Inhibition of zinc-dependent peptidases by Maillard reaction products

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Leticia Missagia de Marco

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List of Abbreviations

ACE	Angiotensin converting enzyme
AGE	Advanced glycation end product
AGP	Arabinogalactan-proteins
AUC	Area under the curve
C/N ratio	Proportion between the carbon and the nitrogen contents
cb0 to cb4	Coffee extracts obtained from coffee beans of increasingly roast degree
CEL	N^{ε}-carboxyethyllysine
ChC	Clostridium histolyticum collagenase
CM- / CE-AP	Carboxymethyl- /carboxyethyl-alalylproline
CM- / CE-GA	Carboxymethyl- /carboxyethyl-glycylalanine
CM- / CE-GL	Carboxymethyl- /carboxyethyl-glycylleucine
CM- / CE-GP	Carboxymethyl- /carboxyethyl-glycylproline
CM- / CE-IA	Carboxymethyl- /carboxyethyl-isoleucylalanine
CM- / CE-IP	Carboxymethyl- /carboxyethyl- isoleucylproline
CM- / CE-IPP	Carboxymethyl- /carboxyethyl-isoleucylprolylproline
CM- / CE-IW	Carboxymethyl- /carboxyethyl-isoleucyltryptophan
CM- / CE-LL	Carboxymethyl- /carboxyethyl-leucylleucine
CML	N^{ε}-carboxymethyllysine
DMSO	Dimethyl sulfoxide
ESI-MS	Electrospray ionization mass spectroscopy
FT -Raman	Fourier transformed Raman spectroscopy
GPC	Gel permeation chromatography
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HHL	Hippuryl-histidyl-leucine, synthetic substrate for ACE assay
HPLC	High performance liquid chromatography
IC$_{50}$ value	Inhibitor concentration needed for 50% inhibition of a enzyme activity
IEC	Ion exchange chromatography
LAL	Lysinoalanine
lmw0 to lmw4	Low molecular weight fraction isolated from coffee beans of increasingly roast degree

m/z	Mass/charge ratio
MMP	Matrix metalloprotease
MMPI	Matrix metalloprotease inhibitor
mRD0 to mRD4	Melanoidin samples isolated from coffee beans of increasingly roast degree
MRP	Maillard reaction product
MWCO	Molecular weight cut off
NMR	Nuclear magnetic resonance
NNGH	N-isobutyl-N-(4-methoxyphenyl-sulfonyl)-glycyl hydroxamic acid, positive control for MMP assays
PzPLGL$_D$R	4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-$_D$Arg, synthetic substrate for ChC assay
RD0 to RD4	Coffee beans samples of increasingly roast degree
TCL	Thin layer chromatography
TRIS	2-amino-2-(hydroxymethyl)-1,3-pro-panediol
UV	Ultraviolet (absorption)
ZBG	Zinc binding groups

1 Introduction

The Maillard reaction is a network of different non-enzymatic reactions between carbonyl groups of reducing sugars and amino groups from amino acids, peptides, or proteins, which progresses in three major stages and originates a very heterogeneous mixture of reaction products. It is also known as non-enzymatic browning, due to the brown macromolecular pigments formed in the final stage of the reaction. The chemistry underlying the Maillard reaction is complex. It encloses not only one reaction pathway, but a whole network of various transformations. As virtually all foods contain both proteins and carbohydrates, Maillard reaction products are present in the daily diet in considerable amounts. The endogenous formation of Maillard reaction products, especially related to ageing and diabetes, aroused intense discussions about the health consequences of the "glycation", the term that describes the in vivo reaction corresponding to the Maillard reaction in foods.

Melanoidins are the final brown products of the Maillard reaction. They are responsible for the color formed during the heat processing of foods like coffee, bread, malt, and beef. Melanoidins are high molecular weight polydisperse polymers containing nitrogen. Their structure is largely unknown. Coffee melanoidins, which are object of the present study, contain thermally transformed polysaccharides, proteins, and phenolic compounds. Since the mechanisms involved on the formation of these macromolecules, and the chemical transformations which take place during the heat treatment are not completely elucidated, key structural features were analyzed. Especially the incorporation of chlorogenic acids in the melanoidin skeleton was object of attention of the present work.

Another major aim of this work was to investigate the influence of the Maillard reaction on the inhibitory potential of food components against zinc metalloproteases. The studied enzymes were three human matrix metalloproteases (MMP-1, -2 and -9), which are able to degrade matrix proteins and participate in many physiological processes, including tissue turnover and repair, but also constitute important targets in malignant and degenerative diseases. A microbial collagenase from *Chlostridium histolyticum* was chosen due to its subtract similarity to MMPs. Furthermore, Angiotensin Converting Enzyme (ACE), which plays a central role in cardiovascular pathologies such as hypertension and cardiac hypertrophy, was investigated. As a prototypical Maillard reaction product, coffee melanoidin was adopted.

Due to the roast dependent inhibitory activity of the coffee melanoidin fractions against matrix metalloproteases, the functionalization caused by the non-enzymatic browning was closer investigated. N^{α}-carboxyalkylated derivatives of a sequence of relevant peptides were synthesized, in a variation of the process-induced formation of N^{ε}-carboxymethyllysine, a major advanced glycation end-product (AGE). The inhibitory activity against zinc metalloproteases of the sequence of selected peptides and their N^{α}-carboxymethyl- (CM-) and N^{α}-carboxyethyl- (CE-) derivates was investigated.

2 Background

2.1 Maillard reaction in food

The Maillard reaction is an intricate pathway of interlacing transformations, which happen principally during thermal processing of food by the interaction of reducing sugars and their degradation products with available nucleophilic amino groups of amino acids, peptides and proteins. The many parallel and consecutive reactions promote the formation of an extraordinarily complex mixture of compounds, which are present in very different amounts *(van Boekel, 2001; Ledl and Schleicher, 1990)*. Furthermore, it is affected by factors such as reactants type and concentration, temperature, time, pH, and water activity *(Huang et al., 2005)*. It is decisive for the sensorial quality of food, being responsible for the color and characteristic aroma developed during backing, cooking or roasting, and altering the stability of food stuff. Therefore it is called non-enzymatic browning.

With the identification of a non-enzymatically glycosylated variant of hemoglobin in diabetic patients, it was found that the non-enzymatic reaction pathways known from food processing are also present *in vivo*, although the process in food is always much more intense, principally due to the drastic conditions of food processing. This reaction net can be defined as a post-translational modification of amino acids and protein, being known in living systems as "glycation" or "non-enzymatic glycosylation". Glycation alters the structure, function and stability of body proteins, constituting an important cause of the physiologic aging and some pathological processes *(Hipkiss, 2006; Henle, 2007)*. It is also considered as a type of covalent damage of proteins, not only due to the functional loss, but also to the development of potential pathogenic molecules *(Cloos and Christgau, 2002)*.

For reasons of clarity, the Maillard reaction is usually divided in three stages, designated "early", "advanced" and "final" Maillard reaction, although actually all reactions can occur simultaneously and influence each other *(Henle, 2005)*. Classically, the reaction begins with condensation between an amino group of amino acids, peptides or proteins and the carbonyl group of a reducing carbohydrate. In food, the amino group most frequently involved in the Maillard reaction is the ε-amino group of lysine side chains, but also the guanidino group of arginine and free α-amino groups from peptides and proteins are possible nucleophilic reagents *(Ledl and Schleicher, 1990; Silván et al., 2006)*. The product is an unstable imine,

the Schiff base, which can rearrange to a glycosylamine, cyclizates to an N-glycoside, or undergoes keto-enol tautomerism to give an enaminol, in a proton catalyzed reaction. The following step is the Amadori rearrangement (in case of aldoses), to give a 1-amino-1-desoxyketose (Amadori product), or Heyns (for ketoses) rearrangement, taking to 2-amino-2-desoxyaldose (Heyns product). Amadori products cyclizate in solution in similar anomers proportions as the original ketose *(Ledl and Schleicher, 1990; Henle, 2005)*. An example of formation of 1-amino-1-desoxy-fructose from glucose and a general amine can be seen in Figure 2-1.
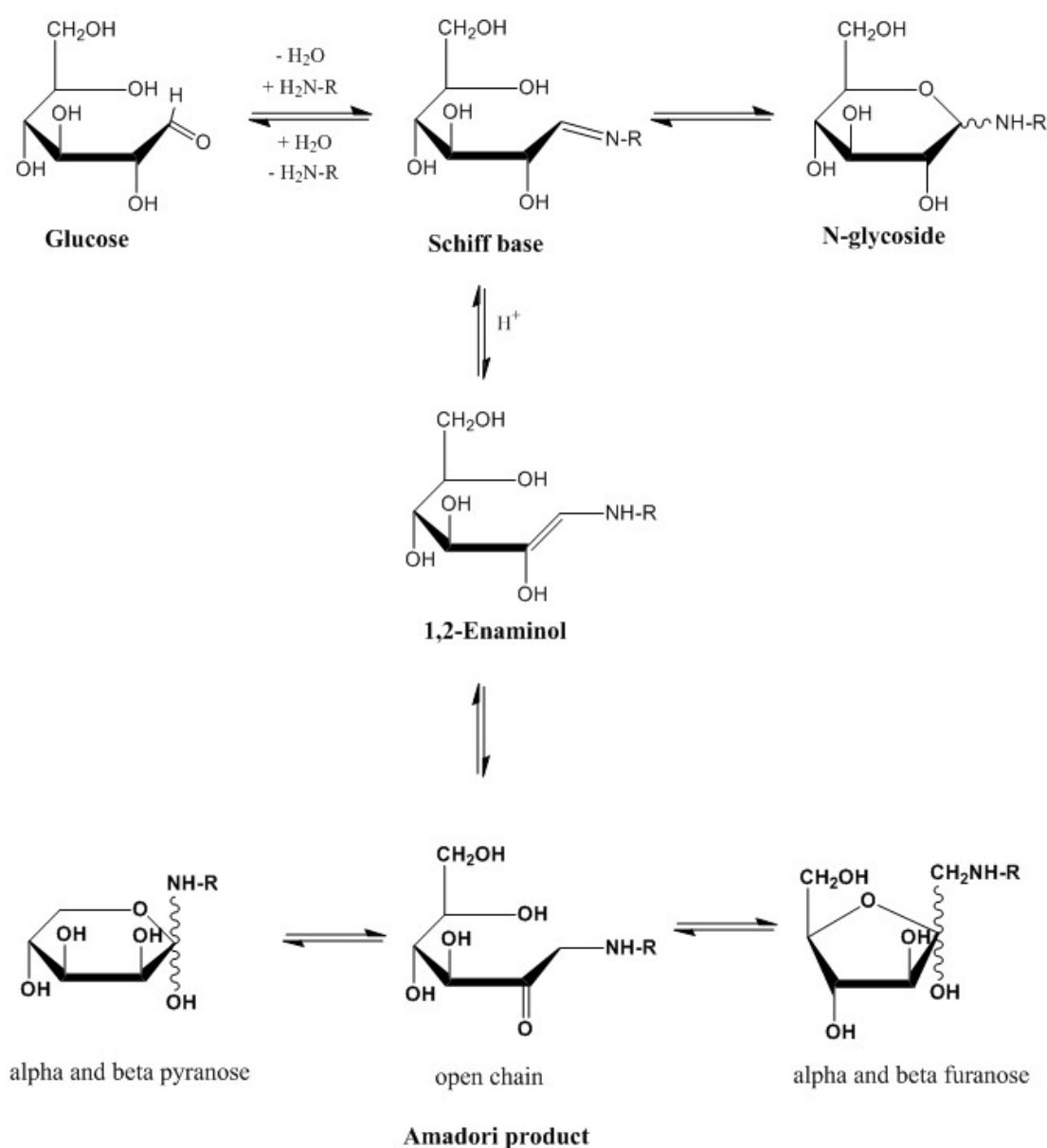
Figure 2-1: Early phase of the Maillard reaction, leading to formation of the first detectable product (Amadori product) in the example of the formation of 1-amino-1-desoxy-fructose from glucose and an amino group. Adapted from *Belitz et al. (2009)* and *Hellwig (2011)*.

Amadori and Heyns products are the first stable products of the Maillard reaction and are commonly used as marker for the evaluation of the intensity of the reaction. Early products of the Maillard reaction do not absorb visible or UV light *(Krause, 2005)*.

In the advanced stage, Schiff bases and Amadori products are degraded by enolisation, water elimination, oxidation and retro aldol cleavages, leading to formation of very reactive intermediate 1,2-dicarbonyls. Especially important are 3-deoxyosone, 1-deoxyosone and the 4-deoxyosone, and retro-aldol fission products such as methylglyoxal and glyoxal, which initiate a cascade of reactions to give a myriad of secondary products *(Belitz et al., 2009)*.
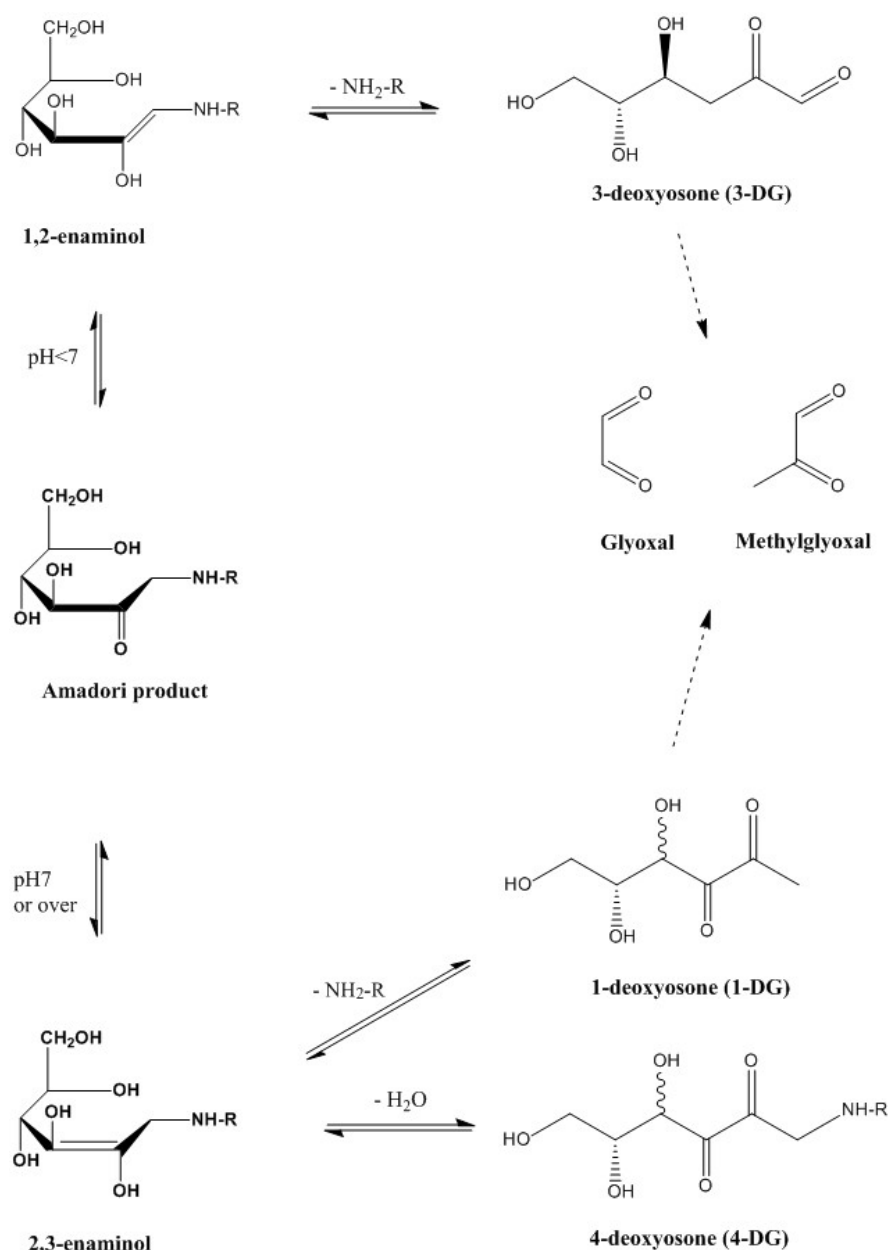
Figure 2-2: Formation of deoxyosones from Amadori products. Example from glucosones. Adapted from *Belitz et al. (2009)* and *Hellwig (2011)*.

Moreover, 1,2-dicarbonyls may also be formed from the degradation of triosephosphates or via lipid peroxidation and by caramelization of sugars in absence of amino groups *(Henle, 2005)*. In the advanced stage of the Maillard reaction, an increase in the UV-absorbance and fluorescence, as well as in color, can be observed *(Krause, 2005)*.

The final stage of the Maillard reaction is marked by degradation, polymerization and reaction of the high reactive dicarbonyl compounds and other sugar degradation products, giving a plethora of advanced glycation end products (AGEs), mostly heterocyclic and colored. Side chain amino groups of proteins can react with 1,2-dicarbonyls to form stable peptide-bound amino acid derivatives and cross-links. Through the reaction of dicarbonyl compounds with the α-amino group from free amino acids, with following Strecker degradation of the formed Schiff bases, are originated at last aroma active aldehydes. If the nucleophile is the N-terminal nitrogen from peptides and proteins, a cyclization to fluorescent pyrazinones is possible *(Krause et al., 2004)*.

The first amino acid derivative of the advanced Maillard reaction, which was detected in food, was N^ε-carboxymethyllysine (CML) *(Henle, 2005)*. CML is a major glycation end-product, even though its contents in food can be overestimated due to the formation of from fructosyllysine during hydrolysis the hydrolytic step of the classical analytical method *(Hartkopf et al., 1994)*. It is not only formed by fragmentation of Amadori product but also by a diversity of other mechanisms are possible *(Gruber and Hofmann, 2005)*. Common is the reaction of lysine residues with glyoxal, which can also originate via lipid peroxidation or caramelization. The formation of N^ε-carboxyethyllysine CEL succeeds by analogous reactions with methylglyoxal *(Henle, 2005; Hellwig, 2011)*.

The best characterized and most common AGE products are, besides CML and the similarly formed N^ε-carboxyethyllysine (CEL), pentosidine, pyrraline, imidazolinone, glucosepan, Arg-pyrimidine and several arginine–lysine cross-links formed by glyoxal, methylglyoxal, and 3-deoxyglucosulose *(Henle, 2005)*. Thus, most known AGEs are relatively stable compounds that are not changed during the isolation and purification process, and do not react further. Some structures with markedly involvement on the hypotheses of the present study are illustrated in Figure 2-3.
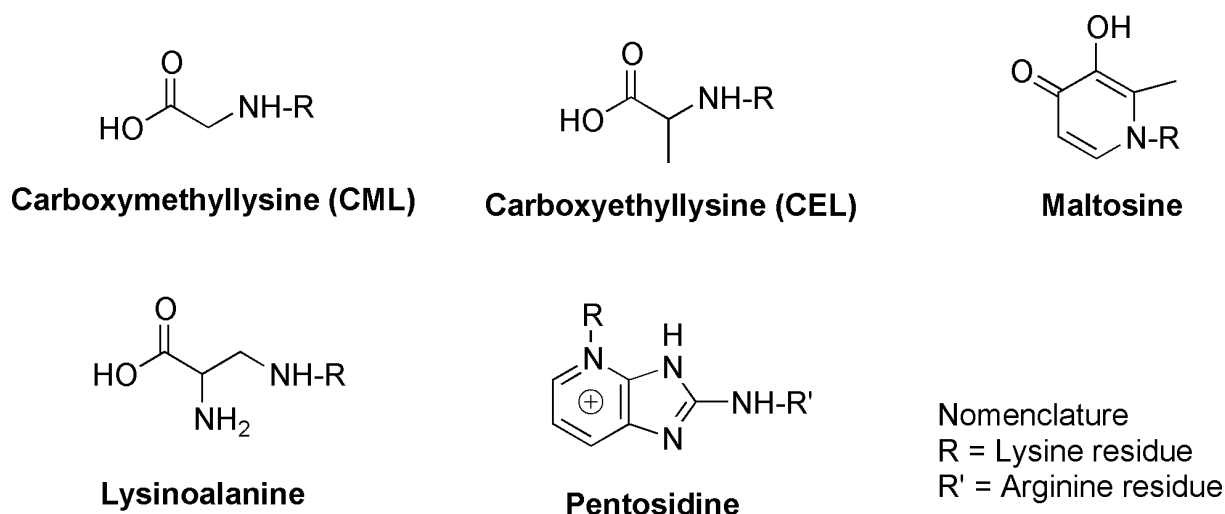
Figure 2-3: Example of AGEs of defined structure with direct relevance for the present work.

The Maillard reaction results, in the final stage of the glycation, after the complex series of cyclizations, dehydrations, retroaldolizations, rearrangements, isomerizations, and condensations of low molecular weight Maillard-reaction products, in brown-colored nitrogenous high molecular weight melanoidins *(Ledl and Schleicher, 1990; Hofmann, 1999; Wang et al., 2011)*. Due to the importance of this group of products for the present work, they are closer described.

Possible detrimental action of the ingestion of food rich in Maillard reaction products has been speculated. The Maillard reaction has nutritional consequences, as loss of physiologically absorbable essential amino acids and diminished digestibility of proteins *(Ledl and Schleicher, 1990)*. Furthermore, many moieties formed during the Maillard reaction show also strong affinity for metallic cations, a fact discussed in the past as a nutritional risk, as the micro-minerals supply could be compromised *(Wang et al., 2011; Wijewickreme and Kitts, 1998; Wijewickreme et al., 1997; O'brien and Morrissey, 1997)*. In vivo, glycation alters the structure, function and stability of body proteins, possibly contributing to aging and some pathological processes *(Hipkiss, 2006; Henle, 2007)*. As AGEs in living organisms were found to increase during aging and diabetes, and were directly linked to the pathophysiology of several diseases like cataract, atherosclerosis, and uremia they are also considered as a type of covalent damage of proteins, not only due to the functional loss, but also to the development of potential pathogenic molecules *(Cloos and Christgau, 2002; Henle, 2003)*.

In spite of these early inquietudes concerning possible negative health effects of the dietary Maillard reaction products, increasingly evidence strikes the disadvantageous action attributed to these substances. The positive impact of the formation of antioxidant moieties (reductones) through non-enzymatic browning is nowadays unambiguous *(Bianchi et al., 2010; Hwang et al., 2011)*. The metal chelation properties of MRPs may also show positive perspectives for health. A low-dose chelation therapy has been even proposed as a useful clinical tool for prevention and treatment of diabetes complications *(Nagai et al., 2012)*, and even a neuroprotective effect in Alzheimer and Parkinson's disease and other neurodegenerative conditions *(Hegde et al., 2009; Cuajungco et al., 2000; Kell, 2010; Weinreb et al., 2010)*. Actually, the antioxidant capacity developed in course of the Maillard reaction is believed to be due not only to direct reductive activity, but also to the sequestration of metals *(Wijewickreme and Kitts, 1998)*. Likewise, a protective effect of AGEs in cataract lenses against the UV-induced macular degeneration, the main cause of age related blindness, has been evidenced in the last years *(Klein et al., 2012; Casparis et al., 2009)*. In conclusion, as humans have been adapting their biochemical repertoire to the consumption of heated food for over 100.000 years, we believe to have enough reasons to think that deleterious effects of products of common household cooking on human health should be rather limited. Even the endogenous glycation, which has been seen as a covalent damage to body proteins and a risk factor to health, should be evaluated in light of the evolutionary adaptation.

2.1.1 **Melanoidins**

The Maillard reaction results, in the final stage of the glycation, after the complex series of cyclizations, dehydrations, retroaldolizations, rearrangements, isomerizations, and condensations of low molecular weight Maillard-reaction products in brown-colored nitrogenous high molecular weight melanoidins *(Ledl and Schleicher, 1990; Hofmann, 1999; Wang et al., 2011)*. These macromolecules are not only responsible for the development of color in heat-processed food products. High antioxidant capacity is associated to the formation of melanoidins *(Gu et al., 2010; Vignoli et al., 2011; Liu and Kitts, 2011; Rufián-Henares and Morales, 2007; Delgado-Andrade and Morales, 2005; Borrelli et al., 2002; del Castillo et al., 2002; Nicoli et al., 1997)*, which are also able to bind metals in food system *(Wijewickreme et al., 1997; Gomyo and Horikoshi, 1976; Takenaka et al., 2005; Wen et al., 2005; Morales et al., 2005; Rufian-Henares and de la*

Cueva, 2009; Nunes and Coimbra, 2007; Nunes and Coimbra, 2007), acting as preservatives and protecting the quality during storage *(Wijewickreme and Kitts, 1998; Liu and Kitts, 2011; Delgado-Andrade and Morales, 2005; Lindenmeier et al., 2002; Morales, 2009)*. Melanoidins may also contribute to food texture *(Zamora and Hidalgo, 2005; D'Agostina et al., 2004)*, foamability and foam stability *(D'Agostina et al., 2004; Nunes et al., 1997; Redgwell et al., 2005)*, and play a role in the binding of flavor compounds *(Hofmann et al., 2001; Hofmann and Schieberle, 2002)*.

Different hypotheses on the structural backbone of melanoidins have been proposed *(Bekedam et al., 2006; Cämmerer et al., 2002; Cämmerer and Kroh, 1995; Adams et al., 2005)*. *Heyns and Hauber (1970)* and *Tressl et al. (1998)* suggested that melanoidins are a complex macromolecular structure consisting of repeating units of furans and pyrroles, linked to linear oligomers by polycondensation reactions. *Hofmann (1998b)* detected low-molecular-weight colored substances, which were able to crosslink proteins via ε-amino groups of lysine or arginine, and proposed that they originate high-molecular-weight colored melanoidins. *Kato and Tsuchida (1981)* and, more recently, *Cämmerer et al. (2002)* and *Cämmerer and Kroh (1995)*, suggested that the melanoidin skeleton is mainly built up of sugar degradation products, formed in the early stages of a Maillard reaction, polymerized through aldol-type condensation, and possibly branched by amino compounds. However, these proposals have been made based on model studies and the complexity and structures of melanoidins depend on the nature and number of possible reactants and the reaction conditions, such as treating temperature and time, pH, and solvent *(Wang et al., 2011)*.

In food, amines and sugars are often proteins and polysaccharides, but also carbonyl groups formed by oxidation of fatty acids during heat treatment – for instance, during roasting or frying of nuts and seeds - may react with amino group much more efficiently than the carbohydrate carbonyl group. Also other structures, as phenolic compounds, are important reactants on the melanoidin formation, as exemplified by the incorporation of chlorogenic acid in coffee melanoidins *(Morales et al., 2012)*. Actually, not even a single food melanoidin structure has been isolated and fully characterized so far. The only information available in the literature regards to the contribution of major compounds to the backbone structure of food melanoidins *(Morales et al., 2012)*. As remarked by *Van Boekel (1998)*, it is quite ironical that the Maillard reaction is called "non-enzymatic browning reaction", while so little is known about the actual browning part.

As a result of their thermal induced formation, melanoidins are prevalent in human diet, comprising a substantial proportion of several food products, such as coffee, bread, roasted malt, cocoa, soy sauces, balsamic vinegar, breakfast cereals, and meat - among which coffee and bakery products represent definitely the main dietary sources *(Morales, 2009; Morales et al., 2012; Fogliano and Morales, 2011)*. Since the molecular structure of food melanoidins are not fully characterized and reference material is lacking, estimations on the contents of melanoidins in different foods and dietary intake are missing *(Morales et al., 2012; Fogliano and Morales, 2011)*.

Increasingly evidence has been demonstrating beneficial physiological activity of the largely indigestible melanoidins *(Wang et al., 2011; Morales et al., 2012)*. Even though bioavailability is a general prerequisite for bioactivity of ingested compounds, the effect inside the gastrointestinal tract is a remarkable exception *(Morales et al., 2012)*. Both soluble and insoluble melanoidins can be considered antioxidant dietary fiber due to their abundance of reducing functional groups *(Morales et al., 2012)*, which can quench reactive species of oxygen and nitrogen either present in diet or formed endogenously by digestion and microflora in gastrointestinal tract *(Babbs, 1990)*. Antioxidant compounds, including scarcely-absorbed high molecular weight food melanoidins, are shown to maintain a reduced environment in the intestinal lumen, diminishing oxidative damage to intestinal mucosa, and potentially reducing the risk of colon cancer and diverticular disease *(Bianchi et al., 2010; Gökmen et al., 2009; Vitaglione et al., 2008; Vitaglione et al., 2012; Garsetti et al., 2000)*. Moreover, prebiotic potential of food melanoidin has been described in the literature, where the fermentability of melanoidins by intestinal flora, particularly Bifidobacteria strains, was reported *(Gniechwitz et al., 2008a; Reichardt et al., 2009; Borrelli and Fogliano, 2005)*. The metal chelating properties of melanoidins has been studied over decades. This property is believed to be greatly responsible for the antimicrobial activity. In low concentrations, melanoidins can chelate iron ions, acting bacteriostatic, and at high concentration they can act bactericide, promoting rupture of the extracellular membranes due to removal of Mg(II) ions *(Rufian-Henares and de la Cueva, 2009; Rufián-Henares and Morales, 2008)*. Furthermore, melanoidins, especially coffee melanoidins, show anti-adhesive and anti-biofilm activity and may reduce development of dental plaque and caries *(Daglia et al., 2002; Stauder et al., 2010)*. *Hiramoto et al. (2004)* observed that protein-derived melanoidin significantly suppresses colonization of *Helicobacter pylori*, a major etiological agent of gastritis, gastric ulcers and possibly gastric

cancer, by inhibiting bacterial adhesion to host mucosa. The authors suggested that foods containing melanoidins may be an alternative to antibiotic-based therapy. In summary, the positive impact of melanoidin-rich diets to human health may be localized in oro-gastrointestinal tract, but are of remarkable relevance.

2.2 Coffee

The term "Coffee" means the beans and cherries of the trees of several species of the genus *Coffea*, mostly *Coffea arabica* and C*offea canephora*, whether parchment, green or roasted, and includes ground, decaffeinated, liquid and soluble coffee *(International Coffee Organization, 2007)*. The infusion of ground, roasted coffee beans is one of the most widely consumed beverages in the world. It is a complex chemical mixture reported to contain more than a thousand different chemicals, including carbohydrates, lipids, nitrogenous compounds, vitamins, minerals, alkaloids and phenolic compounds *(Belitz et al., 2009)*. Although venerated for its aroma and flavor, the psychoactive alkaloid caffeine is probably at least partially responsible for its popularity *(Higdon and Frei, 2006)*.

2.2.1 General aspects

Coffee belongs to the botanical family *Rubiaceae*, which has over 600 genera and 13000 species *(Davis et al., 2009)*. Coffee was first discovered in Eastern Africa and its cultivation was restricted to Arabia until 1600 *(Oestreich-Janzen, 2010)*. Nowadays, coffee is cultivated in over 70 countries throughout the tropics, primarily in equatorial Latin America, Southeast and South Asia and Africa. The two economically most important species of coffee are *Coffea arabica*, known by the trade name "Arabica coffee", and C*offea canephora*, the "Robusta coffee" *(Clifford and Willson, 1985)*.

Coffee is most widely traded tropical agricultural commodity, accounting for exports worth an estimated US$ 15.4 billion (93.4 million bags) in 2009/10. Its cultivation, processing, trading, transportation and marketing provide employment for hundreds of millions of people worldwide *(International Trade Centre, 2012)*. Coffee is crucial to the economies and politics of many developing countries. Brazil is currently responsible for over 35% of the world total coffee production. Global coffee consumption has increased on average 1.2% annually since the early 1980s, rising to more than 2% in recent years. Despite the adverse macroeconomic turbulent scenario, the global coffee consumption remained high

and reached 135 million bags in 2010, thus confirming the inelasticity of demand for coffee *(International Coffee Organization, 2012a)*.

2.2.1.1 Coffee production

In Figure 2-4, the sequence of transformations, which make from the tropical primary product a beverage drink around the world, mostly in high developed countries *(International Coffee Organization, 2013; International Coffee Organization, 2012b)*, can be seen.

Figure 2-4: Sequence of coffee production: Transformation of the tropical crop to the beverage ready to drink. **A** shows coffee cherries of increasingly ripeness. **B** shows a worker inspecting unroasted beans in the producing developing land. **C** shows coffee beans of various roast degrees, **D** shows a common "working hours" form of the beverage, mostly consumed in industrial lands (Images obtained from *Fujisaki (2010)*, *David Silverman/Getty Images (2012)*, *Food wallpaper (2012a)*, and *Food wallpaper (2012b)* , respectively).

Figure 2-4 A shows the maturation cycle of the coffee cherries. The deep red ripe cherries are harvested either by selectively picking by hand, or strip picked by hand or using mechanical harvesters.

The fruits are subsequently submitted to drying operation, a process of crucial importance for the final quality of the green coffee. It can be done by either dry or wet methods. The dry method is the oldest, simplest and requires little machinery and water, but demands a uniform flowering and predominance of ripe coffee. This method implicates spreading the cherries out in the sun to dry naturally. The coffee is periodically turned to ensure uniform

drying and to help prevent deterioration caused by fungi and bacteria. Once proper drying has occurred, all the remaining outer layers of the dried cherry are removed by a hulling machine. In the 'fully washed' wet method, the coffee cherries are washed to remove stones and other foreign matter, and passed through a depulper, removing the exocarp (skin) and most of the mesocarp (pulp). The remaining mesocarp is removed by fermentation, using pectinases to accelerate the process, when needed, to avoid formation of undesirable flavor. The 'semi washed' wet method is similar to the 'fully washed', but a partial or reduced fermentation is used, and the coffee is then washed to remove the remaining pulp. From both wet methods, the resultant beans are dried to uniform moisture using natural or mechanical methods. After drying, the coffee is further mechanically processed to remove the endocarp (parchment) and the spermoderm (silverskin) before grading. The wet process generally produces coffee of more uniform quality and flavor. After being processed by any of the above methods, the coffee is brought to the desired 11–13% moisture range. When properly stored, the quality of unroasted green coffee can be maintained for up to a year *(Wasserman et al., 2007)*. The unroasted green coffee beans are illustrated in the Figure 2-4 B.

The pleasant organoleptic characteristics from coffee are developed by the roasting process. Roasting is defined as the dry heating of the green coffee beans, normally at atmospheric pressure. The roasting process of coffee beans to produce high quality coffee is carried out under mild conditions, achieving temperatures between 180°C and 240°C in 8 to 15 minutes, depending on the degree of roast required, mostly in contact (drum) roasters or in convection (hot-air) roasters *(Packert, 1993)*. Industrially, coffee roasting is commonly realized at temperatures from 240 °C to 270 °C for 6 min to 3 min. Eventually, even higher temperatures can be used *(Packert, 1993; Murkovic and Derler, 2006)*.

The roasting process of coffee beans can be divided in two steps. The first stage is endothermic, demands 80% of the roasting time and is responsive for the loss of free water. The second stage is characterized by an abrupt inflation and occasional bursting of the beans, with a volume increase of up to 100%, depending on variety and roasting conditions. This happens due to a quick increase in the roaster temperature caused by massive exothermic reactions, such as pyrolytic fragmentations. Also the Maillard reaction takes place, originating the brown melanoidins. These extreme complex reactions produce the desired color, flavor and texture of the coffee. The loss of carbon dioxide, other volatile substances, and bound water, accounts for most of weight loss during the second stage, which can reach up to 6%. Most of the lipid, caffeine, inorganic salts, and polymeric

carbohydrate survive the roasting process *(Wasserman et al., 2007)*. An experienced and skilled roasting operator can optimize color and aroma development and minimize over-roasting damage *(Belitz et al., 2009)*. The treatment is conducted until the desired degree of roast is achieved. Differently intensive roasted beans can be seen in Figure 2-4 C.

The best way to make and drink coffee depends not only on cultural or personal preferences. It is both a ritual and a practical necessity and can vary from day to day and even change according to the momentary circumstances. The drip or filter method is possibly the most widely used method today, but espresso and cappuccino are the fastest growing methods of making coffee. The plunger method (cafetière) has also an increasing market, probably due to its convenience and effective extraction of flavor from ground beans. Other methods, such as the Turkish method and the percolator, have decreasing predominance, as part of the coffee flavor is lost during the preparation and the market is increasingly concerned about the quality of the beverage. The market of soluble coffee did not show important changes in the last years *(International Trade Centre, 2012; International Coffee Organization, 2012b)*. A very popular way of drinking coffee during the working hours can be seen in Figure 2-4 D.

2.2.1.2 General chemical composition

Coffee is composed of over thousand different substances among soluble and insoluble carbohydrates, acids and phenolics, lipids, proteins and other nitrogenous compounds, and minerals. The composition of the natural product is affected not only by coffee variety, but also by climate conditions and postharvest handling *(Belitz et al., 2009)*. Not only carbohydrates and proteins, but also polyphenols compounds, take directly part in the formation of coffee high molecular weight melanoidins during the roasting of the coffee beans *(Nunes and Coimbra, 2007; Bekedam et al., 2006; Nunes and Coimbra, 2010; Bekedam et al., 2008a; Bekedam et al., 2008c)*. This section provides a short overview on these components, which are protagonists on the non-enzymatic browning, and the main chemical transformations they undergo during the thermal treatment. Caffeine will be briefly mentioned due to its attractiveness for the consumer and to its relevance in the discussion related to health issues.

Green beans contain around 50% carbohydrates, mostly polysaccharides of differing molecular weights *(Oestreich-Janzen, 2010; Redgwell and Fischer, 2006)*. Positive

characteristics of coffee beverage are developed upon roasting and are related to the complex chemical changes, which carbohydrates undergo during the heating process. Roasting is responsible for the degradation of up to 40% of the polysaccharides present in green coffee beans *(Redgwell and Fischer, 2006; Oosterveld et al., 2003; Redgwell et al., 2002b)*. The polysaccharides extracted by brewing are directly involved in the viscosity, creaminess, foam stability, and the capacity of retaining volatile substances of the beverage *(Redgwell et al. 2002a)*. A schematic representation of the main carbohydrates found in green coffee beans can be seen in Figure 2-5.

Figure 2-5: General structure of main polysaccharides from green coffee beans extractable in hot water: (a) galactomannans and (b) arabinogalactans (polysaccharide moiety of arabinogalactan-proteins). Adapted from *Moreira et al. (2012)*.

In mature grains, sucrose compounds 5-12% of the dry weight and is practically the only free sugar present *(Redgwell and Fischer, 2006)*. In general, Robusta beans contain 30% less sucrose than Arabica varieties *(Oestreich-Janzen, 2010)*. However, this disaccharide is readily degraded during roasting and its content is minimal in roasted coffee *(Oosterveld et*

al., 2003; Bekedam, 2008). The polysaccharide fraction of green beans is mainly composed by mannans and galactomannans, arabinogalactan-proteins (AGPs) and cellulose. Small amounts of pectin and xyloglucan are also present *(Redgwell and Fischer, 2006; Redgwell et al., 2002a; Oosterveld et al., 2004)*.

Arabinogalactan-protein and peptides are widely distributed in the plant kingdom, acting in plant growth and development, including signaling, embryogenesis, and programmed cell death *(Sanchez et al., 2008; Clarke et al., 1979)*. Coffee arabinogalactans consist of an extremely heterogeneous mixture of molecules formed as backbones of β-(1→3) linked galactosyl residues, substituted in O-6 position with various combinations of arabinosyl and galactosyl residues *(Redgwell and Fischer, 2006; Oosterveld et al., 2003; Redgwell et al., 2002a; Fischer et al., 2001)*. Most of the arabinogalactans are covalently bound to proteins, being more correctly referred to as arabinogalactan-proteins (AGPs) *(Redgwell et al., 2002b; Redgwell et al., 2002a)*. The AGPs present an average molecular weight of 650 kDa and are negatively charged due to the presence of 6 to 10% glucuronic acid residues as non-reducing terminal units, linked on β-(1→6) galactosyl side chains *(Redgwell et al., 2002a)*. The carbohydrates of AGPs are extensively degraded or chemically modified during roasting and the fragmentation of their protein backbone is also probable *(Bekedam et al., 2007)*. Especially the arabinose residues seem to be prone to thermal degradation *(Moreira et al., 2012)*. AGPs from roasted coffee beans show high solubility at common brewing conditions *(Oosterveld et al., 2003)*.

Plant galactomannans are reserves from both carbon and solvatation water for germinating seeds *(Dey, 1978)*. Coffee galactomannans consist of polymers formed by β-(1→4) linked mannosyl residues, with different grades of α-O-6 substitutions with single galactosyl residues *(Oosterveld et al., 2004)*. In green beans, the galactomannans are most insoluble *(Oestreich-Janzen, 2010)*. Determinant for galactomannan solubility is the frequency of galactose substitutions on the mannan backbone *(Redgwell and Fischer, 2006)*. Besides the galactosyl side chains, acetyl groups can also be found linked O-2 and O-3 to the mannosyl residues in the soluble fraction of galactomannans. O-6 substitutions on the backbone with single L-arabinosyl and D-glucosyl can also be observed *(Nunes et al., 2005; Nunes et al., 2006)*. The general extractability of galactomannans is enhanced after roasting, being highest at medium roast *(Bekedam et al., 2008c; Redgwell and Fischer, 2006)*. Although the degrees of branching and polymerization of the galactomannans decreases upon roasting,

and modification of the reducing ends can be observed, these polysaccharides are more stable than the arabinogalactans *(Nunes and Coimbra, 2010; Nunes et al., 2005; Nunes et al., 2006; Nunes and Coimbra, 2002)*.

