enzymatic modifications were noted (Figure 4-37B).

4.3.2.5 The changes in molecular weight by electrophoresis

The characterization of the molecular properties of β-LG was judged by the SDS-PAGE method, and data are presented in Figure 4-38. Under reducing conditions, the unmodified β-LG protein showed a molecular weight of 18 kDa. The covalent bound CQA with β-LG leads to an increase in molecular weight, where the β-LG modified at alkaline conditions (samples 5 and 7) appears to be more strongly modified compared to those modified with PPO enzyme from apple juice (samples 3, 4 and 6). This change is visually observed in the increased intensity of the protein bands just about the β-LG band (Figure 4-38).

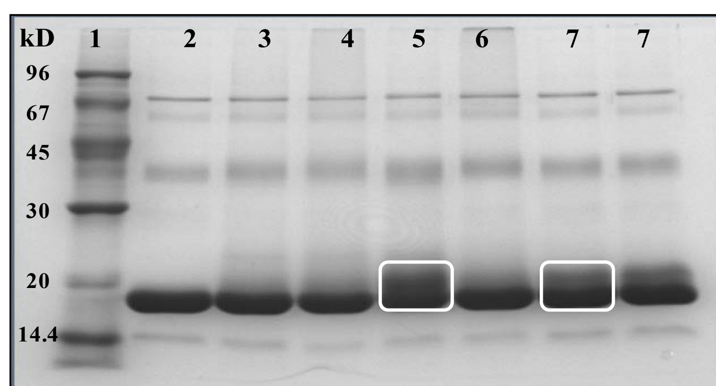


Figure 4-38 SDS-PAGE of β-Lactoglobulin-chlorogenic acid derivatives under reducing conditions

Where: β-LG, β-Lactoglobulin; 1, Standard; 2, unmodified β-LG; 3, β-LG + 3 ml juice + coffee extract; 4, β-LG + 6 ml juice + coffee extract; 5, β-LG + pH 9 + coffee extract; 6, β-LG + 3 ml juice + CQA solution and 7, β-LG + pH 9 + CQA solution

The high molecular weights were still observed even though SDS and mercaptoethanol were added during sample preparation. This is an indication that the CQA covalently bound with β-LG. These results are in the line with Rawel *et al.* (2002b) and Rohn *et al.* (2005). They reported that the molecular weights of bovine serum albumin and bromelain proteins were increased after incubation with phenolic compounds (e.g. CQA). Moreover, the reaction products thus formed may react further with other β-LG molecules, resulting in polymerized products, as identified by SDS-PAGE, where bands with molecular masses between 30 and 96 kDa were found (Figure 4-38).

4.3.2.6 MALDI-TOF-MS analysis

The changes in molecular weights in the modified β-LG protein were also studied by Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS), and

the exemplary MALDI mass spectra of unmodified β-LG and modified β-LG in coffee extract under alkaline and enzymatic, 6ml apple juice, modifications is presented in Figure 4-39. The results show the presence of two β-LG fractions; the determined molecular weight of the main β-LG fractions was 18351.7 Da, agreeing with data calculated from its sequence. The reaction of β-LG with coffee extract and commercial CQA (MW = 352.3 Da) at both modification methods, alkaline and enzymatic, formed a new product with a molecular mass of 19035.4 Da, which in turn could account for the addition of about 684 Da to one β-LG molecule (curve 2 and 3, Figure 4-39). This mole mass addition may correspond to a dimer of two molecules of CQA (Namiki *et al.* 2001, Prigent *et al.* 2007, Schilling *et al.* 2008).

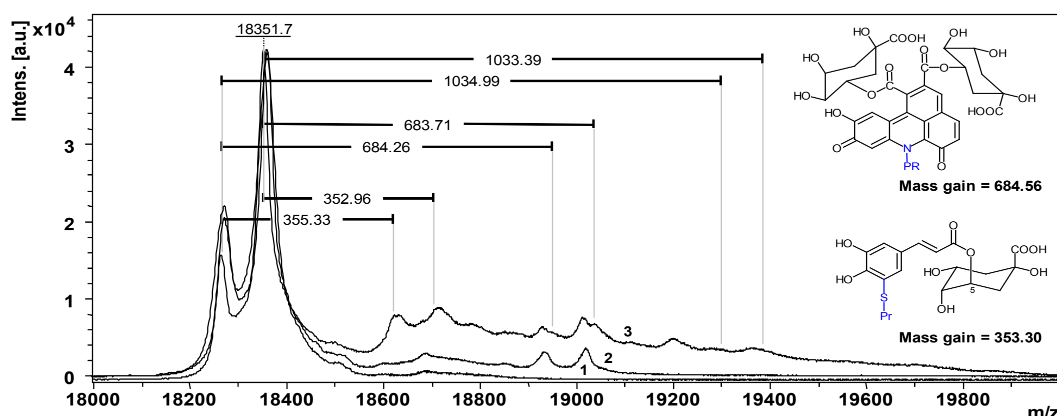


Figure 4-39 MALDI-TOF-MS of modified β-Lactoglobulin depending on modification conditions

Where: 1, unmodified β-LG; 2, β-LG + 6 ml juice + coffee extract and 3, β-LG + pH 9 + coffee extract

Furthermore, new peaks with a lower intensity compared to the peaks of covalent addition of dimer CQA were observed (curve 3, β-LG modified at alkaline conditions). Moreover, an addition of one and three molecules of CQA, presenting new components with 18704.6 and 19385.1 Da respectively, were noticed. These results are in agreement with Rawel *et al.* (2001a). In addition to this, the interaction of CQA with amino acid lysine produced covalent modified lysine by one, two and three CQA (Prigent *et al.* 2008, Schilling *et al.* 2008). At alkaline conditions, 1-3 CQA molecules bounded covalently with BSA and lysozyme proteins (Rawel *et al.* 2000, Rawel *et al.* 2002b, Prigent *et al.* 2007). Accordingly, based on these observations, a tentative list of possible adduct structures was derived (Figure 4-6) and proposed allocations based on an intact protein MALDI-TOF-MS analysis of the CQA derivatives of β-LG are provided for the samples modified at both modification methods with

commercially available CQA (Appendix, Figure 7-11). The results showed similar modifications, in addition β-LG modified with CQA solution at alkaline conditions was also more modified than under enzymatic conditions. Therefore, the results again confirm the SDS-PAGE results, where the β-LG modified at alkaline conditions appears to be more strongly modified compared to those modified at enzymatic conditions.

4.3.2.7 Identification of the sites and types of modification in β-LG - CQA derivatives

In order to get some more information on the types and sites of β-LG modification, the proteins were digested by trypsin. The peptides thus produced were analyzed with MALDI-TOF-MS to produce a peptide mass fingerprint, which was then submitted to a Mascot MS search in the data bank Swiss-Prot. On the basis of the preliminary identification and comparison of the derived sequence with the available complete primary structure for β-LG bovine, variant A in the protein knowledgebase (UniProtKB; http://www.uniprot.org/), the following candidate was found to have the best match: P02754 (sequence details see Appendix, Figure 7-12).

Table 4-12 Identification of sites and types of modification in β-Lactoglobulin-CQA derivatives

Modified peptides	β-LG + 6 ml juice + coffee extract		β-LG + pH 9 + coffee extract	
	Modification sites	Modification types*	Modification sites	Modification types
71-91	K77	CQA2	K77	CQA2
84-100	K91	CQA1	-	-
136-148	K138	CQA4	K138	CQA4
41-60	K47	CQA1/3/4	K47	CQA1/4
102-124	C121	CQA2	C121	CQA2

* Modification types were shown in Figure 4-6

Therefore, further analysis with regard to the identification of the type and site of modification in the protein with CQA was performed, applying this P02754 complete model sequence. The tentative classification of the modification type and sites is provided (Figure 4-40- based on the proposed different types of modifications given in Figure 4-6). In summary, the peptide analysis shows that a few peptides are liable to modification (Table 4-12) and the sites of modification are ε-amino groups of lysine (K77, K91, K138, K47) and thiol group of cysteine (C121). The allocation of the first three modification sites to peptides results in molecular weights that coincide with those which are also released by the tryptic digestion of the

unmodified β-LG. Therefore, their allocation can be predicted, but further MS/MS analysis is needed to confirm this result.

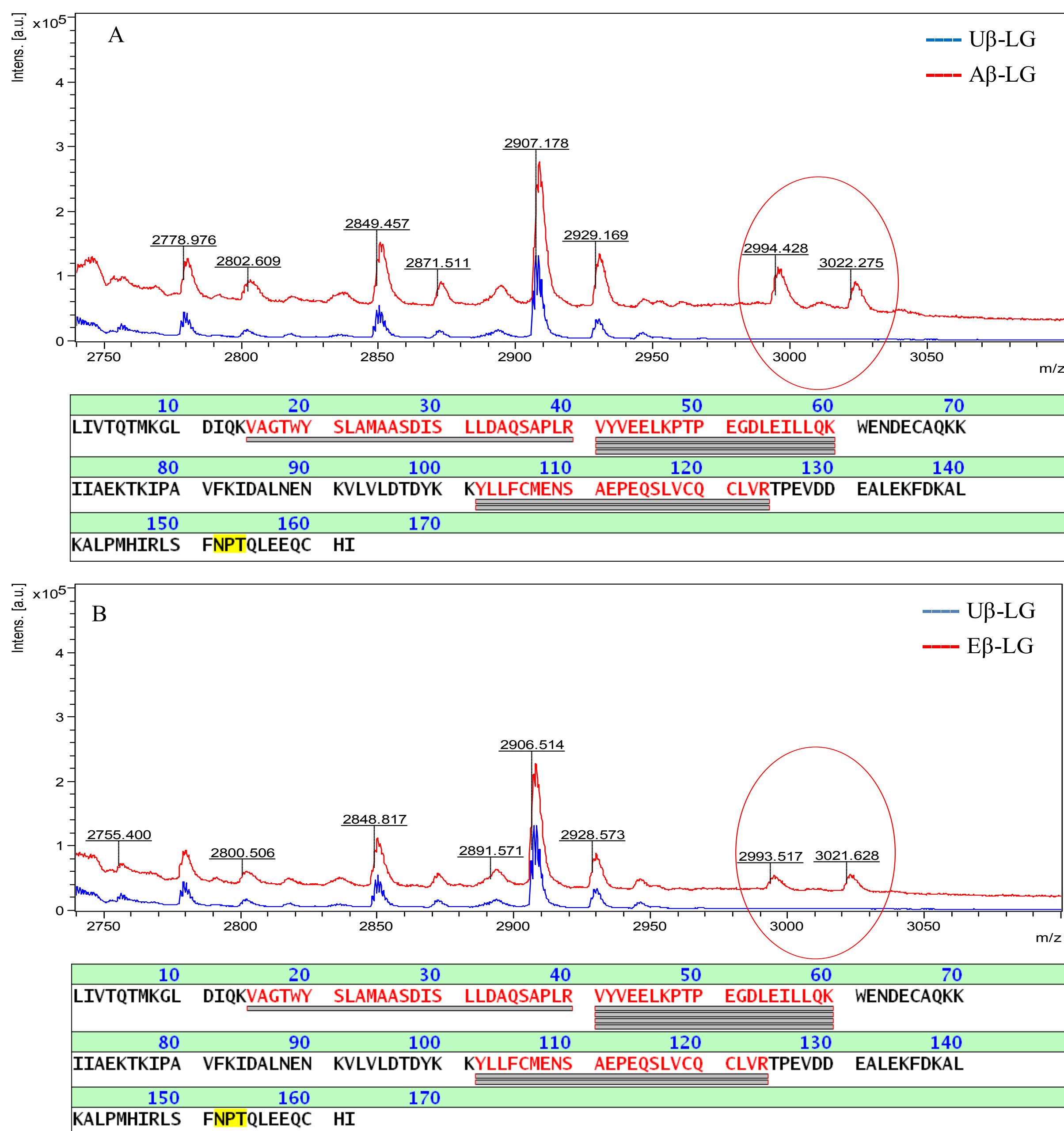


Figure 4-40 Peptide allocation and identification of probable type of β-Lactoglobulin-chlorogenic acid adducts and the corresponding modification sites

Where: Uβ-LG, unmodified β-LG; Aβ-LG, β-LG + pH 9 + coffee extract (A) and Eβ-LG, β-LG + 6 ml juice + coffee extract (B)

The peptide fingerprints of both the modified β-LG showed two new peptide peaks (Figure 4-40), which could not be allocated as products of tryptic digestion of β-LG even when

considering a higher number of missed cleavages (or partials accounting for tolerated internal missed cleavage sites in the matching peptides).These two peptides contain two sites (K47 and C121), which we may assume as modified although a high mass tolerance (the peptide mass error) needed to be applied. Thus, the nature of modification can be predicted to be a benzacridine derivative with lysine after dimerization of CQA or the direct addition of di-CQA and that with cysteine resulting in an adduct (Figure 4-6) as already reported (Schilling *et al.* 2008). The possibility that these two peptides may originate from coffee extract or apple juice can be discarded, since they were also found when investigating the modification of β-LG with pure CQA under alkaline conditions (Appendix, Figure 7-11). From the sequence of P02754, it can be observed that two tryptophan residues - W19 and W61 - are present. W19 is in a polar environment and contributes to 80% of total fluorescence, while W61 is partly exposed to aqueous solvent and has a minor contribution to tryptophan fluorescence (Belatik *et al.* 2012).This observation can be confirmed by the results for β-LG (Figure 4-34). The modification leads to a significant decrease, and can be assumed to come from the interaction between CQA and protein, since it remains low even in the presence of urea. Whether tryptophan side chains themselves are involved in the interactions remains unclear, since both peptides containing W19 and W61 were released by tryptic digestion of both modified Eβ-LG and Aβ-LG (Figure 4-40).

4.3.2.8 Tentative modeling of the modification

In order to understand the change in the structural behavior of β-LG, it is necessary to consider the structure of β-LG in more detail. β-LG consists of 162 amino acids folded into a compact globular conformation, stabilized by two intramolecular disulphide bonds, one inside the molecule (C106-C119) and one near the C-terminus (C66-C160). Further, it contains one free thiol group (at C121), which in the native molecule is buried in the hydrophobic part, but becomes exposed and more reactive on dissociation of dimers and/or partial unfolding of the molecule, e.g. with increasing temperature (Otte *et al.* 1997). We propose that the disulfide-thiol exchange in the C terminus (C66–C160) may be initiated by the redox conditions provided in the presence of CQA. The protein structure thereupon becomes more disordered as simulated by molecular dynamic calculation (34 ns), with the results shown in Figure 4-41A. Protein properties computed after this simulation support the previously documented

increase in the hydrophilic surface area from experiments applying ANS binding and tryptophan fluorescence. This unfolding process may additionally be supported by the reaction of the CQA at the proposed sites of modification of ε-amino groups of lysine (K77, K91, K138 and K47) and the thiol group of cysteine (C121). The data thus correlates with the observed structural changes obtained for Eβ-LG and under the progressed reactivity for the Aβ-LG sample. The results of molecular modeling indicate the accessibility of these reaction sites as illustrated in Figure 4-41B.

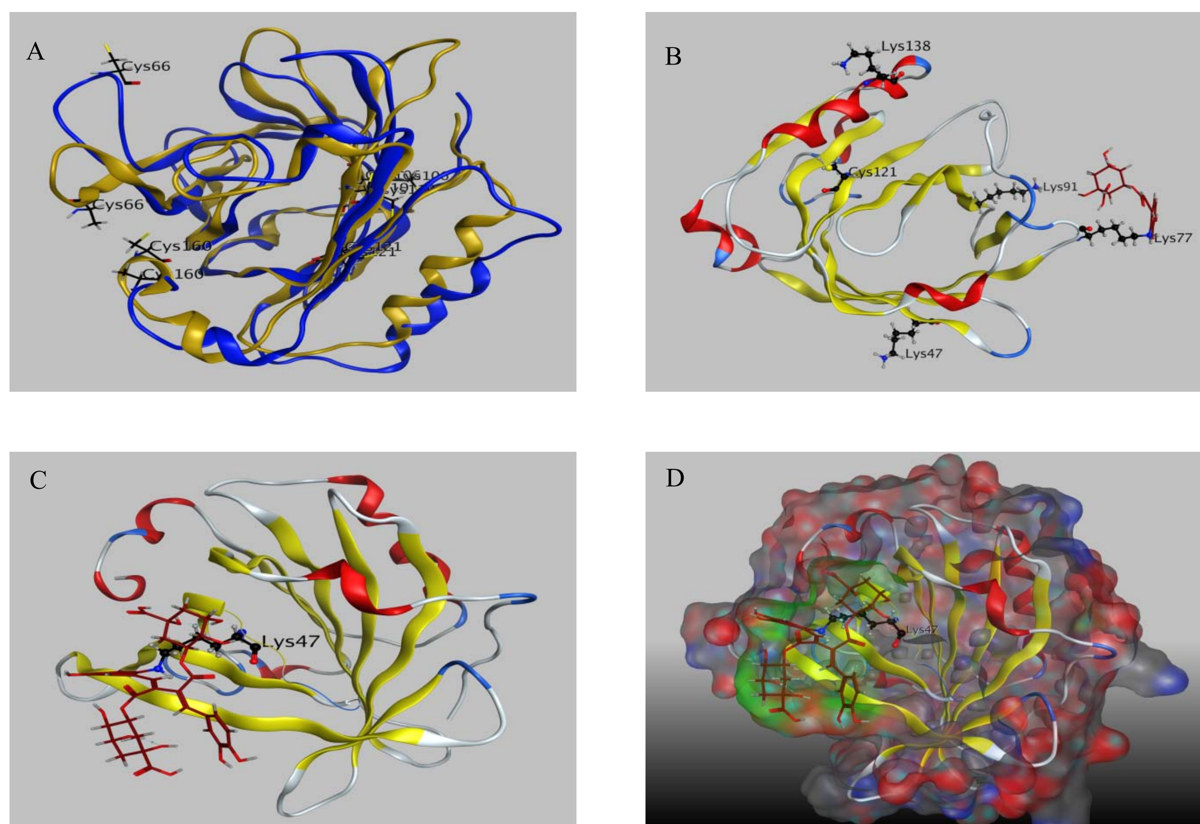


Figure 4-41 Molecular modeling of modified β-Lactoglobulin

Where: (A) = disulfide/thiol exchange (C66-C160) as provoked by presence of CQA leading to initial enfolding (blue coloured protein conformation) of the initial protein molecule (gold coloured protein conformation), (B) = proposed sites of modification of ε-amino groups of lysine (K77, K91, K138, K47) and the thiol group of cysteine (C121) in β-LG; the simulation also illustrates the modification at the lysine site K77, (C) = modeling of CQA modification at the lysine site 47 in β-LG, and (D) = modeling of CQA modification at the lysine site 47 in β-LG

In the next step, the accessibility of two reaction sites (K47 and K77) was simulated by molecular modeling to underline the possibility of these modifications (exemplary with the corresponding modification types CQA5 and CQA2 as well as considering the possibility of

a further reaction of CQA5 to CQA1 at K47, Figure 4-6). Thereafter, the pharmacophores, describing the molecular features which are necessary for molecular recognition for a ligand to react in the vicinity of K47 and K77 in the protein, were defined. On basis of this definition, the modification of the sites was conducted, such that the docking distance of under 4Å for different poses could be achieved. The results of these simulations confirm the possibility of the proposed reactions at K77 (Figure 4-41B) and K47 (Figure 4-41C). The simulation also shows exemplary the exposure of the charged molecular surface of the protein after the modification of the site K47 (Figure 4-41D).

4.3.3 The effect of CQA on the functional properties of proteins

4.3.3.1 The change in solubility

Solubility is a prerequisite for an ingredient protein and is critically necessary for functional products such as beverages. Whey proteins remain soluble during the precipitation of casein at pH 4.4-4.6. Thus, the loss in solubility at this pH is commonly used to access the extent of protein denaturation (Rawel *et al.* 2001a). The effect of enzymatic and alkaline modifications on the solubility of β-LG, GMP and BZ1 proteins as a function of pH was investigated after 24h modification at room temperature, and the results are ordered in Figure 4-42.