Cellulose, the main constituent in cell walls of plants, is fibrous, tough and water insoluble *(O'Sullivan, 1997)*. Coffee cellulose is mostly not extractable in coffee brew and remains unchanged along roasting *(Redgwell and Fischer, 2006; Redgwell et al., 2002b)*.

The protein fraction of coffee has not been object of intensive investigation. The protein content in green coffee beans is relatively high, representing up to 12% of coffee dry weight *(Montavon et al., 2003)*, where approximately 45% of total grain protein is estimated to be 11S storage protein *(Rogers et al., 1999)*. On the other hand, the concentration of free amino acids can be as low as 0.3% on dry weight basis, being higher in Robusta than in Arabica beans. The extremely low concentrations on free amino acids in the coffee restrict their importance in the thermally induced formation of aroma or flavor *(Murkovic and Derler, 2006; Montavon et al., 2003)*. The amino acid content decreases rapidly during roasting and only negligible amounts of free amino acids can be detected in roasted coffee *(Bekedam, 2008)*. The composition of coffee proteins is intensely changed with progress of the thermal treatment. A decrease in total amino acids amount of coffee beans is also observed, especially of arginine, cysteine, lysine, and serine, as well as an increase in the racemization *(Casal et al., 2005)*. However, limited knowledge on the resulting structures is available *(Nunes and Coimbra, 2002)*. *Montavon et al. (2003)* studied the changes in coffee protein profile during roasting and found a complete degradation of non storage proteins within the first minutes of heating. The storage protein showed a slow pattern of degradation and the authors suggested the participation of these proteins, together with chlorogenic acids, on the origination of melanoidins.

Green coffee beans are one of the richest dietary sources of chlorogenic acids, a class of compounds belonging to food polyphenols *(Crozier et al., 2009)*. Chlorogenic acids are a family of esters of trans-cinnamic acids, such as caffeic, ferulic, and p-coumaric acid, with quinic acid *(Clifford et al., 2006)*. Green coffee beans are very rich in chlorogenic acids; their contents are up to 7.5% in Arabica beans. Plant phenolic acids play a role in stress adaptation, but the high levels found in coffee seeds point to specific physiological functions of these compounds in the plant, possibly related to seed germination and cell growth, as well as other already unknown roles *(Farah and Donangelo, 2006)*. Although 45

different chlorogenic acids have been identified in green Arabica coffee beans, and 69 in Robusta beans *(Jaiswal et al., 2010)*, only nine kinds of these trans-cinnamic acids account for 80% of the total, being 5-caffeoylquinic acid, illustrated in Figure 2-6, by far the most prevalent one, being simply referred to as "chlorogenic acid" *(Nunes and Coimbra, 2010; Bekedam et al., 2008a; Clifford et al., 2006)*. This is accompanied by significant amounts of 3-O- and 4-O-caffeoylquinic acid, the three analogous feruloylquinic acids and 3,4-O-, 3,5-O- and 4,5-O-dicaffeoylquinic acids *(Crozier et al., 2009)*. *Clifford et al. (2006)* identified also minor classes of chlorogenic acids in coffee beans, such as diferuloylquinic acids, di-p-coumaroylquinic acids, dimethoxycinnamoylquinic acids, and others, which together constitute less than 1% of total chlorogenic acid content. Chlorogenic acids and their derivatives contribute considerably to coffee flavor, conferring acidity, astringency, and bitterness to the beverage *(Frank et al., 2008; Farah et al., 2006)*.
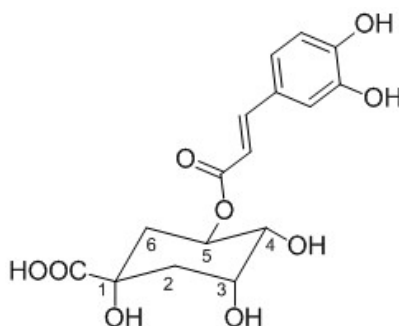
Figure 2-6: 5-Caffeoylquinic acid, the principal polyphenolic compound from coffee beans, commonly referred to simply as chlorogenic acid.

During coffee roasting, the content of detectable chlorogenic acid in beans decreases rapidly *(Bekedam et al., 2008a; Bekedam et al., 2008b; Perrone et al., 2012)*. Different explanations are found in the literature including acyl migration, hydrolysis, oxidation, fragmentation, polymerization, and association with denatured and degraded proteins *(Bekedam et al., 2008b)*. Those products are not yet fully characterized. However, it is already known that they are diverse and range from simple phenols to condensation products of high structural complexity *(Nunes and Coimbra, 2010)*. Early in roasting, when the water content is still sufficient, isomerization occurs, accompanied by hydrolysis, releasing the cinnamic acids and quinic acid. Later, the free quinic acid epimerizes and lactonizes, and several chlorogenic lactones including 3-O- and 4-O-caffeoyl-1,5-quinide are formed. The cinnamic acids may be decarboxylated and transformed to a number of simple phenols and a range of phenylindans, probably via decarboxylation and cyclization of the vinylcatechol

intermediate *(Crozier et al., 2009)*. In autoxidation model studies, chlorogenic acids showed in solution the ability to form lignin-like products, probably via phenolate anion *(Cilliers and Singleton, 1989; Cilliers and Singleton, 1991)*. But not all the "lost" chlorogenic acid has undergone a complete degradation of its original molecular structure. Nowadays, the incorporation of preserved chlorogenic acid molecules in the melanoidin structure is well evidenced *(Nunes and Coimbra, 2007; Adams et al., 2005; Nunes and Coimbra, 2010; Bekedam et al., 2008a; Gniechwitz et al., 2008b)*. The retention of the antioxidant activity and phenolic characteristics of coffee chlorogenic acids upon incorporation in coffee melanoidins was recently reported *(Bekedam et al., 2008b)*. The role of chlorogenic acids on the formation of coffee melanoidins is discussed in detail in Section 2.2.2.1.

The caffeine content in dry matter base is not significantly affected by postharvest processing and roasting. A small amount of this alkaloid sublimate during the thermal treatment, but this effect is compensated by the general organic weight loss. In coffee brews prepared from 55 g coffee powder for one liter beverage, the caffeine content obtained from Arabica beans varies between 50 and 88 mg/L and from Robusta beans 77 to 160 mg/L *(Oestreich-Janzen, 2010)*.

The quantitative changes of key chemical components of Arabica coffee beans upon roasting can be seen in Table 2-1. Despite the relatively advanced age of the original publication, these values are still taken as a compilation of the available data and are still a solid reference *(Bekedam, 2008)*.

Table 2-1: Alterations on the quantitative chemical composition of Arabica coffee beans upon roasting (% of dry matter).

	Green beans	**Roasted beans**
Proteins	11.0 – 13.0	13.0 – 15.0
Amino acids	2.0	–
Polysaccharides	50.0 – 55.0	24.0 – 39.0
Oligosaccharides	6.0 – 8.0	0.0 – 3.5
Chlorogenic acids	5.5 – 8.0	1.2 – 2.3
Caffeine	0.9 – 1.2	~ 1.0
Lipids	12.0 – 18.0	14.5 – 20.0
Minerals	3.0 – 4.2	3.5 – 4.5

Data extracted from *(Bekedam, 2008)* were originally produced by *Clifford (1975)*.

The melanoidins formed during coffee roasting are object of the present work and this topic is presented in more detail in the Section 2.2.2.1.

2.2.1.3 Coffee and health

Although coffee beverages have been consumed for already thousand years *(Leeb, 2008)*, it still fascinates and challenges the science. Throughout this time, innumerous postulations about its possible effects on health have been done, but only in the last 15 years rigorous scientific conclusions have been reached *(Illy and Pizano, 2004)*. For decades, solely detrimental influence of coffee consumption was object of attention of the scientific community, particularly concerning the effects of caffeine on the cardiovascular system. At the present, there is merely modest evidence of health risks of moderate consume of coffee. Early assumptions, as the deleterious influence of coffee ingestion on chronic diseases, have been progressively refused with the advance of the research *(Floegel et al., 2012)*. In contrast, voluminous data pointing to health benefits of coffee has been accumulated *(Higdon and Frei, 2006)*. Large epidemiological investigations reported an inverse association between coffee consumption and total and cause-specific mortality, where the beneficial effect of coffee ingestion was especially remarkable for deaths caused to heart disease, respiratory disease, stroke, injuries and accidents, diabetes, and infections *(Tamakoshi et al., 2011; Freedman et al., 2012)*.

Contrary to the early anxiety, a protective effect of moderate coffee consumption against stroke and heart failure is nowadays well established. In a meta-analysis review, *Mostofsky et al. (2012)* evidenced that moderate coffee consumption is inversely associated with risk of heart failure, with the largest inverse association observed for consumption of 4 servings per day. A Japanese follow-up study observed a strong inverse association between coffee consumption and mortality due to coronary heart disease in women, and no correlation in men *(Sugiyama et al., 2010)*, while a Dutch follow-up found a slight risk reduction due to moderate coffee consumption *(de Koning Gans et al., 2010)*. To date, no consistent detrimental effects of habitual coffee consumption on arterial pressure could be reported. Although a raise in plasma homocysteine concentration, a controversial risk factor for cardiovascular disease, could be observed in trial studies involving heavy coffee drinking *(Urgert et al., 2000; Grubben et al., 2000)*, no alteration was measured for moderate intake *(Esposito et al., 2003)*. Putative risk factor for atherosclerosis and subsequent hypertension

is elevated serum concentrations of total cholesterol, especially low-density lipoprotein cholesterol. A meta-analysis of randomized controlled clinical trials found only a very little increase in serum cholesterol caused by consumption of filtered coffee, although coffee prepared by other brewing methods were shown to slightly increase serum levels of total and LDL cholesterol *(Jee et al., 2001)*. *Kempf et al. (2010)* recently found no evidence for adverse effects of filtered coffee ingestion on proatherogenic lipids. In line with these biochemical investigations, no increased risk of hypertension was associated with a habitual coffee consumption in recent epidemiologic systematic reviews and meta-analyses of long-term prospective studies *(Mesas et al., 2011; Zhang et al., 2011)*.

Various epidemiologic studies attest a positive effect of coffee against neoplasic diseases of different sites. In a meta-analysis of cohort studies *Yu et al. (2011)* found that coffee consumption may reduce the total cancer incidence and it has also an inverse association with some type of cancers. They observed lower cancer occurrence in regular coffee drinkers, particularly in high drinkers. Overall, an increase in consumption of 1 cup of coffee per day was associated with a 3% reduced risk of cancers. The protective effect of coffee ingestion on prostate cancer is well established. A strong inverse association between coffee consumption and risk of lethal prostate cancer, apparently related to non-caffeine components of coffee, was evidenced in a follow-up study *(Wilson et al., 2011)*. In a recent British prospective cohort study, coffee consumption was likewise observed to reduce the risk of aggressive prostate cancer *(Shafique et al., 2012)*. The cohort study from *Discacciati et al. (2013)* reported a clear inverse association between coffee consumption and risk of localized prostate cancer, especially among overweight and obese men. A population-based case–control study supports the inverse association between coffee and lethal and high-grade prostate cancer *(Wilson et al., 2013)*. A strong protective effect of coffee intake on risk of endometrial cancers has been reported in several cohort and follow-up studies, and in meta-analyses *(Yu et al., 2011; Arab, 2010; Nkondjock, 2009; Giri et al., 2011; Je and Giovannucci, 2012; Je et al., 2011)*. A recent meta-analysis showed that coffee consumption has protective effects on esophageal cancer *(Zheng et al., 2013)*. An inverse association of coffee intake with oral and pharyngeal cancer mortality was reported in a large prospective study *(Hildebrand et al., 2013)*. Coffee drinking may help reduce death due to liver cancer, as shown by numerous meta-analyses. A high lifetime coffee consumption was negatively associated with development of hepatocellular carcinoma in the hospital-based case–control study from *(Jang et al., 2013)*. Also *Larsson and Wolk*

(2007) observed a reduction in the risk of liver cancer caused by increased coffee consumption. The meta-analysis from *Bravi et al. (2007)* evidenced a real reduction in hepatocellular carcinoma risk among coffee drinkers, and suggested a continuum of the favorable effect of coffee on liver function. Some of the protective components of coffee reported in the literature are caffeine, affecting cell cycle, proliferation, and apoptosis; cafestol and kahweol, which decrease mutagenesis and tumorigenesis in animal models; and chlorogenic acid, an antioxidant reported to decrease DNA methylation *(Arab, 2010)*. Other components of coffee have been related to favorable modifications in liver enzymes such as γ-glutamyltransferase and aminotransferase activity. Also the detoxification metabolism *via* induction of glutathione-S-transferase and inhibition of N-acetyltransferase is modified by coffee ingestion *(Gomaa et al., 2008)*.

Of particular interest for the present work are the effects of coffee intake on cancers of the digestive tract. The relationship between coffee consumption and colorectal cancer risk has been extensively examined over the last decades. Although *Arab (2010)* affirmed that the evidence for cancer reduction caused by coffee ingestion is weak and differences between coffee consumers and non-consumers are possibly a threshold effect, *Galeone et al. (2010)* described a 17% lower risk of colorectal cancer for regular coffee drinkers than for non- or occasional drinkers. The protection was about 30% for the highest coffee drinkers. A significant reduction in the risk of rectal cancer with decaffeinated coffee has also been reported in a prospect cohort study *(Michels et al., 2005)*. Results from case–control studies suggest a significant decrease of the risks of colorectal cancer and colon cancer by coffee consumption *(Li et al., 2013)*. In a recent large cohort study, coffee was inversely associated with colon cancer, particularly proximal tumors *(Sinha et al., 2012)*. *Tian et al. (2013)* confirmed, in a dose-response analysis, a significant association between coffee consumption and decreased risk of colorectal and colon cancer. The meta-analysis of cohort studies conducted by *(Yu et al., 2011)* also showed coffee drinking to be consistently associated with a reduced risk of colorectal cancer. Although some studies question the relevance of the protective impact, even small effects on colorectal cancer in persons could have a large impact on public health, because of the high consumption of coffee *(Yu et al., 2011; Je et al., 2009)*.

Furthermore, a negative association between regular moderate coffee consumption and a reduced risk of developing type-2 diabetes is well-documented in several studies. An extensive meta-analysis found an inverse log-linear relationship between coffee

consumption and subsequent risk of diabetes *(Huxley et al., 2009)*. A population-based cohort study found coffee consumption to be associated with a substantially lower risk of this disease *(van Dam, 2008; van Dam and Feskens, 2002)*. In a prospective cohort investigation, *van Dieren et al. (2009)* found the notable reduced risk of developing type-2 diabetes (about 42% protection by 3 daily cups of coffee) not to be caused by caffeine. Also *Bhupathiraju et al. (2013)* described a prospective association of coffee intake with a lower risk of type-2 diabetes, irrespective of the caffeine content. A multi-ethnical prospective cohort showed a protective effect of coffee consume against diabetes, more explicit in women than in men *(Doo et al., 2013)*. Moderate coffee consumption was also significantly associated with lower prevalence of metabolic syndrome *(Matsuura et al., 2012; Takami et al., 2013)*, condition known to increase the risk of developing cardiovascular disease and diabetes.

Unambiguous inverse relation of coffee consumption and Parkinson's disease has been described in the literature. An up to 5-fold reduced incidence of Parkinson's disease for those drinking more than 4 cups of coffee a day was observed in prospective investigations *(Ross et al., 2000)*. The most probable mechanism for the antiparkinsonian effect is the antagonist action of caffeine over adenosine A_2A receptors, which are located in the striatum, the cerebral region involved in locomotion control and motor function, dramatically impaired in Parkinson disease *(Fenu and Morelli, 1998; Chen et al., 2001; Morelli et al., 2009)*. In fact, meta-analysis found that the overall risk of developing Parkinson diminished up to 32% per 300 mg increase in caffeine intake (approximately 3 cups of coffee). This inverse correlation could not be explained by bias or uncontrolled confounding factors *(Hernán et al., 2002; Costa et al., 2010)*. An overview on the numerous data is available in comprehensive reviews *(Hernán et al., 2002; Costa et al., 2010; Elbaz and Moisan, 2008; Butt and Sultan, 2011)*.

Also well established is the protective effect of coffee intake against age related dementia. Numerous epidemiological studies suggest that coffee could be effective against Alzheimer's disease *(Arendash and Cao, 2010; Maia and De Mendonça, 2002; Lindsay et al., 2002; Eskelinen et al., 2009; Cao et al., 2012)* and reduce the risk, or delay the onset, of dementia *(Hernán et al., 2002; Cao et al., 2012; Rees et al., 1999)*. A systematic literature review of human studies estimates a lower cognitive decline of coffee consumers in comparison with non-consumers, especially among women *(Arab et al., 2013)*.

Coffee drinking seems to have anticaries effects, which can be due to the interference with bacterial adhesion and coaggregation mechanisms or inhibition of key enzymes in biofilm development *(Gazzani et al., 2012)*. The importance of melanoidins has been proposed *(Stauder et al., 2010)*. Also polyphenols are believed to contribute to oral health *(Varoni et al., 2012)*

A general anxiety issue concerns possible negative effects of maternal caffeine ingestion on fetal, neonatal and maternal outcomes. In a recent cohort study neither fetal growth restriction nor reduced gestation length mediated by coffee consumption, nor developmental programming influences of intrauterine exposure to caffeine on children behavior could be observed *(Loomans et al., 2012)*. A systematic Cochrane review found insufficient evidence to confirm or refute the effectiveness of caffeine avoidance on birth weight or other pregnancy outcomes *(Jahanfar and Sharifah, 2013)*.

In general, although the epidemiological research approaching the influence of coffee consume in health issues is just beginning, and discrepant results and extremely heterogeneous quality of experimental designs difficult unequivocal statements about the correlation of coffee ingestion and the health of populations, the presence of coffee in the diet seems to have a clear positive overall contribution to life quality.

2.2.2 Coffee melanoidins

The characteristic aroma, taste, and color of coffee beverage are developed upon roasting of the beans. Coffee melanoidins are final products of the Maillard reaction, constituted of a heterogeneous macromolecular material containing nitrogen, which responds for the brown color of the coffee brew. They account for around 25% of total solids in coffee infusions, making these beverages one of the main sources of melanoidin in human diet.

2.2.2.1 Chemistry of coffee melanoidins

The chemical structure and properties of coffee melanoidins are very complex and not completely elucidated. It is even unclear whether proteins or polysaccharides are the backbone in coffee melanoidins. On account of the obscure nature of food melanoidins, their amount in coffee is often equated to the proportion of structurally unknown compounds *(Gniechwitz et al., 2008b)*. Probably all the structures proposed for melanoidins until today, briefly presented in Section 2.1.1, are found among coffee melanoidins, and they may even occur

within the same melanoidin complex *(Bekedam et al., 2006; Bekedam et al., 2008c; Gniechwitz et al., 2008b)*. Despite the uncertainty about their exact structure, voluminous scientific production assert that coffee brew melanoidins are anionic, structurally diverse polymers and contain thermally transformed polysaccharides, proteins, and phenolic compounds, showing metal chelating capacity *(Nunes and Coimbra, 2007; Bekedam et al., 2006; Reichardt et al., 2009; Nunes and Coimbra, 2010; Bekedam et al., 2008a; Bekedam et al., 2008c; Bekedam et al., 2007; Gniechwitz et al., 2008b; Rawel et al., 2005)*. In sequence, a short recapitulation of the principal facts respective to the chemical structure and origination of coffee melanoidins is presented.

Takenaka et al. (2005) observed that the metal-chelating brown melanoidinic polymer from coffee is derived from chlorogenic acids, carbohydrates and proteins. Even though it is difficult to differentiate proteins, polysaccharides and melanoidins in the high-molecular-weight fraction of coffee brews, *Nunes and Coimbra (2007)* isolated a whole range of melanoidin fractions from coffee brews by means of differentiated precipitation ethanol solutions, anion exchange chromatography and immobilized copper-chelating chromatography. They concluded that coffee melanoidins are a heterogeneous mixture, differing in characteristics and contents of carbohydrates, proteins, and unknown compounds (chromophores, phenolics). In his PhD thesis *Bekedam (2008)* concludes that "no melanoidin molecule is alike" and suggests the major relevance of the investigation of general structural properties of coffee melanoidins in detriment to chasing the elucidation of the exact chemical structure of a melanoidin molecule. As a matter of fact, intensive research has been done focusing the involvement of chlorogenic acids and carbohydrates in coffee melanoidin formation as well as on digestibility, ionic charge properties, molecular size properties, and acidifying properties of melanoidins *(Bekedam et al., 2008c)*.

The two main polysaccharides populations in coffee brews are galactomannans and arabinogalactans; the latter are predominantly present in arabinogalactan-proteins. *Nunes et al. (2006)* identified brown-colored structures at the reducing end of galactomannans, showing that galactomannans may be involved in melanoidin formation. However, the contribution of these structures to the overall melanoidins content is rather limited, since each galactomannan molecule has only one reducing group. Furthermore, the increase in galactomannan content in the extractable fraction of coffee cannot be correlated to the increase in melanoidin contents of the brews. Therefore, the results imply that galactomannans are not the main polysaccharide involved in melanoidin formation. With

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respect to arabinose, it can be suggested that this carbohydrate is involved in melanoidin formation in form of arabinogalactan-proteins *(Bekedam et al., 2007)*. An inverse correlation between arabinose content and melanoidin level could suggest that chromophores might be formed from or attached to the arabinose moiety from arabinogalactan-proteins upon roasting *(Bekedam et al., 2008c)*. However, melanoidins can vary respectively their total carbohydrate contents and proportions, degree of branching of the arabinogalactans, and conservation of the AGP structures *(Nunes and Coimbra, 2007; Bekedam et al., 2006; Bekedam et al., 2007)*. Contrary to earlier broadly assumed theory, *Bekedam et al. (2007)* found that the notorious negatively charged groups of coffee melanoidins, responsible for characteristics as metal chelation and aroma binding, are not originated exclusively from uronic acids or from amino acids from original arabinogalactan-proteins (described in Section 2.2.1.2). The authors concluded that other acid groups, at that time still of unknown nature, must be incorporated to the melanoidin structure.

Differently to the carbohydrate fraction, just a few studies investigate the role of coffee proteins in melanoidin formation. During roasting, the protein fraction of coffee beans undergoes substantial changes and acquires negative charge density *(Nunes and Coimbra, 2007)*. *Montavon et al. (2003)* observed a synchronized disappearance of the intracellular 11S storage protein and chlorogenic acids upon roasting. The protein subunits were observed to be integrated into the polymeric structure of melanoidins. *Nunes and Coimbra (2007)* found a significant amount of coffee melanoidins to be originated from cell walls, which indicates the involvement of other proteins besides 11S in the formation of coffee melanoidins. Also proteins from arabinogalactan-proteins, which contain hydroxyproline, are incorporated in melanoidins *(Bekedam et al., 2007)*. After roasting, alanine, aspartic acid/asparagine, glutamic acid/glutamine, and glycine are the most abundant amino acids in the melanoidin fraction, whereas histidine, lysine, methionine, and tyrosine are rather rare and arginine is not present at all *(Nunes and Coimbra, 2007; Bekedam et al., 2006; Moreira et al., 2012)*. A roast-dependent presence of Maillard reaction products derived from lysine (N^ε-fructosyllysine, N^ε-carboxymethyllysine and N^ε-carboxyethyllysine) in the high molecular fraction of coffee brews has been reported as well *(Nunes and Coimbra, 2007)*.

The decrease in the content of water extractable chlorogenic acid by more than 50% upon coffee roasting is not explained by single formation of flavor compounds *(Moon et al., 2009)*. Also their incorporation in other molecules is responsible for the loss of detectable free phenolic compounds. Although the participation of chlorogenic acids and their

derivatives in the genesis of coffee melanoidin has been suspected for decades *(Maier et al., 1968; Heinrich and Baltes, 1987)*, recent research shows a far more important role of chlorogenic acid incorporation in coffee melanoidin than expected until now *(Reichardt et al., 2009; Bekedam et al., 2008a; Montavon et al., 2003; Gniechwitz et al., 2008b)*. *Montavon et al. (2003)* suggested a linkage of polyphenols to subunits of the storage protein S11 as important for the formation of coffee melanoidins. In the light of the state of knowledge at that time, the authors presumed the incorporation into the polymeric matrix to happen via ester, ether, and peroxy links. Also *Rawel et al. (2005)* described an increasingly formation of covalent linkages between coffee polyphenols and proteins upon roasting. *Takenaka (2005)* detected several phenolics in the decomposed products of alkaline hydrolyses of the high molecular weight melanoidinic material isolated from coffee. *Nunes and Coimbra 2007)* proposed likewise the involvement of phenolics condensation in melanoidin formation. In this study, no release of monomeric phenolic compounds could be detected after alkaline hydrolysis of the high-molecular-weight melanoidins, neither at room temperature nor at 100°C. Not even by pyrolysis-GC-MS or after treating the samples with NaCl, aiming to release ionic-bound phenolic compounds, monomeric phenolic compounds could be observed. As the only method able to release small amounts of monomeric phenols was alkaline fusion, which is a method known to release condensed phenolic structures, the authors suggest a covalent incorporation of the chlorogenic acids in the melanoidin structure.

The extensive study from *Bekedam et al. (2008a)* brought many important facts to the discussion. Quinic acid was shown to correlate directly with the negative charge of melanoidins, supporting the hypothesis that quinic acid is not linked via its carboxyl group, i.e. that chlorogenic acids are not esterified in the melanoidin skeleton. Quinic acid level correlated also directly with total phenolics, measured by Folin-Ciocalteu, indicating that quinic was incorporated to a similar extent as the polyphenolic moiety, possibly being the whole chlorogenic acid molecule incorporated in the melanoidins. Enzymatic experiments confirmed the incorporation of intact chlorogenic acids to the melanoidin skeleton. They propose the incorporation of intact chlorogenic acids in melanoidins upon roasting via caffeic acid moiety, through mainly nonester linkages.

On the other hand *Gniechwitz et al. (2008a* and *b)* questioned the incorporation of intact hydroxycinnamates from chlorogenic acids in coffee melanoidins, supposing their incorporation as condensed phenolics. They suggested an incorporation via reactions

leading to loss or modification of their propenyl side chains, as these could not be detected in by NMR-spectroscopy using two-dimensional heteronuclear single quantum coherence (HSQC).

Also the work from *Perrone et al. (2012)* described the intensive involvement of polyphenolic compounds in the formation coffee melanoidins. They supported the proposal that the incorporation mechanism occurs via the caffeic or ferulic acid moiety, mainly through nonester linkages. The incorporation of chlorogenic acid lactones into melanoidins is likewise proposed. The authors explained the roast dependent increase in the dihydrocaffeic acid content in melanoidin samples, detected after saponification, as a sign of oxidation of chlorogenic acids posterior to incorporation.

Recent reviews summarize the most important findings relative to the formation of coffee melanoidins and try to schematically represent the knowledge cumulated in the last years *(Nunes and Coimbra, 2010; Moreira et al., 2012)*. A simplifying illustration can be seen in Figure 2-7.

In conclusion, it can be said that that proteins and chlorogenic acids are primarily involved in melanoidin formation, and arabinogalactans seem to be more involved in melanoidin formation than galactomannans *(Bekedam et al., 2006; Bekedam et al., 2008a; Bekedam et al., 2007)*. Despite the recent advances in the field, the nature of the meanwhile uncontested covalent linkage of chlorogenic acids to proteins, as part of the structural skeleton of the coffee melanoidins, is yet unknown *(Nunes and Coimbra, 2010)*. As the phenolic acid moiety from chlorogenic acid is far more prone to oxidative changes than the quinic acid, it is generally assumed that the phenolic acid is likely the moiety which participates in the chemical reactions during roasting *(Bekedam, 2008)*. However, the formation of phenolate radicals and modification of the otherwise quite stable aromatic moiety may not be the main incorporation mechanism, as still assumed, probably due to the (biosynthesized) examples of condensed polyphenols found in nature, as lignins and melanins. Additionally, a recent model study questioned the importance of oxidative reactions in the formation of heat degradation products of polyphenols *(Moon and Shibamoto, 2010)*. Possibly, the incorporation of the intact chlorogenic acids in the melanoidins structure occurs through paths already not investigated.
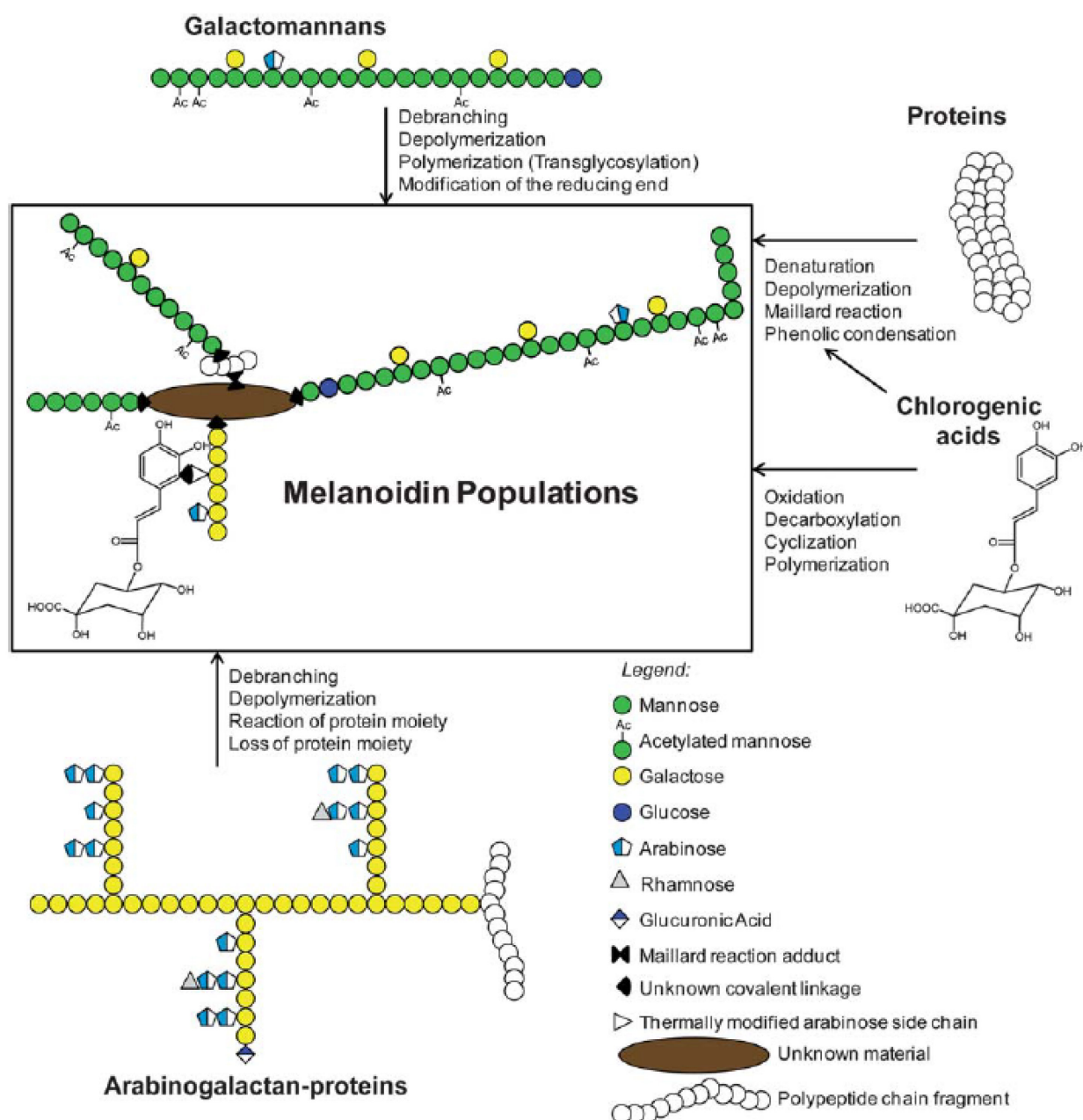
Figure 2-7: Systematization of the state of knowledge relative to the formation of coffee melanoidins. Illustration extracted from *Moreira et al. (2012)* modifying *Nunes and Coimbra (2010)*

2.2.2.2 Properties of coffee melanoidins

Coffee is the main melanoidin source in human diet, attributing primordial relevance to their biological activities and impact on health. Also due to water solubility and abundance, they are the most extensively studied food melanoidin system. Melanoidins are of interest because of their contribution to the color of coffee brew but also for their flavor-binding properties *(Hofmann et al., 2001; Hofmann and Schieberle, 2002)*, antioxidative capacity *(Delgado-Andrade and Morales, 2005; Borrelli et al., 2002; Wen et al., 2005)*, metal-

chelating properties *(Takenaka et al., 2005; Morales et al., 2005)*. Also their high content on polyphenols can have a positive impact on health. Many of the health promoting activities of coffee described in Section 2.2.1.3 can be most probably attributed to the melanoidin component of the brew.

Garsetti et al. (2000) reported a coffee consumption-dependent antioxidant capacity of human feces and attributed the effect to the coffee melanoidins. They noted that the ingestion of whole grain, fruit and vegetable did not cause a detectable alteration. In fact, the antioxidative properties of coffee melanoidin are unquestionable. During the roasting of coffee beans, the antioxidative capacity of coffee increases consistently *(Vignoli et al., 2011; Delgado-Andrade and Morales, 2005; Borrelli et al., 2002; del Castillo et al., 2002; Nicoli et al., 1997; Bekedam et al., 2008b; Sacchetti et al., 2009; Delgado-Andrade et al., 2005)*. The modification of antioxidant properties of coffee and coffee melanoidins caused by the thermal treatment has been object of intensive investigation in recent years. The antioxidant effect is ascribed to the ability of melanoidins to donate hydrogen and break radical chain, to reduce hydroperoxide to non-radical products, or to scavenge oxygen radical but also their actuation as metal chelator may play a role on the measured antioxidant capacity *(Wijewickreme and Kitts, 1998; Moreira et al., 2012; Morales and Jiménez-Pérez, 2004)*. Dependent of the chemical principle of the assay, the improvement in the antioxidant activity persists until the end of roasting *(Richelle et al., 2001)* or decreases from medium to dark roast *(del Castillo et al., 2002; Nicoli et al., 1997)*. Many authors considered the formation of antioxidant Maillard reaction products as responsible for the maintained antioxidant activity of coffee brew over roasting process, compensating the decrease on detectable chlorogenic acids caused by the thermal treatment *(Delgado-Andrade and Morales, 2005; Delgado-Andrade et al., 2005)*. Since the proposal of the incorporation of chlorogenic acids in the melanoidin skeleton as a partial cause of the loss upon roasting of detectable coffee polyphenolics, the crucial contribution of chlorogenic acids to the antioxidant properties of coffee melanoidins has been established. In addition, the formation of roast-induced antioxidative structures is still recognized, although the relative importance of the different moieties is still unclear *(Nunes and Coimbra, 2007; Adams et al., 2005; Bekedam et al., 2008a; Bekedam et al., 2008b; Delgado-Andrade et al., 2005)..*

Dietary phenolic compounds have shown to influence positively the pathology of degenerative diseases *(Crozier et al., 2009)*. Even though most of the chlorogenic acid incorporated in high molecular weight melanoidins cannot be absorbed and will pass to the

large intestine, it can still have positive impact on both colonic health and the colonic microbiota *(Crozier et al., 2010)*. The indigestible coffee melanoidins can hence be defined as antioxidant dietary fiber, which diminishes oxidative damage to intestinal mucosa, and potentially reducing the risk of colon cancer and inflammatory conditions *(Bianchi et al., 2010; Gökmen et al., 2009; Vitaglione et al., 2008; Vitaglione et al., 2012; Garsetti et al., 2000)*.

Although innumerous model studies investigated the metal chelating ability of Maillard reaction products and it is long accepted that ketones and hydroxy groups of pyranones and pyridines can be present in melanoidins and act as chelating moieties *(Wijewickreme and Kitts, 1998; Takenaka et al., 2005; Rufian-Henares and de la Cueva, 2009)*, it was recently reported that the classical cromophoric groups formed through the Maillard reaction are not the main coordination sites for metal complexation in coffee melanoidin *(Morales et al., 2005)*. *Takenaka (2005)* observed a positive correlation between chlorogenic acid content and zinc chelating abilities in melanoidin models. They suggested the polyphenols to be a key component for the metal binding capacity of coffee melanoidin.

Rufian-Henares and Morales (2007) described the inhibition of Angiotensin Converting Enzyme (ACE), an upmost important target in the treatment of hypertension, caused *in vitro* by coffee melanoidins. The effect was significantly higher for melanoidins formed at more severe heating conditions and could not be reverted by removal of non-covalently bound chlorogenic acids. ACE is a zinc-dependent peptidase and the drugs designed to control its activity pursue a zinc binding moiety. The causal relation between metal chelation ability and enzyme inhibition can be speculated.

Probably, the antimicrobial effect of coffee melanoidin is also closely related to their metal-chelating activities. In low concentrations, they seem to sequestrate essential minerals, impairing the growth of microorganism. In high concentration they chelate the stabilizing $Mg(II)$, causing membrane disruption *(Rufian-Henares and de la Cueva, 2009)*. While the presence of coffee melanoidins seem to inhibit the growth of microbiological pathogens, prebiotic potential of food melanoidin has been described in the literature, where the fermentability of melanoidins by the intestinal flora, particularly by Bifidobacteria strains, was reported *(Gniechwitz et al., 2008a; Reichardt et al., 2009; Borrelli and Fogliano, 2005)*.

As already mentioned, the regular ingestion of pure coffee may reduce development of dental plaque and caries, as the melanoidin fraction show anti-adhesive and anti-biofilm activity, diminishing the invasion in host dental tissue *(Daglia et al., 2002; Stauder et al., 2010)*.

Evidence of anti-inflammatory action of coffee melanoidins has been reported. *Goya et al. (2007)* described a protective effect of coffee melanoidins against oxidative stress in hepatoma cells. *Vitaglione et al. (2010)* observed a reduction of pro-inflammatory cytokines and increase of anti-inflammatory cytokines caused by coffee melanoidins ingestion in a rat model steatohepatitis. High molecular weight Maillard reaction products formed upon coffee roasting were recently shown to induce cytoprotective enzymes by activating the transcription factor Nrf2 (Nuclear factor erythroid-derived 2-related factor 2) in different cell types and in intact gut tissue, having potentially beneficial effects on gut barrier dysfunction, intestinal mucosal injury, some intestinal inflammation types as well as possibly on prevention of colorectal cancer. The effect was discussed as caused by the antioxidative capacity of these products *(Sauer et al., 2013)*.

The research field involving the effect of coffee and coffee melanoidins is just emerging. The improvement of the knowledge about the mechanism of formation, chemical features and structural characteristics of coffee melanoidins, will increasingly inspire life science hypotheses respective to the influence of coffee melanoidins on human health. Reciprocally, new experimental and epidemiological findings arises chemical questions and investigations in a very exciting collaboration work.

2.3 Zinc metallopeptidases

Based on the mechanism of catalysis, mammalian proteases are classified into five distinct classes: aspartic, cysteine, serine, threonine, and metalloproteases *(Zucker and Cao, 2010)*. Metalloproteases are the most diverse of the four main types of protease, with more than 50 families identified to date *(Rawlings and Barrett, 1995)*.

Zinc metalloproteases are the hydrolases in which the nucleophilic attack on the scissile peptide bond is mediated by a water molecule coordinated to a Zn(II). The catalytic metal ion is generally tetrahedrally bond to three donor groups present within the active site from the enzyme, most frequently the side chain moieties of the amino acid His, Glu, and Asp.

Histidine coordinates via its imidazole substituent as a neutral donor, while glutamate and aspartate coordinate via their anionic carboxylate substituents. The catalytic properties of enzymes are influenced by the nature of the donor groups and the different spacer lengths between the coordinating residues. Typically, two of the coordinating amino acids are separated by 1-3 residues, while the third is separated by a long spacer of 5-196 residues *(Rawlings and Barrett, 1995; Gupta, 2007; Supuran et al., 2002)*

This broad class of peptidases represent high-value medicinal targets, as their disturbed activity is associated with many illnesses such as cancer and inflammatory, infectious, cardiovascular, and neurodegenerative diseases. The presence of the metal ion in these enzymes has been frequently exploited for the development of synthetic inhibitors. The vast majority of metalloprotein inhibitors, either under investigation or in clinical use, employ metal-binding groups, for interacting with the active site metal ion, coupled to small-molecule, which mimics of the natural peptide substrates and promotes an efficient interaction with substrate domains of the enzyme. Nowadays, metalloprotease inhibitors are not limited to substrate derived compounds. Also non-peptidomimetic structures are object of intensive investigation aiming to increase activity and specificity, reducing the collateral effects of the pharmacological therapy *(Jacobsen et al., 2010; Rouffet and Cohen, 2011)*.