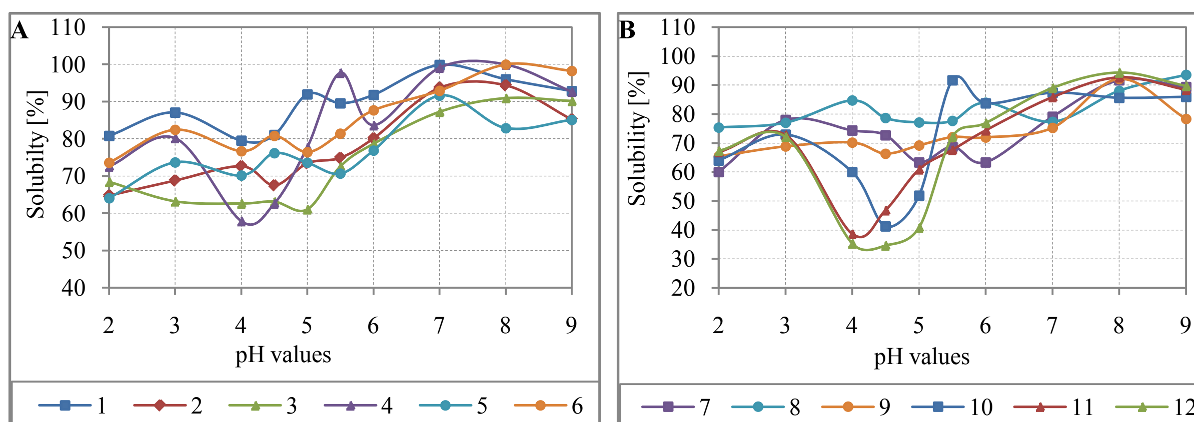


Figure 4-42 Solubility of β-Lactoglobulin (A), Glycomacropeptide and BioZate1 (B) -chlorogenic acid derivatives as a function of pH

Where: β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution; 7, unmodified GMP; 8, GMP + pH 9 + coffee extract; 9, GMP + 3 ml juice + coffee extract; 10, unmodified BZ1; 11, BZ1+ pH 9 + coffee extract and 12, BZ1 + 3 ml juice + coffee extract

When the effects of alkaline modification on solubility are compared with the effects of PPO modification, it can be seen that both methods decreased the solubility of the proteins. The β-LG modified with coffee extract at pH 9 showed a decrease in solubility between 15 - 27% at pH from 4 to 5, demonstrating a denaturation due to modification. On the other hand β-LG modified with PPO enzyme exhibited diminished solubility between pH 2-5, where % decrease ranged from 15 – 33.6% (Figure 4-42A). These results are in accordance with previous studies (Rawel *et al.* 2000, Rawel *et al.* 2002a, Rawel *et al.* 2002b, Rohn *et al.* 2005, Prigent *et al.* 2007), who reported that the solubility of proteins decreased when the protein is modified with phenolic compounds. Furthermore, β-LG modified with CQA solution using both methods of modification showed a slight change in the solubility compared to an unmodified sample. The modified GMP at pH 9 showed an increase in solubility at pH from 4 to 6, while in the case of modified GMP with PPO it seems to be unaffected (Figure 4-42B). In contrast, a significant decrease at $P < 0.05$ at pH 4 and 5.5 was found in case of BZ1, where a decrease in the solubility of ca. 35.6 and 41.1 % at pH 4 and 26.3 and 21.4 % at pH 5.5 for modified BZ1 with alkaline and enzymatic modifications, respectively was noted. This behavior can be attributed to the decrease in the number of charged groups followed by the covalent binding of phenolic compounds to the free amino groups. Furthermore, an introduction of an aromatic ring(s) structure contributing to the hydrophobic nature of the samples could also explain the changes in solubility (Rohn *et al.* 2005).

4.3.3.2 The change in antioxidative capacity

In recent years, the relationship between the antioxidants and consumption of foods was the subject of many epidemiological studies, while few studies dealt with the effect of covalent bonds between proteins and phenolic compounds on the antioxidative properties of protein. The total antioxidant capacity of whey protein (WP) products, β-LG, GMP and BZ1, modified with coffee extract and commercial CQA at different conditions, enzymatic and alkaline solutions, was determined using DPPH and TEAC assays. The data of both assays are outlined in Figure 4-43 and Figure 4-44. The modification of proteins at both conditions consequently leads to a significant increase in the antioxidative capacity of the WP products compared to the controls. These results are in agreement with Rohn *et al.* (2004) and Aewsiri *et al.* (2009), who reported that the antioxidative activity of modified bovine serum albumin (BSA) and

gelatin proteins, by covalent attachment of phenolic compounds, was increased compared to unmodified samples.

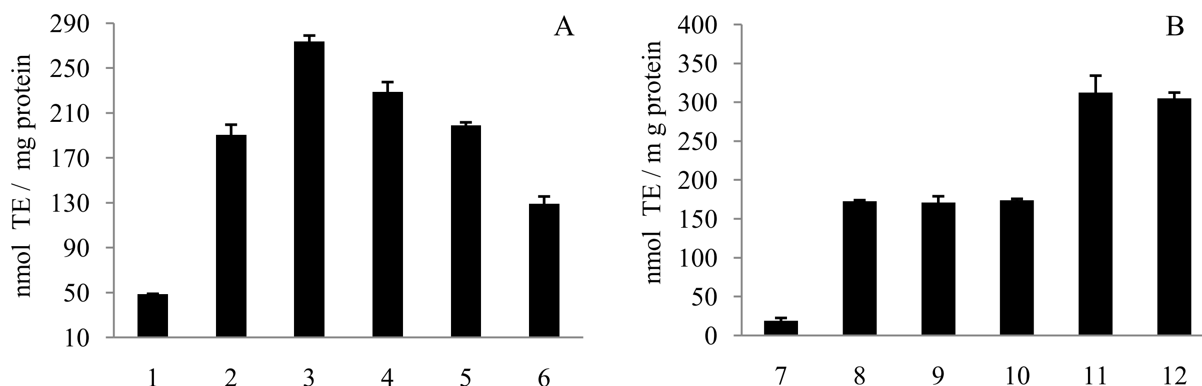


Figure 4-43 Antioxidative capacity of β-Lactoglobulin (A), Glycomacropeptide and BioZate1 (B) - chlorogenic acid derivatives (nmol TE/mg protein) by TEAC assay

Where**:** β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution; 7, unmodified GMP; 8, GMP + pH 9 + coffee extract; 9, GMP + 3 ml juice + coffee extract; 10, unmodified BZ1; 11, BZ1+ pH 9 + coffee extract and 12, BZ1 + 3 ml juice + coffee extract

As shown in Figure 4-43A, a large variation between samples was observed. β-LG modified at enzymatic conditions showed the highest antioxidative capacity, compared to proteins modified at alkaline conditions and unmodified proteins. Therefore, β-LG modified with coffee extract and 6 ml juice provides a protein with a higher antioxidative power, followed by β-LG modified at alkaline conditions, where the values were 273.85 and 228.8 nmol TE/mg protein respectively, in comparison with 48.48 nmol TE/ mg for unmodified protein. GMP and BZ1 also showed an increase in the antioxidative capacity after modification at room temperature for 24h with coffee extract, where the values were increased from 18.96 to 172.51 and 171.10 nmol TE/mg protein for unmodified GMP and modified GMP at alkaline and enzymatic conditions, respectively. The antioxidative capacity increased from 173.99 to 312.49 and 305.02 TE/mg proteins for unmodified BZ1 and modified BZ1 at alkaline and enzymatic conditions, respectively (Figure 4-43B). Moreover, it can be noticed that the antioxidative capacity for unmodified BZ1 was higher than unmodified β-LG, where the values were 173.99 compared to 48.48 nmol TE/mg protein, respectively. These variations might be explained by that enzymatic hydrolysis which occurs during BZI preparation and provides a strategy in the improvement of the antioxidant potential of protein (Dryakova *et al.*

2010, Yang *et al.* 2011), e.g. the antioxidant potential of whey and soy sauce proteins was increased after hydrolysis by alcalase. Additionally, total antioxidative capacity values obtained by the DPPH assay showed a similar trend in their results when compared with the TEAC assay, but the values obtained were lower (Figure 4-44). The difference in the mechanisms of the procedures of each assay may explain these variations in values. The results indicate that the hydroxyl groups, which are produced from the reduction of free quinones of oxidized phenolic compounds, could provide hydrogen to DPPH radicals (Aewsiri *et al.* 2009).

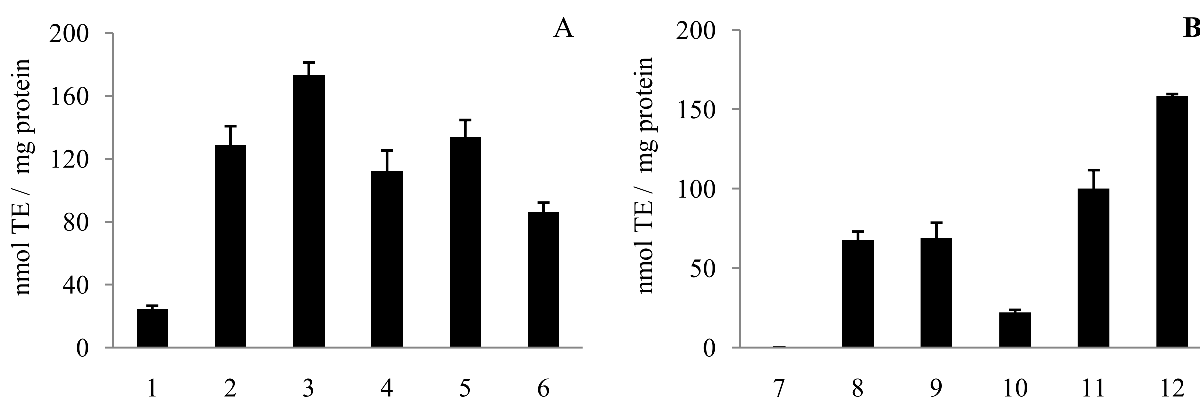


Figure 4-44 Antioxidative capacity of β-Lactoglobulin (A), Glycomacropeptide and BioZate1 (B) - chlorogenic acid derivatives (nmol TE/mg protein) by DPPH assay

Where**:** β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution; 7, unmodified GMP; 8, GMP + pH 9 + coffee extract; 9, GMP + 3 ml juice + coffee extract; 10, unmodified BZ1; 11, BZ1+ pH 9 + coffee extract and 12, BZ1 + 3 ml juice + coffee extract

Finally, an explanation for the antioxidative ability of the whey protein products- CQA derivatives can be hypothesized in the oxidized state of the reaction products, where CQA can be oxidized to its respective quinone, which can then react covalently with the nucleophilic side chains of proteins. A covalent modification of proteins preferably takes place at the ε-amino group of lysine and thiol groups of cysteine as documented for 5-CQA in presence of tyrosinase (Namiki *et al.* 2001, Yabuta *et al.* 2001, Prigent *et al.* 2008, Schilling *et al.* 2008). This interaction goes on to form a protein-phenol derivative, which is still a potential antioxidant because of its capability to become a free radical, which can then scavenge ABTS radicals.

4.3.3.3 The change in emulsification properties

Oil in water emulsions were prepared with modified and unmodified proteins. Furthermore, the emulsions were emulsified in the presence of lutein ester and their properties were studied:

4.3.3.3.1 Droplet size

The particle diameter of oil droplets of 10% MCT oil - in - water emulsion stabilized using 0.2% unmodified and modified β-LG with coffee extract at alkaline and enzymatic conditions, 6 ml apple juice, is presented in Figure 4-45. The droplet size was measured subsequently, and the mean particle diameter of the oil droplet was calculated and expressed as a 10th, 50th and 90th percentile. Good emulsifying properties of unmodified β-LG were not significantly affected by the reaction of CQA, as documented by the characterization of emulsion with regard to the 10th and 50th percentile of particles size D (v, 0.1) and D (v, 0.5), where the value ranged from 1.17 to 1.22 µm and 2.89 to 3.22 µm, respectively. These values are higher than the values reported by Khalil *et al.* (2012). These differences may be related with the concentration of protein used, where they used 4% β-LG protein compared to 0.2 % in the current study.

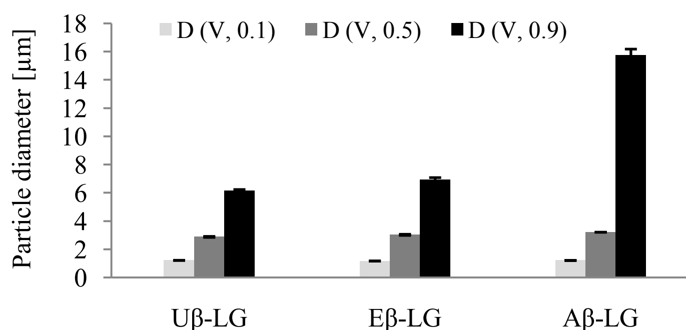


Figure 4-45 Mean particle size of freshly prepared emulsions made with modified β-Lactoglobulin

Where**:** Uβ-LG, unmodified β-LG; Eβ-LG, β-LG + 6 ml juice + coffee extract; Aβ-LG, β-LG + pH 9 + coffee extract; D (v, 0.1), particles with diameters of 10 % of total volume; D (v, 0.5), particles with diameters of 50% of total volume and D (v, 0.9), particles with diameters of 90% of total volume

The droplet size of the emulsion decreased distinctly when the higher concentration of protein was used (Surh *et al.* 2006, Aewsiri *et al.* 2009). On the other hand, a significant change was noticed in the 90th percentile, where the 90th percentile of the particle size D (v, 0.9) showed that a considerable increase in size distribution could be observed upon alkaline modification. The droplets size increased from 6.14 µm in emulsion stabilized with unmodified β-LG

protein to 15.72 μm in emulsion stabilized using modified β-LG protein at pH 9, as shown in Figure 4-45. These results are in line with Aewsiri *et al.* (2009), who reported that the particle size of oil droplets was larger in the emulsion stabilized by cuttlefish skin gelatin modified with tannic acid at pH 9 than the emulsion stabilized by unmodified gelatin. One possible explanation for this observation is that the amount of protein taken on the oil droplet may not be enough because the decrease of surface hydrophobicity may not encourage its migration to the oil droplets (Aewsiri *et al.* 2009). Moreover, flocculation of droplets/aggregation may also be encouraged at the interface as a consequence of interaction with CQA.

4.3.3.3.2 Microscopic images

The emulsions without lutein ester were also studied in the microscopic images of the particles, and the results are shown in Figure 4-46. Although the concentration of β-LG protein used is low compared to other studies, the images showed a normal distribution with a partial aggregation of oil droplets, thereby providing a suitable vehicle for emulsified lutein esters in the next step. This may be ascribing to using β-LG, which has good emulsification properties, as emulsifier. Moreover, the best solubility for the β-LG protein is at around pH 7, which was used to prepare these emulsions (Pelegrine and Gomes 2012, Pelegrine and Gomes 2008).

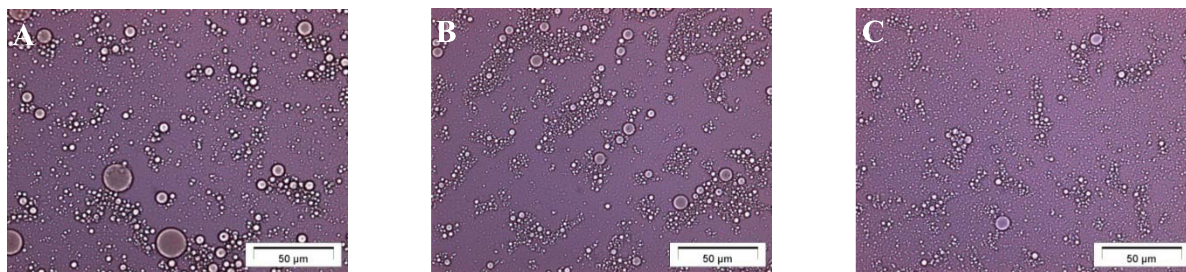


Figure 4-46 Microscopic images for emulsions stabilized by modified and unmodified proteins

Where: (A), unmodified β-LG; (B), β-LG + 6 ml juice + coffee extract and (C), β-LG + pH 9 + coffee extract

4.3.3.3.3 Stability of emulsions

Proteins as emulsifiers have been used in food processing for many years. The density and structure of the adsorption layers of protein on the drop surface effect strongly on the stability of oil-in-water emulsions (Tcholakova *et al.* 2002, Tcholakova *et al.* 2006). So, the idea of these experiments was to study the effect of the modification of β-LG, GMP and BZ1 proteins with CQA on the stability of emulsions against of the centrifugation. The creaming,

aggregation, and coalescence are the main reasons for instability of emulsion. Creaming is caused as a result of poor emulsion forming; moreover, when droplets of emulsion are aggregated, the effective particle size increases (van der Ven *et al.* 2001). The emulsions were centrifuged and the absorbance at 500 nm was recorded (Figure 4-47). The creaming occurred for all the prepared emulsions, where emulsions started to form cream after 5 min centrifugation, and slight changes between them till 40 min were noted. After 40 min centrifugation, clear differences between all emulsions were observed. The results also revealed that in emulsions centrifuged to provoke the formation of the serum layer, modified samples 3, 4 and 6 were more effective in improving the quality of prepared emulsions. Emulsion stabilized with β-LG modified with 3 ml juice and coffee extract (sample 2) had the lowest stability (Figure 4-47A).

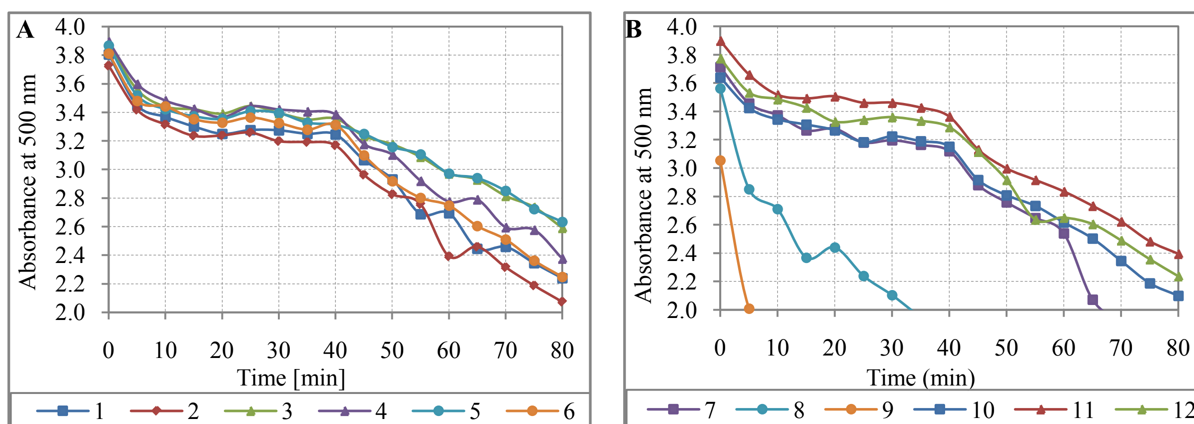


Figure 4-47 Stability of emulsions stabilized by β-Lactoglobulin (A), Glycomacropeptide (B) and BioZate1 (C) - chlorogenic acid derivatives

Where**:** β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1; 1, unmodified β-LG; 2, β-LG + 3 ml juice + coffee extract; 3, β-LG + 6 ml juice + coffee extract; 4, β-LG + pH 9 + coffee extract; 5, β-LG + 3 ml juice + CQA solution; 6, β-LG + pH 9 + CQA solution; 7, unmodified GMP; 8, GMP + pH 9 + coffee extract; 9, GMP + 3 ml juice + coffee extract; 10, unmodified BZ1; 11, BZ1+ pH 9 + coffee extract and 12, BZ1 + 3 ml juice + coffee extract

The solubility of protein results supports these observations, where sample 2 showed the lowest solubility. On the other hand, in the case of emulsions stabilized by BZ1, their stability did not change much till 40 min centrifugation, after which it was decreased (Figure 4-47B). However, no clear differences were found between emulsions stabilized using unmodified and modified BZ1 at enzymatic and alkaline conditions, where the percent of decrease in the stability after 80 min centrifugation were 42.3, 40.7 and 38.6 %, respectively. On the other hand, significant differences were recorded between emulsions stabilized by unmodified GMP

and modified GMP at enzymatic and alkaline conditions, where % of decrease after 80 min was 49.5, 92 and 86.3 %, respectively. Bad emulsifying properties for modified GMP can be explained by the decrease in surface hydrophobicity (Aewsiri *et al.* 2009), and a large droplet size in the emulsions (data not shown) (Tcholakova *et al.* 2002).

4.3.3.3.4 Stability of emulsified lutein ester

Lutein ester is sensitive to UV light, and the most damaging wavelengths to lutein stability were identified in UV range of 200 - 400 nm and at 463 nm in a lutein-fortified model colloidal beverage (Kline *et al.* 2011); this is confirmed in model emulsions by Khalil *et al.* (2012). The stability of emulsified lutein ester in oil-water emulsions stabilized by modified β-LG, GMP and BZ1 with and without CQA was studied, and data are displayed in Figure 4-48. The data showed that emulsified lutein esters with MCT oil exhibited a higher stability against UV light compared to lutein ester in MCT oil extract without emulsification.