2.3.1 **Matrix Metalloproteinases (MMPs)**

Almost 50 years ago, the interstitial collagenase, the first MMP family member identified, was discovered in experiments designed to explain how the collagen-rich tail of the frog is resorbed during metamorphosis. After identification of a similar collagenase in human skin, this protease was named MMP-1. Since then, a whole family of related enzymes has been identified in species from hydra to humans. They were collectively called matrix metalloproteinases (MMPs) because of their dependence on Zn(II) ions for catalytic activity, their potent ability to degrade structural proteins of the extracellular matrix (ECM), and specific evolutionary sequence considerations that distinguish them from other closely related metalloproteinases *(Zucker and Cao, 2010; Klein and Bischoff, 2011; Sternlicht and Werb, 2001)*.

These enzymes have both a descriptive name, typically based on a preferred substrate, and a MMP number, based on the order of discovery. The initial classification according to preferred substrates separates the MMPs in collagenases, which are able to cleave triple

helical collagen at a single site across the three chains, gelatinases, which hydrolyze denatured collagen and gelatin, stromelysins, which have broad substrate specificity and degrade many proteoglycans, and other MMPs, with indefinite biologic functions *(Zucker and Cao, 2010; Sela-Passwell et al., 2010)*. Table 2-2 provides a list of the identified human MMPs. The family reflects the preferred substrates.

Table 2-2: Overview of the identified human matrix metalloproteases and their common names.

Family	MMP	Alternative names
Collagenases	1	Collagenase-1, fibroblast collagenase
	8	Collagenase-2, neutrophil collagenase
	13	Collagenase-3
	18	Collagenase-4
Gelatinases	2	Gelatinase A
	9	Gelatinase B
Stromelysins	3	Stromelysin-1, proteoglycanase
	10	Stromelysin-2
	11	Stromelysin-3
Membrane-type MMPs	14	MT1-MMP
	15	MT2-MMP
	16	MT3-MMP
	17	MT4-MMP
	24	MT5-MMP
	25	MT6-MMP
Other	7	Matrilysin-1, PUMP
	12	Macrophage metalloelastase
	19	RASI-1
	20	Enamelysin
	21	- (human ortholog of *Xenopus* MMP)
	23	CA-MMP
	26	Matrilysin-2, endometase
	28	Epilysin

Adapted from *(Jacobsen et al., 2010; Klein and Bischoff, 2011)*

Most MMPs are secreted and have their function in the extracellular environment *(Hadler-Olsen et al., 2011)*. Together, MMPs cleave and degrade virtually all other ECM

components including laminin, fibronectin, vitronectin, elastin, enactin, and proteoglycans, not only intact fibrillar collagen (types I, II, and III) as initially thought. In addition, they can process a large number of non-ECM proteins, such as growth factors, cytokines, chemokines, cell receptors, serine proteinase inhibitors and other MMPs, and thereby regulate the activity of these compounds *(Hadler-Olsen et al., 2011)*.

The regulation of gene expression can modulate the function of MMPs. A variety of soluble factors, as cytokines, growth factors and glucocorticoids, are described to adjust the expression of MMPs. Also the cell-cell contact and interaction of cells with extracellular matrix components seem to be important in the modulation, as well as epigenetic processes *(Klein and Bischoff, 2011)*. Humans possess 24 MMP genes, but only 23 MMP proteins are expressed, as MMP-23 is coded by two identical genes *(Hadler-Olsen et al., 2011)*. The multiplicity of MMPs, with distinct but somewhat overlapping functions, probably acts as a safeguard against any losses of regulatory control. Although such redundant and compensatory mechanisms are advantageous to the organism, they often confound efforts to fully understand how MMPs function *in vivo (Zucker and Cao, 2010; Sternlicht and Werb, 2001)*.

2.3.1.1 Functions of MMPs

MMPs have been demonstrated to participate in many physiological processes including tissue turnover and repair, as during blastocyst implantation, ovulation, postlactational involution, and bone resorption. MMPs have been described to have biological functions in multiple cellular processes, including proliferation, angiogenesis, migration, invasion, and host defense *(Sternlicht and Werb, 2001)*. A pathological role for MMPs in arthritis, non-healing wounds, aortic aneurysms, congestive heart failure, and other disorders has also been recognized. *(Zucker and Cao, 2010)*. MMPs are invariably upregulated in rheumatoid arthritis and malignant disease, with more severe increases often indicating a worse prognosis. An important characteristic of these diseases is the capacity of cells to cross tissue boundaries and, in the case of cancer, spread to distant sites of the body. Thus ECM-degrading enzymes must be present to break down the structural barriers to invasion *(Sternlicht and Werb, 2001)*. Since collagens represent the major structural proteins of all tissues and the chief obstacle to tumor cell migration, collagenolytic enzymes play pivotal roles in facilitating dissemination of cancer *(Zucker and Cao, 2010)*. Overexpression of many MMPs, including MMP-1, -2, and -9, has been demonstrated in human gastric and

colorectal cancers, being a sign of poor prognosis *(Zucker and Vacirca, 2004; Sinnamon et al., 2008; de Mingo et al., 2007)*.

Production of MMPs by stromal cells within a tumor, as well as cancer cells, is well established. Cancer progression is recognized to be a complex, multistage process in which the transformation from normal to malignant cells involves genetic changes that lead to numerous phenotypic alterations *(Zucker and Cao, 2010)*. MMPs appear to exert a dominant effect and have been implicated in virtually all aspects of cancer progression and dissemination. This proteolytic activity is essential for cell migration in native matrices *(Pavlaki and Zucker, 2003; Packard et al., 2009)*

Human MMP-1 (EC 3.4.24.7) is detected in a variety of physiological processes including embryonic development and wound healing, as well as in a number of pathological processes, including chronic cutaneous ulcers and different types of malignant tumors, including colorectal carcinoma, gastric carcinoma, and malignant melanoma. In cultured cells, MMP-1 is expressed by various normal cells, for example keratinocytes, fibroblasts, endothelial cells, monocytes, macrophages, hepatocytes, chondrocytes, and osteoblasts, as well as by many different types of tumor cells. MMP-1 cleaves several components of the ECM, including collagen of types I, II, III, VII, VIII, and X, aggrecan, as well as serine proteinase inhibitors, and a2 macroglobulin *(Ala-Aho and Kahari, 2005)*.

Two particular members of this family, gelatinases A and B (MMP-2, EC 3.4.24.24 and MMP-9 EC 3.4.24.35), seem to play a notably important role in tumor invasion and metastasis. These two type IV collagenases are the dominant MMPs released by most epithelial and endothelial cells. They are involved in the turnover of basement membrane collagen under basal conditions and of other matrix proteins during angiogenesis, tissue remodeling, and repair *(Demeule et al., 2000)*.

The expression of MMPs in human cancer is the result of a complex interaction between tumor cells and non-malignant stromal cells including fibroblasts, endothelial cells and inflammatory cells, which all actively participate in the production of MMPs in tumor tissue *(DeClerck, 2000)*. More recently, MMPs have also been incriminated in more complicated processes including the liberation of biologically active proteins such as cytokines, growth factors and chemokines from their membrane-anchored pro-forms *(Klein and Bischoff, 2011)*.

2.3.1.2 **Structure of MMPs**

As members of the metzincins superfamily of proteases, all MMPs show a Zn(II) ion at the catalytic center, coordinated by three histidine residues in the zinc-binding consensus sequence, protected in a hydrophobic cleft formed by a strictly conserved methionine containing β-turn, the "Met-turn", which provides a hydrophobic base for the zinc-binding site. In MMPs, the catalytic domain is approximately 165 residues in length, spherical, being the shallow substrate-binding cleft divided into an upper and a lower sub-domain. In the catalytic domain, containing the zinc-binding motif **HEXXHXXGXXH,** the three histidines bind the Zn(II) ion and the glutamate residue activates a zinc-bound H_2O molecule, providing the nucleophile that attacks the polarized carbonyl group in the scissile peptidic bond of substrate, cleaving the molecule *(Zucker and Cao, 2010; Klein and Bischoff, 2011; Hadler-Olsen et al., 2011).*

Most of the matrix metalloproteases are secreted as proenzymes and their activation occurs in the pericellular and extracellular space. MMPs possess a signal peptide, which directs these proteases to the secretory pathway.

A prodomain of around 80 residues, configured as a α-helix globular structure, confers latency to the enzymes. The interaction of the catalytic Zn(II) and a conserved cysteine residue from the propeptide, known as cysteine switch, inactivates the proMMPs, until this blockage of the catalytic center is disrupted, leading to the active enzymes. *In vivo*, a large number of proteinases, such as serine and metalloproteinases, are involved in the activation of MMPs, but several other activation mechanisms are described *(Klein and Bischoff, 2011; Hadler-Olsen et al., 2011).*

All MMPs, except for MMP-7, -23 and -26, contain a C-terminal hemopexin-like domain, containing around 200 residues, with a characteristic 3D disc-like four-bladed β-propeller structure. This domain mediates interactions with substrates and confers specificity of the enzymes. Although MMPs retain catalytic activity toward a wide range of substrates when missing this domain, the hemopexin domain is an absolute necessity for the degradation of triple-helical collagens. Improving the bonding of the substrates gelatin and collagen, the gelatinases (MMP-2 and 9) also contain fibronectin type II domains *(Zucker and Cao, 2010; Klein and Bischoff, 2011; Hadler-Olsen et al., 2011).*

As a matter of exemplification, the full MMP-2 is illustrated in Figure 2-8.
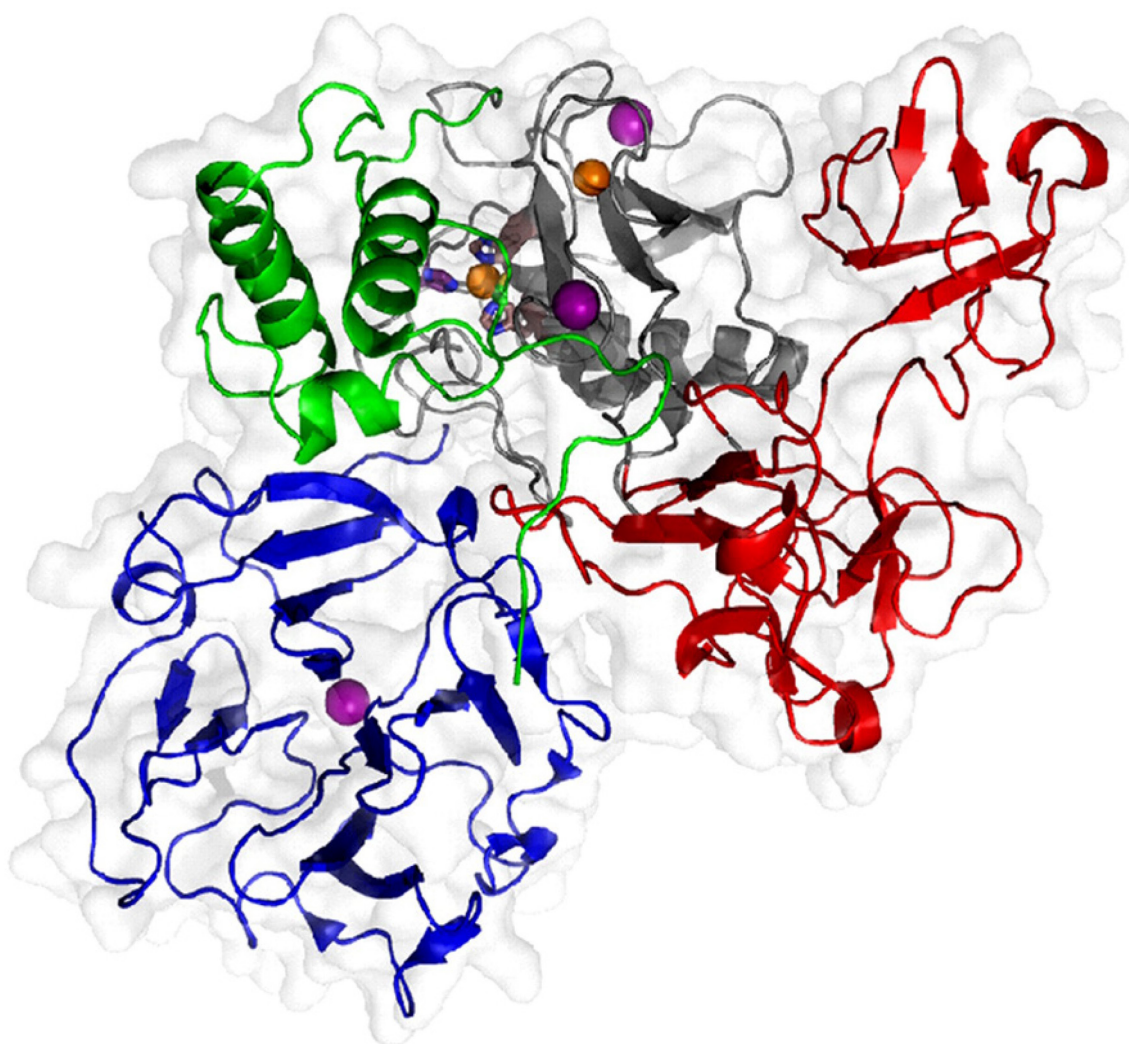
Figure 2-8: Full-length three-dimensional X-ray crystallography structure of pro-MMP-2 (PDB code 1ck7). The first atomic structure of full-length pro-MMP-2 represents the enzyme modular domain organization. The structural and catalytic zinc ions are presented as dark orange and light orange spheres respectively. The catalytic zinc ion is linked to three conserved histidines and cysteine residues. The catalytic domain is presented in gray, facing the extended catalytic pocket. The green ribbon represents the pro-domain, and the dark-blue and red ribbons represent the fibronectin residue and fibronectin repeats, respectively *(Sela-Passwell et al., 2010)*.

MMPs can be classified into two main types, depending on their specific substrate $S_{1'}$ pocket: the deep pocket enzymes (such as MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13), possessing a relatively big S1′ pocket, and the shallower pocket enzymes (MMP-1, MMP-7 and MMP-11 among others), which possess a somehow smaller specificity S1′. The S2′ and S3′ subsites are also important for the binding of inhibitors, as well as for the specificity of such inhibitors towards the different proteases. The S2′ subsite is generally a solvent-exposed cleft with a preference for hydrophobic P2′ residues, in both substrates and MMP inhibitors. The S3′ subsite on the other hand is a relatively ill-defined, solvent

exposed region *(Ilies et al., 2003)*. The active centre of a MMP and the substrate binding pockets can be seen in Figure 2-9. The binding of a collagen chain is also schematic represented.
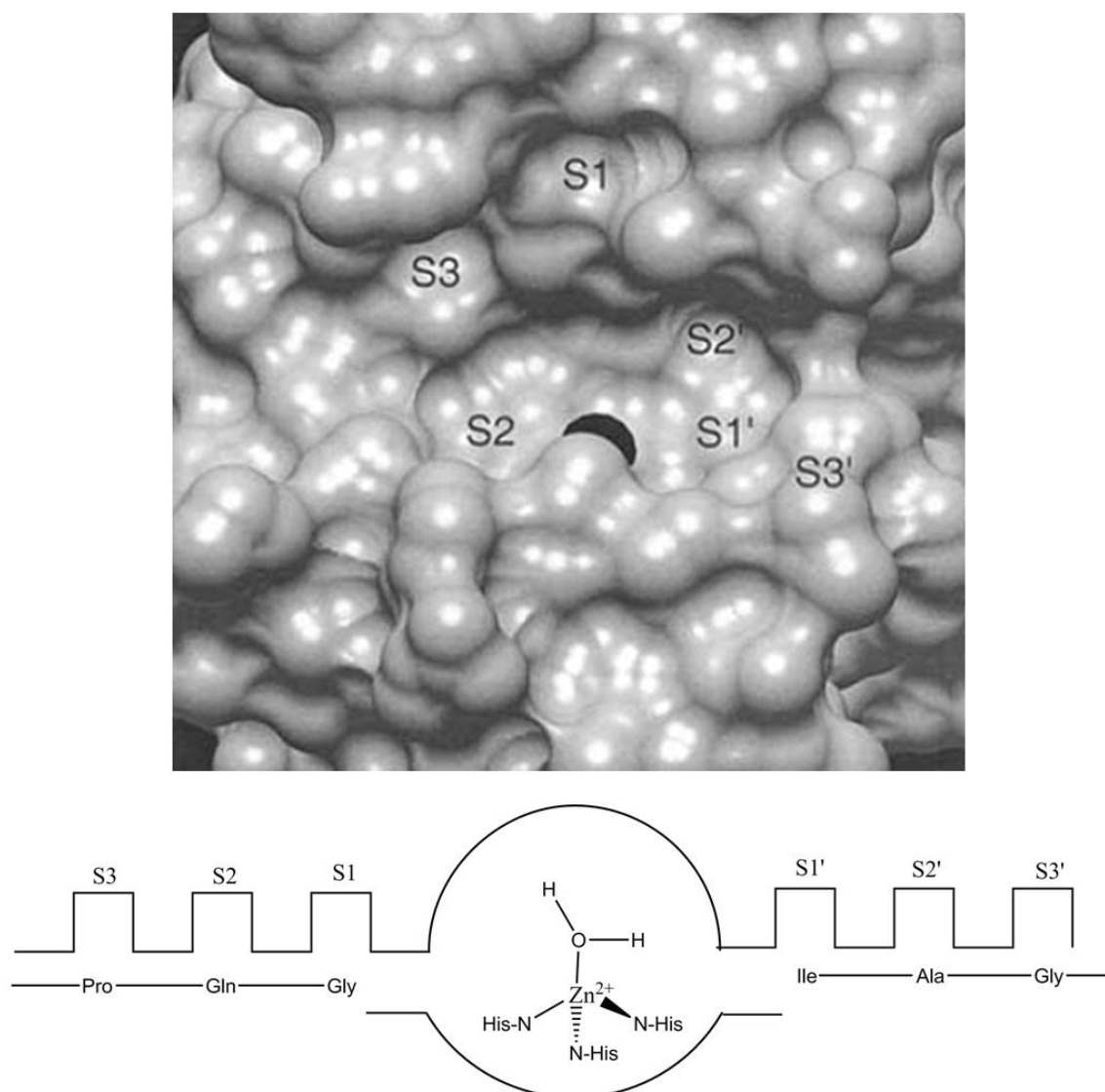
Figure 2-9: Above: Molecular surface diagram of an exemplar MMP (MMP-3). The catalytic Zn(II) is shown in black and locations of subsites are labeled. Below: Schematic figure of the MMP active site with substrate *(Puerta and Cohen, 2004)*.

2.3.1.3 Inhibition of MMPs

In vivo activity of MMPs is under tight control at several levels including gene expression, cellular compartmentalization, proenzyme activation, inhibition by protease inhibitors, and endocytosis *(Zucker and Cao, 2010)*. In the extracellular milieu, the activity of these proteases is tightly regulated by endogenous inhibitors designated as Tissue Inhibitors of

Metalloproteinases (TIMPs). Four members of the TIMP family have been so far described in human and other mammalian species. These inhibitors have many structural and functional properties in common, including a primary amino-acid sequence with 12 disulfide bonded cysteine residues and a tertiary structure that identifies two distinct domains, each containing three overlapping disulfide bonds. The N-terminal domain contains a region of higher homology among the four TIMPs and is responsible for their anti-metalloproteinase activity against all members of the MMP family. TIMPs inhibit MMPs by inserting a conserved anchor into the active site of the target MMP, which directly coordinates the catalytic zinc ion via an N-terminal cysteine residue. This conserved N-terminal segment binds the extended S1′ pocket in a substrate-like manner. Modifications that weaken the metal ion chelating ability of TIMPs, via the addition of an extra Ala at the N-terminus, or carbamylation of the N-terminal amino group, inactivate TIMPs with respect to MMP inhibition. This confirms the mechanistic importance of metal chelation by the N-terminal amino group in metalloproteinase inhibitory activity *(Sela-Passwell et al., 2010; Blavier et al., 1999)*.

Abundant literature supports the concept that TIMPs can suppress not only tumor invasion and metastasis, but also inhibit the growth of the primary tumor, due to their anti-metalloproteinase activity and their protective role on the extracellular matrix. These observations were fundamental in support of the development of synthetic MMP inhibitors in cancer clinical trials *(Sela-Passwell et al., 2010; Blavier et al., 1999)*. Controlling the enzymatic activity of specific individual MMPs by antagonist molecules has an enormous potential for therapeutic applications and has been object of intensive investigations *(Sela-Passwell et al., 2010)*. Actually, MMPs appear to be ideal drug targets, once they are disease-associated, extracellular enzymes with a dependence on zinc for activity *(Fingleton, 2008)*. Several structural classes of matrix metalloproteases inhibitors (MMPIs) have been studied by diverse methods including substrate-based design and combinatorial chemistry. The substrate-based design, which has been the principal approach for the identification of synthetic MMPIs, generally follow a two-component strategy: a peptidomimetic backbone is designed to interact in a non-covalent fashion with the MMP active site, while an appended zinc(II)-chelating moiety (zinc-binding group, ZBG) binds via coordinate–covalent bonds to the hydrolytic zinc(II) ion, rendering the enzyme inactive *(Gupta, 2007; Puerta et al., 2006)*.

The requirements for a molecule to be an effective matrix metalloprotease inhibitor (MMPI) are:

-The presence of a functional group able to chelate the active site Zn(II) ion of the enzyme such as a carboxylic group (-COOH), hydroxamic group (-CONHOH), and sulfhydryl group (-SH). Such a group is referred to as a zinc binding group, ZBG.

-At least one functional group capable of hydrogen bonding with the enzyme backbone.

-One or more side chains that can have effective van der Waals interactions with the enzyme subsites.

In Figure 2-10, the general structure of a zinc-binding inhibitor is illustrated. P1', P2' and P3' represent the groups that interact with the subsites S1', S2' and S3' of the target.
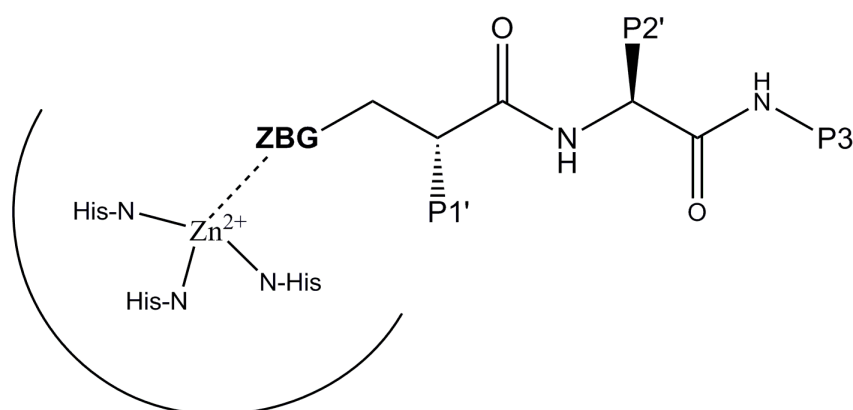
Figure 2-10: Features of a general MMP inhibitor, showing the interaction with the catalytic zinc *(Puerta and Cohen, 2004)*.

Thousands of MPPIs have been synthesized, and some have been tested in clinical trials. It is estimated that more than 90% of MPPIs contain a hydroxamic acid as ZBG. Aiming to improve the pharmacodynamic and pharmacokinetic of MMPIs, the group from Cohen studied a series of heterocyclic zinc-binding moieties and compared their metal affinity. The selected ligands selected consisted of hydroxypyridinones, hydroxypyridinethiones, pyrones, and thiopyrones. In fact, all of them demonstrated a stronger inhibitory activity against MMPs (up to 700 fold lower IC_{50}) as the control acetohydroxamic acid *(Puerta et al., 2006; Puerta et al., 2004)*. The tested moieties can be seen in Figure 2-11.

**Acetohydroxamic acid
(control)**
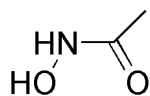
Pyrones
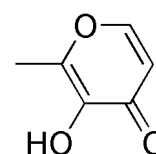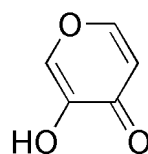
Maltol

Thiopyrones
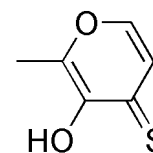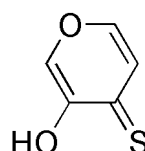
Hydroxypyridinones
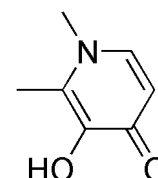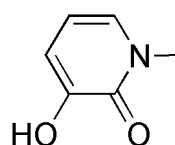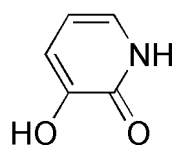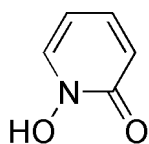
Hydroxypyridinethiones
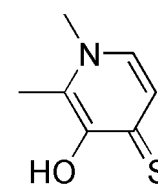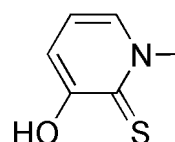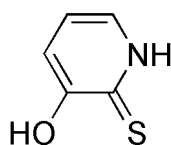
Figure 2-11: Heterocyclic zinc binding group (ZBGs) proposed for use in matrix metalloproteinase inhibitors *(Puerta et al., 2006; Puerta et al., 2004).*

However, blocking the enzymatic activity with synthetic small inhibitors appears to be an extremely difficult task. Despite tremendous efforts to explore individual members of this target family, along with multiple inhibitor classes, effective drugs for inhibiting individual MMPs have not yet emerged *(Sela-Passwell et al., 2010).* Disappointing results from clinical trials of small-molecule MMP inhibitors have prompted reconsideration of strategies for more enzyme-specific MMP inhibition by targeting the "exodomain– substrate" interactions. Such exosites or allosteric sites have been proposed to represent

unique opportunities for the design of selective inhibitors *(Sela-Passwell et al., 2010)*. The lack of clinical efficacy of MMP inhibitory drugs developed until now do not disprove the causal relationship between MMPs and cancer, but rather reflects the incomplete understanding of the complexity of cancer pathobiology and of the functions und the delicate and highly networked physiological control of this family of enzymes *(Sela-Passwell et al., 2010; Zucker and Cao, 2009)*.

2.3.2 *Clostridium histolyticum* collagenase (ChC)

Clostridium histolyticum collagenase (ChC, EC 3.4.24.3) is one of the bacterial collagenases that, like MMPs, also degrades extracellular matrix *(Gupta, 2007; Vanwart and Steinbrink, 1981)*. This enzyme is unique in that it can degrade both water-insoluble native collagens and water-soluble denatured ones, can attack almost all collagen types, and can make multiple cleavages within triple helical regions. Kinetic studies of collagenases have provided insight into the high-ordered structure of collagens *(Matsushita et al., 1998)*.

2.3.2.1 Functions of ChC

Metalloproteases are widely spread in all types of bacteria, being critical virulence factors, and playing various pathogenic roles in infection. In local bacterial infections, such as keratitis, dermatitis and pneumonia, metalloproteases act as decisive virulence determinants, being generated at the site of infection and causing necrotic or hemorrhagic tissue damage through hydrolysis of structural tissular components. In the case of systemic infections, such as septicemia, metalloproteases act as a synergistic virulence factor, causing a disordered proteolysis of various plasma proteins, inducing imbalances of the proteinase-proteinase inhibitor equilibrium, disturbing the physiological homeostasis and leading the host to an immunocompromised condition *(Supuran et al., 2002)*.

2.3.2.2 Structure of ChC

Very little is known about the structure of this zinc dependent protease. The 116 kDa protein belongs to the M-31 zinc-containing metalloproteinase family and is able to hydrolyze triple helical regions of collagen under physiological conditions, as well as an entire range of synthetic peptide substrates *(Gupta, 2007)*. In addition to the catalytic Zn(II) ion, ChC requires two structural Ca(II) ions for full activity *(Bond and Vanwart, 1984; Matsushita et al., 2001)*.

ChC has the conserved **HEXXH** zinc-binding motif, with two histidine residues (His 415 and His 419) acting as Zn(II) ligands, whereas the third ligand seems to be Glu 447, and a water molecule/hydroxide ion acting as nucleophile in the hydrolytic cleavage. ChC is also a multiunit protein, consisting of four segments, S1, S2a, S2b and S3, with S1 incorporating the catalytic domain *(Supuran et al., 2002; Scozzafava and Supuran, 2000b; Scozzafava and Supuran, 2000a)*.

The ChC enzyme catalyzes the cleavage of the Xaa-Gly (Xaa: amino acid residues) peptide bond of the repeating sequence of the native collagen in the triple helical region (-Gly-Pro-Xaa-Gly-Pro-Xaa-). It appears that S3′, S2′, and S1′ subsites of the enzyme are occupied by Gly, Pro, and Xaa, respectively *(Gupta, 2007)*.

2.3.2.3 **Inhibition of ChC**

C. histolyticum is known as an etiological agent of necrotizing fasciitis, gas gangrene and other serious complications. Isolated cases of clostridial infections evoluting to infective endocarditis from injecting drug users has been reported *(Jóźwiak et al., 2006)*. Since bacterial corneal keratitis in humans as well as animals has been reported to be associated with a highly increased bacterial collagenase activity in ocular tissues, some researchers assumed that ChC inhibitors may be of great value for putative ophthalmologic applications *(Gupta, 2007)*. Most important, due to the attributed similarity in the binding of the inhibitors with ChC and MMPs, it has been proposed that compounds which strongly inhibit ChC would also act as potent MMPs inhibitors and many compounds were studied for ChC and MMP inhibitors together *(Gupta, 2007; Ilies et al., 2003; Scozzafava and Supuran, 2000b)*.

Gupta (2007) compared synthetic inhibitors developed against this enzyme and described the following structural elements as important for an effective binding: (a) A strong zinc-binding function (like a carboxylic acid). (b) A relatively compact spacer between this function and the rest of the molecule, i.e., any amino acid moiety. (c) A variant of a benzyl group to interact with S2′site. (d) An arylsulfonyl moiety to interact with S3′ site. An example of these interactions in active centre is illustrated in Figure 2-12.
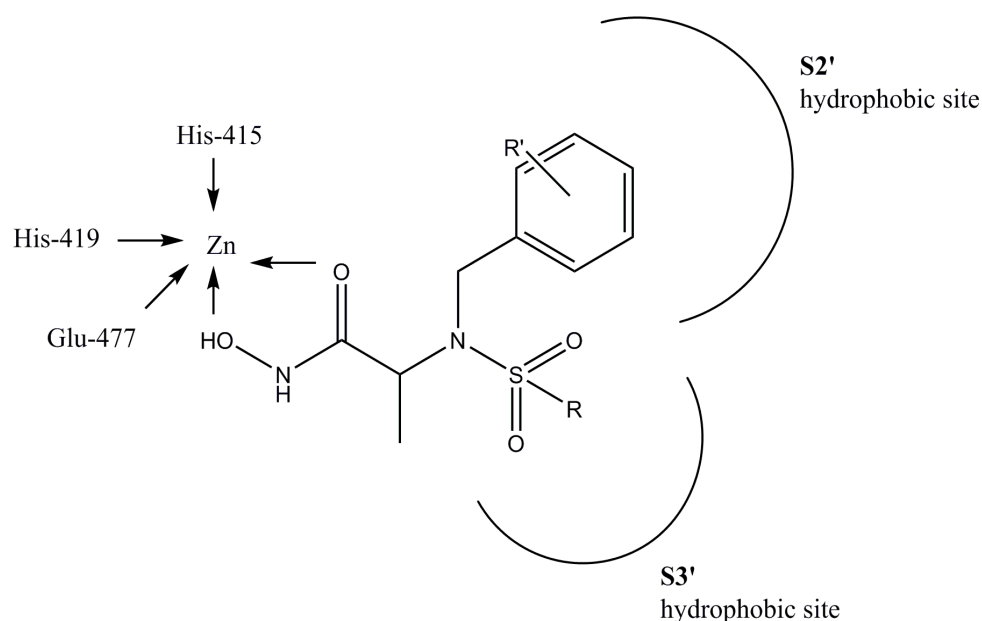
Figure 2-12: Important structural patterns for adequate ChC inhibition. *(Scozzafava and Supuran, 2000a)* adapted by *(Gupta, 2007)*.

2.3.3 Angiotensin converting enzyme (ACE)

Angiotensin converting enzyme (ACE, EC 3.4.15.1) is an evolutionarily conserved transmembrane zinc-dependent dipeptidyl carboxypeptidase expressed on the surface of endothelial cells, in epithelial or neuroepithelial cells, in the brain, and as a soluble form in blood and numerous body fluids, with orthologues known to exist in organisms as diverse as *D. melanogaster, C. elegans* and bacteria *(Lambert et al., 2010; Dive et al., 2009; Shen et al., 2008)*. In human somatic tissues it exists as a glycoprotein consisting of a single large polypeptide chain of 1277 amino acids. In germinal cells, it is synthesized as a lower molecular mass form, containing 701 amino acids *(Natesh et al., 2004; Natesh et al., 2003)*.

2.3.3.1 Functions of ACE

Angiotensin-converting enzyme is a type-I membrane-anchored dipeptidyl carboxypeptidase that is essential for blood pressure regulation and electrolyte homeostasis through the renin–angiotensin–aldosterone system *(Natesh et al., 2003)*. The central role played by ACE in cardiovascular pathologies such as hypertension and cardiac hypertrophy is well established. It hydrolyses substrates such as angiotensin I and bradykinin by removing one or more of their C-terminal dipeptides, and working as a hormone system regulating blood pressure and water balance so that, if the renin-angiotensin-aldosterone-system is too active, blood pressure will be too high. Renin, normally secreted by kidney

when blood pressure is low, converts the angiotensinogen (secreted from liver) into angiotensin I. In sequence this decapeptide is converted to the active octapeptide angiotensin II (Ang II) by ACE, which cleaves the C-terminal dipeptide from angiotensin I in the lungs. Ang II acts directly on vascular smooth muscle cells by working through the angiotensin II type 1 receptor (AT1R). The final result is a potent vasoconstrictor effect leading to higher blood pressure. Furthermore, this peptide binds to AT1R of the adrenal cortex and of noradrenergic neurons. In adrenal cortex, angiotensin II promotes vasoconstriction, aldosterone release, salt retention in the renal proximal tubules, and stimulation of the sympathetic nervous system via receptors in the brain, increasing arterial pressure *(Bader and Ganten, 2008; Lemarie et al., 2008)*. In noradrenergic neurons, this receptor-molecule interaction releases noradrenalin which acts primarily to raise systemic vascular resistance and increase heart rate and coronary blood flow, and thus for everything blood pressure rises. ACE also plays an important role in the regulation of blood pressure by hydrolyzing, and thereby inactivating, the vasodilator bradykinin, a nine amino acid peptide that causes blood vessels to enlarge, thus causing lowered blood pressure *(De Leo et al., 2009)*.

The action of ACE as the major mechanism in the biosynthesis of Ang II has made it an excellent target for therapeutic intervention in the treatment of cardiovascular diseases. While a number of ACE inhibitors have been developed and effectively used for the treatment of hypertension, adverse side effects such as persistent cough and angioedema are associated with ACE inhibition. It is believed that the accumulation of bradykinin is largely responsible for the side effects associated with ACE inhibition.

To testicular ACE (tACE), produced in germinal cells, is attributed a role in sperm maturation and binding of sperm to the oviduct epithelium *(Natesh et al., 2004)*.

2.3.3.2 Structure of ACE

Although no crystal structure for full-length ACE has already been solved, crystallographic studies on individual domains has been important for the elucidation of the enzymatic structure *(Dive et al., 2009)*. Somatic ACE (sACE), expressed on the surface of endothelial and epithelial cells in a wide variety of tissues, is composed of two homologous catalytic domains, each containing the **HEXXH**+E zinc-binding active site motif where the two histidines are zinc ligands, with a glutamate, 24 residues downstream, forming the third

ligand. The ACE ectodomain is composed of two homologous domains (N and C) connected by an interdomain linker region. Each of the distinct domains of ACE pursues a catalytic active site. Although the two domains of ACE are highly homologous, they differ in their substrate specificities, inhibitor and chloride activation profiles, and physiological functions. The active sites of both domains catalyze the hydrolysis of angiotensin I and the vasodilator bradykinin with similar efficiency. However, inhibition of the N-domain has no effect on blood pressure regulation, making the C-domain necessary and sufficient for the maintenance of proper basal blood pressure. Hence the C-domain is viewed as the main site of Ang II generation *(Dive et al., 2009; Natesh et al., 2003)*. Substrate hydrolysis in the C-domain active site is strongly activated by chloride in a substrate-dependent manner, but not the in N-domain. Therefore, Ang II formation is a highly chloride ion-dependent process, whereas bradykinin inactivation is not *(Bhuyan and Mugesh, 2011)*.

Testis ACE (tACE) is identical to the C-terminal half of somatic ACE, except for a 36-residue sequence constituting its amino terminus, making tACE a valuable model for structural investigations *(Natesh et al., 2003)*. An illustration of the structure of tACE binding to an inhibitor can be seen in Figure 2-13.
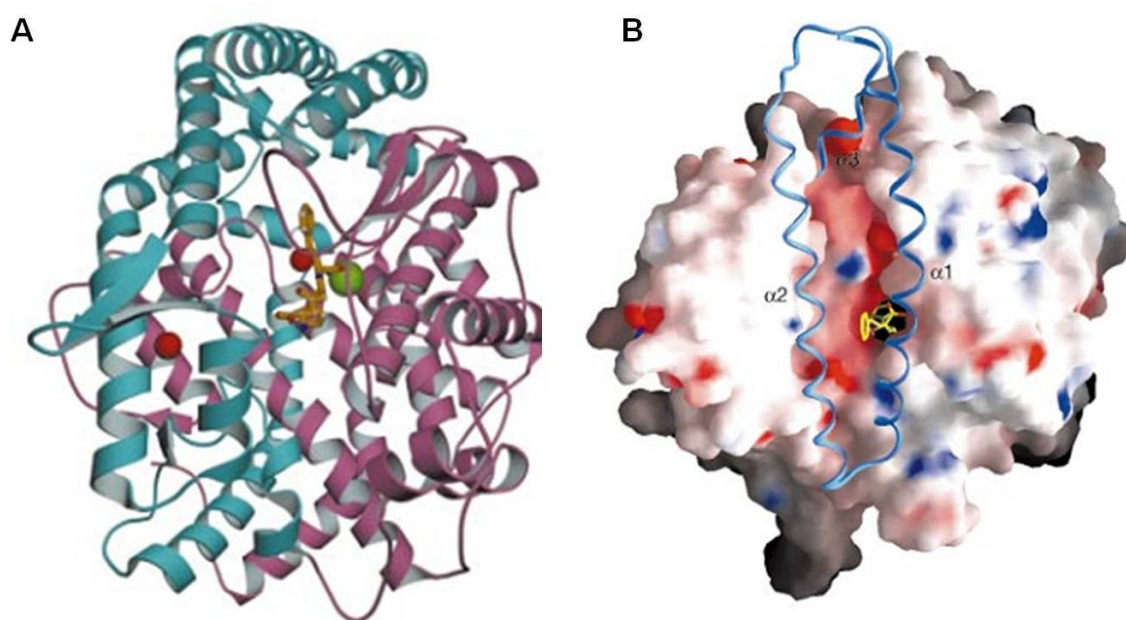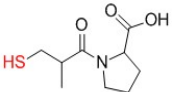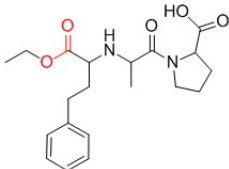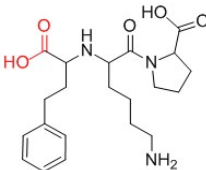
Figure 2-13: Overview of the structure of a truncated version of tACE. **A:** Stereo view of the ribbon representation of the molecule looking down on the active site. The active-site zinc ion and the ligand molecule (lisinopril) are shown in green and yellow, respectively. **B:** Molecular surface representation showing the active-site pocket. The buried lisinopril molecule is shown in yellow. Adapted from *(Natesh et al., 2004; Natesh et al., 2003)*.

2.3.3.3 **Inhibition of ACE**

ACE inhibitors have been effectively used for the treatment of hypertension. The inhibition of ACE prevents the secretion of angiotensin II, allowing the treatment of hypertension as well as congestive heart failure. However adverse side effects such as persistent cough and angioedema are associated with ACE inhibition, probably due to the accumulation of bradykinin, whose degradation is also catalyzed by this enzyme *(Lambert et al., 2010; Dive et al., 2009)*.