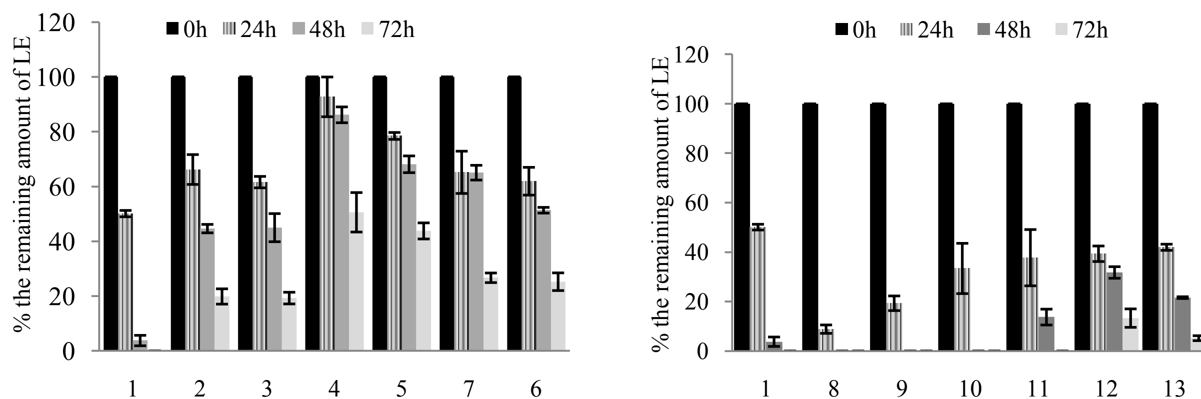


Figure 4-48 Stability of emulsified lutein ester against UV light

Where: β-LG, β-Lactoglobulin; GMP, Glycomacropeptide; BZ1, BioZate1;1, unemulsified MCT oil; 2, unmodified β-LG; 3, β-LG + 3 ml juice + coffee extract; 4, β-LG + 6 ml juice + coffee extract; 5, β-LG + pH 9 + coffee extract; 6, β-LG + 3 ml juice + CQA solution and 7, β-LG + pH 9 + CQA solution; 8, unmodified GMP; 9, GMP + pH 9 + coffee extract; 10, GMP + 3 ml juice + coffee extract; 11, unmodified BZ1; 12, BZ1+ pH 9 + coffee extract and 13, BZ1 + 3 ml juice + coffee extract

All emulsions except, those prepared using modified β-LG with coffee extract and 3 ml apple juice, are significantly different from controls (MCT-oil and emulsion prepared using unmodified β-LG) after 24, 48 and 72 h exposure to UV light. Consequently, emulsions emulsified with modified β-LG at enzymatic, 6 ml apple juice, and alkaline conditions in coffee extract (Figure 4-48A, samples 4 and 5) exhibited a moderate increase in the amount of lutein ester remaining after 24-72 h of UV exposure, where the ratios were 92.7- 86.2 and

78.5-68.1% respectively, compared to 66.2-19.9% for control β-LG, underlining the fact that UV stability should also be given for long term storage and usage of food stuffs fortified by this method. The PPO modulated reaction provides a modified protein sample 4 with a high antioxidative power, and when emulsified with lutein esters, exhibits their higher stability against UV light. Furthermore, MCT oil represents a good medium to protect lutein ester against temperature and emulsion with MCT oil against UV light, both being important factors when considering fortification issues in developing countries, especially those with tropical and sub-tropical climates (Khalil *et al.* 2012). On the other hand, as mentioned in Figure 4-47A, emulsions stabilized by GMP proteins did not remain stable (Figure 4-47B). Therefore, these proteins cannot protect lutein ester against UV light (Figure 4-48B), since all lutein ester was lost after only 24 h. Emulsions stabilized using BZ1 proteins illustrated more protection in comparison with MCT oil and unmodified BZ1. The modified BZ1 showed the highest protection for lutein ester after 48 – 72 h compared to the unmodified sample, where % of the remainder were 31.8 – 13.4 and 21.6 – 5.3% for BZ1 modified at both alkaline and enzymatic conditions respectively, compared to 13.8 -0 % for unmodified BZ1.

Finally, as recently reported, the combined effect of binding of carotenoids to proteins and the process of emulsification may lead to an effective use of selected carotenoids in supplements, provided it is possible to optimize their bioavailability under these conditions (Khalil *et al.* 2012). Therefore, the PPO/CQA modulated reaction may also provide a tool to improve the stability and bioavailability of carotenoids, by using such modified proteins in emulsions.

5. Summary

5.1 English Summary

The phenolic compounds as food components represent the largest group of secondary metabolites in plant foods. The phenolic compounds, e.g. chlorogenic acid (CQA), are susceptible to oxidation by enzymes specially, polyphenol oxidase (PPO) and at alkaline conditions. Both enzymatic and non-enzymatic oxidations occur in the presence of oxygen and produce quinone, which normally further react with other quinone to produce colored compounds (dimers), as well as is capable of undergoing a nucleophilic addition to proteins. The interactions of proteins with the phenolic compounds have received considerable attention in the recent years where, plant phenolic compounds have drawn increasing attention due to their antioxidant properties and their noticeable effects in the prevention of various oxidative stress associated diseases. Green coffee beans are one of the richest sources of chlorogenic acids. Therefore, a green coffee extract would provide an eligible food relevant source for phenolic compounds for modification of proteins. These modified proteins may be use to improve the stability and bioavailability of carotenoids e.g. lutein ester, easily oxidized lipophillic bioactive compounds.

Aims: In this study, we wanted to combine high nutritional quality proteins with the health promoting substances (e.g. phenolic compounds) into a multifunctional ingredient that could be used to design a wide range of food products and at the same time provide a better accessibility. Therefore, the aims of the current study were as follows:

- Characterization of different apple varieties by its PPO activity to select the suitable variety for the next work. Moreover, characterization of the interactions of CQA with model amino components and proteins
- Despite the immense economic importance of coffee, only few studies have dealt with the biochemical and molecular properties of its seed storage proteins. Therefore, we want to increase the knowledge on the composition and structure of green coffee bean proteins and to the changes induced in them especially with regard to their interactions with the phenolic compounds.

- Study the effects of modification with CQA on chemical, structural and functional properties of milk whey protein products. Moreover, the characterization of the modified proteins with respect to their sites and arts of modification.

Materials and methods: Five different varieties of fresh apples (Boskoop, Braeburn, Cox Orange, Golden Delicious and Jonagold) were characterized by PPO activity and amount of chlorogenic acid (CQA) content using HPLC. Protein profiles of the two main commercial species of Coffea genus, *Coffea arabica (CA)* and *Coffea robusta (CR)* from different regions, were compared to faba beans (FB) protein (as control for 11s protein). Protein extraction was optimized using various solvents/additives. Moreover, milk whey protein products (β-lactoglobulin, BioZate1 and glycomacropeptide) were modified (using enzymatic and alkaline conditions) with an aqueous green coffee Uganda *(CR)* extract and commercial CQA under implementation of food relevant processing conditions. The chemical, structural and functional properties of such proteins were characterized with appropriate methods (reversed-phase high-performance liquid chromatography (RP-HPLC), sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS−PAGE), matrix-assisted laser desorption ionization−time of flight mass spectrometry (MALDI−TOF−MS), circular dichroism (CD) spectroscopy, differential scanning calorimetry (DSC), Trolox equivalent antioxidant capacity (TEAC) assay, surface hydrophobicity and emulsification). Furthermore, In-gel digestion combined with MALDI-TOF-MS provided tentative allocation of the probable modification type and site in the protein. On basis of this information, the first available *in-silico* modeling of modified proteins is reported**.** Finally, lutein ester was emulsified using these proteins and the percent of its residual after exposure to UV light at 365 nm was monitored using high-performance liquid chromatography (HPLC).

Results: Chlorogenic acid is the major phenolic compounds in apple varieties tested. The Braeburn variety exposed the highest PPO activity and the lowest CQA content. Moreover, the stability of PPO activity appears to be unaffected after two weeks storage at -20 °C. The interaction between 5-CQA and amino acid lysine showed decrease in both free CQA and amino acid groups and only a slight effect on the antioxidative capacity depending on the reaction time was found. Furthermore, this interaction showed a large number of intermediary substances of low intensities. The reaction of lysine with 5-CQA in a model system initially leads to formation of 3-CQA and 4-CQA (both are isomers of 5-CQA), oxidation giving rise

to the formation of a dimer which subsequently forms an adduct with lysine to finally result in a benzacridine derivative as reported and confirmed with the aid of HPLC coupled with ESI-MS^n. The benzacridine derivative containing a trihydroxy structural element, was found to be yellow, being very reactive with oxygen yielding semiquinone and quinone type of products with characteristic green colors. Finally, the optimal conditions for this interaction as assessed by both the loss of CQA and free amino groups of lysine can be given at pH 7 and 25°C, the interaction increasing with incubation time and depending also on the amount of tyrosinase present.

Green coffee bean has a higher diversity and content of phenolics, where besides the CQA isomers and their esters, other conjugates like feruloylquinic acids were also identified, thus documenting differences in phenolic profiles for the two coffee types (*Coffea arabica* and *Coffea robusta*). Coffee proteins are modified by interactions with phenolic compounds during the extraction, where those from *C. arabica* are more susceptible to these interactions compared to C. *robusta*, and the polyphenol oxidase activity seems to be a crucial factor for the formation of these addition products. Moreover, In-gel digestion combined with MALDI-TOF-MS revealed that the most reactive and susceptible protein fractions to covalent reactions are the α-chains of the 11S storage protein. Thus, based on these results and those supplied by other research groups, a tentative list of possible adduct structures was derived. The diversity of the different CQA derivatives present in green coffee beans complicates the series of reactions occurring, providing a broad palette of reaction products. These interactions influence the properties of protein, where they exposed changes in the solubility and hydrophobicity of proteins compared to faba bean proteins (as control).

Modification of milk whey protein products (primarily β-lactoglobulin) with coffee specific phenolics and commercial CQA under enzymatic and alkaline conditions seems to be affecting their chemical, structural and functional properties, where both modifications led to reduced free amino-,thiol groups and tryptophan content. We propose that the disulfide-thiol exchange in the C-terminus of β-lactoglobulin may be initiated by the redox conditions provided in the presence of CQA. The protein structure β-lactoglobulin thereupon becomes more disordered as simulated by molecular dynamic calculation. This unfolding process may additionally be supported by the reaction of the CQA at the proposed sites of modification of ε-amino groups of lysine (K77, K91, K138, K47) and the thiol group of cysteine (C121).

These covalent modifications also decreased the solubility and hydrophobicity of β-lactoglobulin, moreover they provide modified protein samples with a high antioxidative power, thermally more stable as reflected by a higher Td, require less amount of energy to unfold and when emulsified with lutein esters, exhibit their higher stability against UV light. The MALDI-TOF and SDS-PAGE results revealed that proteins treated at alkaline conditions were more strongly modified than those treated under enzymatic conditions. Finally, the results showed a slight change in emulsifying properties of modified proteins.

Conclusion: This study provides evidence that occurrence of covalent and non-covalent interactions between CQA and proteins. These interactions can occur under alkaline and enzymatic (modulated by polyphenol oxidase) conditions. Moreover, a covalent modification of proteins preferably takes place at the ε-amino group of lysine and thiol groups of cysteine. Both modifications led to changes in chemical, structural and functional properties of tested proteins. Finally, modified proteins provide a tool to improve the stability and bioavailability of carotenoids, where using such modified proteins in emulsions improved the stability of lutein ester against UV light. Positive outcome of these studies would open the possibility for incorporation of these multifunctional ingredients into various food products and enable consumers to benefit from a health promoting effect of phyto-nutrients found in vegetables.