The first approved inhibitor was developed more than thirty years ago under the name captopril. Importantly, this substrate-derived inhibitor interacts with the active site Zn(II) ion by direct coordination through the thiol metal-binding group *(Rouffet and Cohen, 2011)*. Despite the strong zinc-sulfur interaction of captopril, second-generation ACE inhibitors ultimately replaced the thiol group by a carboxylic acid to achieve better pharmacokinetics, and carboxylate is nowadays the most frequent of the three zinc-binding moieties present at currently clinically used ACE inhibitors *(Rouffet and Cohen, 2011; Thunnissen et al., 2002; Yiotakis and Dive, 2009)*. Examples of substrate-derived ACE inhibitors are illustrated in Table 2-3.

Table 2-3: Subsite-binding amino acid residues of substrate-derived ACE inhibitors. ZBGs are marked in red.

Inhibitor	Structure	Residues		
		P1	**P1'**	**P2'**
Captopril			Ala	Pro
Enalapril		Phe	Ala	Pro
Lisinopril		Phe	Lys	Pro

Adapted from *Natesh et al. (2004).*

Many peptides derived from a multitude of plant (maize, wheat, rapeseed, soybean, buckwheat, sunflower, sorghum) and animal (milk meat eggs and fish) foodstuff show in vitro inhibitory activity against ACE, being milk the main source of antihypertensive peptides. Peptides showing inhibitory activity against ACE have been receiving much attention, as they can be a useful tool in the prevention and treatment of hypertension by being ingested alone, or in bioactive foods *(Hartmann and Meisel, 2007)*. Potent ACE inhibitory peptides from caseins and whey proteins are respectively termed casokinins and lactokinins. Encrypted ACE inhibitor peptides may be released from food proteins during in vitro and/or in vivo enzymatic degradation *(De Leo et al., 2009; Hartmann and Meisel, 2007; Hernández-Ledesma et al., 2011)*. Most of the di- and tripeptides active against ACE described in the literature have a proline residue in the C-terminal position, although tryptophan, tyrosine and phenylalanine at this position also contribute positively to the binding. The N-terminal position is usually occupied by a branched amino acid, as isoleucine, leucine or valine *(Ricci et al., 2010; FitzGerald and Meisel, 2000)*. Meanwhile, even epidemiological investigations evidence a beneficial effect of ACE-inhibiting peptides ingestion for hypertensive subjects *(Cicero et al., 2013; Cicero et al., 2010; Geleijnse and Engberink, 2010)*.

3 Experimental section

3.1 Chemicals, materials and equipment

3.1.1 Chemicals

1-Butanol , high purity (min 99%)	0772	Applichem, Darmstadt, Germany
ACE Angiotensin Converting Enzyme of rabbit lung	A6778	Sigma-Aldrich, Steinheim, Germany
Acetic acid glacial, 100%	20104.298	VWR, Darmstadt, Germany
Ala-Pro (L-alanyl-L-proline)	G-1350	Bachem, Bubendorf, Switzerland
Beta-lactoglobulin		Sigma-Aldrich, Steinheim, Germany
BSA (Bovine serum albumin)	28-4038-42	Amersham, Little Chalfont, UK
Brij-35 (polyoxyethylene(23) lauryl ether)	P1254	Sigma-Aldrich, Steinheim, Germany
Captopril (min 99.0%)	21751	Fluka, Taufkirchen, Germany
ChC Collagenase from *Clostridium histolyticum* Type VII	C-0773	Sigma-Adrich, Steinheim, Germany
CH_2Cl_2 (dichloromethane) HPLC grade	9315-02	Mallinckrodt, Deventer, Holland
Chlorogenic acid hemihydrate (min 98%)	25700	Sigma-Aldrich, Steinheim, Germany
DMSO (dimethyl sulfoxide) anhydrous (min 99.9%)	472301	Sigma-Aldrich, Steinheim, Germany
EDTA (ethylenediamintetraacetic acid)		HB Labor- und Feinchemikalien GDR, Germany
Ferritin	28-4038-42	Amersham, Little Chalfont, UK
Fluorogenic MMP substrate OmniMMP (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-Leu-N-3-(2,4-dinitrophenyl)-L-α-β-diamino-propionyl-Ala-Arg-NH_2	BML-P276	Enzo, Lörrach, Germany
Folin-Ciocalteu reagent	F9252	Sigma-Aldrich, Steinheim, Germany
Formic acid 99%	10123	Grüssing, Filsum, Germany
Gly-Ala (L-glycyl-L-alanine)	50150	Fluka, Taufkirchen, Germany
Gly-Leu (L-glycyl-L-leucine)	G 2002	Sigma-Aldrich, Steinheim, Germany
Glyoxylic acid monohydrate pure (min 97%)	50710	Fluka, Taufkirchen, Germany
Gly-Pro (L-glycyl-L-proline)	G2190	Bachem, Bubendorf, Switzerland
H_2 (hydrogen) 5.0, > 99.999%		Air Liquide, Düsseldorf, Germany
HCl (hydrochloric acid), concentrated grade (37%), p.a.	1003172500	Merck, Darmstadt, Germany

HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), min 95%	H-3375	Sigma-Aldrich, Steinheim, Germany
HHL (Hippuryl-histidyl-leucine)	M-1485	Bachem, Heidelberg, Germany
Ile-Ala (L-isoleucyl-L-alanine)	G-2370	Bachem, Bubendorf, Switzerland
Ile-Pro (L-isoleucyl-L-proline)	G-2425	Bachem, Bubendorf, Switzerland
Ile-Pro-Pro (L-isoleucyl-L-prolyl-L-proline)	H-4632	Bachem, Bubendorf, Switzerland
Ile-Trp (L-isoleucyl-L-tryptophane)	G-2435	Bachem, Bubendorf, Switzerland
KI (potassium iodide)99,5%	12044	Grüssing, Filsum, Germany
$KMnO_4$ (potassium permanganate), p.a.		Grüssing, Filsum, Germany
Leu-Leu (L-leucyl-L-leucine)	M-1535	Bachem, Bubendorf, Switzerland
Methanol, HPLC grade	20864.320	VWR, Darmstadt, Germany
MMP-1 Matrix metalloprotease 1 human recombinant catalytic domain	BML-SE180	Enzo, Lörrach, Germany
MMP-2 Matrix metalloprotease 2 human recombinant catalytic domain	BML-SE237	Enzo, Lörrach, Germany
MMP-9 Matrix metalloprotease 9 human recombinant catalytic domain, active	BML-SE244	Enzo, Lörrach, Germany
Na_2HPO_4.2 H_2O (sodium hydrogen phosphate)		Ferak, Berlin, Germany
$NaBH_3CN$ (sodium cyanoborohydride)	8.18053.0025	Merck Henbrumm, Germany
NaCl (sodium chloride), 100%	27810.295	VWR, Darmstadt Germany
NaH_2PO_4 (sodium dihydrogen phosphate) anhydrous 99.99%	1063700050	Merck, Darmstadt, Germany
NaOH (sodium hydroxide), p.a.		Merck, Darmstadt, Germany
NNGH N-isobutyl-N-(4-methoxyphenyl-sulfonyl)-glycyl hydroxamic acid	BML-PI115	Enzo, Lörrach, Germany
Ovalbumin	28-4038-42	Amersham, Little Chalfont, UK
Palladium on carbon, 10% Pd (w/w)	205699	Sigma-Aldrich, Steinheim, Germany
Pyruvic acid, for synthesis, (min 98%)	820170	Merck, Darmstadt, Germany
PzPLGL$_D$R (4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-$_D$Arg) trifluoroacetate salt	M-1715	Bachem, Bubendorf, Switzerland
Thyroglobulin	28-4038-42	Amersham, Little Chalfont, UK
TRIS, (2-amino-2-(hydroxymethyl)-1,3-pro-panediol), buffer grade	A1379	Applichem, Darmstadt, Germany
$ZnCl_2$ (zinc chloride) p.a., 98 %	31650	Sigma-Aldrich, Steinheim, Germany
o-dianisidine (3,3′-dimethoxybenzidine) p.a.		Ferak, Berlin, Germany

3.1.2 **Material**

Pipettes	Various volumes	Eppendorf, Hamburg Germany
Pipette tips	Various sizes	Brand, Wertheim, Germany
Parafilm		Pechiney Plastic Packaging, Chicago, USA
Multichannel pipette	8 channels, 5-50 μL	Abimed, Langenfeld
Cuvettes for visible spectrometry (total phenols)	1 cm path length PMMA, 4.5 mL	Brand, Wertheim, Germany
Syringe membrane filters	13 mm diameter, hydrophilic polypropylene, 0.45 μm pore size	Pall, Craislheim, Germany
Hydrolysis tubes Duran	6 mL, with screw cap and septum	Schott, Mainz, Germany
Eppendorf tubes, safe-lock	1.5 and 2 mL,	Eppendorf, Hamburg, Germany
Disposable syringes	1-10 mL	Braun, Melsungen, Germany
TCL chamber	twin-trough	Camag, Berlin, Germany
Absorption micro cuvettes	Suprasil quartz, 200-2,500 nm spectral range, path length 10 mm	Hellma Analytics, Germany
Centrifugation spin filters	Macrosep, MWCO 10 kDa	Pall, New York, USA
Glass column	Econo 2.5 x 20 cm	BioRad, Munich, Germany
Membrane filters	47 mm diameter, hydrophilic polypropylene, 0.45 μm pore size	Pall, Crailsheim, Germany
Microtiter plate (ACE and ChC assays)	96 wells, flat bottom, transparent	Brand, Wertheim, Germany
Microtiter plate (MMP assay)	96 wells, flat bottom, black	Brand, Wertheim, Germany
Paper filters	No. 288 and 292	Sartorius, Göttingen, Germany
Strongly basic anion exchange resin	Dowex 1x8, 100-200 mesh,	BioRad, Munich, Germany
TCL sheets	precoatet aluminium back, coated with silica gel 60 0.25 mm layer	Merck, Darmstadt, Germany

3.1.3 **Equipment**

Amino acid analyser (general)	S4300	Sykam, Fürstenfeldbruck, Germany
Amino acid analyser	4151 Alpha Plus	Pharmacia, Freiburg, Germany
Amino acid analysis column	Cation exchange LCA K07/Li	Sykam, Fürstenfeldbruck, Germany
Analytical balance	BP 121S, 0.0001 g precision	Sartorius, Göttingen, Germany
Analytical cation exchange	125 x 4.6, 5 μm, PEEK	K. Grüning, Olching, Germany

column (for pentosidine)

Analytical cation exchange column (for LAL)	190 x 4.6, 7 μm	K. Grüning, Olching, Germany
Analytical HPLC column	C18-Eurosphere 100, 5 μm, 150 × 4.6 mm, with integrated guard column	Knauer, Berlin, Germany
Analytical HPLC system	Smartline HPLC system: Manager K5000, pump K1000, DAD detector K2600, autosampler K3950, and column oven	Knauer, Berlin, Germany
Centrifuge (isolation of melanoidins)	5804R	Eppendorf, Hamburg, Germany
Centrifuge (coffee extract)	Cryofuge 6000i	Heraeus, Hanau, Germany
Fluorescence plate reader	Tecan Infinite F 200	Tecan, Crailsheim, Germany
Freeze dryer (small samples)	Alpha 1-2	Christ, Osterode, Germany
Freeze dryer (coffee extracts)	Beta 1-8K	Christ, Osterode, Germany
GPC column	BioSep-SEC-S3000, 300 x 7.8 mm, guard cartridge GFC 3000, (4 x 3.0 mm),	Phenomenex, Aschaffenburg, Germany
GPC system	Smartline HPLC system: manager K5000, pump K1000, DAD detector K2600	Knauer, Berlin, Germany
Hydrolysis oven	WS 986	VEB, Ilmenau, Germany
Incubation oven	ICP 400	Memmert, Schwabach, Germany
Low pressure liquid chromatography system	BioLogic LP, fraction collector 2128	BioRad, Munich Germany
Mill	Retsch GM 100	Retsch, Haan, Germany
Vortex minishaker	MS 1	IKA, Staufen, Germany
pH meter	InoLab Level 1, electrode InLab Semi-Micro	Mettler-Toledo, Weilheim
Preparative HPLC column	C18-Eurosphere 100, 10 μm, 250 × 16 mm, guard column 30 x 16 mm	Knauer, Berlin, Germany
Preparative HPLC system	Smartline HPLC system: manager K5000, pump K1000, UV detector K2500	Knauer, Berlin, Germany
Raman spectrometer	Bruker MultiRam	Bruker Optik, Etlingen Germany
Rotary evaporator	VV 2000, controller WB 2000, water aspirator	Heidolph, Schwabach, Germany

Rotary evaporator	Laborota 4000, pump MZ2C, controller CVC2	Heidolph, Wertheim, Germany and Vacuubrand, Wertheim, Germany
Ultrasonic bath	Sonorex RK 510 Super	Bandelin, Berlin, Germany
UV spectrophotometer	Ultraspec 1000 UV	Pharmacia Biotech, Cambridge, UK
Vacuum concentrator (centrifugal evaporator)	SPD SpeedVac, condensator RVT 4104, vacuum pump OFP 400	Thermo Savant, Holbrook, USA
Water purification system	Purelab plus, 1 µm filter, max conductivity 0.55 µS	USFilter, Ransbach-Baumbach, Germany

3.1.4 Solutions

The ultra pure water used for the preparation of buffers and solutions was obtained using a Purelab plus purification system (USFilter, Ransbach-Baumbach, Germany). All eluent solutions were filtrated through a membrane with 0.45 µm pores and submitted to ultrasonic degassing prior to use. Pure HPLC-grades solvents were sonicated for 15 min. The preparation of eluents, assay puffers and solutions needed for ion exchange chromatography (IEC) are described below:

ACE-assay buffer	HEPES 50 mM, NaCl 200 mM, pH 8.3:
	Solubilize 1.192 g HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) and 1.753 g NaCl in 80 mL bidistilled water, set pH at 37 °C to 8.3 using NaOH 1 M. Complete volume to 100 mL.
MMP-assay buffer	HEPES 50 mM, $CaCl_2$ 10 mM, pH 7.5:
	Solubilize 1.192 g HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), 0.148 g $CaCl_2$ and in 0,05 g Brij-35 in 80 mL bidistilled water, set pH at 37 °C to 7.5 using NaOH 1 M. Complete volume to 100 mL.
ChC-assay buffer	TRIS 50 mM, pH 7.5:
	Solubilize 0.610 g TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol), in 80 mL bidistilled water, set pH at 25 °C to 7.5 using HCl 1 M. Complete volume to 100 mL.
HPLC quantification for ACE assay	Methanol HPLC grade
	0.1% formic acid: Dilute 1 mL formic acid 99% to 1 L with ultra pure water.

HPLC quantification for ChC assay	Methanol HPLC grade
	Phosphoric acid pH 3:
	Set the pH of 900 mL ultra pure water to 3 with H_3PO_4 85%.
Gel Permeation Chromatography	Phosphate 50 mM, NaCl 150 mM, pH 6.5:
	Solubilize 4.45 g Na_2PO_4 and 4.38 g NaCl in ultra pure water to 500 mL.
	Solubilize 7.80 g $NaHPO_4$ and 8.77 g NaCl in ultra pure water 1 L.
	Achieve the desired pH by progressively mixing the solutions.
Ion Exchange Chromatography	
Regeneration	Hydrochloric acid 0.1 M:
	Dilute 83 mL 37% HCl to 1000 mL with ultra pure water.
Conditioning	Sodium hydroxide 0.1 M:
	Solubilize 80 g NaOH with ultra pure water to 2 L.
	Acetic acid 1 M:
	Dilute 57 mL glacial acetic acid with ultra pure water to 1000 mL.
Elution	Acetic acid 0.5 M:
	Dilute 29 mL glacial acetic acid with ultra pure water to 1000 mL.
	Acetic acid 1 M:
	(see Conditioning)
	Acetic acid 1.5 M:
	Dilute 86 mL glacial acetic acid with ultra pure water to 1000 mL.

3.2 Synthesis of N^α-carboxyalkylated peptides

The glycated peptides were not commercially available and were prepared from commercial L,L-dipeptides by methods described in the literature with alterations.

3.2.1 N^α-carboxyalkylation of GP, LL, IA, GA, GL, AP, IP and IPP by reductive alkylation

The carboxyalkylated derivatives of GP, LL, IA, GA, GL, AP, IP and IPP were synthesized by reductive alkylation of peptides with alpha-keto acids using the catalytic hydrogenation

for reduction of the imine intermediates according to *Liardon et al. (1987)* and *Grunwald et al. (2006)*. Commercial peptides (0.500 mmol) and glyoxylic acid monohydrate (0.65 mmol) for CM-derivatization, or pyruvic acid (0.65 mmol) for CE-derivatization, were dissolved in water (20 mL) and the pH of the solutions was adjusted to 10.0 with NaOH (1 M) prior to the addition of 50 mg Pd/C catalyst. The exact amounts of the used reagents are described in section 3.3.3. The reaction mixture was put in a round-bottom flask with a magnetic stirring bar, connected to a flow-control three way glass adapter, closed with a three-way stopcock. After removing the air in the reaction flask by using a vacuum pump connected to one of the adapter's tubes, H_2 was filled in a rubber balloon, which was also connected to the synthesis flask through the adapter. By changing the position of the three way stopcock, the H_2 was let in the reaction flask and the mixture was hydrogenated at room temperature (RT) under magnetic stirring for 48 h. The H_2 was renewed after 24 h. The catalyst was then filtered off. The solutions were directly used for the subsequent purification.

The purification of these derivates was performed by ion-exchange chromatography (IEC), as described in item 3.3.1.

3.2.2 N^α-carboxyalkylation of IW using sodium cyanoborohydride

The syntheses of the N^α-carboxymethyl- and N^α-carboxyethyl-derivatives of IW were realized based on the procedure described by *Shinonaga et al. (1994)*.

For the synthesis of CM-IW, 158.9 mg of Ile-Trp (0.5 mmol) and 147.5 mg of glyoxylic acid monohydrate (1.6 mmol) were dissolved in 20 mL methanol and 31 mg $NaBH_3CN$ was added to the system, which was stirred at 0 °C (ice bad) for 30 min. The solvent was evaporated under reduced pressure.

The residue was dissolved in 30 mL water (in exhaust hood) and the aqueous solution was extracted with 1-butanol in a separatory funnel (4 x 30 mL). The organic phase was dried overnight with Na_2SO_4, filtered and concentrated to dryness. The residue was taken up in ethanol and dried under N_2 flow. The mass spectrum of CM-IW showed the presence of a biscarboxymethylated-IW product (bis-CM-IW), and the mixture was purified by RP-HPLC, as described in item 3.3.2.

The synthesis of CE-IW was performed accordingly, starting from 158.5 mg Ile-Trp (0.5 mmol) and 141.1 mg pyruvic acid (1.6 mmol). The methanolic solution (20 mL) was stirred for 2 h at 0 °C after adding 31 mg NaBH$_3$CN. CE-IW was obtained as mass spectrometric pure light yellowish amorphous powder and no further purification was needed. The products were stored at -20 °C.

3.3 Purification

The separation of target products synthesized by catalytic hydrogenation (section 3.2.1) from the non-modified peptides was realized by ion-exchange chromatography (IEC). The separation from CM-IW from the sub-product bis-carboxymethylated IW (section 3.2.2) was performed by semi-preparative HPLC.

3.3.1 Ion Exchange Chromatographic purification

The purification of the carboxyalkylated peptides was performed by ion-exchange chromatography (IEC) after *Grunwald et al. (2006)*. The pH of the synthesis mixtures was corrected to 3.0 with glacial acetic acid, and the products were applied to the IEC column, which was subsequently rinsed with a small volume of 1 M acetic acid. The chromatographic system and conditions are described below:

System:	BioRad BioLogic LP low pressure liquid chromatography system, with fraction collector 2128
Column:	BioRad Econo glass column (2.5 x 20 cm). filled with strongly basic anion exchange resin Dowex 1x8, (100-200 mesh) 2.5 x 15 cm (approx 75 mL)
Conditioning:	1. NaOH 1 M 1 L 2. Purified water 80 mL 3. Acetic acid 1 M 250 mL 4. Purified water 500 mL
Injection volume:	1 mL
Flow:	0.6 mL/min
Elution:	A: 0.5 M acetic acid, 200 mL B: 1 M acetic acid, 200 mL C: 1.5 M acetic acid, 200 mL
Fractions:	7 mL
Regeneration:	1. HCl 1 M 500 mL 2. Purified water 500 mL

The presence of the product was monitored by spotting 5 µL of each fraction on TLC plates and detection with o-dianisidine after chlorination, as described on the following section (3.3.1.1). The spotting test showed that the products were well resolved from their educts and usually eluted with 0.5-1.0 M acetic acid, being most of them elutable with 1 M acetic acid. Fractions containing the target product were concentrated to dryness, and repeatedly taken up in water and lyophilized until the smell of acetic acid had become imperceptible, to yield white powders or needles. All products were stored at -20 °C. Mass spectra showed only signals related to the target molecules.

3.3.1.1 Spotting test

The resolution of the purification of the glycated peptides using anion exchange chromatography was monitored by spotting test using a variation of the Reindel-Hoppe reagent *(Jork et al., 1994)*.

Each fraction (5 µL) was spotted onto Merck TLC silica gel 60 plates, 0.25 mm layer. A chlorine atmosphere was produced in a twin-trough chamber by pouring 5 mL HCl (6 N) on 0.5 g $KMnO_4$ located in one of the troughs. The plate was placed in the other trough and let there for 10 min. After the excess chlorine has been removed from TCL plates by exposure to air (30 min), they were homogeneously sprayed with the detection solution. The detection solution is prepared by solving 16 mg o-dianisine (3,3′-dimethoxybenzidine) in 3 ml glacial acetic acid, diluting the solution with 50 mL water and adding 100 mg potassium iodide.

3.3.2 HPLC purification of CM-IW

The CM-IW synthesis mixture obtained as described at 3.2.2 was dissolved in 15 mL of a 50% methanol/water solution and 1 mL of the solution was injected in the system and under the conditions described below:

Instrument:	Knauer Smartline HPLC system, composed by Manager 5000, Pump 1000, and UV Detector 2500
Column:	Knauer C18-Eurosphere 100, 10 µm, 250 × 16 mm, with guard column (30 x 16 mm)
Injection volume:	1 mL
Eluents:	A: 0.1% formic acid in purified water
	B: 100% Methanol

Flow: 5 mL/min

Gradient:

Time (min)	Eluent B (%)
0	20
3	20
50	80
55	100
58	100
65	20
70	20

Detection: UV, $\lambda = 220$ nm

Fractionation: Manual

CM-IW eluted between 29 and 36.5 min (maximum 32 min) and bis-CM-IW eluted between 37.5 and 42 min (maximum 40 min). The peak relative to CM-IW was collected, concentrated to dryness, taken up in ethanol and dried under N_2 flow, to yield a grayish-white amorphous powder.

3.3.3 Overview of the synthesis and elution conditions

The exact amounts of peptides and alpha-keto acid used for each catalytic hydrogenation, the elution volumes of target compounds, and their gravimetric amounts after freeze-drying are summarized on Table 3-1.

Table 3-1: Overview of reagents used for the reductive alkylation by catalytic hydrogenation, the elution volumes of each target product and the gravimetric yield after lyophilization.

	Peptide	Alpha-keto acid		Elution	Gravimetric yield
		Glyoxylic acid	Pyruvic acid		
CM-GP	86.1 mg GP (0.500 mmol)	60.0 mg (0.652 mmol)		175 mL-248 mL (0.5 M-1 M acetic acid)	100.2 mg (0.436 mmol)
CE-GP	86.2 mg GP (0.501 mmol)		57.5 mg (0.653 mmol)	161 mL-245 mL (0.5 M-1 M acetic acid)	104.2 mg (0.427 mmol)
CM-LL	122.3 mg LL (0.501 mmol)	59.9 mg (0.651 mmol)		245 mL-329 mL (1 M acetic acid)	72.4 mg (0.239 mmol)
CE-LL	122.4 mg LL (0.501 mmol)		57.1 mg (0.648 mmol)	238 mL-357 mL (1 M acetic acid)	105.5 mg (0.333 mmol)

Cont. Table 3-1

	Peptide	Alpha-keto acid		Elution	Gravimetric yield
		Glyoxylic acid	Pyruvic acid		
CM-IA	101.0 mg IA (0.499 mmol)	59.8 mg (0.65 mmol)		189 mL-238 mL (0.5 M-1 M acetic acid)	80.2 mg (0.310 mmol)
CE-IA	101.0 mg IA (0.499 mmol)		57.1 mg (0.648 mmol)	196 mL-238 mL (0.5 M-1 M acetic acid)	72.5 mg (0.264 mmol)
CM-GA	73.1 mg GA (0.500 mmol)	59.8 mg (0.650 mmol)		203 mL-245 mL (1 M acetic acid)	78.0 mg (0.382 mmol)
CE-GA	73.1 mg GA (0.500 mmol)		57.5 mg (0.653 mmol)	210 mL-245 mL (1 M acetic acid)	84.4 mg (0.387 mmol)
CM-GL	94.3 mg GL (0.501 mmol)	59.8 mg (0.650 mmol)		224 mL-301 mL (1 M acetic acid)	106.2 mg (0.431 mmol)
CE-GL	94.2 mg GL (0.501 mmol)		57.2 mg (0.650 mmol)	224 mL-280 mL (1 M acetic acid)	89.0 mg (0.342 mmol)
CM-AP	93.0 mg AP (0.499 mmol)	60.0 mg (0.65 mmol)		196 mL-266 mL (0.5 M-1 M acetic acid)	100.0 mg (0.410 mmol)
CE-AP	93.1 mg AP (0.500 mmol)		57.3 mg (0.651 mmol)	196 mL-280 mL (0.5 M-1 M acetic acid)	125. mg (0.487 mmol)
CM-IP	114.2 mg IP (0.500 mmol)	60.0 mg (0.652 mmol)		245 mL-350 mL (1 M acetic acid)	134.2 mg (0.469 mmol)
CM-IPP	162.7 mg IPP (0.500 mmol)	60.0 mg (0.652 mmol)		210 mL-224 mL (1 M acetic acid)	60.0 mg (0.157 mmol)
CE-IPP	162.7 mg IPP (0.500 mmol)		57.1 mg (0.648 mmol)	224 mL-231 mL (1 M acetic acid)	10.2 mg (0.026 mmol)

As the reagent proportions and purification conditions of both carboxyalkyl-IW derivatives differed from those used for the catalytic hydrogenation, the exact masses of reagents and target products, as well as the purification method performed are outline on Table 3-2.

Table 3-2: Overview of reagents used for the reductive alkylation of IW, gravimetric yield of target products and performed purification.

	Peptide	Alpha-keto acid		Gravimetric yield	Purification
		Glyoxylic acid	Pyruvic acid		
CM-IW	158.9 mg IW (0.501 mmol)	147.5 mg (1.602 mmol)		38.3 mg (0.102 mmol) (bis-CM-IW 42.5 mg)	HPLC as described at 3.3.2
CE-IW	158.5 mg IW (0.499 mmol)		141.1 mg (1.602 mmol)	161.1 mg (0.414 mmol)	No further purification

3.4 Characterization of carboxyalkylated peptides

3.4.1 Mass spectrometry

Mass spectra of aqueous solutions were recorded with ion trap mass spectrometer Bruker Esquire-LC 00084 instrument (Bruker Daltonics, Billerica, USA) using electrospray ionization. Calibration of the mass scale was established using an electrospray calibrant solution (Agilent, Palo Alto, CA). Two microlitres of 1 mg/mL aqueous sample solutions were injected into the spectrometer by the autosampler from a Hewlett Packard 1100 liquid chromatography system (Hewlett Packard Corporation, Palo Alto, USA) at a flow rate of 0.2 mL/min of methanol with 0.1% ammonium acetate. CM- and CE-IPP were measured in the negative mode. For all other derivates, the positive mode was used. The analyses were conducted at the Institute of Organic Chemistry, Technische Universität Dresden, by Dr. Ingmar Bauer.

3.4.2 Elemental Analysis

Elemental analysis data were obtained with a Euro EA 3000 elemental analyzer (Eurovector, Milano, Italy) at the Institute of Organic Chemistry, Technische Universität Dresden, by Anke Peritz. Elemental analysis was used to calculate the product contents of the preparations. The percentage of nitrogen in the preparation was divided by the theoretical percentage of nitrogen of the target substance and the content was expressed in per cent by weight.

3.4.3 **Analytical characteristics of carboxyalkylated peptides**

The analytical data of the obtained target products are described below.

CM-GP

Elemental analysis: $C_9H_{14}N_2O_5$, 230.1 g·mol^{-1}. Theoretical composition: C 46.95%, H 6.13%, N 12.17%, O 34.75%. Found: C 44.99%, H 5.65%, N 11.10%.

Content of product: 91.21%. Corrected yield: 79.43% (0.397 mmol).

ESI-MS: Fragmentation: + 100 V, m/z = 231.0 ([M+H]$^+$) ; 461.1 ([2M+H]$^+$), 483.1 ([2M+Na]$^+$), 691.0 ([3M+H]$^+$).

CE-GP

Elemental analysis: $C_{10}H_{16}N_2O_5$; 244.1 g·mol^{-1}. Theoretical composition: C 49.17%, H 6.60%, N 11.47%, O 32.75%. Found: C 47.02%, H 7.72%, N 10.76%.

Content of product: 94.27%. Corrected yield: 80.42% (0.403 mmol).

ESI-MS: Fragmentation: + 100 V, m/z = 245.0 ([M+H]$^+$) ; 489.2 ([2M+H]$^+$), 511.1 ([2M+Na]$^+$), 733.0 ([3M+H]$^+$).

CM-LL

Elemental analysis: $C_{13}H_{26}N_2O_5$, 302.4 g·mol^{-1}. Theoretical composition: C 55.61%, H 8.67%, N 9.26%, O 26.46%. Found: C 54.42%, H 8.39%, N 9.03%.

Content of product (N-ratio): 97.52%. Corrected yield: 46.63% (0.233 mmol).

ESI-MS: Fragmentation: + 10 V, m/z = 303.1 ([M+H]$^+$) ; 605.2 ([2M+H]$^+$).

CE-LL

Elemental analysis: $C_{14}H_{28}N_2O_5$, 316.4 g·mol^{-1}. Theoretical composition: C 56.94%, H 8.92%, N 8.85%, O 25.28%. Found: C 55.75%, H 6.21%, N 8.15%.

Content of product (N-ratio): 92.09%. Corrected yield: 61.29% (0.307 mmol).

ESI-MS: Fragmentation: + 75 V, m/z = 317.2 ([M+H]$^+$) ; 633.2 ([2M+H]$^+$); 655.4 ([2M+Na]$^+$).

CM-IA

Elemental analysis: $C_{11}H_{20}N_2O_5$, 260.3 g·mol^{-1}. Theoretical composition: C 50.76%, H 7.74%, N 10.76%, O 30.73%. Found: C 48.95%, H 7.60%, N 10.30%.

Content of product (N-ratio): 95.73%. Corrected yield: 59.06% (0.295 mmol).

ESI-MS: Fragmentation: + 10 V, m/z = 261.0 ([M+H]$^+$) ; 521.2 ([2M+H]$^+$); 543.2 ([2M+Na]$^+$).

CE-IA

Elemental analysis: $C_{12}H_{22}N_2O_5$, 274.4 g·mol^{-1}. Theoretical composition: C 52.54%, H 8.08%, N 10.21%, O 29.16%. Found: C 48.09%, H 6.05%, N 8.92%.

Content of product (N-ratio): 87.36%. Corrected yield: 46.25% (0.231 mmol).

ESI-MS: Fragmentation: + 10 V, m/z = 275.1 ([M+H]$^+$) ; 549.2 ([2M+H]$^+$); 571.2 ([2M+Na]$^+$).

CM-GL

Elemental analysis: $C_{10}H_{18}N_2O_5$, 246.3 g·mol^{-1}. Theoretical composition: C 48.77%, H 7.37%, N 11.38%, O 32.48%. Found: C 47.73%, H 7.35%, N 11.23%.

Content of product (N-ratio): 98.68%. Corrected yield: 84.93% (0.426 mmol).

ESI-MS: Fragmentation: + 25 V, m/z = 247.1 ([M+H]$^+$) ; 493.2 ([2M+H]$^+$), 515.2 ([2M+Na]$^+$), 739.0 ([3M+H]$^+$).

CE-GL

Elemental analysis: $C_{11}H_{20}N_2O_5$, 260.3 g·mol^{-1}. Theoretical composition: C 50.76%, H 7.74%, N 10.76%, O 30.73%. Found: C 47.83%, H 7.27%, N 10.05%.

Content of product (N-ratio): 93.36%. Corrected yield: 63.80% (0.319 mmol).

ESI-MS: Fragmentation: + 25 V, m/z = 261.0 ([M+H]$^+$), 521.2 ([2M+H]$^+$), 781.0 ([3M+H]$^+$).

CM-GA

Elemental analysis: $C_7H_{12}N_2O_5$, 204.2 g·mol^{-1}. Theoretical composition: C 41.18%, H 5.92%, N 13.72%, O 39.18%. Found: C 39.71%, H 5.21%, N 11.90%.

Content of product (N-ratio): 86.74%. Corrected yield: 66.24% (0.331 mmol).

ESI-MS: Fragmentation: + 100 V, m/z = 205.0 ([M+H]$^+$), 227.0([M+Na]$^+$), 409.1 ([2M+H]$^+$), 431.1 ([2M+Na]$^+$), 613.0 ([3M+H]$^+$).

CE-GA

Elemental analysis: $C_8H_{14}N_2O_5$, 218.2 g·mol^{-1}. Theoretical composition: C 44.03%, H 6.47%, N 12.84%, O 36.66%. Found: C 40.92%, H 5.52%, N 11.55%.

Content of product: 89.95%. Corrected yield: 69.62% (0.348 mmol).

ESI-MS: Fragmentation: + 10 V, m/z = 219.0 ([M+H]$^+$), 437.0 ([2M+H]$^+$), 459.1 ([2M+Na]$^+$), 655.2 ([3M+H]$^+$), 677.2 ([3M+Na]$^+$).

CM-AP

Elemental analysis: $C_{10}H_{16}N_2O_5$, 244.3 g·mol^{-1} Theoretical composition: C 49.18%, H 6.60%, N 11.47%, O 32.75%. Found: C 46.75%, H 5.07%, N 10.59%.

Content of product: 92.28%. Corrected yield: 75.64% (0.378 mmol).

ESI-MS: Fragmentation: + 25 V, m/z = 245.0 ([M+H]$^+$), 267.0 ([M+Na]$^+$), 489.2 ([2M+H]$^+$), 411.2 ([2M+Na]$^+$), 673.3 ([3M+H]$^+$), 755.3 ([3M+Na]$^+$).

CE-AP

Elemental analysis:C$_{11}$H$_{18}$N$_2$O$_5$, 258.3 g·mol^{-1}. Theoretical composition: C 51.15%, H 7.02%, N 10.85%, O 30.97%. Found: C 48.61%, H 6.71%, N 9.99%.

Content of product: 92.03%. Corrected yield: 89.54% (0.448 mmol).

ESI-MS: Fragmentation: + 25 V, m/z = 259.0 ([M+H]$^+$), 281.1 ([M+Na]$^+$), 517.2 ([2M+H]$^+$), 539.2 ([2M+Na]$^+$), 775.3 ([3M+H]$^+$), 797.4 ([3M+Na]$^+$).

CM-IP

Elemental analysis: C$_{13}$H$_{22}$N$_2$O$_5$, 286.3 g·mol^{-1}. Theoretical composition: C 54.53%, H 7.74%, N 9.78%, O 27.94%. Found: C 50.80%, H 7.04%, N 8.84%.

Content of product: 90.34%. Corrected yield: 84.62% (0.423 mmol).

ESI-MS: Fragmentation: + 10 V, m/z = 287.1 ([M+H]$^+$), 573.3 ([2M+H]$^+$), 595.3 ([2M+Na]$^+$).

CE-IP

Elemental analysis: C$_{14}$H$_{24}$N$_2$O$_5$, 300.3 g·mol^{-1} Theoretical composition: C 55.98%, H 8.05%, N 9.33%, O 26.63%. Found: C 51.40%, H 7.13%, N 8.51%.

Content of product: 91.21%. Corrected yield: 23.19% (0.116 mmol).

ESI-MS: Fragmentation: + 10 V, m/z = 301.1 ([M+H]$^+$), 601.2 ([2M+H]$^+$), 623.3 ([2M+Na]$^+$).

CM-IPP

Elemental analysis: C$_{18}$H$_{29}$N$_3$O$_6$, 383.4 g·mol^{-1} Theoretical composition: C 56.38%, H 7.62%, N 10.96%, O 25.04%. Found: C 51.59%, H 7.22%, N 10.78%.

Content of product: 98.36%. Corrected yield: 30.80% (0.154 mmol).

ESI-MS: Fragmentation: - 10 V, m/z = 382.0 ([M-H]$^-$), 765.2 ([2M-H]$^-$), 787.1 ([2M+Na-2H]$^-$).

CE-IPP

Elemental analysis: C$_{19}$H$_{31}$N$_3$O$_6$, 397.4 g·mol^{-1} Theoretical composition: C 57.41%, H 7.86%, N 10.57%, O 4.15%. Found: C 52.67%, H 5.91%, N 9.03%.

Content of product: 85.4%. Corrected yield: 4.38% (0.022 mmol).

ESI-MS: Fragmentation: - 10 V, m/z = 395.9 ([M-H]$^-$), 793.2 ([2M-H]$^-$).

CM-IW

Elemental analysis: $C_{19}H_{25}N_3O_5$, 375.4 g·mol^{-1}. Theoretical composition: C 60.79%, H 6.71%, N 11.19%, O 21.31%. Found: C 56.00%, H 5.80%, N 9.38%.

Content of product: 83.78%. Corrected yield: 17.08% (0.085 mmol).

ESI-MS: Fragmentation: + 75 V, m/z = 376.2 ([M+H]$^+$), 398.3 ([M+Na]$^+$), 751.5 ([2M+H]$^+$), 773.5 ([2M+Na]$^+$), 795.4 ([2M+K]$^+$).

CE-IW

Elemental analysis: $C_{20}H_{27}N_3O_5$, 389.2 g·mol^{-1}. Theoretical composition: C 61.68%, H 6.99%, N 10.79%, O 20.54%. Found: C 58.2%, H 7.66%, 8.89N %.

Content of product: 82.35%. Corrected yield: 68.30% (0.341 mmol).

ESI-MS: Fragmentation: + 75 V, m/z = 390.3 ([M+H]$^+$), 412.3 ([M+Na]$^+$), 779.6 ([2M+H]$^+$)

3.5 Preparation of coffee fractions

Although the principal object of study among the coffee samples were the high molecular weight coffee melanoidins, which are product of the Maillard reaction during the thermal treatment of coffee beans, the whole coffee brews and the low molecular weight fractions were also investigated. The roasting process, the extraction and fractionation of the coffee brews are described below.

3.5.1 Roasting conditions

Coffee beans from *Coffea arabica* var. Santos (NY 2/3; screening 17/18) of varying roasting degree (RD) were obtained from a local coffee roaster (K+M Kaffee und Maschinen, Dresden, Germany).

The coffee was submitted to a conventional artisanal roasting process in a drum roaster. The green coffee beans (RD 0) were given to the roaster at room temperature and the drum temperature was progressively increased. RD 1 was taken from the roaster after 10 min treatment and presented light roast (cinnamon roast). The roaster temperature at the time of removal was 174°C. RD 2 was roasted for 12 min, until the drum temperature reached 190°C. This sample presented medium roast (American roast). RD 3 was treated 14.5 min and showed dark roast (French roast). The drum temperature at the time of removal was 194°C. RD 4 is the commercial espresso. It was roasted 16 min until the temperature in the roaster reached 200°C, when the expected roasting characteristics for the final product were

achieved. These beans presented very dark roast (Italian roast). The roasting process and the times of removal of each coffee sample are illustrated on Figure 3-1.
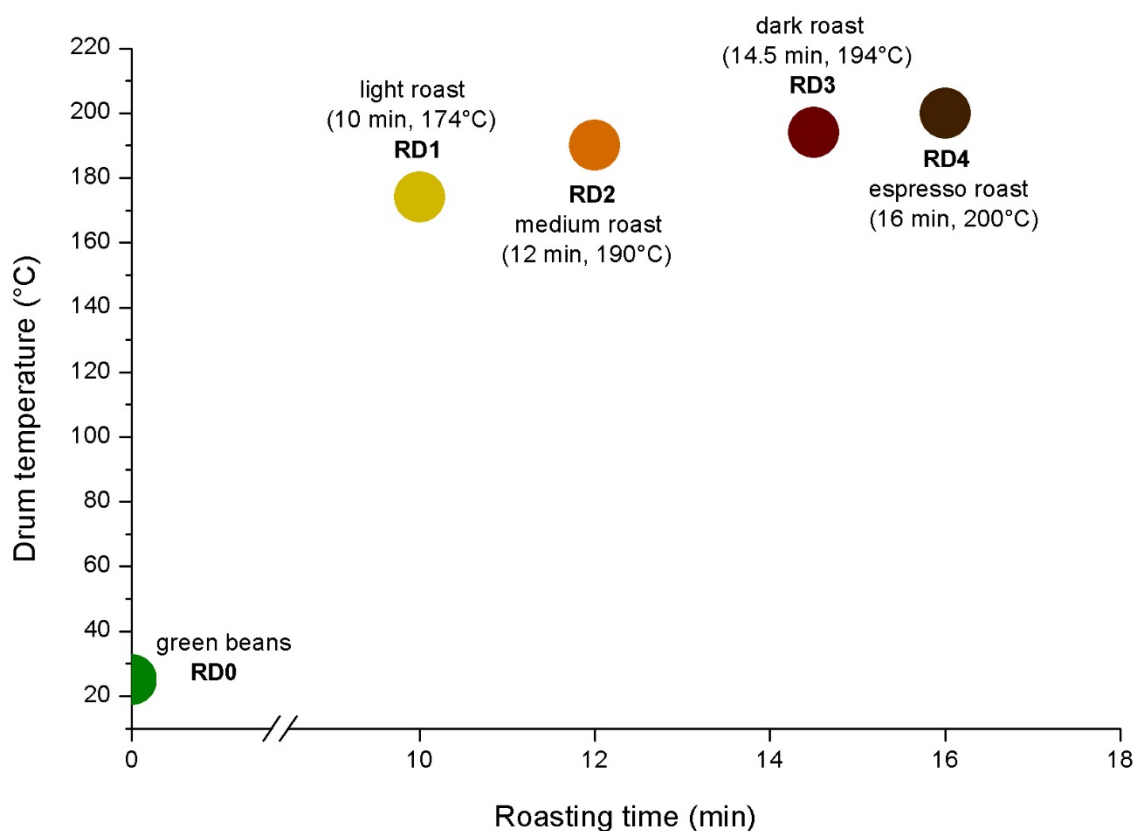
Figure 3-1: Roasting conditions used for preparation of the coffee samples.

The roasted beans were provided as coarse grind powder, appropriate for percolation. The unroasted sample (RD0) was provided in form of beans and was ground in laboratory. 120 g of the green beans were frozen with liquid nitrogen and ground subsequently for 0.5 min at 3000 rpm and for 1.5 min at 10000 rpm using a Retsch GM 100 mill equipped with a 0.74 mm sieve. All samples were stored at -20 °C. The appearance of the coffee samples can be seen on Figure 3-2.
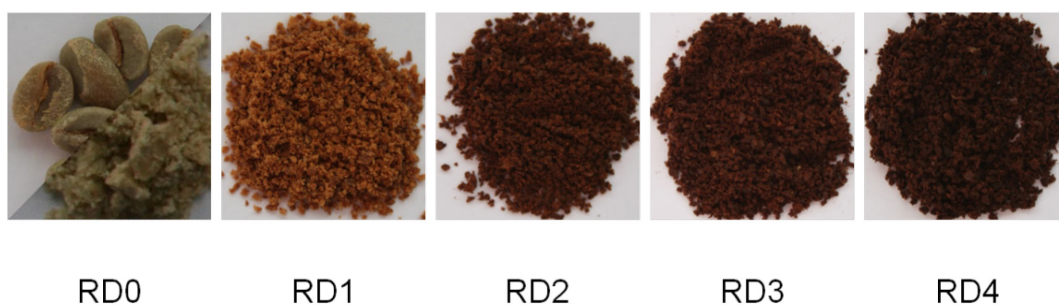
Figure 3-2: Coffee samples of increasingly roast degrees, obtained as described above.

3.5.2 **Fractionation of coffee samples: Isolation of coffee melanoidins**

Coffee extracts were prepared by percolation as described by *Bekedam et al. (2006)* and illustrated in Figure 3-3. For the extraction of RD0, 600 mL of water heated to 90 °C was poured on 100 g of powder. Extracts of the roasted samples RD1 to RD4 were prepared accordingly, using 150 g powder and 900 mL water at 90 °C. After cooling to room temperature, the extracts were centrifuged at 4000 rpm for 10 min and filtered under reduced pressure, using paper filters No. 288 and 292 subsequently. The filtrates (coffee brews), named cb0 to cb4, were freeze dried and used for the next steps.
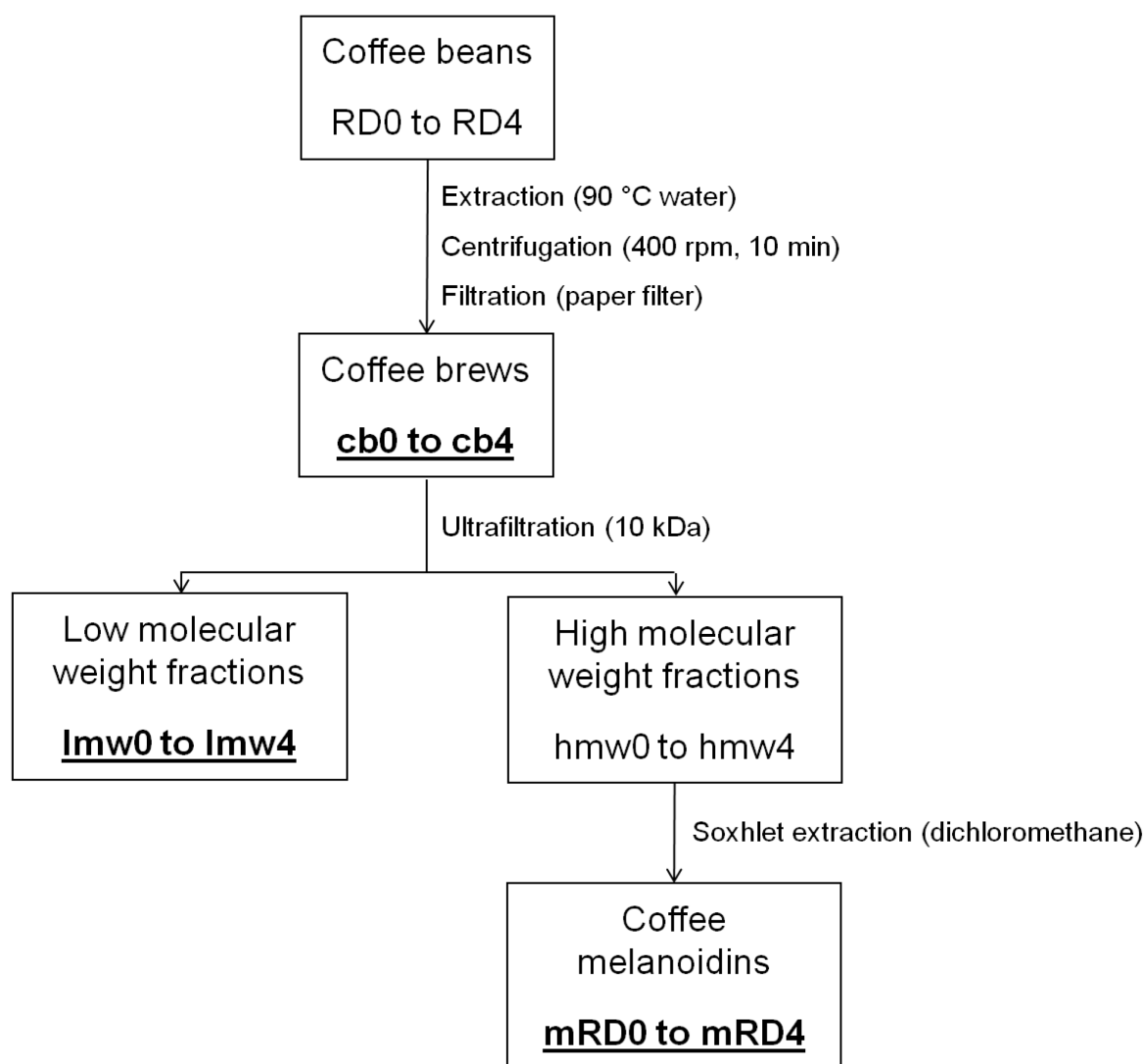
Figure 3-3: Overview of extraction and fractionation of coffee samples. Samples used in following experiments are underlined.

Samples of 5 g of each lyophilized coffee brew were solubilized in 10 mL water and subjected to ultrafiltration using centrifugation spin filters, as described by *Bekedam et al.*

(2006). The use of membranes with MWCO of 10 kDa was based on *Rufian-Henares and Morales (2007)*. The retentates were resuspended in 10 mL water and ultrafiltrated until the filtrates showed absorption below 0.1 at a wavelength of 405 nm, using purified water as blind *(Bekedam et al., 2006)*.

The filtrates were lyophilized and named "low molecular weight fraction" (lmw0 to lmw4), according to the corresponding roast degree. The retentates were likewise freeze-dried and named "high molecular weight fractions" (hmw 0 to hmw4).

The complete hmw were defatted by Soxhlet extraction *(Bekedam et al., 2006)*, for 4 h, using 500 mL dichloromethane. The so obtained pure high molecular weight melanoidins mRD0 to mRD4 were again lyophilized.

All brews and fractions were stored at -20 °C. They are illustrated in Figure 3-4.
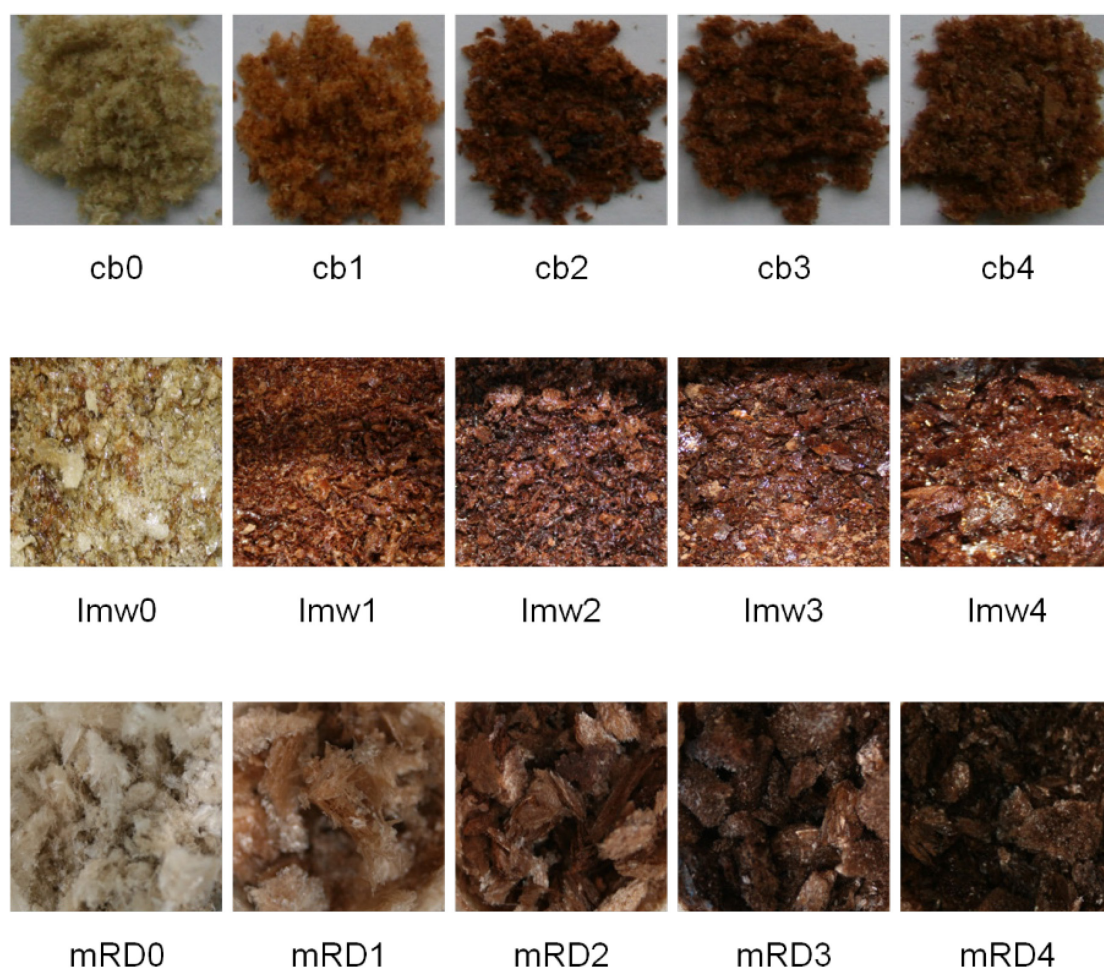
Figure 3-4: Photographic documentation of all studied coffee samples. Cb0-4 are the lyophilized coffee brews obtained from coffee beans of increasingly roast degree. Analogously, lmw0-4 are the low molecular fractions isolated from cb0-4 and mRD0-4 are the correspondent melanoidin fraction.

3.6 Structural studies

Aiming to evaluate key structural changes during formation of the coffee melanoidins with upon thermal treatment, the following aspects were investigated.

3.6.1 Estimation of the molecular weight

The changes on the molecular weight of the components of coffee brews and their fractions along the roasting process were evaluated by gel permeation chromatography (GPC). The chromatographic conditions were developed according to *(Kwak et al., 2005)*. A broad range column was chosen in order to cover the complete range of molecular weights described in the literature for coffee melanoidins.

Samples of coffee brews, low molecular weight fraction and melanoidins of the five studied roast degrees were dissolved in elution buffer at a concentration of 2 mg/mL, filtrated and submitted to the chromatographic analysis under the following conditions:

Instrument:	Knauer Smartline composed by manager K5000, pump K1000, and DAD detector K2600
Column:	BioSep-SEC-S3000 (300 x 7.8 mm), 5-700 kDa separation range, Phenomenex.
Injection volume:	100 µL
Eluent:	50 mM phosphate buffer, containing 0.15 M NaCl, pH 6.5
Flow:	10 min 0.5 mL/min
	11-90 min 0.2 mL/min
Detection:	UV, $\lambda = 220$, 280 and 405 nm, DAD spectrum between 200-500 nm

The molecular weight was estimated after calibrating with thyroglobulin (669.0 kDa, 21.7 min), aldolase (158.0 kDa, 30.2 min), bovine serum albumin (66.0 kDa, 31.6 min), ovalbumin (43.0 kDa, 33.0 min), beta-lactoglobulin (dimeric at pH 6.5, 36.8 kDa, 33.5 min) and lysozym (14.6 kDa, 41.4 min). Dextran blue (2000 kDa, 15.6 min) was also was used for void volume determination. The linear regression $y = -0.08816x + 7.6869$, $r^2 = 0.93537912$ was obtained by plotting the logarithmic molecular weights of the protein standards against their respective retention times. DAD spectra from melanoidins were

registered and used to choose the wavelengths, in which the analysis was carried out. The analyses and reporting were carried out with the softwares Eurochrom 2000 and EZChrom Elite.

3.6.2 C/N ratio

The contents of nitrogen, hydrogen and carbon were quantified using elemental analysis (Elemental Analyser Euro EA 3000, Eurovector, Milan, Italy), as described on section 3.4.2. The C/N ratio was calculated as the proportion between the carbon and the nitrogen contents.

3.6.3 Amino acid analysis

The amino acid analysis was realized in order to evaluate transformations of the amino acid content caused by the roasting of the coffee beans, as well as to investigate the formation of the crosslink amino acids, lysinoalanine (LAL) and pentosidine, which have a potential role on the formation of high molecular coffee melanoidins.

3.6.3.1 Acid hydrolysis

The samples were submitted to acid hydrolysis as described by *Förster (2006)*. Hermetically closed Schott hydrolysis tubes, containing 5 mg of the sample and 5 mL of HCl 6 N under N_2 atmosphere, were heated for 23 h at 110 °C. After cooling down, the samples were filtrated and 1.5 mL aliquots were taken up. The hydrochloric acid was removed from the samples by vacuum concentration.

The residues of the aliquots designated for general amino acid analysis were dissolved in 0.5 mL of lithium citrate buffer (0.12 M, pH 2.2). For the analysis of LAL and pentosidine, the buffer used was sodium citrate 0.2 M (pH 2.2).

3.6.3.2 General amino acid analysis

The amino acid analysis was performed by Karla Schlosser after the method developed by *Henle et al. (1991)*. It consists of a cation exchange chromatographic separation followed by post-column derivatization with ninhydrin and detection at $\lambda = 570$ nm and 440 nm. The samples prepared as described in section 3.6.3.1 were transferred to glass vials and submitted to the chromatography under the described conditions:

Instrument:	Amino acid analyser S433, Sykam
Column:	Cation exchange column LCA K07/Li, Sykam
Injection volume:	80 µL (except for mRD0 = 20 µL)
Eluents:	Sykam lithium citrate buffer system:

	Li$^+$ concentration and pH
Loading buffer:	0.12 M pH 2.20
Buffer A-1:	0.12 M pH 2.90
Buffer B-1:	0.30 M pH 4.20
Buffer C-4:	0.30 M pH 8.00 (citrate-borate)
Regeneration:	LiOH 0.5 M

Flow:	0.45 mL/min

Gradient system:

Time (min)	Buffer A-1 (%)	Buffer B-1 (%)	Buffer C-4 (%)	LiOH	Column temperature (°C)
0	85	15	0	0	42
3	85	15	0	0	42
4	79	21	0	0	42
21	43	57	0	0	42
25	43	57	0	0	42
33	0	100	0	0	42
39	0	0	100	0	42
40	-	-	-	-	60
43	0	0	68	32	60
46	-	-	-	-	74
63	0	0	68	32	74

Derivatization:	Sykam derivatization reagent: 1% ninhydrin, containing 0.08% hydrindantin in a mixture of glycol and 5 M sodium acetate 7:3
Flow:	0.25 mL/min. Coil temperature: 130 °C
Detection:	UV, λ = 570 nm and 440 nm

The peaks were integrated manually using the software Chromstar 6.3.

3.6.3.3 Lysinoalanine

The quantification of the crosslink amino acid LAL on the coffee samples was performed accordingly to *Henle et al. (1993)*. Similarly to the general amino acid analysis, lysinoalanine is determined in samples after acid hydrolysis (Section 3.6.3.1) under the following conditions:

Instrument:	Alpha Plus 4151 LKB-Biochrom, Pharmacia	
Column:	Cation exchange analytical column 200 x4.6 mm, 7 µm, Grüning	
Injection volume:	80 µL	
Eluents:	Sodium citrate system:	

	Na$^+$ concentration and pH
Loading buffer:	0.12 M pH 2.20
Buffer 1:	0.2 M, pH 3.20, 7% isopropanol
Buffer 2:	0.2 M, pH 3.20
Buffer 3:	0.2 M, pH 4.20
Buffer 4:	Buffer 3 + Buffer 5, 1/1 (v/v)
Buffer 5:	1.2 M, pH 6.45
Buffer 6:	NaOH 0.4 M

Flow: 0.27 mL/min

Gradient system:

Time (min)	Buffer	Column temperature (°C)
0	1	85
5	1	85
18.5	2	85
32.5	3	85
40.5	4	60
50.5	4	60
61.5	5	60
69.5	5	85
75.5	6	85

Derivatization: Sykam derivatization reagent: 1% ninhydrin, containing 0.08% hydrindantin in a mixture of glycol and 5 M sodium acetate 7:3

Flow: 0.18 mL/min. Coil temperature: 135 °C

Detection: UV, $\lambda = 570$ nm

The peaks were integrated with EZChrom 1.3 and the quantification was done after external calibration.

3.6.3.4 Pentosidine

Pentosidine, another important crosslink amino on the non-enzymatic browning in food, was determined after acid hydrolysis (Section 3.6.3.1) using the method described at *Henle et al. (1997)* with the elution program described at *Henle et al. (1991)*.

Instrument:	Alpha Plus 4151 LKB-Biochrom, Pharmacia
Column:	Cation exchange analytical column 125 x4.6 mm, 5 μm, Grüning
Injection volume:	80 μL
Eluents:	Sodium citrate system.

	Na^+ concentration and pH
Loading buffer:	0.12 M pH 2.20
Buffer 1:	0.2 M, pH 3.20
Buffer 2:	0.2 M, pH 4.25
Buffer 3:	1.2 M, pH 6.45
Buffer 4:	1.2 M, pH 8.00
Buffer 5:	NaOH 0.4 M

Flow: 0.33 mL/min

Gradient system:

Time (min:s)	Buffer	Column temperature (°C)
0:00	1	55
5:00	1	55
5:05	2	55
5:30	1	55
5:35	2	55
6:00	1	55
6:05	2	55
6:30	1	55
6:35	2	55
7:00	1	55
7:10	2	55
7:30	1	55
7:40	2	55
8:00	1	55
27:00	2	55
32:00	3	65
53:00	3	90
58:00	3	90
80:00	4	90
92:00	5	90
104:00	1	90

Detection: Flurescence, $\lambda_{ex}/\lambda_{em}$ = 335/385 nm

Quantification after external calibration was realized with the software EZChrom 1.3.

To calculate the content of pentosidine on protein basis, the nitrogen content, determined by elemental analysis (Section 3.6.2), and the universal conversion factor 6.25 were used to estimate the protein content of the samples.

3.6.4 **Total phenols**

This was determined with the Folin-Ciocalteu reagent according to *Singleton et al. (1999)*, with modifications. Solutions from all coffee samples and the standard chlorogenic acid solutions were prepared in purified water. The method conditions are described below:

Instrument:	Ultraspec 1000 UV-spectrophotometer, Pharmacia
Sample solutions:	Coffee brews: 0.5 mg/mL Low molecular weight fractions: 1.5 mg/mL Coffee melanoidins: 1.0 mg/mL
Calibration solution:	Chlorogenic acid 0.2 to 0.02 mg/mL
Folin-Ciocalteu reagent:	0.2 N
Sodium carbonate solution:	0.7 M

Pipetting scheme:

	Volume (μL)	
	Calibration	Test
Water	40	-
Sample	-	40
Folin-Ciocalteu reagent	200	200
Na_2SO_4 solution	160	160

Reaction conditions:	2 h, room teperature
Detection:	Absorption, λ= 750 nm (Blank = water)

Absorption was measured in suprasil quartz absorption cuvettes, 10 mm pathlength. Concentration of phenolics compounds was calculated as chlorogenic acid equivalents in the total sample dry matter (mg/mg).

3.6.5 **Raman spectroscopy**

The Raman spectroscopy was conducted on the Institute of Wood and Plant Chemistry, Technische Universität Dresden by Alexander Feldner. FT Raman spectra of the sample in Raman quartz cuvettes were recorded on a Bruker MultiRam spectrometer (Bruker Optik GmbH, Etlingen Germany) with a liquid-nitrogen cooled Ge diode as detector. The samples were dissolved in water.

A cw-Nd:YAG-laser with an exciting line of 1064 nm was applied as light source for the excitation of Raman scattering. The Raman spectra were recorded over a range of 3500-150 cm^{-1} using an operating spectral resolution of 4 cm^{-1}. A laser power output of 100 mW was used. Every sample was analyzed under the same conditions three times and 100 scans were accumulated. An average spectrum was formed as final spectrum of the corresponding sample.

An iterative baseline correction (32 iterations) was executed and the spectra were submitted to a min-max normalization in the region between 1200-1000 cm^{-1}. Spectra treatment and peak integration were carried out with help of the operating spectroscopy software OPUS Ver. 6.5 (Bruker Optik). The analysis of the data was executed with Excel.

3.7 Study on inhibition of zinc metalloproteases

3.7.1 Inhibition of ACE

The inhibitory potentials against ACE of the studied peptides and their carboxyalkylated derivatives, as well as of coffee melanoidins gained from coffee samples of increasingly roast degree, were determined using the method described by *Martin et al. (2008)*, with some modifications. It is based on the hydrolysis of the synthetic substrate hippuryl-L-histidyl-L-leucine by the ACE of rabbit lung, releasing hippuric acid, which is chromatographic quantified.

3.7.1.1 General enzymatic assay

Angiotensin converting enzyme (ACE from rabbit lung, Sigma-Aldrich, Steinheim, Germany) was dissolved in bidistillated water to reach an activity of 0.4 mU/mL and stored in aliquots at -18 °C. The synthetic substrate hippuryl-histidyl-leucine (HHL, Bachem, Switzerland) was dissolved in assay buffer (50 mM HEPES and 300 mM NaCl at pH 8.3, section 3.1.4) to give a 5 mM solution.

Dilution rows of inhibitor solutions were prepared with 6 concentrations, comprising at least three logarithmic units in the relevant range for the inhibition (from 100 to 0% residual activity of the enzyme, when possible).

The assays were realized in flat-bottom transparent 96 wells microtiter plates, following the procedure described in Table 3-4, for the assays involving peptides and alkylated derivatives, and in Table 3-5, for the study of the inhibitory potential of coffee melanoidins.

Each vertical row of the plate had a negative control, which was prepared by replacing the inhibitor solution by the same volume of water. As positive control, captopril (3 nM, Fluka Taufkirchen, Germany) was periodically tested. All assays were performed at least in duplicate.

The inhibitor concentration necessary to reduce the enzymatic activity to 50 % (IC_{50}) was calculated as described in section 3.7.4.

3.7.1.1.1 Inhibition of ACE by N^{α}-carboxyalkylated peptides

All peptides and derivates were diluted in ultra pure water and membrane filtered for dilution. The concentration of the test substances in assay comprised the ranges listed in Table 3-3. The exact final concentrations, used for the calculation of IC_{50} (Section 3.7.4) considered the content of the target substance, determined by the elemental analysis (Section 3.4.2).

Table 3-3: Ranges of the inhibitor concentrations used for the determination of IC_{50} values of native and glycated peptides against ACE.

Concentration in assay (µM)	Test substance
10^{-2} to 10^{2}	CE-IA
10^{-1} to 10^{2}	CM-IW, CE-IW
10^{-1} to 10^{3}	CM-GL, CE-GL, CM-IA, CM-GP, CE-GP, CM-AP, CE-AP, CM-IP, CE-IP, CM-LL, CE-LL, CM-IPP, CM-GA, CE-GA
10^{0} to 10^{2}	CE-IPP, IW
10^{1} to 10^{3}	AP, IP, GP; GL GA, IA IPP, LL

The determination of the ACE activity in absence (negative control) and presence of inhibitors (test sample) was realized as follows:

Table 3-4: Scheme of the enzymatic assay for the determination of *in vitro* inhibition of ACE by peptides and carboxyalkylated derivatives.

	Volume (µL)	
	Negative control	**Test sample**
Enzyme solution: ACE 0.4 mU/mL	10	10
Test sample:	-	10
Purified water:	10	-
10 min pre-incubation at 37 °C		
Substrate solution: HHL 5 mM	80	80
2 h incubation at 37°C		
Stopping solution: HCl 1 M	100	100
HPLC quantification		

The amount of enzymatically liberated hippuric acid was measured via RP-HPLC as described in section 3.7.1.2.

3.7.1.1.2 Inhibition of ACE by coffee melanoidins

From each sample, 5 g was weighed and dissolved in 1 mL water in an Eppendorf tube. Due to the difficult resolubilization of the high molecular melanoidins after lyophilization, the tube was sonicated for 30 min, centrifuged at 10,000 rpg for 10 min, and the supernatant was carefully transferred into a new tube, used to prepare the dilution for the assay. The insoluble residue was freeze-dried and subsequently reweighed. The actual concentration of solution was calculated by difference.

In order to achieve higher melanoidin concentrations in the assay as permitted by the solubility of the samples, the volumes were slightly adapted, as can be seen in Table 3-5.

Table 3-5: Scheme of the enzymatic assay for the determination of *in vitro* inhibition of ACE by coffee melanoidins.

	Volume (µL)	
	Negative control	**Test sample**
Enzyme solution: ACE 0.4 mU/mL	10	10
Test sample:	-	10, 20 or 30
Purified water:	30	20,10 or 0
10 min pre-incubation at 37 °C		
Substrate solution: HHL 5 mM	60	60
2 h incubation at 37°C		
Stopping solution: HCl 1 M	100	100
HPLC quantification as described in section 3.7.1.2.		

In spite of the lower concentration, the excess on substrate was retained and the system provided reliable results.

3.7.1.2 **Quantification**

The HPLC system used for the quantification of the hippuric acid liberated to the hydrolytic activity of ACE on the synthetic substrate hippuryl-histidyl-leucine (HHL) is described below:

Instrument: Knauer Smartline composed by manager K5000, pump K1000, DAD detector K2600, autosampler K3950, and column oven.

Column: C18-Eurosphere 100, 5 µm, 150 × 4.6 mm, Knauer. 25 °C

Injection volume: 30 µL

Eluents: A: 0.1% formic acid in purified water
B: 100% Methanol

Detection: UV, λ = 228 nm

For the determination of IC_{50} of peptides and glycated derivatives, the elution was as follows:

Flow: 1 mL/min

Gradient:	Time (min)	Eluent B (%)
	0	15
	5	40
	7	80
	9	80
	10	15
	11	15

The analyte hippuric acid eluted at 7.6 min elution time and the substrate HHL at 8.9 min.

In order to prevent co-elution of coffee constituents and hippuric acid, a longer elution was realized for the study of the inhibitory potential of coffee melanoidins, as described below:

Flow: 1 mL/min

Gradient:	Time (min)	Eluent B (%)
	0	15
	2	15
	10	25
	21	80
	22	80
	24	15
	27	15

Using this gradient, hippuric acid eluted at 8.9 min elution time and the substrate HHL at 10.4 min. The evaluation software was ChromGate V3.3.1. The product peak was integrated to calculate the ACE activity in the absence or presence of inhibitors.

3.7.2 Inhibition of MMP-1, -2 and -9

The determination of the enzymatic activity of matrix metalloproteases was realized using the synthetic fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, where Mca is the fluorophore substituent (7-methoxycoumarin-4-yl)-acetyl- and Dpa is the quencher group N-3-(2,4-dinitrophenyl)-L-α-β-diamino-propionyl *(Knight et al., 1992)* and recombinant human MMP-1, -2 and -9. After cleavage of the Gly-Leu bond, the fluorescence of the unquenched peptide Mca-Pro-Leu liberated can be measured. The enzymatic activity is reflected by the increase on the system fluorescence at 393 nm emission upon excitation at 328 nm *(Troeberg and Nagase, 2004)*.

The inhibitory potential of peptides and derivatives was tested and IC_{50} from coffee fractions against the enzymes was established.

3.7.2.1 General enzymatic assay

Recombinant human MMP-1, -2 and -9 active domains were commercially available (Enzo, Lörrach, Germany). The supplied solutions were diluted in assay puffer (50 mM HEPES, 10 mM $CaCl_2$, 0.05 % Brij-35, pH 7.5, Section 3.1.4) to the following activities: MMP-1: 0.765 U/µL, MMP-2: 0.058 U/µL, MMP-9: 0.045 U/µL. The resulting solutions were divided in aliquots and stored at -80°C until use. The substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Enzo) was dissolved in DMSO to give a 400 µM solution, which was also divided in aliquots, stored at -80 °C and diluted with assay puffer to 40 µM prior to use. As positive control, a 6.5 µM N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl hydroxamic acid (NNGH, Enzo) solution was prepared. This control inhibitor was stored as a 1.3 mM solution in DMSO, which was diluted 1:200 in assay buffer prior to use. The preparation of test solutions is described in sections 3.7.2.1.1 and 3.7.2.1.2. The assays were realized in flat-bottom black 96 wells microtiter plates, following the procedure described in Table 3-6.

Table 3-6: Scheme of the enzymatic assay for the *in vitro* determination of the MMP inhibition.

	Volume (µL)		
	Negative control	**Positive control**	**Test sample**
Enzyme solution: MMP-1 (0.765 U/µL), or MMP-2 (0.058 U/µL), or MMP-9 (0.045 U/µL).	20	-	20
Test sample:	-	-	x
Standard inhibitor: NNGH 6.5 µM	-	10	-
Assay puffer:	70		70 - x
60 min pre-incubation at 37 °C			
Substrate solution:	10		10
30 s shaking			
Measurement of fluorescence (λ_{ex} = 328 nm, λ_{em} = 393 nm) in 1 min intervals for 15 min			

Each vertical row of the plate had a negative and a positive control. All assays were performed at least in duplicate.

The fluorescence was measured by using a Tecan Infinite F 200 plate reader and Tecan-i-Control software. The curve was plotted and the slope of the linear region of the regression curve (initial velocity) was used to compare the activities between the samples.

All assays were performed at least in duplicates. The inhibitor concentration necessary to reduce the enzymatic activity to 50 % (IC_{50}) was calculated as described in section 3.7.4.

3.7.2.1.1 Inhibition of MMPs by N^{α}-carboxyalkylated peptides

The peptides and alkylated derivatives were tested in a screening in their highest concentrations, as described in Table 3-3. The whole concentration ranges of IA and LL derivatives were used for the determination of $IC_{50.}$

3.7.2.1.2 Inhibition of MMPs by coffee fractions

Solutions were prepared as described on section 3.7.1.1.2 for the coffee melanoidins, using assay puffer in place of water to solubilization. Due to the high inhibitory potential of melanoidins measured, all other coffee fractions were accordingly investigated.

The concentration range tested was between 0.0005 and 2 mg/mL in the enzyme solution. The concentrations above 0.5 mg/mL were achieved by giving 20 or 30 µL test solution to the system, as described in table, to the limited solubility of the samples.

3.7.2.2 Effect of zinc addition on the inhibition of MMP-1 by melanoidins

In order to study if inhibition of MMPs by melanoidins is due to zinc sequestration, $ZnCl_2$ was dissolved in MMP-assay buffer (Section 3.1.4) to a concentration of 0.5 mM. This solution was diluted to 0.05 and 0.005 mM with the same buffer. The MMP-1 inhibition assay was conducted as described above (Sections 3.7.2.1 with observations of section 3.7.2.1.2), substituting 10µL of buffer by 10 µL of each of the zinc solutions, providing zinc concentrations of 0.5, 5 and 50 µM in the assay. The melanoidins were tested at a concentration of 0.5 mg/mL. Negative and positive controls were also measured. The inhibitory activity of each sample was calculated as described in section 3.7.4. All assays were performed in duplicate.

For the estimation of the inhibitory potential against ChC of the peptides and the carboxyalkylated derivatives, as well as of coffee melanoidins gained from coffee samples of increasingly roast degree, a chromatographic method was developed based on a spectrophotometric method *(Wunsch and Heidrich, 1963)*. In this method, the synthetic substrate 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg-OH dehydrate (PzPLGL$_D$R) is hydrolyzed by the microbial collagenase between the residues Leu-Gly, liberating the more hydrophobic molecule PzGL, which can be separated chromatographically from the more hydrophilic original peptide.

3.7.3 Inhibition of ChC

3.7.3.1 General enzymatic assay

Collagenase from *Clostridium histolyticum* (ChC, Type VII, Sigma-Aldrich, Steinheim, Germany) was dissolved in bidistillated water to an activity of 0.4 FALGPA mU/mL (FALGPA = N-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala) and stored in aliquots at -18 °C. A 4 mM solution of the substrate was prepared by dissolving 62.1 mg of the PzPLGL$_D$R (Bachem, Switzerland) in 200μL methanol, adding 19800 μL of assay puffer (50 mM HEPES pH 7.5, Section 3.1.4) and mixing until complete solubilization.

The assays were realized in flat-bottom transparent 96 wells microtiter plates, following the procedure described in Table 3-7, for the assays involving peptides and alkylated derivatives, and in Table 3-8, for the study of the inhibitory potential of coffee melanoidins. Each vertical row of the plate had a negative control, which was prepared by replacing the inhibitor solution by the same volume of water. As positive control, EDTA (1 mM) was periodically tested. All assays were performed at least in duplicate.

The calculation of IC$_{50}$, the inhibitor concentration necessary to reduce the enzymatic activity to 50 %, is described in section 3.7.4.

3.7.3.1.1 Inhibition of ChC by N$^\alpha$-carboxyalkylated peptides

The peptides, both IW derivatives and CE-IPP were studied in the highest concentrations described in Table 3-3. All other alkylated derivatives were tested in the concentrations described in the same table. The enzymatic assay is described in Table 3-7.

Table 3-7: Scheme of the enzymatic assay for the determination of *in vitro* inhibition of ChC by peptides and carboxyalkylated derivatives.

	Volume (µL)	
	Negative control	**Test sample**
Enzyme solution: ChC 0.4 mU/mL	10	10
Test sample:	-	10
Purified water:	10	-
10 min pre-incubation at 25 °C		
Substrate solution: PzPLGL$_D$R 4 mM	80	80
15 min incubation at 25°C		
Stopping solution: Methanol	100	100
HPLC quantification as described in section 3.7.3.2.		

3.7.3.1.2 Inhibition of ChC by coffee melanoidins

Aqueous melanoidin solutions were prepared as described on section 3.7.1.1.2 . In order to achieve higher melanoidin concentrations in assay system, the procedure was slightly changed. The adapted scheme can be seen in Table 3-8.

Table 3-8: Scheme of the enzymatic assay for the determination of *in vitro* inhibition of ChC by coffee melanoidins.

	Volume (µL)	
	Negative control	**Test sample**
Enzyme solution: ChC 0.4 mU/mL	10	10
Test sample:	-	25
Purified water:	25	-
10 min pre-incubation at 25 °C		
Substrate solution: PzPLGL$_D$R 4 mM	65	65
15 min incubation at 25°C		
Stopping solution: Methanol	100	100
HPLC quantification as described in section 3.7.3.2.		

3.7.3.2 Quantification

The amount of enzymatically liberated 4-phenylazobenzyloxycarbonyl-Pro-Leu-OH (PzPL) was measured via RP-HPLC. The chromatographic system was as follows:

Instrument: Knauer Smartline composed by manager K5000, pump K1000, DAD detector K2600, autosampler K3950, and column oven.

Column: C18-Eurosphere 100, 5 µm, 250 × 4.6 mm, Knauer, 35°C.

Injection volume: 30 µL

Eluents: **A**: H_3PO_4 solution pH 3 / **B**: 100% Methanol

Gradient:

Time (min)	Eluent B (%)
0	15
2	15
8	25
12	80
30	80
34	15
37	15

Flow: 0.75 mL/min

Detection: UV, $\lambda = 320$ nm

The analyte (PzPL) could be detected at 23.1 min elution time and the substrate $PzPLGL_DR$ at 18.0 min.

The evaluation software was ChromGate V3.3.1. The product peak was integrated to calculate the enzymatic activity in the absence or presence of inhibitors. IC_{50} values were calculated as described at 3.7.4.

3.7.4 Calculation of IC_{50}

To calculate the inhibitor concentrations needed for 50% inhibition of the enzyme activity (IC_{50} values), the residual activity of the enzyme in presence of a test solution was transformed to the relative inhibition [Inhibition (%) = 100%- residual activity (%)].

Inhibition was plotted against the inhibitor concentration (in logarithmic scale). The obtained sigmoidal curve was submitted to a non linear regression using a four-parameter logistic model with the software Origin 6.1. The concentration of inhibitor at 50% enzymatic activity (IC_{50}) was calculated using the formula (1):

$$y = \frac{A_1 - A_2}{1 + \left(\dfrac{x}{x_0}\right)^p} + A_2$$

When y = 50%, x = IC$_{50}$

Solving for x $\longrightarrow$ $\qquad IC_{50} = x_0 \cdot \sqrt[p]{\dfrac{A_1 - A_2}{50\% - A_2} - 1}$ $\qquad$ **(1)**

Where,

A1 = initial value (left horizontal asymptote),

A2 = final value (right horizontal asymptote),

X0 = center (point of inflection),

p = power (parameter that affects the slope of the area about the inflection point).

The significance of the observed differences was calculated by one-way ANOVA using the software Origin 6.1 (OriginLab, Northampton, USA).

4 Results and Discussion

This work investigates the influence of the Maillard reaction on the inhibitory potential of food components against zinc metalloproteases. As a prototypical Maillard reaction product, coffee melanoidins were chosen. Since the mechanisms involved on the formation of these macromolecules and the chemical transformations which take place during the heat treatment are not completely elucidated, key structural features were analyzed. Due to the impressive IC_{50} values observed, the role of the functional group introduced by the non-enzymatic browning on the inhibition the metalloproteases was studied. Specific advanced glycation end-products of relevant peptides were synthesized and submitted to the enzymatic assays.

4.1 Coffee melanoidins

4.1.1 Isolation of coffee fractions

The coffee beans used in this study were submitted to roasting under the mild conditions conventionally used for the production of high quality espresso beans. The extracts were obtained by extraction with hot water in conditions proportions often used to prepare the beverage at home, so that the results are expected to represent realistically the daily ingested brew. The whole extracts were lyophilized and named "coffee brew" (cb) 0 to 4, where the numbers reflect the intensity of thermal treatment. The attribute "0" describes the non-treatment and the samples with roast degree "4" were obtained from the beans roasted until they were ready to proportionate a high quality espresso. Numbers "1" to "3" represent the intermediary roast intensities. The methods for preparation of coffee brews of increasingly roast degrees (cb0-cb4) and the isolation of the respective low molecular weight fractions (lmw0-lmw4) and high molecular weight melanoidins (mRD0-mRD4) is described in details in section 3.5.