5.2 Zusammenfassung

Für die Verbesserung von Nahrungsmitteleigenschaften können Modifikationen an verschiedenen Inhaltsstoffen vorgenommen werden. Beispielsweise werden bereits Proteine miteinander verknüpft und bilden sogenannte „Crosslinks" oder vernetzte Biomoleküle. Diese werden für die Herstellung fester, viskoelastischer Produkte, die zum Verdicken als auch zum Stabilisieren von Emulsionen oder Schäumen eingesetzt werden, genutzt. Da die Verbraucher sich Zunehmens mit gesundheitsfördernden Lebensmitteln befassen, ist das Einbringen von gesundheitsfördernden Inhaltsstoffen wie z.B. phenolische Verbindungen, immer mehr in den Fokus der Forschung gerückt. Demnach ist das wissenschaftliche Bestreben phenolische Verbindungen in die Vernetzung von Proteinen mit einzubeziehen und deren positive Wirkungen (antioxidativ) auszunutzen, vorteilhaft.

Als Phenole werden Verbindungen bezeichnet, die eine oder mehrere Hydroxygruppen am Benzolring aufweisen. Phenole liegen in der Enolform vor, da diese, bedingt durch den Erhalt des aromatischen Benzolringes, energetisch begünstigt ist. Kaffeesäure ist eine Hydroxyzimtsäure und in Kaffeebohnen zu finden. In Früchten sind Hydroxyzimtsäuren meist mit China- oder Weinsäure verestert oder liegen als Glycoside vor. Der am häufigsten anzutreffende Ester besteht aus Kaffee- und Chinasäure. Der einfachste Vertreter ist die Chlorogensäure (5-Caffeoylchinasäure, 5-CQA), die in vielen Pflanzenteilen enthalten ist. Chlorogensäure und ihre Derivate besitzen ebenfalls antioxidative Eigenschaften. Zusätzlich wirken sie auf Enzyme, die an entzündlichen- oder allergischen Reaktion teilnehmen, inhibierend.

Während Verarbeitungs- und Lagerungsprozessen können phenolische Komponenten pflanzlicher Lebensmittel mit den Aminosäuren der Proteine in Lebensmitteln reagieren. Solche Reaktionen können die physikalisch-chemischen Eigenschaften von Proteinen verändern und deren ernährungsphysiologische Wertigkeit vermindern. Proteine weisen verschiedene reaktive Seitengruppen (Sulfhydryl-, Hydroxyl-, Aminogruppen) auf, mit denen sie über kovalente und nicht-kovalente Wechselwirkungen mit Phenolen Verbindungen eingehen können. Zu den nicht-kovalenten Verbindungen gehören u. a. Wasserstoffbrückenbindungen, hydrophobe Wechselwirkungen und Ionenbindungen. Phenole (z.B. Chlorogensäuren) können bei Anwesenheit von Sauerstoff enzymatisch bzw. nichtenzymatisch oxidiert werden. Die Autoxidation ist ein nicht-enzymatischer Prozess, der

in Anwesenheit von Sauerstoff und z.B. unter alkalischen Bedingungen phenolische Gruppen verändern kann. Voraussetzung dafür ist die Bildung von reaktiven elektrophilen Chinonen, die mit Hilfe der nukleophilen Addition an reaktive Seitenketten (z.B. Aminogruppe des Lysins, Thiolgruppe des Cysteins) der Proteine binden können. In Gegenwart von Enzymen wie z.B. Polyphenoloxidasen (PPO) werden von Chlorogensäuren ebenfalls unter Verbrauch von Sauerstoff reaktive Chinone gebildet. Diese bilden anschließend mit reaktiven Thiol- bzw. Aminogruppen von Proteinen Addukte.

Die Erfassung dieser verschiedenen Facetten von Interaktionen stellt somit die primäre Forschungsaufgabe im Rahmen dieser Arbeit. Daraus ergeben sich nicht nur Ansatzpunkte zur Bewertung von sekundären Pflanzenstoffen hinsichtlich ihrer ernährungsphysiologischen und präventiven Bedeutung, sondern auch Aussagen über die Veränderung der Eigenschaften von Proteinen. Die primäre Aufgabe der vorliegenden Arbeit besteht demzufolge in der Etablierung der Analysen- und der Charakterisierungsmöglichkeiten solcher Wechselwirkungen (Bindung) pflanzlicher Verbindungen bzw. deren Reaktionsprodukten mit Proteinen u.a. über massenspektrometrische Methoden. Aufgrund ihrer hohen Genauigkeit, Auflösung und Sensitivität stellt besonders die MALDI-TOF-MS (Matrix-unterstützte Laserdesorptions / Ionisations-Flugzeit-Massenspektrometrie) hier eine elegante und integrative Plattformtechnologie dar. Da die Wechselwirkung mit Proteinen auch zu Veränderungen der Proteinstruktur führt, können deren funktionelle Eigenschaften auch verändert sein. Dies soll anhand der Messung von isolierten Proteinen die an der Wechselwirkung beteiligt sind, nachgewiesen werden. Anschließend sollen über Docking-Untersuchungen die entsprechenden Bindungsstellen näher charakterisiert werden. Die Arbeit besteht daher aus drei Studien:

1. Gewinnung von PPO-reicher Extrakte aus Äpfeln, deren Charakterisierung und Modeluntersuchungen von CQA mit einfachen Aminosäuren und Proteinen.
2. Charakterisierung der Phenol-Protein Reaktionen in Kaffee und Einschätzung ihrer Bedeutung.
3. Nachmodulierung der Reaktionen in Kaffee an Hand von beta-Lactogobulin – Charakterisierung der strukturellen und funktionellen Eigenschaften

Durch die vorliegenden Ergebnisse wurden mögliche Reaktionen von phenolischen Verbindungen mit Proteinen, näher charakterisiert. Es wurde festgestellt, dass die Apfelsorte

Braeburn über die höchste PPO- Enzymaktivität beim gleichzeitigen niedrigen CQA Gehalt im Vergleich zu den anderen untersuchten Sorten verfügt. Die PPO/Tyrosinase modulierte Reaktionen zwischen CQA und Lysine wurden in Abhängigkeit der vorherrschenden Bedingungen optimiert und die Reaktionsprodukte analysiert.

In dem zweiten Teil wurden solche Reaktionsmöglichkeiten in den Grünen Kaffeebohnen lokalisierte und modelliert. Dazu wurden die sortenabhängige CQA-Zusammensetzung ermittelt und die möglichen Reaktionen mit den Hauptspeicherproteinen des Kaffees dargestellt.

Im letzten Teil wurden dann diese Reaktionen mit Molkenproteinen simuliert und Einflüsse auf die Struktur und die funktionellen Eigenschaften erfasst. Die Ergebnisse belegen eine umfangreiche und sehr heterogene Adduktbildung mit den Aminoseitenketten des Lysins und Cysteins. Ein Katalog der unterschiedlichen Reaktionsprodukte wurde erstellt und am Protein modelliert. Die entsprechende Veränderung an die Proteinstruktur wurde experimentell belegt und der Einfluss wurde in den technofunktionelle Eigenschaften (wie die Löslichkeit, Emulgierbarkeit usw.) wiederspiegelt. Ein Anstieg des antioxidativen Potentials der Proteine wurde erreicht und diese so modifizierten Proteine wurden weiter zur Stabilisierung und Produktentwicklung getestet. Die ersten Ergebnisse eröffnen Nutzungsmöglichkeiten der modifizierten Proteine zur Verkapselung von bioaktiven Sekundären Pflanzenstoffen.

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7. Appendix

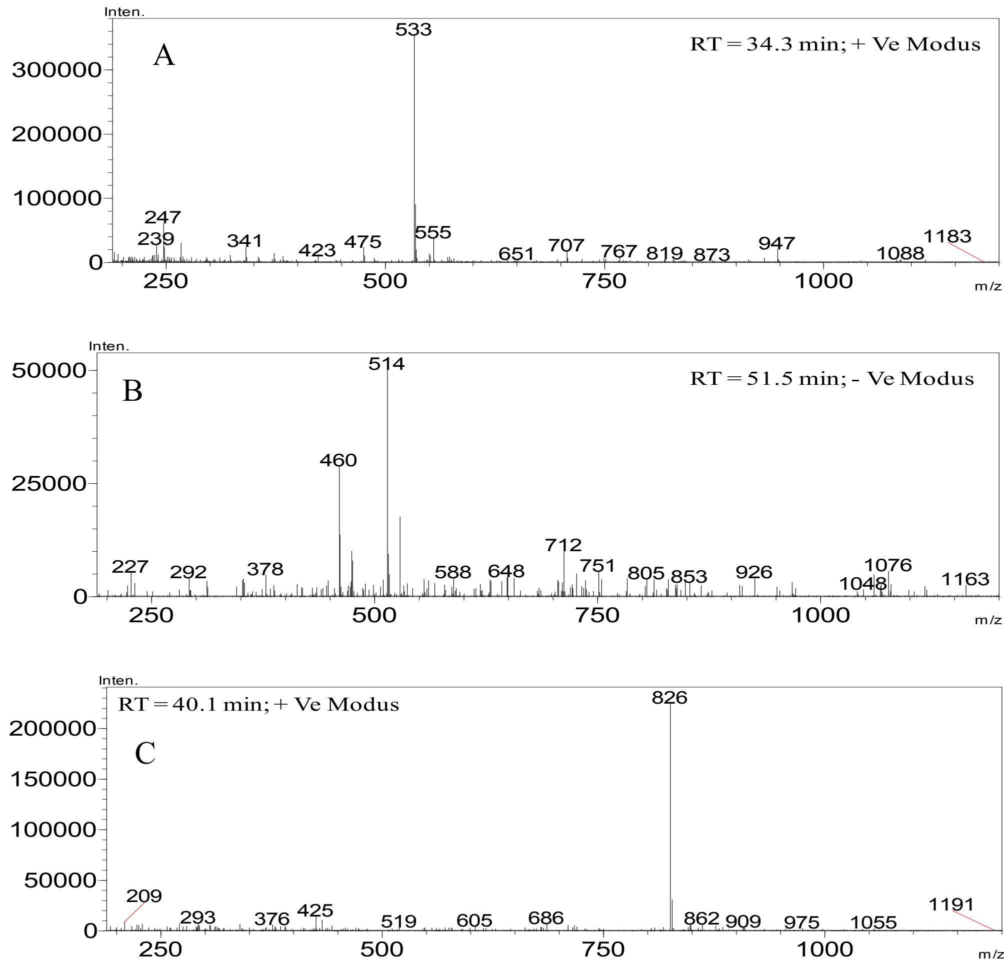


Figure 7-1 Mass-Spectra of lysine-CQA reaction products

Where: (A) CQA dimer minus a quinic acid; (B) Di-CQA; (C) lysine CQA dduct and caffeoylquinic acid (CQA)

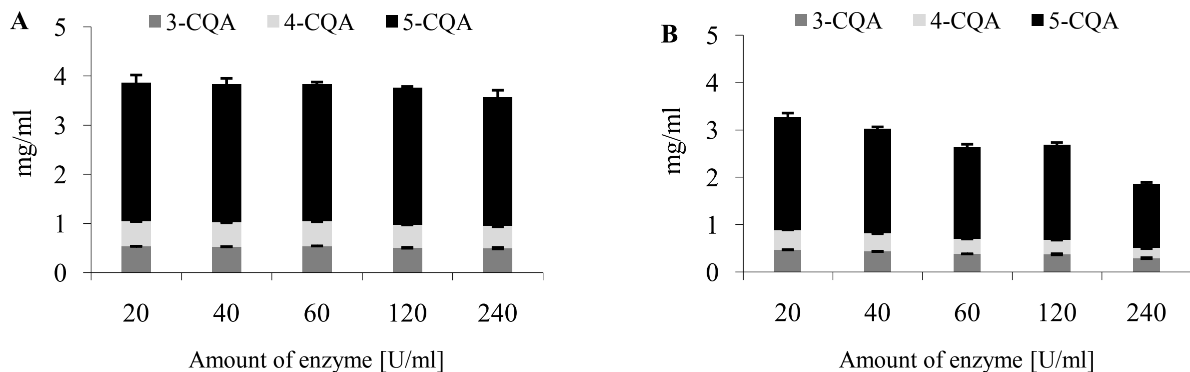


Figure 7-2 Effect of reaction of 5-CQA with lysine on composition of CQA (mg/ml) depending on the amount of tyrosinase at pH 7

Where: (A) control experiment without tyrosinase; (B) sample with tyrosinase and caffeoylquinic acid (CQA)

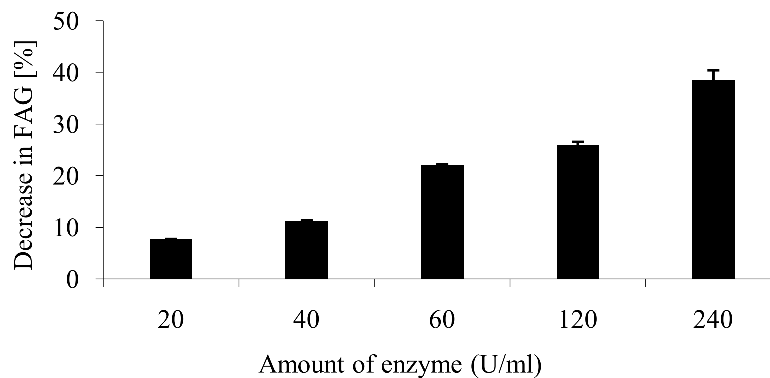


Figure 7-3 Effect of reaction of 5-CQA with lysine on decrease in the amount of free amino groups (FAG) depending on the amount of tyrosinase

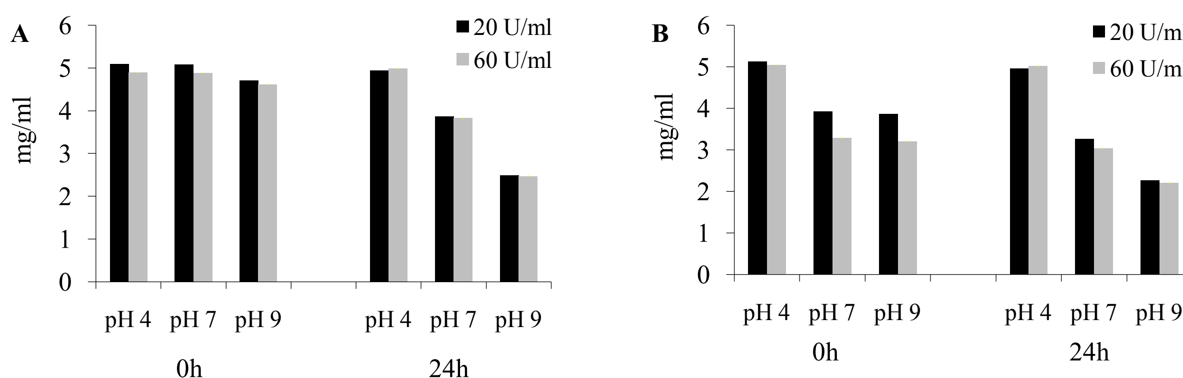


Figure 7-4 Effect of reaction of 5-CQA with lysine on total CQA depending on the pH and the amount of tyrosinase,

Where: (A) control experiment without tyrosinase; (B) sample with tyrosinase and caffeoylquinic acid **(**CQA**)**

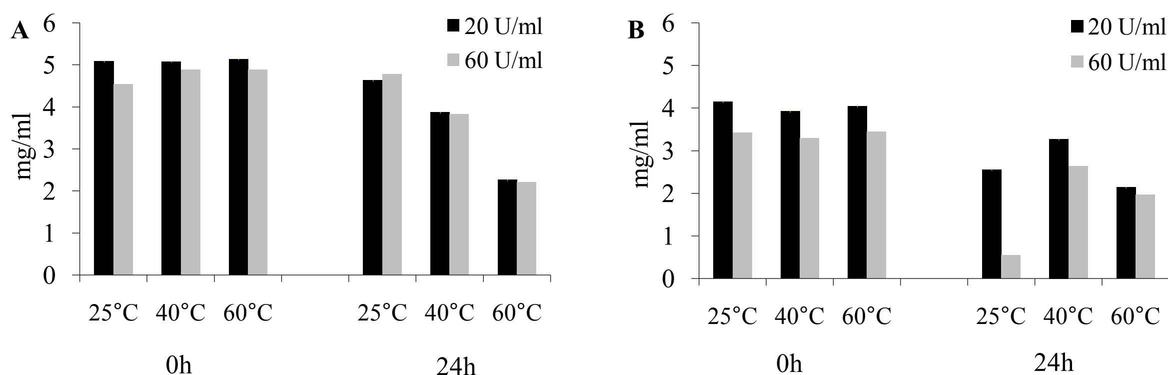


Figure 7-5 Effect of reaction of 5-CQA with lysine on total CQA depending on incubation temperature and the amount of tyrosinase

Where: (A) control experiment without tyrosinase; (B) sample with tyrosinase and caffeoylquinic acid (CQA)

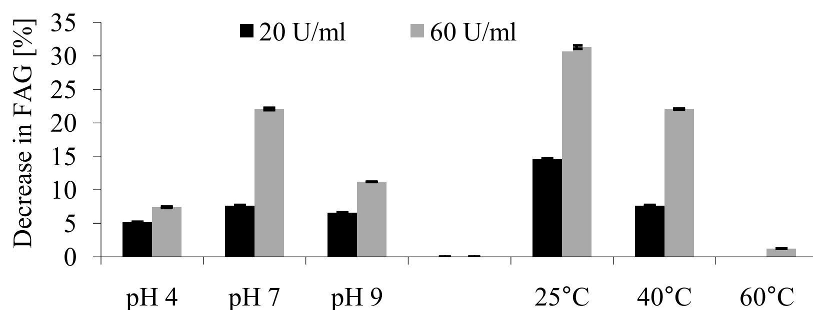


Figure 7-6 Effect of reaction of 5-CQA with lysine on decrease in the amount of free amino groups (FAG) depending on the pH, incubation temperature and the amount of tyrosinase

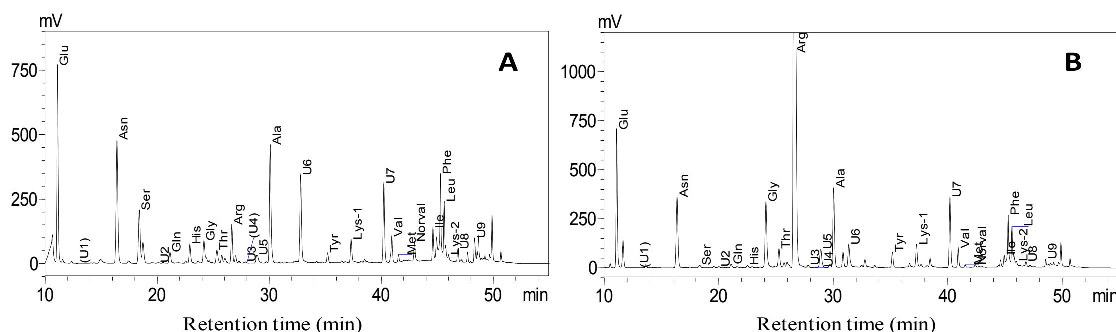


Figure 7-7 Exemplary chromatograms of amino acids in green coffee from Brazil (A) and faba bean from Giza (B)

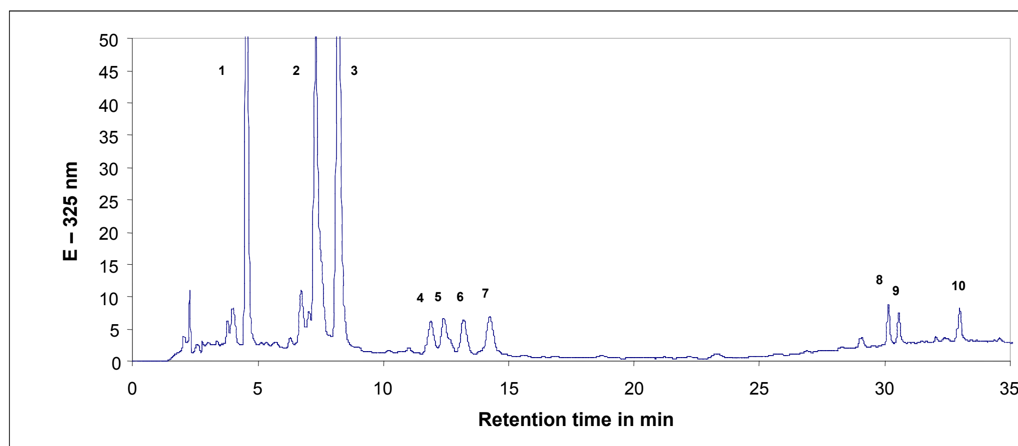


Figure 7-8 A typical HPLC Chromatogram of roasted coffee Columbia extract -at 325 nm

Table 7-1 Identification parameters used to analyze the main phenolic compounds in roasted Columbia coffee