The yields on coffee brew after lyophilization can be seen in Table 4-1. From 100 g non roasted coffee beans were obtained almost 20 g extract. The extractability of coffee solids decreases with progression of roast. From the beans with cinnamon roast (RD 1, removed from roaster after 10 min thermal treatment, which presented 174 °C at the moment of removal), 17 g extract (cb1) were obtained from 100 g coffee powder. From the beans with American roast (RD 2, 12 min, 190 °C), 16 g of cb2 were obtained, and the French roast

beans (RD 3, 14.5 min, 194 °C) proportionate only 15 g of cb3. The final Italian roast beans (RD 4, 16 min, 200 °C) gave over 16 g of cb4. The observed variation in the contents of soluble solids of coffee upon roasting is well known. Initially, a decrease on the extractable matter from coffee powder is observed, most due to denaturation and aggregation processes of native proteins of the green beans, which diminishes the solubility of the macromolecules in hot water. At the end of the roasting process, a slight increase on the soluble solids can be noticed, due to fragmentations caused by the heat treatment, and formation of polar and charged functional groups, which contributes to the general extractability. The yield of coffee brews are consistent with literature results, where values between 17 and 23% (w/w) dry matter, depending on roast grade and brew method, are described *(Borrelli et al., 2002; Bekedam et al., 2006; Bekedam et al., 2008c; Nunes and Coimbra, 2002). Borrelli et al. (2002)* obtained 14 g dry extract from 100 g green coffee beans and 17 g dry extract/100 g roasted beans, from brews prepared by solid-liquid extraction with hot water. *Bekedam et al. (2006, 2008c)* describe yields from 16 g/100 g and 19 g/100 g for extracts obtained from green and roasted beans, respectively, at the same conditions as the used in the resent work.

Table 4-1: Amount of extractable matter (coffee brew) of coffee samples of increasingly roast degrees and respective isolated fractions (low molecular weight fractions and melanoidins over 10 kDa), represented in g dry matter per 100 g coffee beans powder. Values in brackets are the relative amounts of each fraction in relation to the respective coffee brew total solids, which corresponds to column "Total", represented as g to 100 g coffee brew total solids.

Dry mass (g/100 g)			
Roast degree	**Total**	**Low molecular weight fraction (lmw)**	**Melanoidins (mRD)**
0	19.73	16.18 (82.0%)	2.27 (11.5%)
1	17.35	15.78 (90.9%)	1.27 (7.3%)
2	16.32	13.92 (85.3%)	1.39 (8.8%)
3	15.21	12.92 (85.0%)	1.75 (11.5%)
4	16.43	14.16 (86.2%)	2.02 (12.3%)

There is no consensus in the literature concerning the molecular weight of melanoidins. The exclusion limit for their isolation is still rather arbitrary chosen, ranging in the literature

between 2 and 100 kDa. In the present study, a 10 kDa cutoff was used in order to better compare our results, once this is the most often nominal pore size choice in investigations involving melanoidins obtained from coffee. In addition, preliminary work showed a similar behavior of melanoidins over 3 kDa and over 10 kDa in respect to their inhibitory activities.

The low molecular weight fraction is the quantitatively most important fraction of the coffee brew, accounting for 80 to 90% of the entire extracts (Table 4-1, values in brackets). As the main components from coffee extract, the yields with increasingly roasting come along with the tendency for the whole extracts, the amount in soluble low molecular weight compounds decreases with progress of roasting until the last roasting stage, where a slight increase can be noticed. In most studies the low molecular weight fraction cannot be precisely quantified, as the fractions are separated often by dialysis, with disposal of dialysate. *Bekedam (2008c)* found 82% of the coffee brew solids isolated by ultrafiltration to be smaller than 3 kDa *(Bekedam et al., 2008)*.

The content of coffee melanoidins over 10 kDa isolated in the present work represented between 1.3 and 2.3% of the coffee powder weight (Table 4-1). Based on coffee brew dry matter, the melanoidins respond for 12.3 % of the solids, considering the espresso sample, which is the commercial coffee. Melanoidin contents in coffee brew up to 25% have been described in the literature *(Gniechwitz et al., 2008b)*. This variation is due to the fact that the definition of melanoidins is not good established, permitting a broad choice of isolation procedures. Parameters of the fractionation procedure, as method (dialysis, ultrafiltration or GPC) and material and cut-off of the membrane used, strong influence the proportion of low and high molecular weight fractions *(Bekedam et al., 2007)*. In addition, purification procedures will inevitably influence the yield.

In the melanoidin fraction, the effect of the roasting on the solubility of the coffee solids is clear. With beginning of the heat treatment, the amount on extractable high molecular weight components decreases due to denaturation and aggregation of proteins and polysaccharides. With progression of roasting, the macromolecules are continuously modified and acquire hydrophilic functional groups, showing an improved solubility in hot water. Similar results were observed by *Vignoli et al. (2011)*, who describes an initial reduction in the high molecular weight fraction of brews, which increases again in advanced heat treatment, achieving the highest ratio in the final roasted beans.

4.2 Inhibition of zinc-dependent peptidases by coffee fractions

This section describes the *in vitro* effect of coffee brews and coffee fractions with progress of the roasting on selected zinc-dependent peptidases. The main objective was to evaluate the food borne inhibitory activity in coffee fractions, especially in the melanoidin fraction, as they arise from the thermal treatment, i.e. they are by definition Maillard reaction products. The following enzymatic models were investigated:

- three human matrix metalloproteases (MMP-1, -2 and -9), which participate in many physiological processes including tissue turnover and repair, and are involved in numerous pathological conditions, especially in malignant diseases. The catalytic domain has an extended zinc-binding motif, **HEXXHXXGXXH**, where the three histidine residues bound the zinc atom. A conserved methionine provides a hydrophobic base for the zinc-binding site.

- *Clostridium histolyticum* collagenase (ChC), which is a zinc-containing bacterial metalloproteinase that is also able to degrade extracellular matrix. ChC has the conserved **HEXXH** zinc-binding motif, where two histidine and a glutamic acid residues coordinate the catalytic zinc.

- Angiotensin converting enzyme (ACE), which is a zinc-dependent dipeptidyl carboxypeptidase, essential for blood pressure regulation and electrolyte homeostasis playing a central role in cardiovascular pathologies such as hypertension and cardiac hypertrophy. Somatic ACE is composed of two homologous catalytic domains, each containing the **HEXXH**+E zinc-binding active site motif.

4.2.1 Inhibition of MMPs

In the present study, a continuous fluorimetric enzymatic assay based on the enzymatic hydrolysis of the fluorogenic peptide was used. The synthetic substrate (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH$_2$ (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$) contains a highly fluorescent 7-methoxycoumarin (Mca) fluorophore and a dinitrophenyl quencher (Dpa) located on opposite sides of the susceptible peptide bond. The excitation peak of the quencher overlaps with the emission peak of the fluorophore, allowing the quencher to absorb the energy from the fluorophore in a distance-dependent manner. This prevents

fluorescence of the intact substrate by a process of fluorescence resonance energy transfer (FRET). Upon cleavage of the susceptible Gly-Leu bond, the quencher and the fluorophore become physically separated, leading to a 190-fold increase in fluorescence of the Mca (λ_{ex}=328 nm, λ_{em}=393 nm) *(Knight et al., 1992; Troeberg and Nagase, 2004)*. The structure of the fluorogenic substrate can be seen in Figure 4-1.

The option for a kinetic measurement instead for a stop-assay indented principally to diminish the interference of samples, as the final products of the non-enzymatic browning are known for their UV- and fluorescence activities. As the measured parameter was the increase in the fluorescence over the time, and not its absolute value, the intrinsic fluorescence of the different samples should not alter the slope in the initial rate period (linear ascending region) of the plotting of time versus fluorescence. In fact, no ground sample disturbance in measurements was observed.

Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂
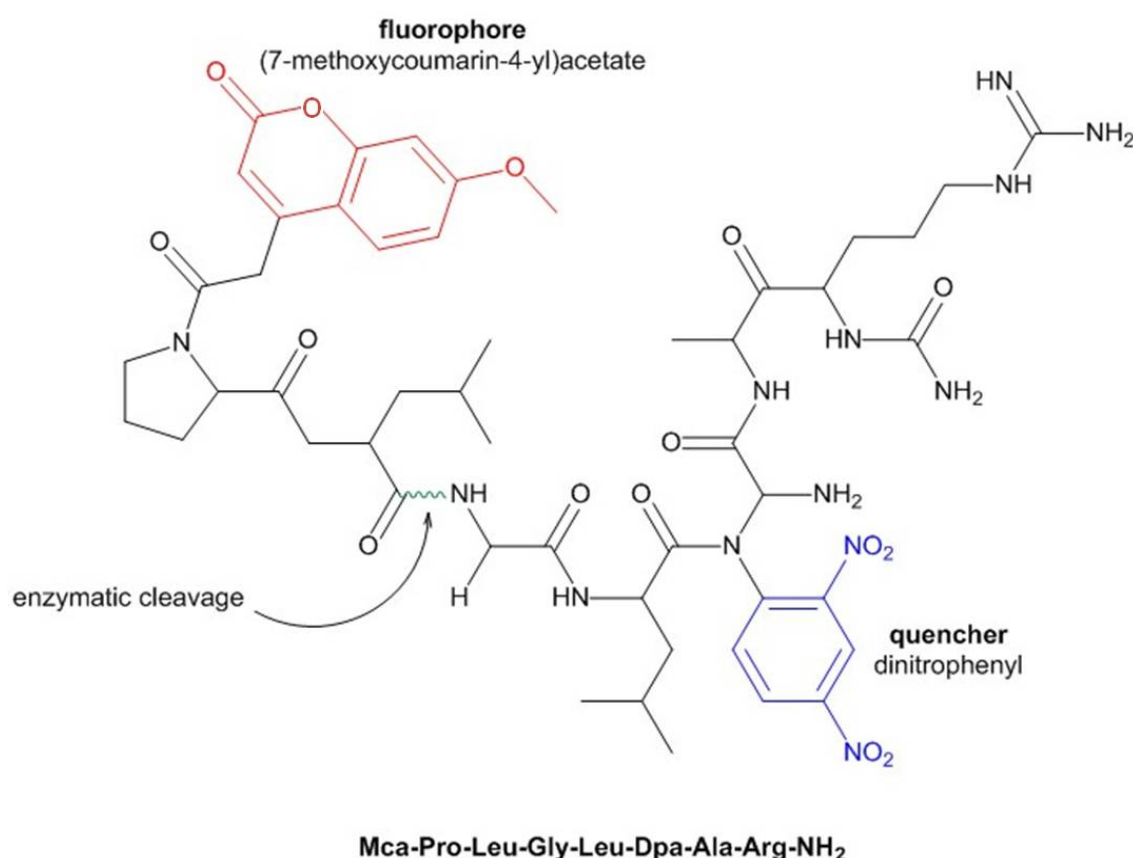
Figure 4-1: Fluorogenic substrate used for the quantification of MMP activities (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitro-phenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH₂ (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂). The susceptible peptide bond is represented by a green wavy bond.

The activity of the high molecular weight melanoidins formed during roasting of coffee beans against MMP-1, MMP-2 and MMP-9 was investigated. Figure 4-2 shows the IC_{50} values of the melanoidin preparations obtained from progressively roasted coffee beans.
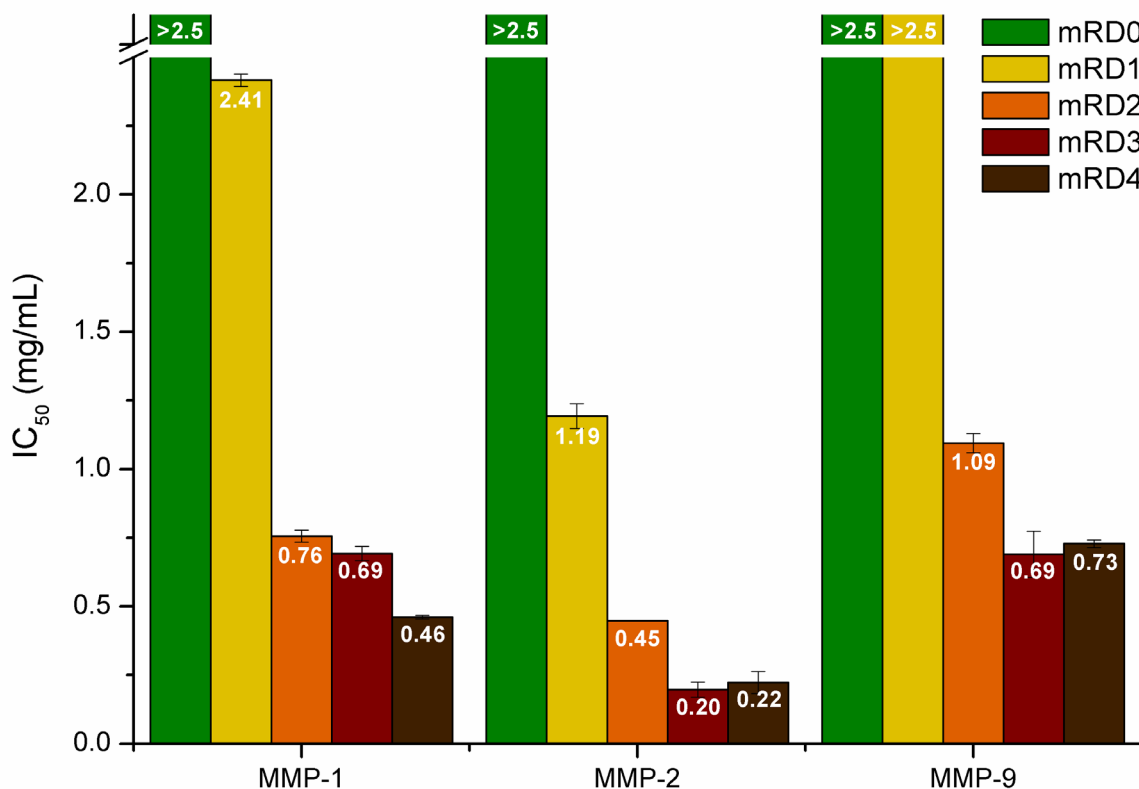
Figure 4-2: IC_{50} values of melanoidins extracted from coffee brews of increasing roast degrees against MMP-1, -2 and -9. Values are means of triplicates (±SD). Assay is described in section 3.7.2.

An evident increase in the inhibitory potential of samples, depending on the roasting degree, can be observed. The high molecular fraction obtained from green coffee beans (mRD0) showed no inhibitory activity against any of the studied enzymes at concentrations up to 2.5 mg/mL in assay system. With beginning of the thermal treatment, the onset of inhibitory activity can be seen. The most lightly roasted melanoidin sample, mRD1, shows weak inhibitory potential against MMP-1 ($IC_{50} = 2.42 \pm 0.02$ mg/mL) and against MMP-2 ($IC_{50} = 1.19 \pm 0.05$ mg/mL), although no inhibitory activity against MMP-9 (up to 2.5 mg/mL) could be detected.

With advance of roasting, a significant improvement of the inhibition can be seen. The inhibitory potential of the melanoidins increases continuously until reaching the dark roast grade of mRD3. From mRD3 to mRD4 is the difference significant ($p<0.05$) only for

MMP-1. The melanoidins from the final roasted product (mRD4) provided IC_{50} values of 0.461 ± 0.006 mg/mL against MMP-1, 0.224 ± 0.040 mg/mL against MMP-2; and 0.728 ± 0.014 mg/mL against MMP-9.

These results indicate that the thermal treatment during roasting has a direct impact on the formation of MMP-inhibiting structures within the complex melanoidins. In fact, drastic changes on the enzymatic inhibition of food protein after induced Maillard reaction has been described in the literature. In experiments involving the IgE-binding activity of peanuts and cherry proteins, strong alteration on the binding behavior of the proteins after induced browning was reported *(Gruber et al., 2004; Gruber et al., 2005)*. The authors suggested that carbohydrate-induced modification of nucleophilic amino acid side chains of proteins reaction resulted in molecules with altered binding ability. The capacity of Maillard reaction products to inhibit metalloenzymes has been receiving increasingly attention. The inhibition of apple polyphenoloxidase by model MRP was reported already over 20 years ago *(Tan and Harris, 1995; Nicoli et al., 1991; Billaud et al., 2003; Brun-Merimee et al., 2004)*. Inhibitory effect of CML and of the arginine derivatives carboxymethylarginin and carboxyethylarginine against enzymes implicated in the generation of NO, with possible repercussion in chronic vasculopathy, was reported *(Lai et al., 2010)*. *Rufian-Henares and Morales (2007)* described the inhibition *in vitro* of ACE by food melanoidins, especially by the ones isolated from coffee. Also *del Castillo et al. (2007)* observed the inhibition of ACE by Maillard reaction products obtained in gluten–glucose model systems. This effect is probably related to the formation of metal-chelating characteristic of Maillard reaction products, as carboxymethyl- and carboxyethyl- modification in the side chains of proteins, as well as heterocyclic structures as maltosin. Particularly carboxylic groups, like the generated with the formation of CML and CEL, and hydroxypyridinones, including the 3-hydroxy-4-pyrone maltol, another well known Maillard reaction product, show high affinity for the catalytic zinc in the active sites of numerous metalloproteases, constituting promising ZBGs for the development of new inhibitors of MMPs *(Rouffet and Cohen, 2011; Puerta and Cohen, 2004; Puerta et al., 2006; Puerta et al., 2005; Jacobsen et al., 2011; Lewis et al., 2003)*.

Nevertheless, the highly complex coffee melanoidins cannot be reduced to melanoidins produced in model system by heating together amino acids and sugars. Recently, *Miyake et al. (2011)* described a dose-dependent inhibitory effect of green tee catequins against MMP-7. Strong inhibitory effect of chlorogenic acid on MMP-9 activity (IC_{50} of 30–50 nM)

in a concentration-dependent manner without relevant effect on cell viability was reported in a hepatocarcinoma model *(Jin et al., 2005)*. A correlation between polyphenol content in food and *in vitro* inhibition of ACE has already been observed *(Kwon et al., 2007)*. *Narita and Inouye (2011)* also studied the effect of chlorogenic acids on the activity of metalloenzymes and observed inhibition in mili- or high micromolar concentrations for all the tested compounds. They found the inhibitory capacity of polyphenols to be dependent on the number of caffeic acid moieties or the number of hydroxyl groups introduced. A roast-induced formation of diverse groups with potential zinc binding ability, as a result of the degradation of caffeoylquinic acids as well as of the Maillard reaction (mostly di- and trihydroxybenzenes) was described *(Muller et al., 2006)*. High coordination ability of hydroxycinnamates and phenol-based derivatives in the active site of zinc-dependent enzymes have recently been reported in a series of kinetic studies *(Durdagi et al., 2011; Şentürk et al., 2011; Innocenti et al., 2010a; Innocenti et al., 2010b)*. *In silico* investigations suggested that mono and polyphenols, including caffeic acid, may anchor at the Zn(II)-coordinated water molecule. By binding more externally within the active site cavity, they interact with various amino acid residues in the active site of the studied enzymatic system *(Durdagi et al., 2011; Şentürk et al., 2011; Innocenti et al., 2010a; Innocenti et al., 2010b; Beyza Öztürk Sarıkaya et al., 2010)*. As a matter of fact, studies on squid-ink melanin, a pigment formed from polycondensed polyphenols, also evidenced the binding of metallic cation on both carboxylic acid and catecholate binding sites *(Liu et al., 2004)*. In conclusion, polyphenols are incorporated to melanoidin skeleton upon roasting *(Nunes and Coimbra, 2007; Bekedam et al., 2008a; Gniechwitz et al., 2008b)*, providing other chelation possibilities to the macromolecular fraction of coffee. Therefore, the incorporation of chlorogenic acid in the melanoidin structure seems to be at least partially responsible for the observed roast-dependent inhibitory activity of this fraction.

The differences of IC_{50}-values of one melanoidin sample among the MMPs cannot be discussed in terms of inhibition specificity, as the inhibitory potentials range in the same order of magnitude. Although the specificity of a classical low molecular weight drug for a MMP is influenced by the interactions with the named 'selectivity subsites', the catalytic site and binding pockets of MMPs are structurally rather very similar *(Overall and Kleifeld, 2006a; Overall and Kleifeld, 2006b; Borkakoti, 2004)*. This characteristic is reflected in the overlapping functions of many of the family members, which partially cleave the same substrates, even though with distinct affinities *(Zucker and Vacirca, 2004; Fingleton, 2008)*.

As complex high molecular weight compounds, melanoidins provide a multitude of interaction possibilities, and binding points to each MMP are potentially found also outside of the catalytic center. Therefore, the specificity within the family in mostly improbable and inhibitory property of the melanoidins against MMPs will be discussed as the inhibition of a group of enzymes, not against individual components of the MMP family.

Due to the encouraging results observed for the coffee melanoidins, the evaluation of the effect of roasting on the inhibitory activity of the complementary low molecular weight fractions and of the whole coffee brews was undertaken.

Contrary to the high molecular weight fraction, where enzymatic inhibition can only be measured after onset of thermal treatment, the low molecular weight fraction from non-roasted coffee beans shows already impressive inhibitory effect, as illustrated in Figure 4-3.
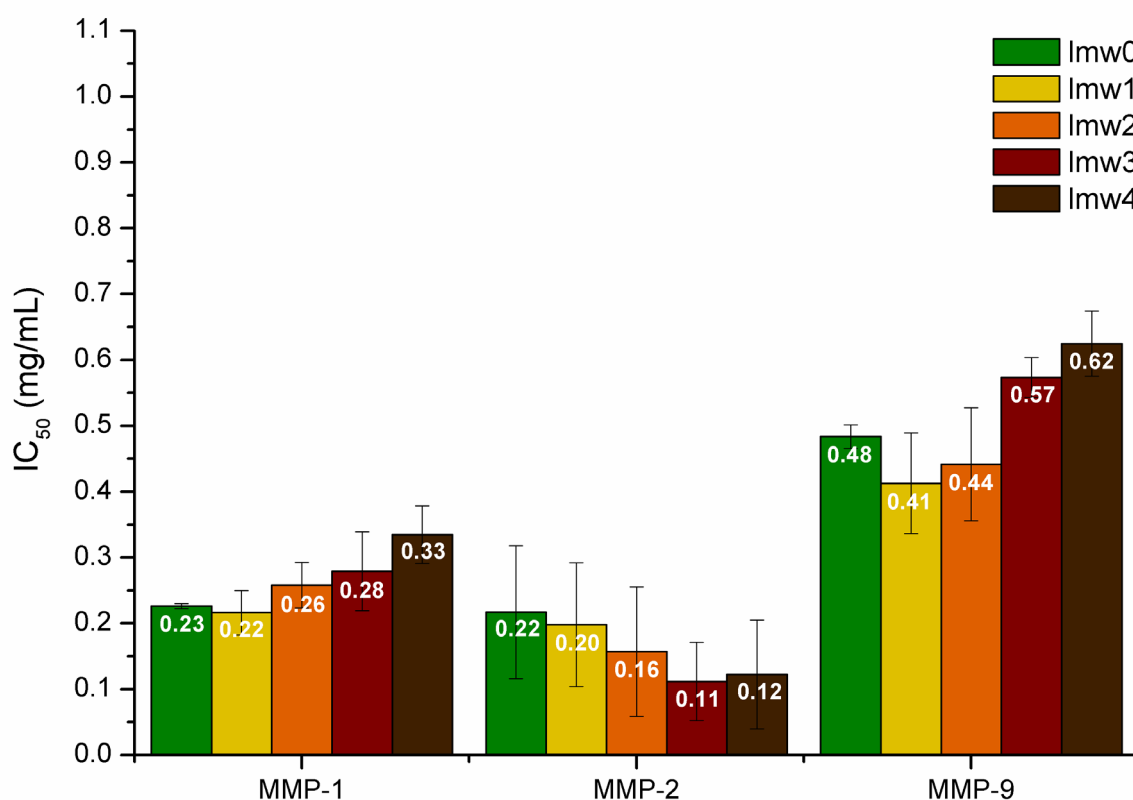

Figure 4-3: IC_{50} values of low molecular weight fractions of coffee brews of increasing roast degrees against MMP-1, -2 and -9. Values are means of triplicates (±SD). Assay is described in section 3.7.2.

Furthermore, the effect of the thermal treatment over the inhibitory potential of the low molecular weight compounds did not follow the trend found for the melanoidins. Except for

MMP-2, which seems to be better inhibited by thermally treated samples of both high and low molecular weight fractions of coffee brew, the roasting exerts a rather attenuating effect on the bioactivity of the low molecular weight fraction of coffee.

After an initial decrease in IC_{50} values, with beginning of the roasting process, the inhibitory activity of the low molecular weight samples worsens with progress of the treatment. While the low molecular weight fraction isolated from non roasted coffee beans (lmw0) provided IC_{50} values of 0.226 ± 0.004 mg/mL against MMP-1, 0.217 ± 0.101 mg/mL against MMP-2; and 0.484 ± 0.018 mg/mL against MMP-9, the low molecular weight compounds obtained from the final roasted product (lmw4) inhibit the studied enzymes with IC_{50} values from 0.335 ± 0.044, 0.123 ± 0.083 and 0.625 ± 0.049 mg/mL against MMP-1, -2 and -9, respectively. The thermally induced decrease of the fraction can possibly be due to a loss in naturally present polyphenols, which can coordinate zinc and possibly impair the hydrolytic function of matrix metalloproteases. It is known that the heat degradation of chlorogenic acids gives a variety of lactones and volatile di- and trihydroxyphenols, quinones and catechols, which contribute to the formation of aroma and flavor of roasted coffee *(Moon et al., 2009; Moon and Shibamoto, 2010; Muller et al., 2006; Sharma et al., 2002)*. The decrease in the inhibitory activity of the low molecular weight fraction upon roasting can thus be caused, to some extent, by the pyrolytic degradation of polyphenols, with formation of structural features less effective for the zinc coordination and, therefore, in samples less prone to impede the enzymatic activity. However, as the roasting temperatures used to prepare the coffee samples used in the present study did not exceed 200°C, temperature at which pyrolysis of chlorogenic acids begins in model systems, the migration of the coffee free polyphenols to the high molecular weight fraction, caused by their incorporation in melanoidin skeleton, can be the principal cause of depletion of chelating moieties in the lmw.

Beholding whole brews, it is evident that the quantitative dominance of the lmw fraction on the extracts shapes the behavior of brew, overshadowing the observed increase in the bioactivity of the melanoidin fraction. The brew from non-roasted coffee beans (cb0), presented IC_{50} values from 0.271 ± 0.079, 0.253 ± 0.043 and 0.547 ± 0.018 mg/mL against MMP-1, -2 and -9, respectively, as represented in Figure 4-4. The effect of roasting over the inhibitory potential of the samples was initially slightly positive, showing cb0 IC_{50} values of 0.207 ± 0.047, 0.180 ± 0.023 and 0.370 ± 0.003 mg/mL against MMP-1, -2 and -9, respectively. However, with progress of the roasting, the inhibitory capacity of the samples

decreased continuously, until IC_{50} values reached about the levels for cb0, except for MMP-2, where the effect of the roasting on the enzyme inhibition was not accentuated. IC_{50} values of brew from espresso beans (cb4) were 0.333 ± 0.092, 0.154 ± 0.054 and 0.614 ± 0.039 mg/mL against MMP-1, -2 and -9, respectively.
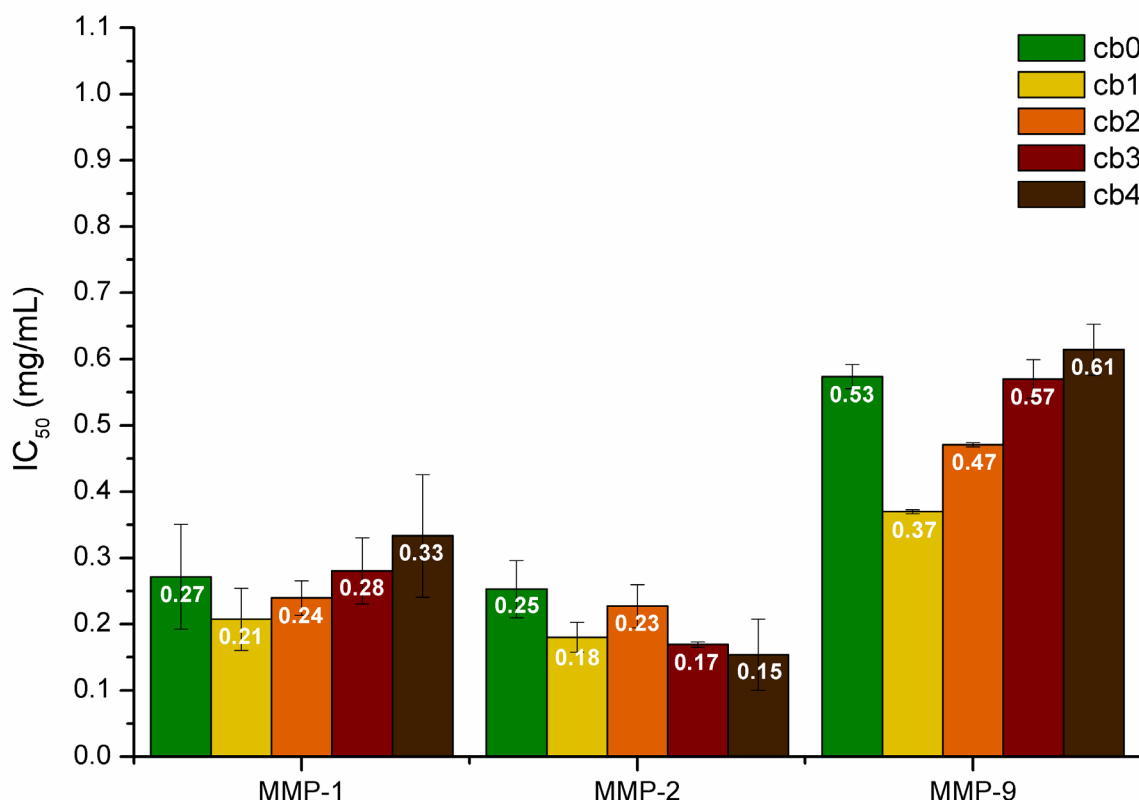
Figure 4-4: IC_{50} values of coffee brews of increasing roast degrees against MMP-1, -2 and -9. Values are means of triplicates ($\pm$SD). Assay is described in section 3.7.2.

The results presented in this section are an evidence of the process-induced formation of compounds with inhibitory activity against metalloproteases in coffee beans. Although this effect cannot be perceived by considering only the inhibitory potential of the whole coffee brews, it is very clear by the analysis of isolated high molecular weight melanoidins, which are formally food borne molecules, that the thermal treatment originates characteristics which were not present in the fraction ahead the heating. However, coffee is a very complex matrix and roasting promotes not only the classical Maillard reaction, but also extensive chemical transformations of polyphenolic compounds, which represent around 7% of green coffee beans dry matter and are almost completely 'degraded' after roasting. These include pyrolysis, polymerization and incorporation in high molecular weight compounds. In summary, the thermal treatment leads to a functionalization of macromolecular fraction by

incorporation of low-molecular compounds with efficient zinc-binding moieties, such as carboxylic, catechol and hydroxypyridinone groups.

The generated non-dialyzable macromolecules cannot be absorbed in intestinal tract, accumulating in lumen, were they can show an important dietary contribution in the protection against intestinal dysplastic formations. We dare to propose that much of the beneficial effects of coffee consumption described in section 2.2.1.3 are caused by the melanoidin fraction. Principally the well-established protective property on colorectal cancer can be a reflex of the action of these final products of the Maillard reaction. A general discussion about the physiological relevance of the observed inhibition is outlined in section 4.2.3.

Effect of zinc addition

As it is known that melanoidins are able to chelate metal ions, it had to be elucidated whether simple zinc complexation, resulting in a removal of the zinc ion from the active center of the metallopeptidases, might be responsible for the observed inhibitory activity of coffee melanoidins, rather than specific interactions of the macromolecules with the enzymes. To check this hypothesis, inhibition of MMP-1 by the melanoidin isolates was measured in the presence of varying amounts of zinc. Melanoidins isolates were tested at 0.5 mg/mL, which is a concentration around the IC_{50} of mRD4 against this enzyme. Zn(II) concentrations were increased only until no impairment on enzymatic function could be measured in the controls. The exact assay conditions are described in section 3.7.2.2. The effect of the zinc addition on the inhibitory potential of the melanoidin samples can be seen in Figure 4-5.

It can be noticed that the inhibitory potential of the melanoidins was not negatively affected by increasingly zinc concentrations. Although the already very low inhibitory effect of mRD0 (0.4%) could not be measured after zinc addition, with progress of roasting the inhibitory potential was even positively affect by the increasingly zinc concentrations; although the differences were not significant under the assay conditions. The reduction of 17.0 ± 3.7 % of the MMP-1 activity caused by the melanoidin isolate mRD1, measured in absence of zinc, increased up to 46.1 ± 4.2 % in presence of 50 μM Zn(II). Under the described conditions, no trend of a restorative impact of zinc on the enzyme activity could be noticed in isolates of more intensively roasted beans. The 51.0 ± 3.9 % metalloprotease

inhibition caused by 0.5 mg/mL of mRD2 in absence of zinc, changed to 46.9 ± 13.8 % in presence of 0.5 µM Zn(II), 42.5 ± 12.4 % at 5 µM Zn(II), and 46.5 ± 1.1 % in presence of 50 µM zinc. Similarly, mRD3 reduced the activity of MMP-1 on 46.2 ± 8.3%, 41.6 ± 20.2 %, 38.9 ± 4.8 %, 44.5 ± 14.9 % and mRD4 on 59.7 ± 1.7 %, 49.4 ± 4.8 %, 56.6 ± 9.9 %, 49.3 ± 3.4 %, in presence of 0 µM, 0.5 µM, 5 µM, and 50 µM Zn(II), respectively.
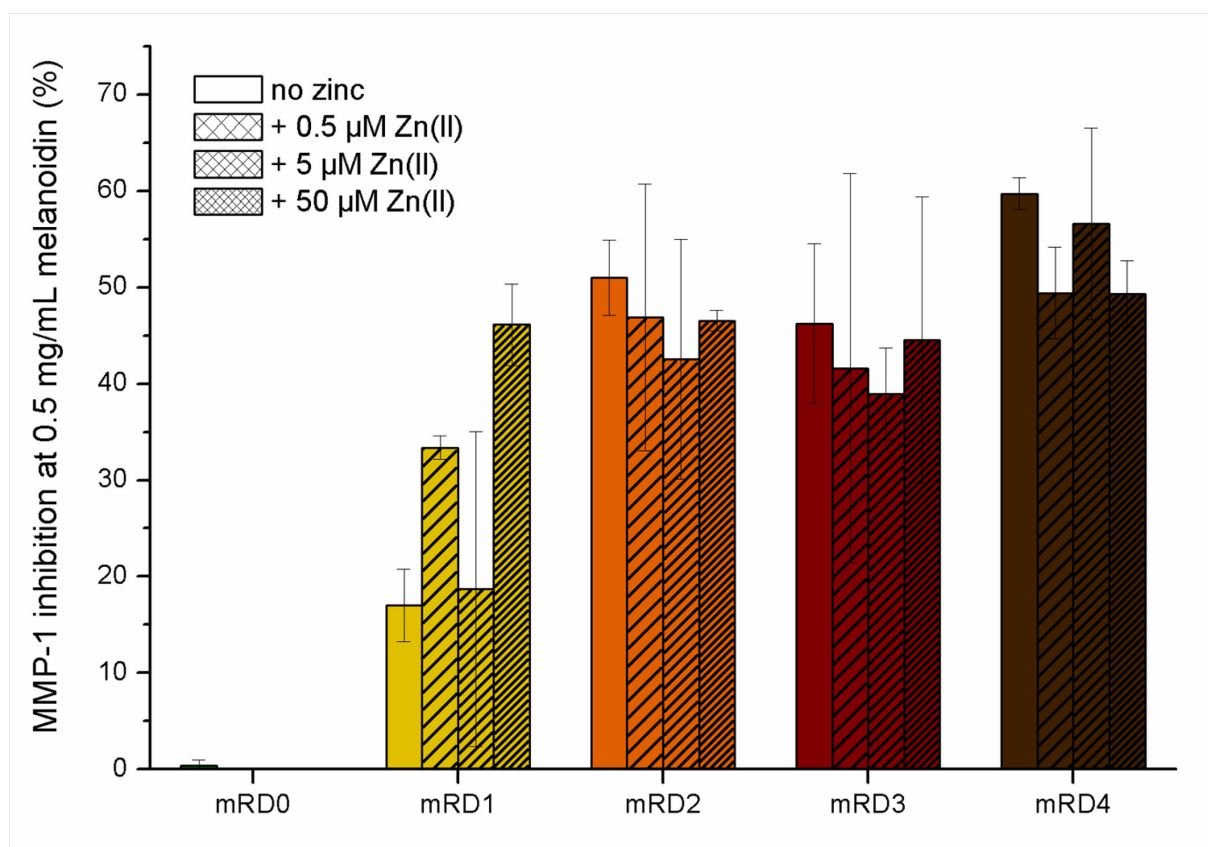
Figure 4-5: Influence of the Zn(II) concentration on the inhibition of coffee melanoidins against MMP-1. Values are means of duplicates (±SD). Assay is described on section 3.7.2.2.

Once the decrease in MMP activity due to the presence of melanoidins cannot be restored by zinc addition, specific molecular interactions between the inhibitor and the active center of the enzymes are supposed.

4.2.2 Inhibition of other zinc metalloproteases

Aiming to proof the specificity of the heat-induced inhibitory properties of the observed for coffee melanoidins against MMPs, two other zinc metalloproteases were tested, namely ChC, which shows relevant substrate similarity to MMPs, and ACE, which plays a central

role in cardiovascular pathologies such as hypertension and cardiac hypertrophy. The two enzymes have a catalytic Zn(II) coordinated to two histidine and a glutamic acid residue. Both assays were based on a time-controlled hydrolysis of a synthetic substrate followed by the chromatographic separation, UV-detection and quantification of the product, according to section 3.5.2.

The high molecular weight melanoidins isolated from coffee beans of increasingly roast degrees did not show any detectable inhibition against ACE in concentrations up to 1.5 mg/mL, which was the highest concentration obtainable in assay. No effect of the roasting on the inhibitory potential could be observed in this concentration. Likewise, the inhibition against ChC was tested up to concentration of 1.25 mg/mL. Once more, no inhibition of the enzymatic activity was found for any of the studied melanoidin isolates.

Rufian-Henares and Morales (2007) reported inhibitory activity of coffee melanoidins of different roast intensities against ACE. The authors found that, at a concentration of 2 mg/mL, the high-molecular coffee melanoidins showed an inhibition of ACE ranging from 36.8% (light roasted) via 43.1% (medium roasted) to 45.1%. This observation reinforces the hypothesis of the present work, that coffee melanoidins have the potential of reducing the activity of zinc metalloproteases. However, this very high concentration could not be reproduced in the present study due to insufficient sample solubility.

In summary, concentrations needed to inhibit MMPs are significantly lower compared to concentrations needed to inhibit the zinc-containing peptidases ACE and ChC, indicating a specific inhibition mechanism based on a molecular interaction between the melanoidins and the catalytic centre of the MMPs rather than simple zinc chelation.

4.2.3 **General considerations**

An undoubtedly increase in the inhibitory activity due to roasting was described in this chapter. Upon roasting, modifications in the chemical structure of the high molecular weight fraction of coffee brews progressively increased the inhibitory potential of the brown non-enzymatic glycation products against zinc-dependent MMPs. Nevertheless, the classical Maillard reaction products may not be the only possible cause of the enhanced inhibitory potential. The incorporation of chlorogenic acids, which are known for inhibiting MMPs in a dose-response manner *(Demeule et al., 2000)*, on the melanoidin structure is good documented *(Adams et al., 2005; Nunes and Coimbra, 2010; Bekedam et al., 2008a;*

Bekedam et al., 2008c). The thermally-induced inhibitory potential of coffee melanoidins towards MMPs is probably an additive effect of new groups formed by through the routes of the Maillard reaction, and the incorporation of molecules present in the green beans, principally hydroxycinnamic acids.

MMPs have physiological inhibitors, a family of 22–28 kDa proteins that form stoichiometric inhibitory complexes with MMPs *(Packard et al., 2009)*. In fact, an increasing number of studies have shown that processing of some protein and peptide substrates by MMPs requires that the substrates interact not only with the active site, but also with regions outside the active site. Such regions are referred to as non-catalytic sites or exosites, which can be motifs localized in the catalytic domain or in one of the other domains. An important role of the exosites may be to orient the substrate properly for cleavage and, for some substrates, exosite-binding is an absolute requirement for degradation *(Hadler-Olsen et al., 2011)*. This observation may explain the preference of the matrix metalloproteases for very complex and voluminous structures, which are able to occupy simultaneously diverse binding pockets inside and outside of the catalytic centre.

This study evidences additionally, that the observed inhibitory potential is not primarily due to the zinc depletion, as the activity of the zinc-containing metallopeptidases ACE and ChC were not affected by melanoidins, and the addition of zinc to the MMP-1 assay system did not restore the enzymatic activity in the presence of coffee melanoidins. The detailed structural background of the interactions between MMPs and melanoidins in order to explain the MMP-selective inhibition remains to be elucidated.

In order to evaluate whether the observed *in vitro* activity of coffee melanoidins are possible to cause physiological consequences, some theoretical estimations were done. The concentration of melanoidins on coffee brew depends on the degree of roast, the type and conditions of extraction, and the strength of the brew. Furthermore, the amount of ingested melanoidins will also depend on the serving size and drinking habits *(Fogliano and Morales, 2011)*. According to the isolation method used in the present study, 100 g of roasted coffee provides around 2 g of soluble high molecular weight melanoidins. Therefore, a 200 mL cup of coffee brew, prepared from 50 g of roasted beans per liter of water, contains around 200 mg of high molecular melanoidins, under the conditions used in the present work. In other words, a cup of coffee supplies 200 mg mRD4, which presents the inhibitory activity presented in Figure 4-2.

Considering the fact that the colon accumulates its content over at least 24 h within its maximum volume of 2 L *(Rogalla et al., 2005)*, the concentration of the non-absorbable high molecular weight melanoidins reaches 0.2-0.3 mg/mL in the lumen, following the ingestion of 2 to3 cups of this coffee brew. This concentration is in the range of the IC_{50} values measured for MMPs. In lumen, a direct contact of the melanoidins with the MMPs secreted by tumoral tissues may happen, proportioning the possibility of effective inhibition. In other words, even conventional drinking habits can result in intestinal melanoidin concentrations, which may lead to a significant inhibition of MMPs. This estimation concerning an uptake of melanoidins is comparable with recently published data *(Fogliano and Morales, 2011)*. Nevertheless, it must be underlined that this is a very conservative estimation. Under normal conditions, the total human colon content comprises only around 200g *(Mackay et al., 1997)*, a fact which indicates that actual much higher concentrations of coffee melanoidins in colonic lumen are commonly reached.

4.3 Structural studies on coffee melanoidins

The chemical structure of coffee melanoidins is largely unknown. Despite several efforts, an exact definition of these food polymers is still impossible *(Nunes and Coimbra, 2010)*. The involvement of proteins, polysaccharides and chlorogenic acids and the influence of heat treatment to form polymers of varying size have been already demonstrated *(Nunes and Coimbra, 2007; Cämmerer et al., 2002; Nunes and Coimbra, 2010; Bekedam et al., 2008a; Bekedam et al., 2008c; Bekedam et al., 2007; Nunes and Coimbra, 2002; Manzocco et al., 2001)*. Melanoidins are formed by cyclizations, dehydrations, retroaldolizations, rearrangements, isomerizations, and condensations of initial Maillard reaction products *(Martins et al., 2000)*, but none of the processes have been fully characterized yet. Structural information is quite limited, mainly because the Maillard reaction in real foods is far more complicated than in model systems, since many more possible reactants are present *(Bekedam et al., 2007)*.

In this context, aiming to elucidate the main chemical transformations caused by the non-enzymatic browning on the high molecular weight fraction of the coffee brew, to chemically characterize the isolated melanoidins, and to localize the groups responsible for the enzymatic inhibition described in section 4.2, it was necessary to draw an exploratory sequence of structural studies, which are reported in the following section.

4.3.1 **Gel permeation chromatography**

In the present work, a silica-based gel filtration column developed for protein and peptide separations was used. The determination of melanoidin molecular weight by conventional methods, including gel permeation chromatography, can be much inaccurate, due to the complexity of melanoidins molecules, with indefinite, most likely irregular structure and shapes. It makes the choice of standards for the estimation of their molecular weight crucial, as the common standards do not show structural similarity to melanoidins. In addition, aggregation phenomena are frequent in solution, difficulting the interpretation of the obtained apparent molecular weights *(Borrelli et al., 2002; Gniechwitz et al., 2008b)*. Since the reports concerning the molecular weight of melanoidin material are very controversial, some of them describing the main fraction around 3 kDa, some describing relevant fraction above 100 kDa *(Borrelli et al., 2002; Reichardt et al., 2009; Gniechwitz et al., 2008b)*, a broad separation range was preferred. Under native conditions, the exclusion sizes are 5-700 kDa, adequate range to analyze such molecules with still largely unknown structure. The most adequate elution was obtained with isocratic flow of 50 mM phosphate buffer, containing 0.15 M NaCl, pH 6.5. The protein standards were used as they exhibited a similar behavior to the melanoidins during the development of the method, with alteration of their elution times caused by the modification of ion force or pH of eluent, in the same manner as melanoidins. Dextran standards were also tested, but they did not show any similarity to the melanoidins elution pattern. This observation suggests that the brown food-borne polymers still maintain a strong protein character, rather than a carbohydrate preponderance.

UV-absorption from high molecular weight fractions of coffee were registered using diode array (DAD) detection of samples submitted to GPC, with the objective of selecting the wavelengths for the measurement sequences. In Figure 4-6 the spectra of the unroasted sample mRD0 and from the melanoidins obtained from coffee with the final roast (mRD4) are illustrated.

In the spectrum of the high molecular weight fraction of green beans (mRD0) three individual peaks can be seen in the high molecular weight region. The maximum around 220 nm can be explained principally by the presence of proteins. Low molecular weight components with pronounced absorption in this region could not be detected. With onset of thermal treatment, the distinct peaks seen at mRD0 give place to an individual peak, which

elutes around 30 min. The 220 nm maximum is still marked, however the high molecular weight compounds are progressively functionalized, acquiring UV-active character along the roasting. The final melanoidin isolate (mRD4) shows, besides the 220 nm absorption, maxima at 320 nm, possibly explained by the presence of chlorogenic or caffeic acid *(Bekedam et al., 2006)* and at 420 nm, due to the formation of chromophores.
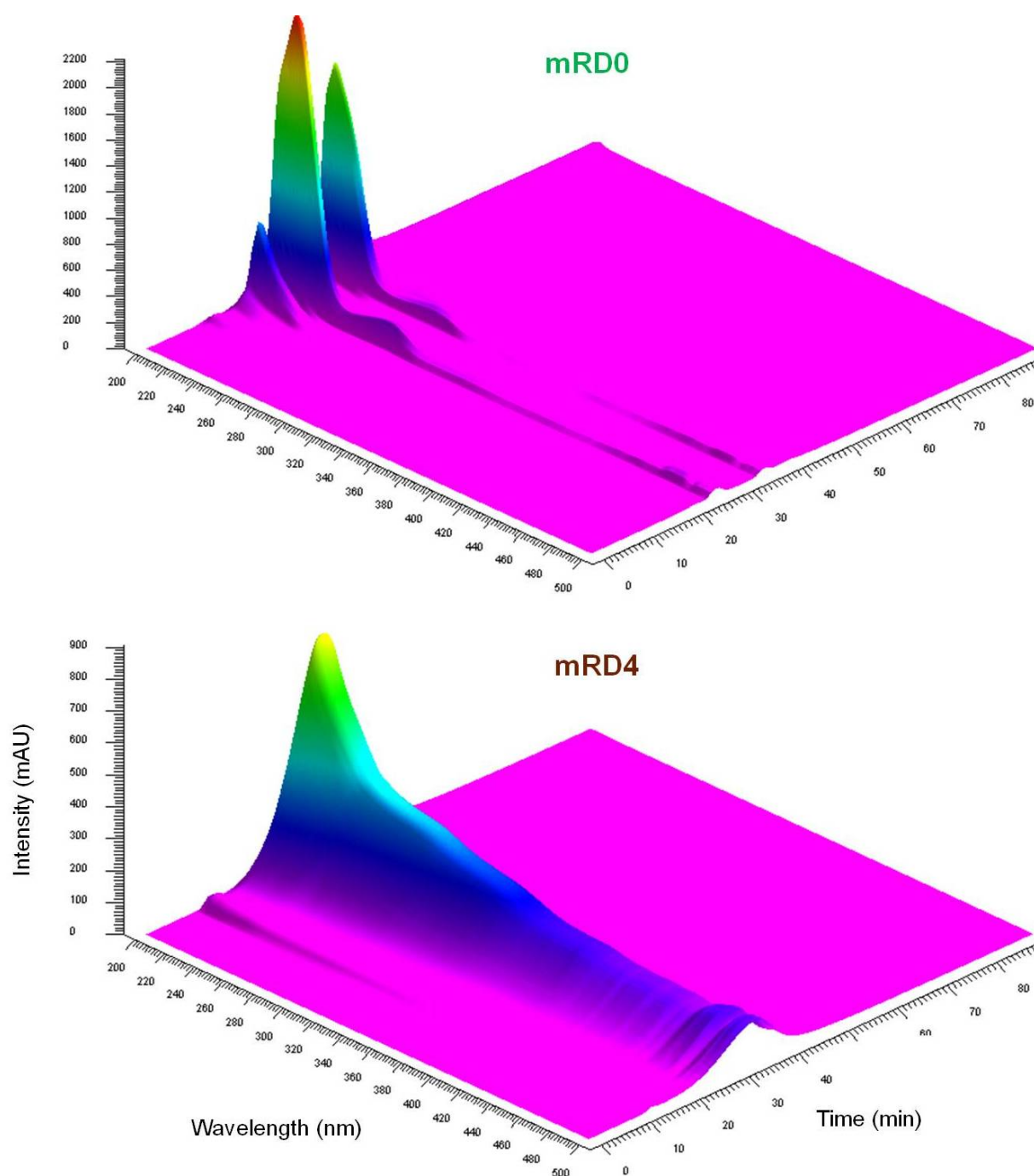
Figure 4-6: DAD spectra of the high molecular weight fraction of green coffee beans (mDR0) and melanoidins from final roasted coffee (mRD4)

The general absorption is less intensive at 405 nm than at 220 nm, but the analysis of the chromatogram can be more specific for melanoidins at the higher wavelength, once the absorption of light at 405 nm is characteristic for these brown compounds and widely used in the literature *(Borrelli et al., 2002; Hofmann et al., 2001; Bekedam et al., 2006)*. In this work, 405 nm was thus the wavelength chosen to accompany the changes in the melanoidin profile. The absorption of peptide bounds of the assumed protein skeleton of the melanoidins (220 nm) constitutes a stable base for comparisons. The absorptions at 280 nm were likewise registered, in order to follow the formation/incorporation of aromatic systems in the melanoidin complex.

The gel permeation chromatograms of the coffee brews, obtained as described in Section 3.6.1 and detected at 220 nm and 405 nm, can be seen in Figure 4-7. The elution times of the standards, used for the estimation of the apparent molecular weight of the peaks, are pointed out.

With beginning of the roasting, major modifications on the profile of the coffee brew can be observed. The peaks at 23 and 34 min from cb0, corresponding to 181 and 22 kDa, respectively, are intensive in cb0, but absent in cb1. Probably, these macromolecules are proteins, which were denatured and aggregated with beginning of the thermal treatment and could not be extracted by hot water. Noteworthy is the roasting-dependent increase of the peak around 39 min. This peak corresponds to emerging melanoidinic structures, product of the non-enzymatic browning. A closer look at this fraction will be taken following.

Also the continuously decreasing peak at 55 min must be noticed. Even though the used column does not separate analytically within this molecular weight range, the region corresponds to the elution of chlorogenic acid. This fact suggests the decomposition or condensation of the free polyphenolic compound, or the reaction of these with other coffee components, forming structures of higher molecular weight. It can therefore be concluded, by means of this figure, that the detectable alterations at 405 nm are principally relative to the formation of high molecular weight compounds and the decomposition of polyphenols or their migration in other structures.
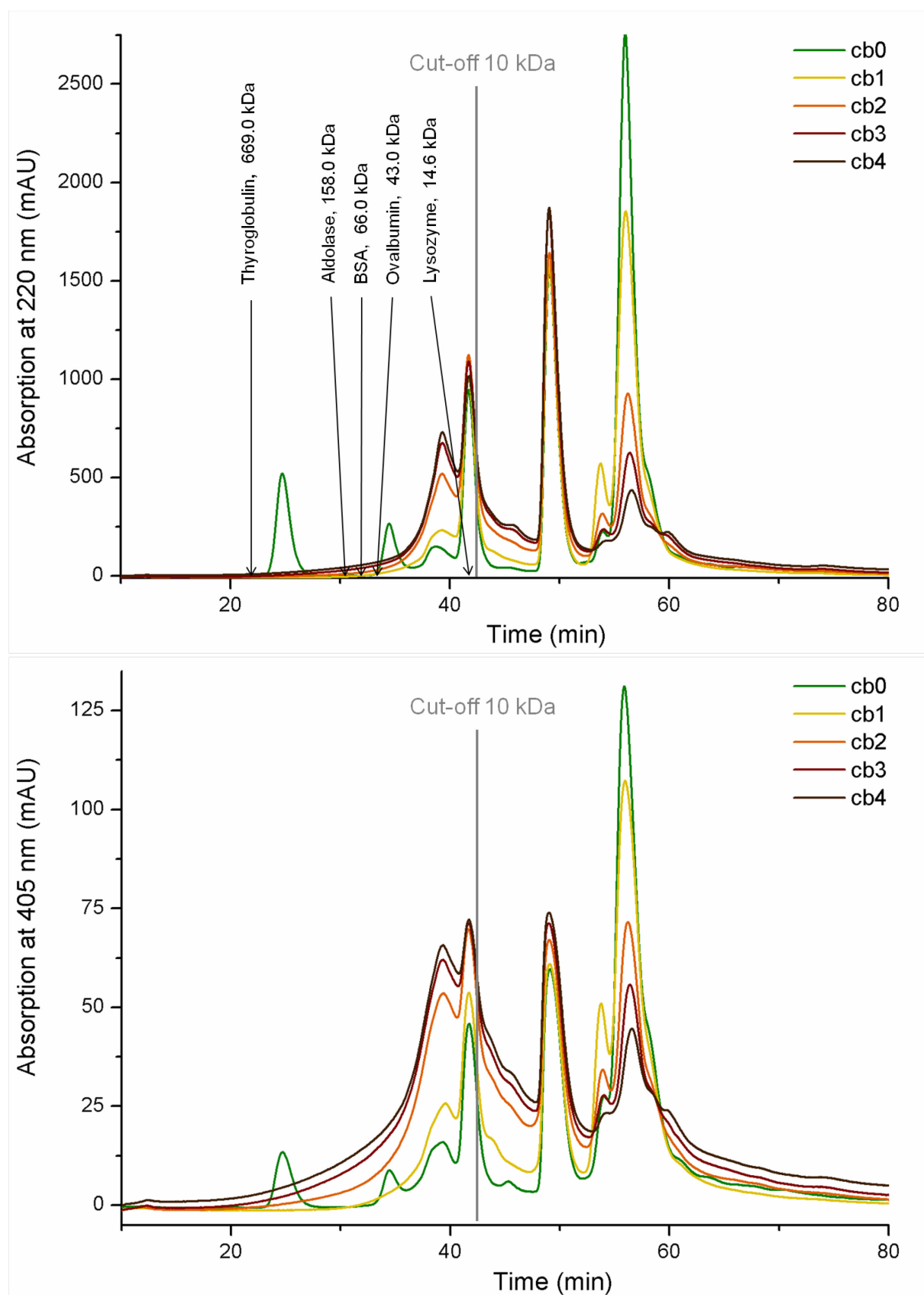
Figure 4-7: Analytical gel permeation chromatogram of coffee brews (cb) of different roast grades, detected at 220 nm and 405 nm. Conditions are described in section 3.6.1. The elution times of the standards used for the calibration are pointed out, as well as the elution time corresponding to the MWCO of the membranes used for isolation of coffee fractions.

In the Figure 4-8, the elution patterns of the low molecular weight fractions are shown. The profile of each lmw isolate is very similar to the respective original coffee brew. This is a consequence of the quantitative dominance of low molecular weight compounds in coffee extract. The fractions used in the present work were isolated by exhaustively resuspending the retentates in water and repeating the ultrafiltration, until the absorption at 405 nm of the filtrate was found to be below 0.1. Since all filtrate was collected, the recovery was almost complete, what is impracticable by using dialyzation as separation method. This extensive isolation procedure led to the recovery of nearly all detectable peaks of the coffee brews in the low molecular weight fraction, which responds also for around 90% of the total coffee extract dry matter.

Figure 4-8: Analytical gel permeation chromatogram of low molecular weight fractions (lmw) obtained from coffee brews of different roast grades, detected at 220 nm. Conditions are described in section 3.6.1. The elution time corresponding to the MWCO of the membrane used for isolation (10 kDa) is shown.

By examining the analytical chromatograms of the lmw fraction, it can be noticed that quantitatively relevant peaks with apparent molecular weights far above the nominal pore size of the membranes used for ultrafiltration (10 kDa) can be seen. This fact was expected,

as, for proteins, a MWCO at least 3 times smaller than the molecular weight of the solute being retained should be chosen to ensure that the target molecules will end up in the filtrate. Furthermore, retention of a molecule by an ultrafiltration membrane is determined by a variety of chemical and physicochemical characteristics, among which its molecular weight should be used merely as orientation. Many other factors including molecular shape, electrical charge, sample concentration, sample composition, and operating conditions influence enormously the separation. However, as the intention of this separation was to obtain pure melanoidin preparations, free from any low molecular weight compounds, the isolation was regarded as successful and adequate to the aims of this work, as can be seen in Figure 4-9. In fact, the high molecular weight fraction (mRD) is composed virtually exclusively by molecules over 10 kDa. No free phenolics or low molecular weight compounds could be chromatographically detected, what shows that the exhaustive washing during ultrafiltration is an efficient tool to gain pure high molecular weight melanoidins.

The high molecular weight isolate obtained from the green coffee beans (mRD0) showed three distinct peaks with apparent molecular masses of 446, 181, and 22 kDa. On the basis of literature facts, it can be supposed that the first peak is composed by arabinogalactan-proteins and the two other peaks with apparent molecular masses of 181 and 22 kDa are basically of a proteinous nature *(Redgwell et al., 2002a; Nunes et al., 2005; Nunes and Coimbra, 2002)*.

Upon roasting, a distinct change can be observed for the chromatographic profiles. Already for mRD1, only one broad peak with a mean molecular mass of approximately 14 kDa was detectable, indicating transformations and degradation of the high molecular polymers from the green coffee beans. Due to the high temperatures, denaturation and aggregation of proteins and polysaccharides occurred, leading to insoluble products *(Bekedam et al., 2008c)*. With progress of roasting, a gradual increase in the apparent mean molecular mass of this melanoidin peak can be noticed, increasing from 14 (mRD1) to 17 (mRD2), 21 (mRD3), and 28 kDa (mRD4). This progression can be better observed in the chromatograms of mRDs detected at 405 nm. This confirms findings by *(Wen et al., 2005)*, who correlated the molecular size of polymers from coffee with the progress of roasting.

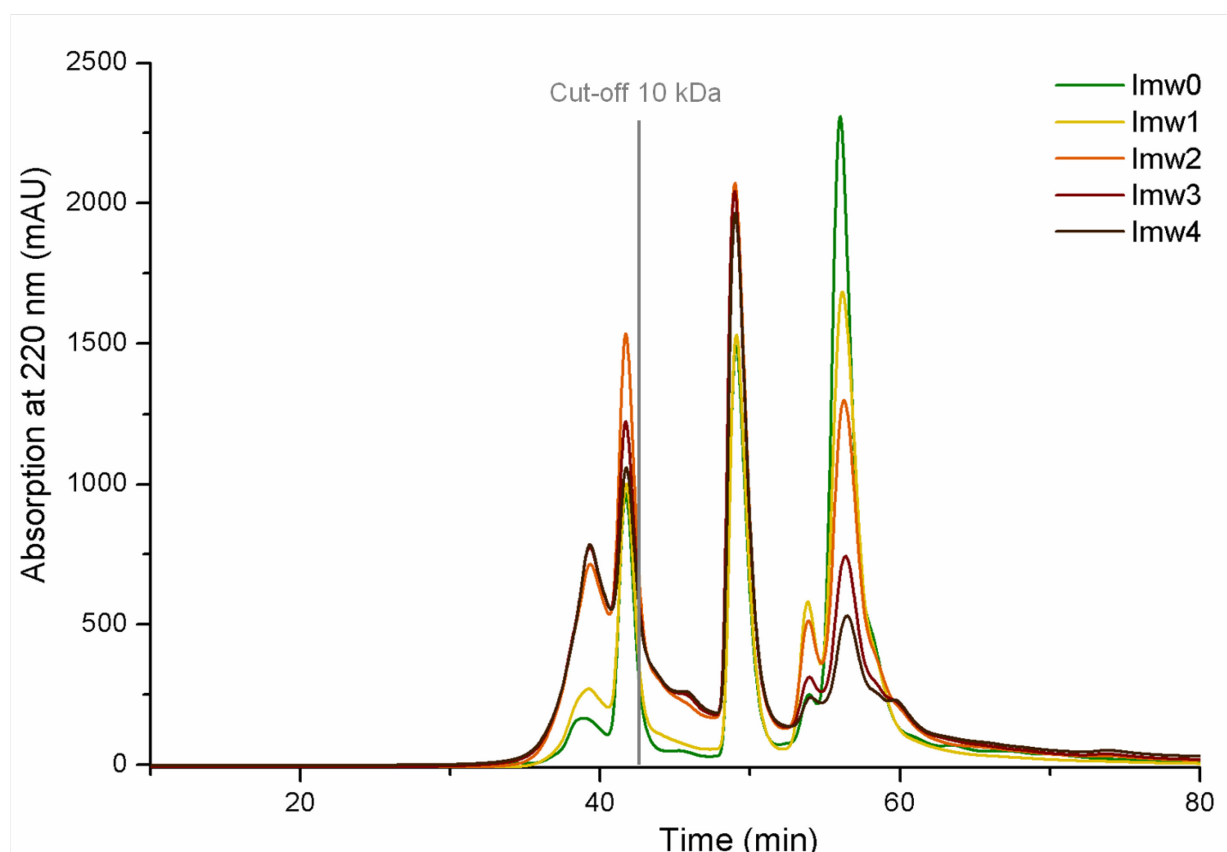**Figure 4-9:** Analytical gel permeation chromatogram of melanoidin fractions (mRD) obtained from coffee brews of different roast grades, detected at 220 nm and 405 nm. Conditions are described in section 3.6.1. The elution time corresponding to the MWCO of the membrane used for isolation (10 kDa) is shown.

The absence of very high molecular weight peaks in the melanoidin chromatograms, even by using a broadband column, corroborates the most recent studies. Coffee extracts or melanoidin isolates obtained by GPC fractionations have been subject of several studies during the last years. Although an important fraction of melanoidins have been reported to show molecular weights above 100 kDa *(Borrelli et al., 2002)*, this observation could not be supported by many groups *(Gniechwitz et al., 2008a; Reichardt et al., 2009)*. *Hofmann et al. (2001)* fractionated coffee brew in a Sephadex G-25 column and obtained melanoidin peaks with molecular weight between 3 and 60 kDa. *Bekedam et al. (2006)* observed that chromatography of this same material, which is broadly used in investigations with coffee melanoidins, showed very poor reproducibility, and concluded that the elution of coffee components on this material was not only based on size exclusion but also on physicochemical interactions. Therefore, they opted for a size exclusion separation in a broadband polymethacrylate column, using an HPLC system. *Gniechwitz et al. (2008b)* isolated from coffee brews, using a Sephadex LH-20 column, a fraction with molecular weight above 100 kDa and another with the components between 3 and 10 kDa. They found both isolates to have similar chromatographic and electrophoretic behaviors and no relevant differences in carbohydrate and amino acid contents or composition could be observed. The authors suggest that extremely-high-molecular-weight coffee melanoidins (>100 kDa) may not be real but are confounded with non-covalent complexes, made up of melanoidins with molecular masses around 3–22 kDa and high-molecular-weight carriers. In line with these findings, *Takenaka et al. (2005)* estimated a molecular weight of about 35 kDa to the zinc-chelating melanoidinic fraction of coffee.

In addition to the raise of the mean molecular mass, a continuous increase of the peak areas was shown. As the enlargement in the melanoidin content in coffee (yield, Table 4-1) was not as intense as the intensification of the AUC for the same isolates, this can be explained as a progressive increase in the absorption coefficient of the melanoidin isolate due to intensified formation of chromophores in the melanoidin complex.

But not only compounds absorbing at 405 nm are formed during the roasting. The formation of compounds absorbing at 280 nm has also been reported *(del Castillo et al., 2002)*. On Figure 4-10 the gel permeation chromatograms of the isolated coffee melanoidins obtained at 280 nm can be seen.
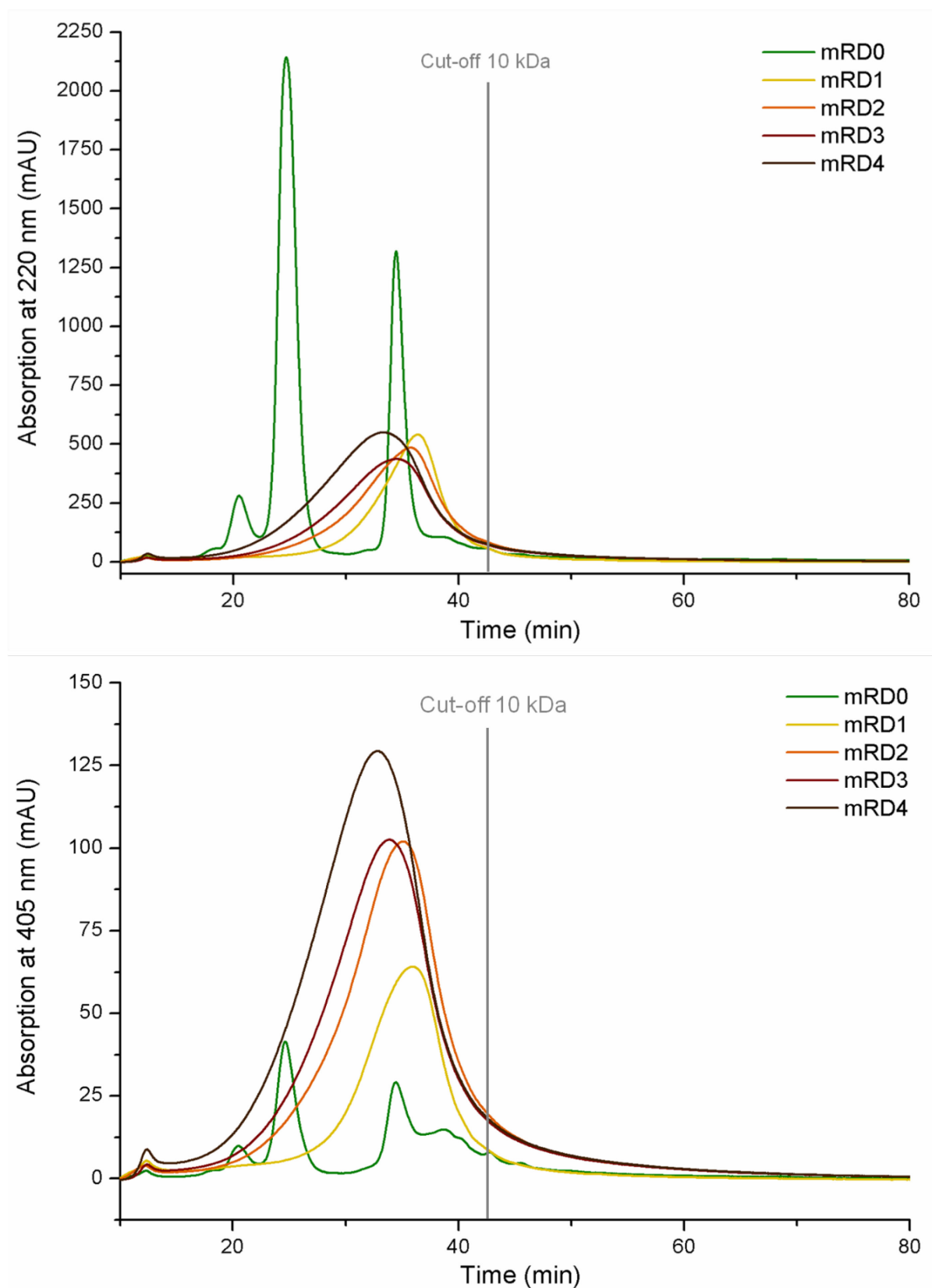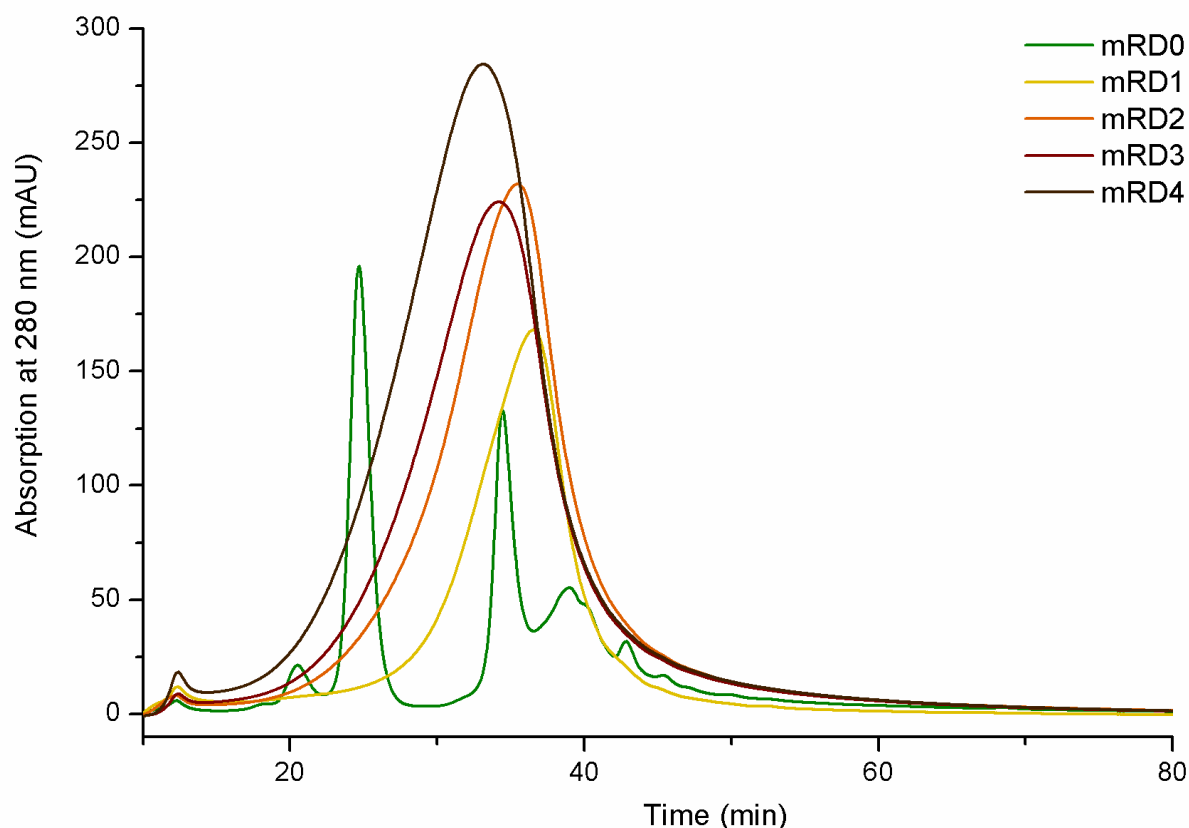
Figure 4-10: Analytical gel permeation chromatogram of melanoidin fractions (mRD) obtained from coffee brews of different roast grades, detected at 280 nm. Conditions are described in section 3.6.1.

The chromatographic profiles of the melanoidin isolates, measured at 280 nm, are qualitatively comparable to the ones detected at 405 nm. The extinction at 280 nm is generally used, in coffee melanoidin investigations, as a basis of comparison, being considered a sign of protein presence. However, also at this wavelength a progressive increase in the absorption coefficient can be observed with progress of roasting, suggesting an incorporation of unsaturated systems to the melanoidin skeleton simultaneously to the development of the pigmentation. This fact led the decision for not using the extinction at 280 nm as the standard absorption for comparisons relative to the roast intensity, as the extinction at 220 nm is less prone to be altered by functionalization.

The general progression of the area under the curve of melanoidin peaks can be seen in Figure 4-11. The alterations reflect the changes in the extinction coefficient of the melanoidins, once the injected solutions presented the same the concentration. It is evident that the formation of characteristic melanoidin chromophores and of unsaturated systems occurs. Their incorporation on the macromolecular structures, manifested as the increase on

the absorptions at 405 and 280 nm of the high molecular weight fraction, are parallel heat-induced phenomena.
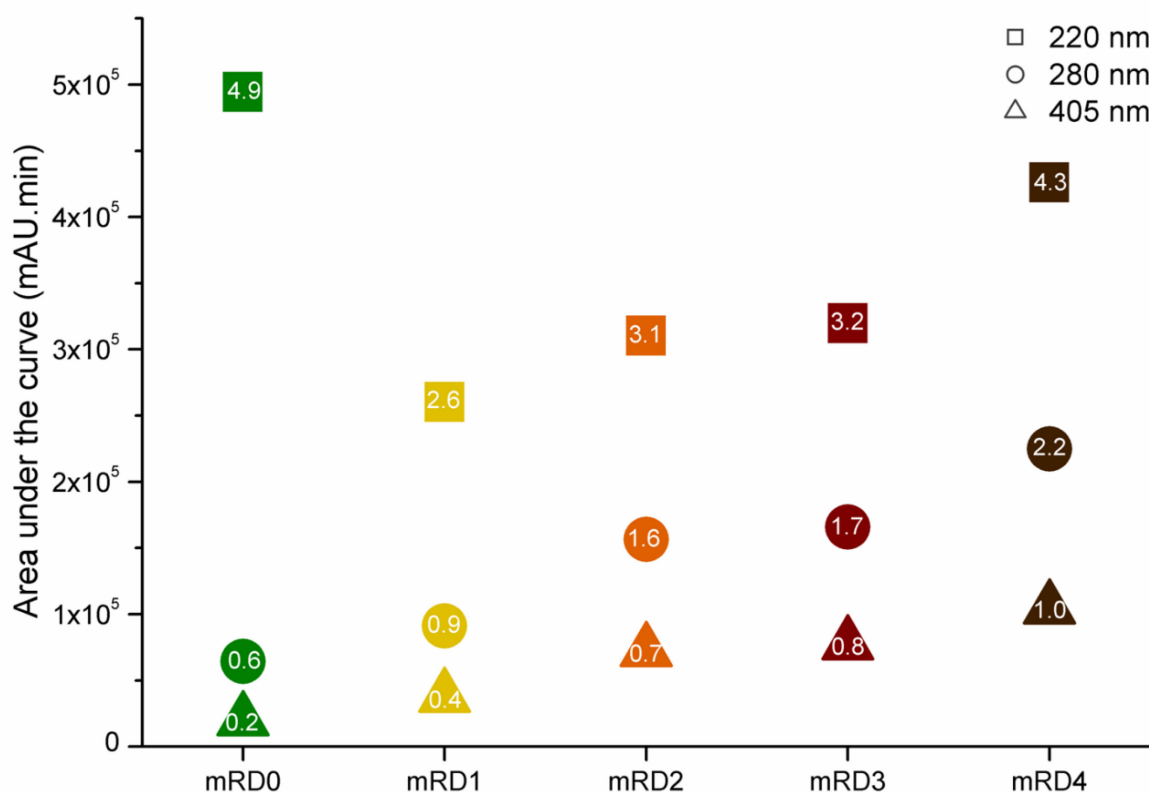
Figure 4-11: Area under the curve of melanoidins gel permeation chromatographic peaks in the different detected wavelengths. mRD0 to mRD4 are the melanoidin isolates obtained from coffee samples of increasingly roast grades. The areas of all mRD0 peaks were summed.

Hofmann (1998a) developed a method for the assessment of chromophores formation during Maillard reaction systems, based on the visual threshold of colored fractions. This method has been currently used in the literature to estimate the melanoidin character of a sample *(Gniechwitz et al., 2008b). Bekedam et al. (2006)* introduced specific extinction coefficients, aiming to determine the relative amount of chlorogenic acid and/or proteins compared to the amount of melanoidins coffee samples. The authors suggested the calculation of extinction constants by comparing the extinction of the samples at 280, 325 and 405 nm. However, they did not take in account that UV-active compounds are formed through the Maillard reaction and other conjugated systems apart from chlorogenic acid can distort the expected prediction.

By means of the absorption areas described in Figure 4-11, another estimation parameter can be suggested. By comparing the area under the curve detected at 405 nm with the area at the more constant "basis" absorption (220 nm) a "browning index" can be proposed[*]. The Figure 4-12 illustrates the evolution of the browning index of the coffee melanoidins isolated in the present work with progress of the roasting.
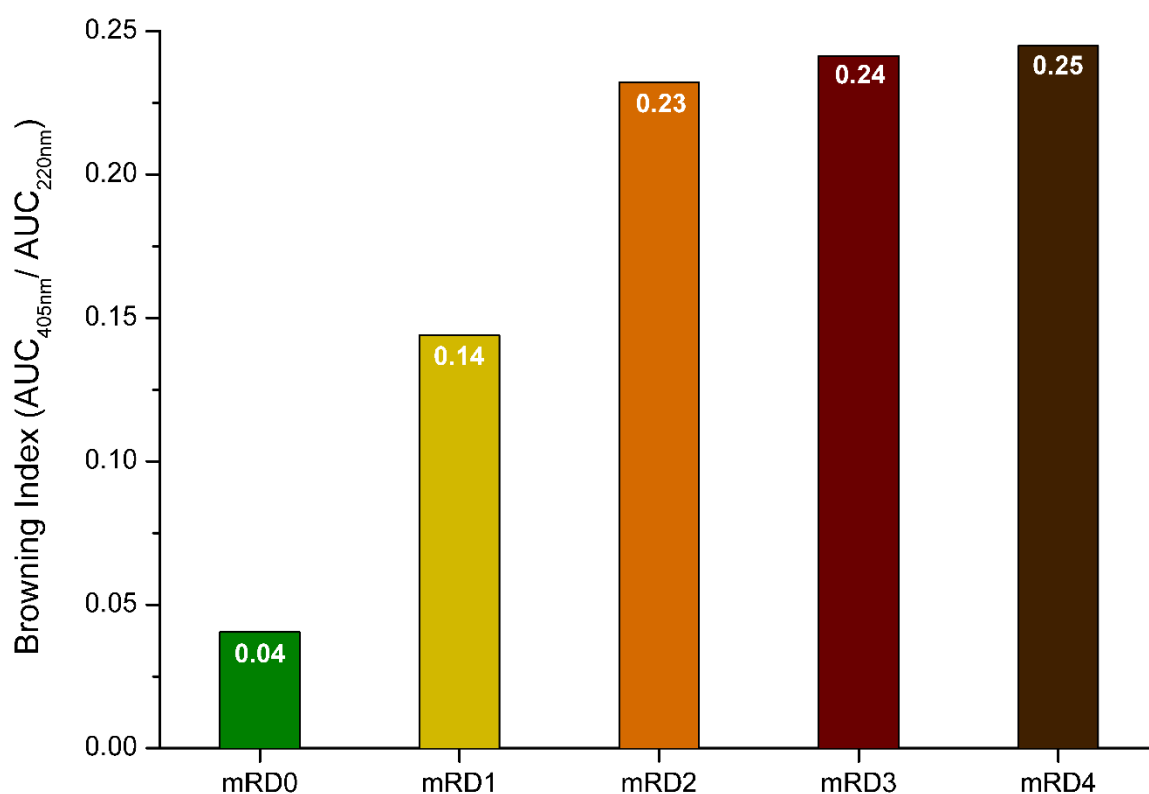
Figure 4-12: Browning index of coffee melanoidin samples. The index is obtained by diving the area under the curve of the melanoidin peak detect at 405 nm by the area under the curve using a 220 nm detection. Values used for the calculation are described in Figure 4-11. mRD0 to mRD4 are the melanoidin isolates obtained from coffee samples of increasingly roast grades.

The suggested estimation seems to qualitatively follow the same trend as the transformations observed in the gel-permeation chromatographic investigations presented until now, as well as with the results to be reported in the sections 4.3.2. and 4.3.4. Following the rapid initial changes of the high molecular weight structures until RD2, a slower progression of the modifications is observed in the last stage of the roasting. This

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[*] $Browning\ Index = \dfrac{AUC_{405}}{AUC_{220}}$

observation correlates also with the fact that the last three melanoidin isolates were obtained from coffee beans in roast stages commercially available, ingested according to personal and cultural preferences. As described in section 3.5.1, the 'American roast' of RD2 correspond to the mildly roasted product, while RD3 is the dark 'French roast', and RD4 is the very dark 'Italian roast', the adequate point for the production of a quality espresso coffee. A validation of the proposed method should be done.