Peak	1	2	3	4	5	6	7	8	9	10
m/z*	353	353	353	367	335	335	367	515	515	515
UV/Vis (nm)	280/325	280/325	280/325	280/325	280/325	280/325	280/325	325	325	325
Identity	**3-CQA**	**4-CQA**	**5-CQA**	**FQA 1**	**CQL 1**	**CQL 2**	**FQA 1**	**3,4-di-CQA**	**3,5-di-CQA**	**4,5-di-CQA**

* affirmed with HPLC-MS, CQA, Caffeoyl-Quinic Acid; FQA, Feruloyl-Quinic Acid; CQL, Caffeoyl-Quinic Acid lactone and di-CQA, di Caffeoyl-Quinic Acid)

Table 7-2 Estimation of the individual contributions of the CQA coffee fractions to the total antioxidative capacity (mmol TE/100g DM) by TEAC Assay

Samples	*Coffea Arabica* (CA)				*Coffea Robusta (CB)*	
	Columbia (C)		Brazil (B)	Guatemala (G)	Uganda (U)	Indonesia (I)
Phenols	Green	Roasted	Green	Green	Green	Green
3-CQA	0.8	0.6	0.7	0.7	1.1	1.3
4-CQA	1.0	0.9	1.1	0.9	1.5	1.4
5-CQA	9.1	1.4	6.6	6.3	8.1	6.7
FQA 1	0.7	0.0	0.7	0.7	1.0	1.2
FQA 2	2.9	1.0	1.8	1.7	4.4	4.7
3,4-di-CQA	0.7	0.3	0.7	0.6	1.5	1.5
3,5-di-CQA	1.6	0.3	1.3	1.1	1.4	1.4
4,5-di-CQA	1.1	0.4	0.9	1.0	2.6	2.6
CQL 1	Nd	0.7	Nd	Nd	Nd	Nd
CQL 2	Nd	1.4	Nd	Nd	Nd	Nd
Unknown	Nd	27.5	Nd	Nd	Nd	Nd
Sum	17.9	6.9	13.8	13.1	21.7	20.8
Total*	22.6	34.4	11.4	10.2	18.0	18.5

* As determined in the complete extract (see Table 4-2)

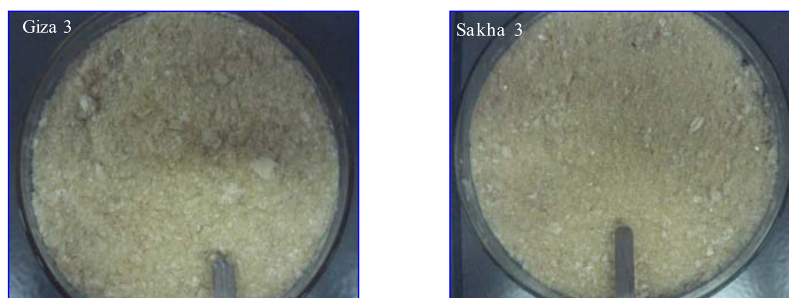
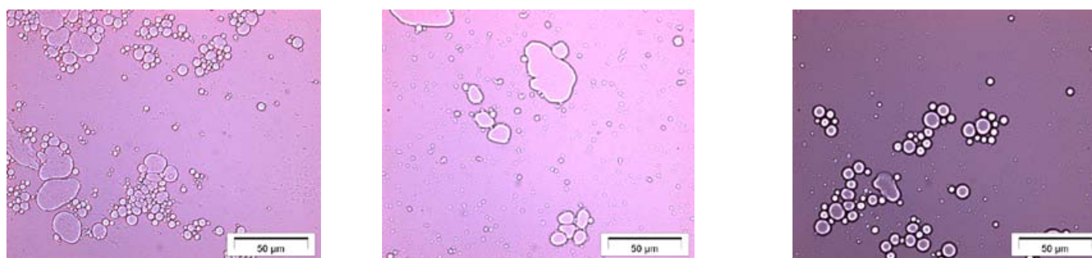


Figure 7-9 Color of reference proteins from faba beans

Where: Proteins were extracted with Tris-HCl pH 8 buffer



The image of emulsion using faba protein | The image of emulsion using coffee protein Brazil | The image of emulsion using coffee protein Indonesia

Figure 7-10 Microscopic images for the particle distribution in emulsions stabilized using coffee and faba proteins

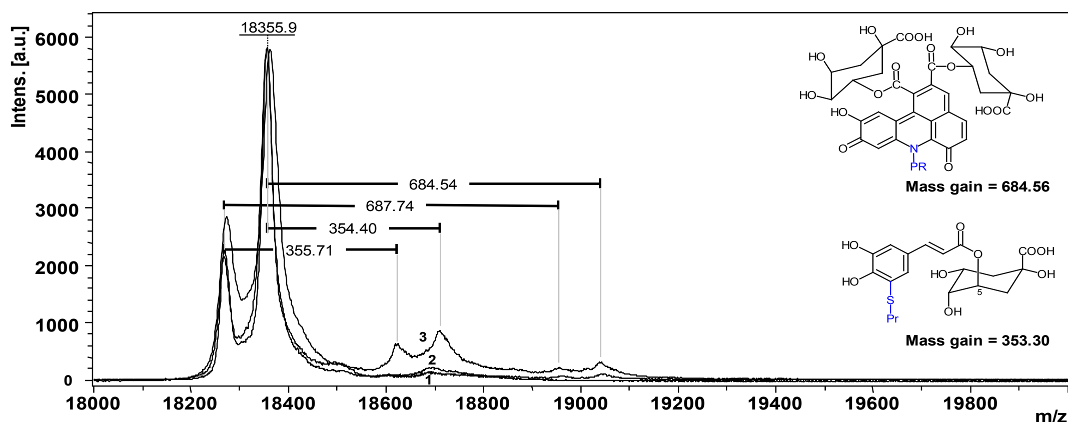
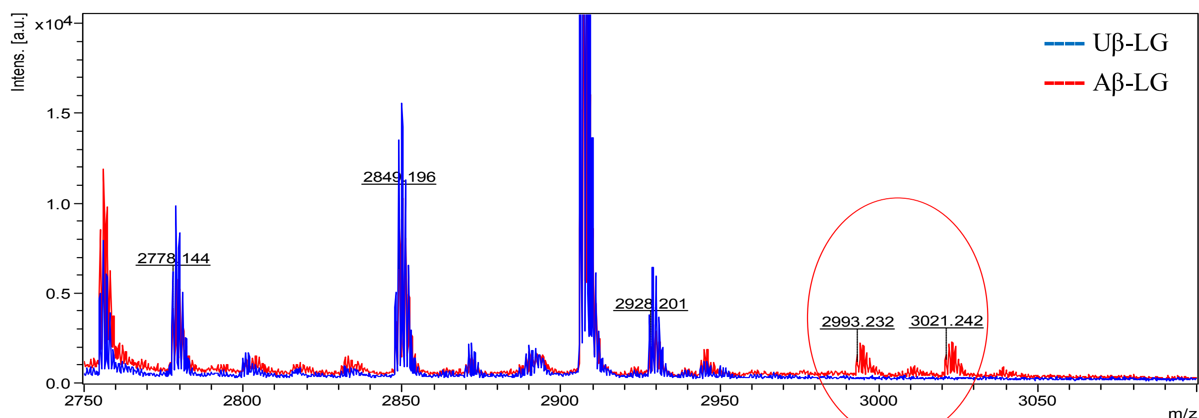


Figure 7-11 MALDI-TOF-MS of modified β-Lactoglobulin (β-LG) with commercial CQA depending on modification conditions

Codes: 1, unmodified β-LG; 2, β-LG + 3 ml juice + CQA solution and 3, β-LG + pH 9 + CQA solution

10	20	30	40	50	60	70
LIVTQTMKGL	DIQKVAGTWY	SLAMAASDIS	LLDAQSAPLR	VYVEELKPTP	EGDLEILLQK	WENDECAQKK
80	90	100	110	120	130	140
IIAEKTKIPA	VFKIDALNEN	KVLVLDTDYK	KYLLFCMENS	AEPEQSLVCQ	CLVRTPEVDD	EALEKFDKAL
150	160	170				
KALPMHIRLS	FNPTQLEEQC	HI				

Figure 7-12 Sequence of β-LG-bovine, variant a – UbiProtKB/Swiss-Prot code: p02754



10	20	30	40	50	60	70
LIVTQTMKGL	DIQKVAGTWY	SLAMAASDIS	LLDAQSAPLR	VYVEELKPTP	EGDLEILLQK	WENDECAQKK
80	90	100	110	120	130	140
IIAEKTKIPA	VFKIDALNEN	KVLVLDTDYK	KYLLFCMENS	AEPEQSLVCQ	CLVRTPEVDD	EALEKFDKAL
150	160	170				
KALPMHIRLS	FNPTQLEEQC	HI				

Figure 7-13 Peptide allocation and identification of probable type of β-Lactoglobulin - chlorogenic acid adducts and the corresponding modification sites

Legend: Uβ-LG, unmodified β-LG and Aβ-LG, β-LG + pH 9 + CQA solution

8. Acknowledgements

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9. Curriculum vitae

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Publications:

- **Ali, M.**, Homann, T., Khalil, M., Kruse, H.-P., Rawel, H. **(2013).** Milk Whey Protein Modification by Coffee Specific Phenolics – Effect on Structural and Functional Properties. Journal of Agricultural and Food Chemistry, 61 (28), 6911–6920.

- **Ali, M.**, Homann, T., Kreisel, J., Khalil, M., Puhlmann, R., Kruse, H.-P. & Rawel, H., **(2012)**. Characterization and Modeling of the Interactions between Coffee Storage Proteins and Phenolic Compounds. Journal of Agricultural and Food Chemistry, 60 (46), 11601-11608.
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Poster Publications:

- **Ali, M.,** Khalil, M., Kruse, H.-P., Krause, J.-P. and Rawel, H. **(2011)**. Comparison of the Properties of Proteins and Phenolics from Different Green Coffee. Proceedings the 48th Scientific Congress of the German Nutrition Society. 15**,** p 87.
- **Ali, M.,** Khalil, M., Kruse, H.-P., Homann, T., Krause, J.-P. and Rawel, H. **(2011).** Comparison of the properties of proteins and phenolics from different green coffee. 6. Symposium on Medicinal and Aromatic Plants, diversity and innovation benefits (6. Fachtagung für Arznei- und Gewürzpflanzen, Innovation Vielfalt und Nutzen), Berlin, 19th - 22th September 2011

Conferences

2013 The 19th International Scientific Meeting, 15th - 16th April 2013, IGV Institute for Cereal Processing Ltd, Nuthetal, Germany.

2013 The 17th EuroFoodChem Conference, 7th - 10th May 2013, Turkey