4.3.2 Elemental analysis: C/N ratio

With the study of the evolution of the ratio between carbon and nitrogen contents of isolated melanoidins upon roasting, measured by elemental analysis (described in section 3.4.2), we aimed to get clear evidence about the chemical nature of the melanoidin backbone and the main transformations taking place in the high molecular weight fraction of coffee during the thermal treatment. The results are illustrated in Figure 4-13.
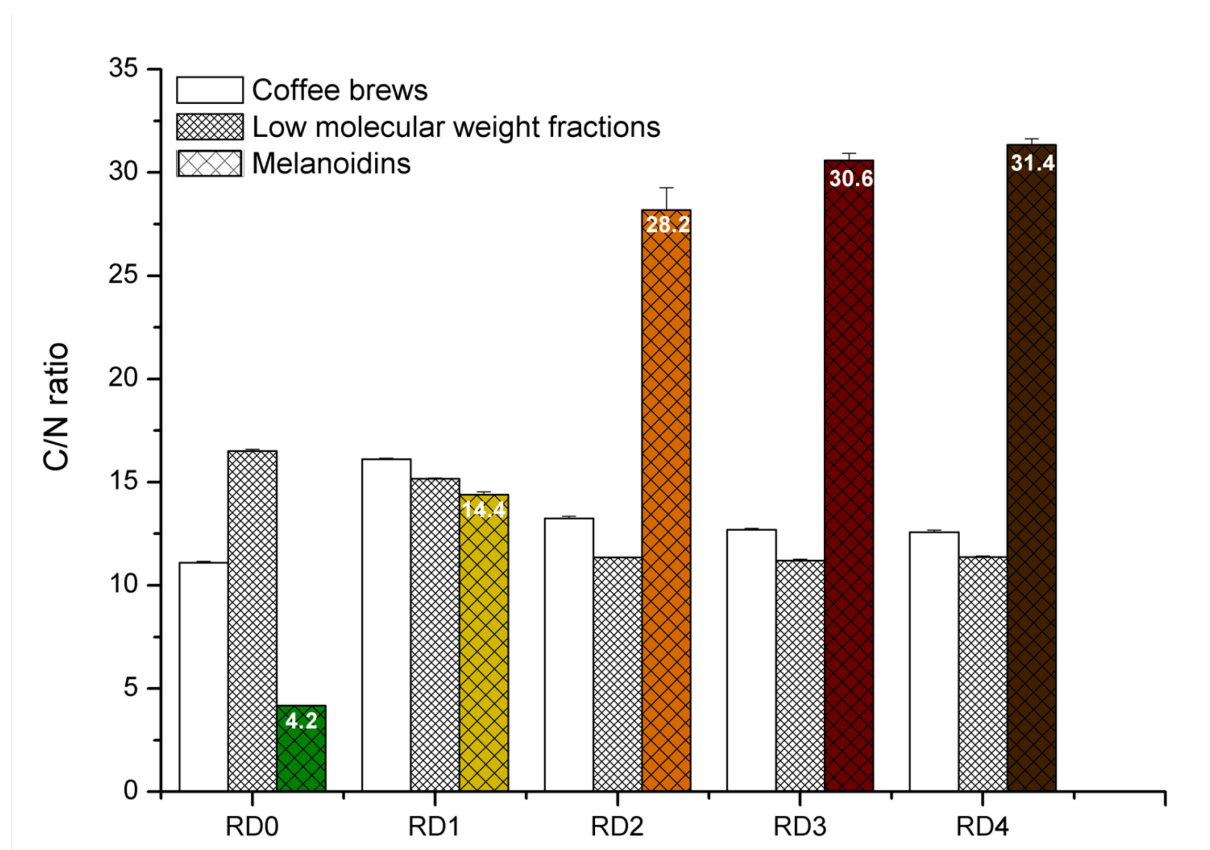
Figure 4-13: Ratio between carbon und nitrogen contents (C/N ratio) in whole coffee brews of increasing roast degree and their respective low molecular weight and melanoidin and their fractions. (RD, roast degree).

Once again, the influence of the low molecular weight fraction on the behavior of the whole coffee brew can be seen. With beginning of the heating, the carbon-rich low molecular weight fraction undergoes a relative content loss of carbon in comparison to nitrogen. This fact can be explained by the initial degradation of sugars with cleavage of the carbon chain and formation of volatile fragmentation products, which, by the way, play a considerable role in aroma formation substances *(Ledl and Schleicher, 1990)*. The pyrolytic loss due to decarboxylation of many other low molecular with is also a much probable event. Also the incorporation of C-rich molecules in the melanoidin complex through the Maillard reaction or other chemical processes, causing carbon depletion in the lmw fraction, should be considered. In roasted beans, the most nitrogen-containing low molecular weight compounds are caffeine and some trigonelline, but also nitrogen from glycated amino acids and peptides is present in the fraction *(Bekedam et al., 2008)*. Except for trigonelline, which shows marked thermolability, partially originating volatile compounds as pyridine and pyrazines, it is likely that the nitrogen composing low molecular weight molecules do not migrate from the fraction. Thereby, the decrease in C/N-ratio is clear.

In regard to the melanoidin fractions, the pattern is opposed. A continuous increase in the C/N ratio can be seen. The changes in the C/N ratio are significant when mRD0 (4.2) and mRD1 (14.4) as well as mRD1 and mRD2 (28.2) are compared. The drastic increase in the carbon content relative to nitrogen suggests a sudden loss or degradation of nitrogen-rich substances, such as amino acids, peptides, and proteins via reactions such as deamidation. Furthermore, an incorporation of carbon-rich substances, such as carbohydrates, sugar degradation products and polyphenols, are much likely to occur, resulting in the final polymeric melanoidin structure.

With further roasting progress from mRD2 to mRD3 or from mRD3 to mRD4, the increase in the C/N ratio is less pronounced. The principal reaction in the formation of a high molecular melanoidin structure, therefore, should be coupling of carbon-rich compounds such as carbohydrates, polyphenols and their degradation products, to a nitrogen-rich protein skeleton. Similar results were found by *Bekedam et al. (2008c)*, who demonstrated a strong carbohydrate-like component on high molecular weight melanoidins, describing a positive correlation between the roasting and the content of galactomannans for the melanoidin fraction of a coffee brew. In the same paper, the authors reported a close relation

of nitrogen and melanoidin levels, indicating a direct involvement of nitrogenous compounds in melanoidins formation.

Interestingly, Maillard reaction products with high C/N ratios have been related to higher metal affinity when compared to counterparts of lower C/N ratios in model studies *(Wijewickreme and Kitts, 1998; Wijewickreme et al., 1997)*.

The N contents, measured by means of elemental analysis, were used to estimate the "protein content" of the coffee brews and their fractions. The universal conversion factor 6.25 was used. The calculated results can be seen on Table 4-2.

Table 4-2: Estimated protein content of coffee samples, calculated using the nitrogen content determined as described in Section 3.6.2 and the universal conversion factor (6.25).

	"Protein" content on dry matter (%)				
	RD0	**RD1**	**RD2**	**RD3**	**RD4**
Coffee brews	24.22 ± 0.13	16.59 ± 0.04	20.41 ± 0.13	21.34 ± 0.13	21.13 ± 0.18
lmw	14.88 ± 0.09	16.47 ± 0.04	22.66 ± 0.04	22.69 ± 0.18	22.41 ± 0.13
Melanoidins	68.19 ± 0.27	17.72 ± 0.22	9.06 ± 0.35	8.81 ± 0.09	8.63 ± 0.09

The values are means of duplicates (±SD) and are based on dry matter (%, w/w).

It can be noticed that the early denaturation and aggregation removes proteinaceous material from coffee brew, reducing its protein content on almost 1/3. This effect disappears with continuation of the roasting process, what can be interpreted as an evidence of resolubilization of denatured proteins with prolonged roasting. The improved extractability could have been caused by chemical modifications of the protein, like chlorogenic acid incorporation.

In the lmw fraction, no initial decrease in protein content can be seen. It must have been caused by the fact that most low molecular weight nitrogen compounds from coffee are not of protein origin, like caffeine and trigonelline, which are not significantly altered. With continuance of the treatment, degraded proteinous material turn extractable, and C-rich low molecular weight compounds, like sugar degradation products and phenolic compounds, migrate to the high molecular weight fraction.

In the melanoidin fraction, the loss in "protein" is abrupt and continuous. The initial decrease is, as discussed above for the coffee brews, due to the diminished extractability of proteins after the initial roasting. The following steps bring C-rich compounds to the originating melanoidinic structure, changing the relative contents of C and N and giving the impression of a decrease in protein content. This observation is in line with the observations of *Nunes and Coimbra (2007)*, who reported a decrease in the amount of protein-like material in the high molecular weight melanoidins quantified by amino acid analysis after acid hydrolysis.

Bekedam et al (2008c) related an increase of the nitrogen amount in all molecular weight ranges, including the high molecular weight fraction, at the final step of roasting process. These results are not in conformity with the values presented in Table 4-2. This contradiction is most probably due to the roasting method. Although the author does not describe the roast conditions of the studied samples, the common industrial process uses temperatures of about 250 °C. The coffee used in the present work was roasted under gentle conditions, aiming to preserve the full aroma and foamability of the espresso beans. Apparently, the mild treatment prevented the extreme degradation of coffee compounds frequently related in the literature for dark roasted coffees.

4.3.3 **Amino acid analysis**

To better understand the transformation of the proteinaceous material of coffee melanoidins, the coffee brews and their fraction were submitted to amino acid analysis after acid hydrolysis, according to section 3.6.3. The Figure 4-14 illustrates the changes in total amino acid content of the melanoidin fractions along the roast treatment.
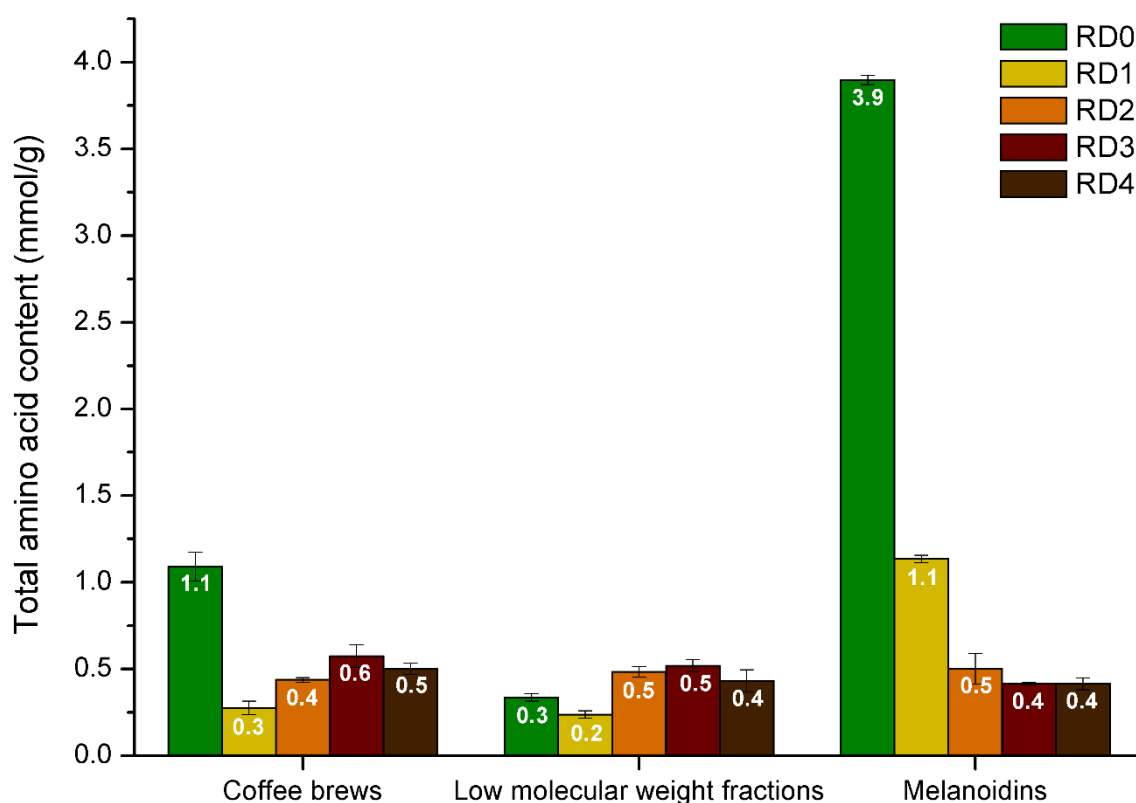
Figure 4-14: Total amino acids content after acid hydrolysis of coffee brews of increasingly roast degrees (RD0-RD4) and their fractions.

The thermal treatment caused an impressive decrease in the amount of detectable amino acids, in particular for the high molecular weight components of green coffee. This decrease from RD0 to RD1 may be due to aggregation and denaturation processes, leading to limited protein extractability. This alteration is visible principally in the melanoidin fractions, and is so intensive, that reflects in the pattern observed for the whole brew. With advance of roasting, increasingly amounts of amino acids can be measured in the lmw fractions, probably as result from fragmentations of higher molecular weight compounds. Even if the decrease in the amino acid content of the melanoidin fraction can partially be explained by the migration of protein fragments in the lmw fraction, the losses of the high molecular weight fractions are also to be explained by the participation of amino acids as nucleophiles in the Maillard reaction, during which they can be blocked or transformed, originating, for example, heterocyclic AGEs. In general, it can be noticed that the course of the relative alterations are very similar to the data observed in Table 4-2, where the protein content was estimated using the nitrogen contents based on elemental analysis.

Also *Bekedam et al. (2008c)* described a massive initial loss of the proteinaceous material of green coffee. Investigating nitrogen and protein losses, this author estimated that 25% of the total amino acid degradation in the high molecular weight fraction should be due to participation of amino acids in Maillard-like reactions and that 75% should be due to limited solubility of denatured proteins. *Cämmerer et al. (2002)* found a marginal liberation of intact amino acids after acid hydrolysis of melanoidins, obtained from heating amino acids and sugars in model systems. The authors interpreted this observation as evidence for either the incorporation of only small amounts of proteins in the melanoidins skeleton, or for an amino acid depletion caused by decomposition and participation in the branching of sugar degradation products. *Nunes and Coimbra (2007)* reported both reduction in the recoverable protein-like components of melanoidins upon roasting and the formation of CML in this fraction. Based on literature discussions and results of this work, it can be suggested that the melanoidins are not poor on protein, as suggested in earlier investigations, but rather the protein backbone loses its native character, and the nitrogen components loses quantitative importance due to massive incorporation of carbon-rich moieties.

Pentosidine

Pentosidine is a crosslink amino acid formed at the final phase of the Maillard reaction between lysine and arginine, and is thus an interesting marker for protein oligomerization induced by glycation reactions. As illustrated in the Figure 2-3, pentosidine contains an imidazo-pyridine ring, which confers to the molecule a characteristic fluorescence (λex = 335 nm, λem = 385 nm). Although its presence in food is limited, the detection is very sensitive, and its presence can be also an indicator of intensity of food processing *(Schwarzenbolz et al., 2000)*.

The Table 4-3 provides an overview on the pentosidine contents in the coffee samples and their fractions, quantified by fluorescence, after acid hydrolysis and chromatographic separation, as described in Section 3.6.3.4. This cross-link amino acid could only be detected from middle roasted samples on. It is remarkable that, after the suddenly initial increase, the pentosidine content stayed almost constant until the end of the roasting process. This observation evidences the major importance of the carbohydrate-dependent non-enzymatic protein cross-link for the initial stages of roasting, which however, is not

observed with advance of roasting, suggesting an early depletion of the available reactive structures in protein skeleton.

Table 4-3: Pentosidine content in coffee samples of increasingly roast degrees (RD0 to 4), analyzed and calculated as described in section 3.6.3.4.

	Pentosidine content (mg/kg protein)				
	RD0	**RD1**	**RD2**	**RD3**	**RD4**
Coffee brews	n.d.	n.d.	1.40*	1.84 ± 0.10	2.10 ± 0.49
lmw	n.d.	n.d.	1.54 ± 0.28	1.83 ± 0.47	1.93 ± 0.61
Melanoidins	n.d.	n.d.	7.96 ± 1.31	9.06 ± 1.05	8.77 ± 0.52

Results are means of duplicates (±SD). n.d.= not determinable: No pentosidine peak was present on chromatogram. * = Single determination. Protein content was calculated on the basis of nitrogen content, measured by elemental analysis.

Higher values were described by *Henle et al. (1997)*, who found contents between 11 and 40 mg/kg protein (1-4 mg/kg coffee) in roasted coffee. As already discussed in section 4.3.2, the coffee samples used in the present study were especially mildly treated, and the lower temperatures can possibly have favored different kinds of derivatization.

The prevalence of this cross link amino acid in the high molecular weight fraction of coffee, in comparison with the low molecular weight fraction, can also be noticed. The high molecular weight protein of coffee extracts are at least four times more intensively cross-linked than in the nitrogenous components in low molecular weight isolate, probably due to the abundant presence of non-protein nitrogen in this last fraction, which cannot be glycated.

In summary, the observed pentosidine contents point to the participation of protein cross-link mediated by carbohydrates in the formation of high molecular melanoidins in the early stages of the roasting, forming a nucleus, which later undergoes different modifications through other mechanisms.

Lysinoalanine

The crosslink amino acid lysinoalanine (LAL) was quantified using ion exchange chromatography, as described in section 3.6.3.3. No lysinoalanine peak could be detected,

suggesting that crosslinks involving lysine are not important features in the formation and "growth" of melanoidins, what can have been caused by the acidity of coffee beans and the short heating time, unfavorable conditions for LAL formation. It can be said that protein cross-link in absence of carbohydrates plays no role in the formation of high molecular coffee melanoidins.

Taken together, these results point to multiple mechanisms, other than the single Maillard reaction, described for model system. The consideration of polyphenols as one part of the process is essential.

4.3.4 Total phenolics

In the recent years, growing evidence points to a crucial role of coffee phenolics on the formation of melanoidins upon roasting *(Nunes and Coimbra, 2007; Nunes and Coimbra, 2007; Nunes and Coimbra, 2010; Bekedam et al., 2008a; Gniechwitz et al., 2008b)*. Aiming to improve the knowledge about this important parameter, and, principally, to characterize the samples used in this work, the total contents of polyphenols of the coffee brews of increasingly roast degrees and their respective low molecular weight fractions and high molecular weight melanoidins were measured using Folin-Cioucalteu reagent, as described in Section 3.6.4. The Folin-Ciocalteu reagent may give positive results with reductones or other reducing or metal-chelating substances formed during glycation, possibly overestimating the total phenolic content of a food sample. Especially in coffee samples, were the high levels of polyphenolic antioxidants are expected to dominate, a precise quantification of the contribution of Maillard reaction products over the total antioxidant activity of coffee samples is difficult *(Delgado-Andrade and Morales, 2005; Bekedam et al., 2008b; Delgado-Andrade et al., 2005)*. However, studies comparing various antioxidant capacity assays found the Folin-Ciocalteu results to be related to the reducing capacity of the coffee beverage and its radical scavenging potential *(Vignoli et al., 2011)*. Although the chemical principals of the reaction are not completely elucidated, the convenience, simplicity and reproducibility of the Folin-Cioucalteu reaction made it the routine assay in studying phenolics, in particular on coffee melanoidins (*Borrelli et al., 2002; Bekedam et al., 2008b; Gniechwitz et al., 2008b; Huang et al., 2005*; *Delgado-Andrade et al., 2005*).

The results are illustrated in Figure 4-15. With beginning of the thermal treatment, an increase in the total phenol content of the coffee brew was observed, changing from

0.22 mg/mg chlorogenic acid equivalents for green beans brew (cb0) to 0.27 mg/mg for the brew after initial roasting (cb1). This tendency was inverted with progress of roasting and the total phenol content decrease continuously to reach 0.20 mg/mg at the most intensively roasted sample (cb4).
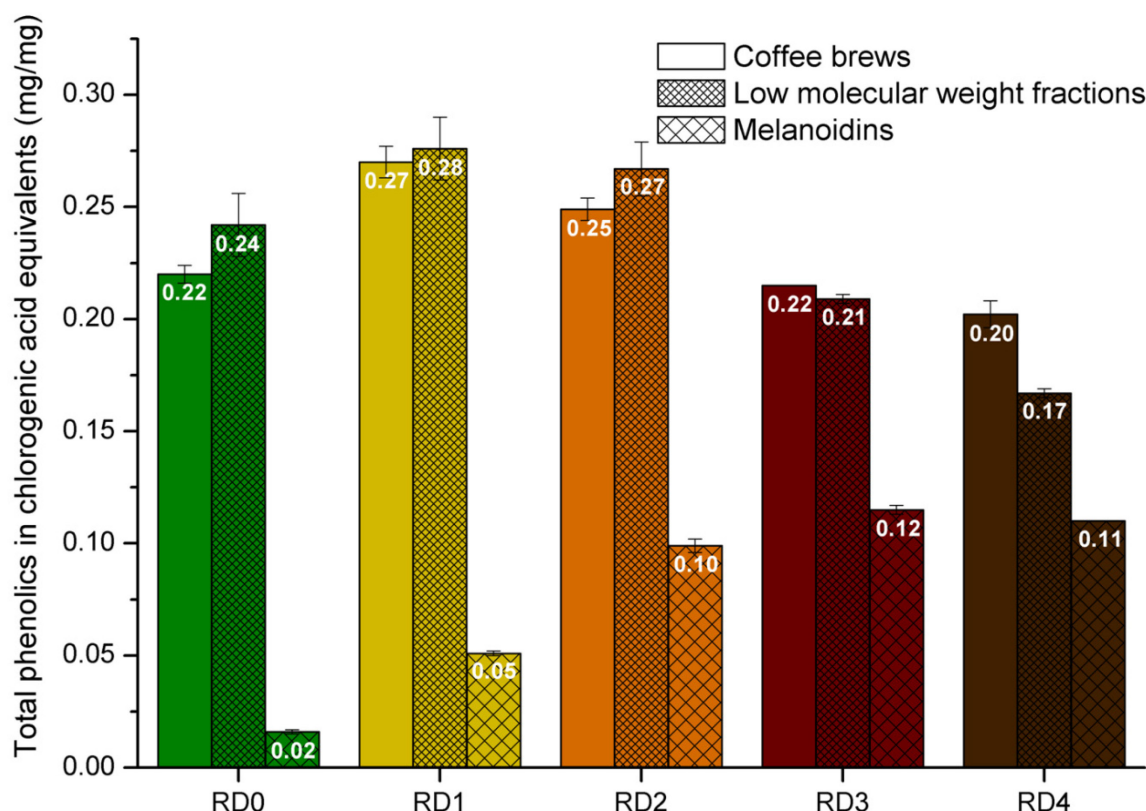
Figure 4-15: Total phenol content of coffee brews and their fractions, determined by Folin-Cioucalteu reagent and represented as chlorogenic acid equivalents (mg/mg). RD0 to RD4 correspond to the increasingly roast degree of the samples. Values are means of triplicates (± SD).

In line with the present results, *del Castillo et al. (2002)* reported a decrease in chlorogenic acid contents between light and medium roasting samples. Also *Sacchetti et al. (2009)* described a decrease in total phenolics content of whole coffee brews with the increase of the intensity of thermal treatment, using Folin-Cioucalteu reagent.

With respect to the low molecular weight fraction of coffee brews, a similar curve profile was observed. After a slight initial increase from 0.24 mg/mg chlorogenic acid equivalents in lmw0 to 0.28 mg/mg in lmw1, a continuously decrease in phenolics content could be observed, reaching 0.17 mg/mg chlorogenic acid equivalents for lmw4.

In relation to the melanoidin fraction, the principal object of study of the present work, the course of the curve deviated from the other samples studied. The initial rapid increase in the apparent polyphenol content, from 0.02 mg/mg chlorogenic acid equivalents in the high molecular weight fraction of green beans (mRD0) to 0.10 mg/mg in the medium roasted sample (mRD2), was substituted by a flatter progression, achieving 0.11 mg/mg in the melanoidins obtained from final espresso roasted beans (mRD4). The observed increase in the total phenolics content in the high molecular weight fraction is most probably prevalently due to covalent incorporation of chlorogenic acids into the melanoidin structures, as the contribution of the roasting-induced antioxidants to the overall antioxidant activity of coffee brews is rather limited in comparison to the predominating antioxidant impact of the phenolic compounds *(Bekedam et al., 2008b)*.

The nature of the incorporation of polyphenols on the melanoidin backbone has been object of intensive investigations, and the interpretations of the findings are often contradictory. The most likely linkage between the phenolic compounds and melanoidins has been supposed to be via the protein fragments incorporated in the coffee melanoidins during the roasting process *(Nunes and Coimbra, 2007; Bekedam et al., 2006)*. By submitting a high molecular weight isolate of coffee extract to hydrolysis under weak conditions, such as acid or alkaline hydrolysis and alkaline decomposition in glycerol, *Takenaka et al. (2005)* found that phenolic compounds are not present in esterified form in the melanoidins, as no effective degradation of could be found. Alkaline fusion is a method commonly used in the literature to liberate covalently bound phenolics (*Takenaka et al., 2005; Nunes and Coimbra, 2007*), in which the dry sample is heated at 350°C with excess of solid NaOH, in presence or not of zinc dust. Under these conditions, double bonds and ether bindings are also hydrolyzed, and not only the ester linkages, as in the conventional saponification. Due to the low amount of recoverable phenolic compounds by alkaline fusion found in the investigation of *Nunes and Coimbra (2007)*, the suggestion of incorporation of condensed hydroxycinnamic acids into the melanoidins complex by non-ester chemical bounds was corroborated. A series of NMR studies realized by *Vignoli et al. (2011)* refuted the existence of intact caffeic acid or ferulic acid moieties as integral units of the melanoidins and favors the concept of condensed phenolics as melanoidin constituents. In his extensive chromatographic investigations about the incorporation of chlorogenic acids in coffee melanoidins, *Bekedam et al. (2008a)* demonstrated the presence of intact chlorogenic acids in the melanoidin backbones, and proposed incorporation to take place upon roasting via

caffeic acid through mainly non-ester linkages. *Gniechwitz et al. (2008b)* questioned the existence of intact caffeic acid or ferulic acid moieties as integral units of the melanoidins, once the propenyl side chains could not be detected in by NMR-spectroscopy. They defended the concept of condensed phenolics as melanoidin constituents, integrated via reactions leading to loss or modification of their conjugated double bound. *Perrone et al. (2012)* also reported on the involvement of polyphenolic compounds in the formation coffee melanoidins via the caffeic or ferulic acid moiety, mainly through nonester linkages and propose likewise the incorporation of chlorogenic acid lactones into the melanoidin structure. The observed roast-dependent increase in the dihydrocaffeic acid content in melanoidin samples, detected after saponification, was discussed as a sign of oxidation of chlorogenic acids posterior to incorporation. As the phenolic acid moiety from chlorogenic acid is far more prone to oxidative changes than the quinic acid, it is generally assumed that the phenolic acid is likely the moiety which participates in the chemical reactions during roasting *(Bekedam, 2008)*. Phenolic acid can be oxidized to quinone in alkaline milieu, which in turn can undergo attack by nucleophiles such as lysine, methionine, cysteine and tryptophan moieties in a protein chain *(Kroll et al., 2003)*. However, the coffee matrix has a strong acid character, and, furthermore, the importance of oxidative reactions in the heat degradation of polyphenols has been recently questioned *(Moon and Shibamoto, 2010)*. Possibly, the incorporation of the intact chlorogenic acids in the melanoidin structure occurs through reactions still not investigated.

In conclusion, the present section shows a progressive increase in the total phenol content of the melanoidin fraction of coffee brew upon roasting, quantified using Folin-Ciocalteu reagent. This evolution follows the same tendency as the inhibitory potential of coffee melanoidins against MMPs, as described in section 4.2. This corroborates the suggestion that incorporation of polyphenols to the melanoidin structure can have been at least partially responsible for the observed emerging of inhibitory activity of this fraction against MMPs.

4.3.5 Correlation between total phenols content and C/N ratio in coffee melanoidins

By comparing the results presented in sections 4.3.2 and 4.3.4, a direct correlation between the C/N-ratio and the total phenol content of melanoidins of increasing roasting degrees can be assumed. In order to proof this presumption, total phenol contents were plotted against C/N ratio for each melanoidin sample. The results can be seen in Figure 4-16.
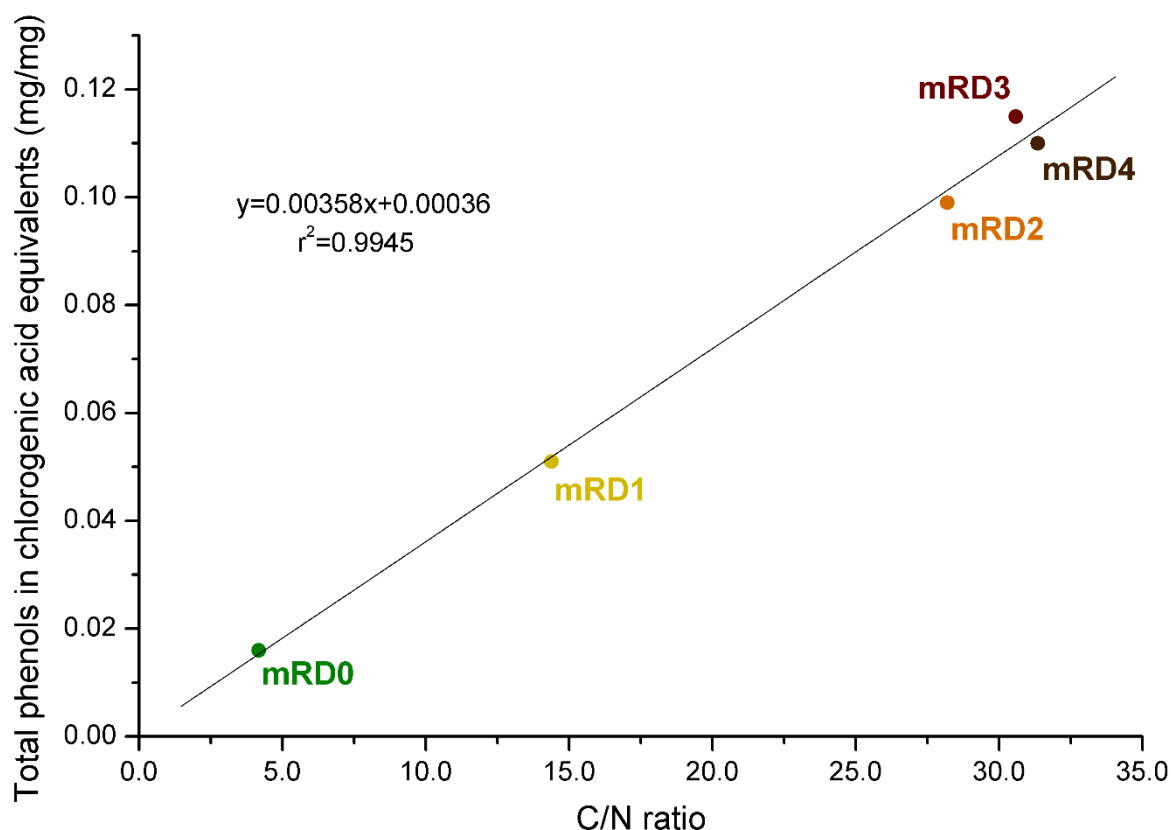
Figure 4-16: Correlation between the increase in C/N ratio and total phenol content in coffee melanoidins with progress of the roasting. mRD0 to mRD4 are the melanoidin isolates of increasingly roast degree.

A linear correlation between the total phenolics contents and C/N ratio of the melanoidin fractions of coffee brews with progress of roasting was observed (y = 0.00358 x + 0.00036, r^2 = 0.9947). This correlation evidences the simultaneity of the thermally induced increase of the proportion of carbon rich compounds in the high molecular weight fraction of brews, and the increase of total phenol contents of the same fraction, strengthening the suggestion of a progressive incorporation of chlorogenic acid into the melanoidin polymer upon roasting, although the thermally induced incorporation of carbohydrates, mainly arabinogalactans, to coffee melanoidin structure also takes place *(Bekedam et al., 2007)* and probably contributes for the increase in the C/N ratio.

4.3.6 **Raman spectroscopy**

The aim of this preliminary experiment was to evaluate the changes on the signal intensity of Raman spectra of the coffee samples of increasing roast intensities, trying to find some evidence about the character of the chemical transformations which take place in the polyphenolic structure upon heating, and the mechanism of chlorogenic acid incorporation

into the melanoidin structure. Unfortunately, due to massive background intensity, the isolated melanoidins could not be analyzed under the conditions described in section 3.6.5. The results will thus be interpreted indirectly, based on the differences between the alterations in coffee brews and the low molecular weight fraction, assuming that the responses of the melanoidin fraction correspond to the "lacking piece".

The Figure 4-17 shows the Raman spectrum of standard chlorogenic acid and the relevant signals for the present investigations, normalized in the region between 1200-1000 cm^{-1}. The band at 1632 cm^{-1} corresponds to the signal from the conjugated carbonic acid (or its ester) and the signal at 1606 cm^{-1} is originated by the aromatic ring of caffeic acid.
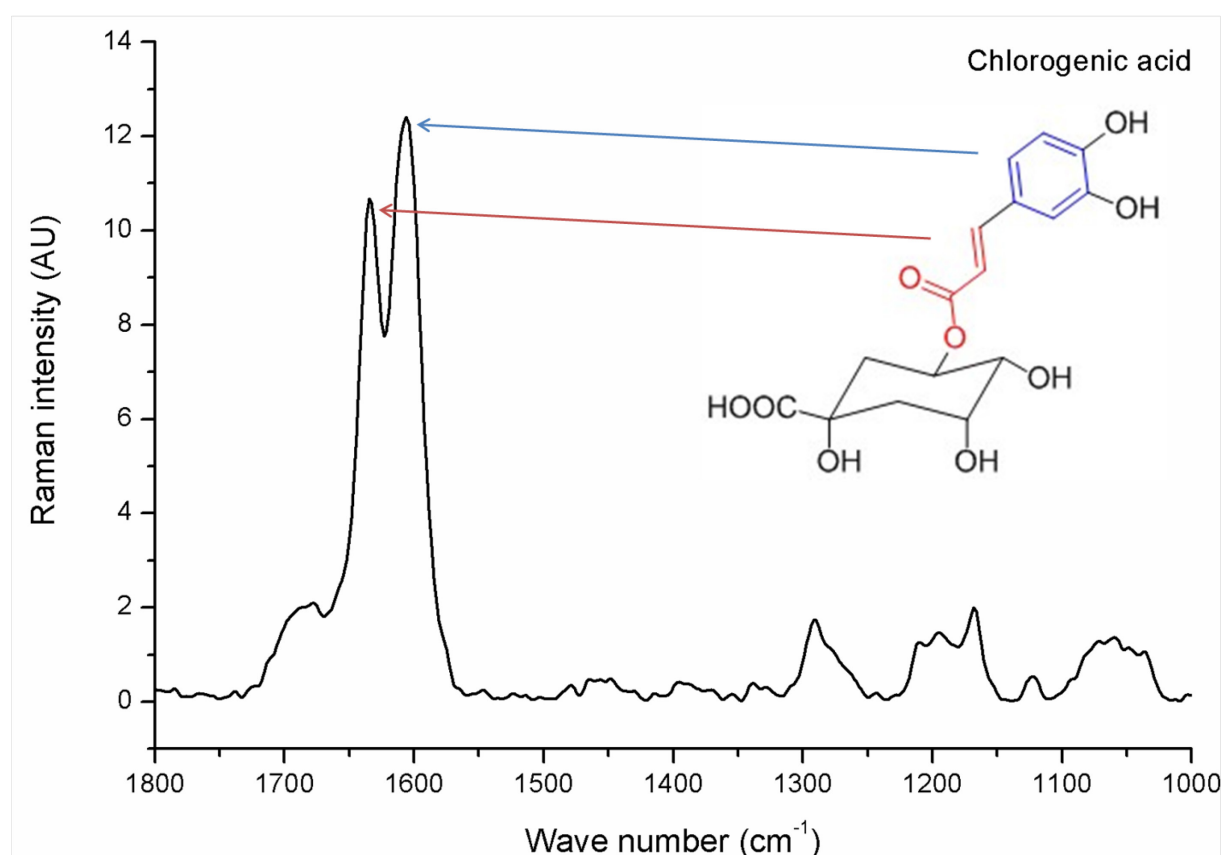
Figure 4-17: Raman spectrum of 5-caffeoylquinic acid. Experimental conditions are described in section 3.6.5.

The Figure 4-18 illustrates the spectroscopic patterns of the whole coffee brews (above) and the respective low molecular weight fractions (below). Considering the whole coffee brews (cb), a marked and continuous roast-dependent decrease in both signals can be noted.
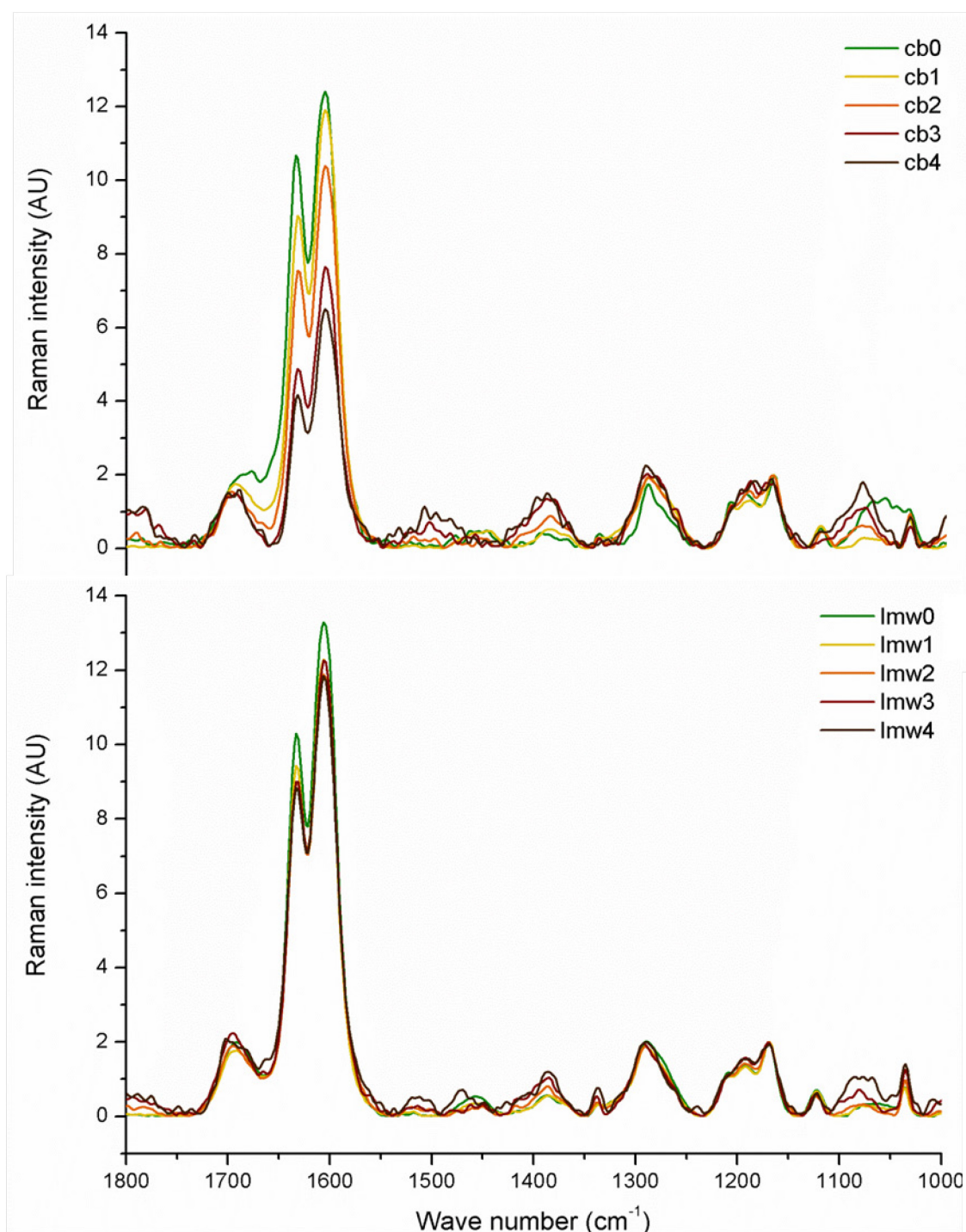
Figure 4-18: Raman spectrum of coffee brews of increasingly roast degrees (cb0 to cb4) and their respective low molecular weight fractions (lmw0 to lmw4). Experimental conditions are described in section 3.6.5.

This observation denotes the degradation of the studied groups. This fact could be explained by oxidation and pyrolytic degradation of the polyphenols, if the decrease of both signal intensities were similar. As can visually be suggested and will be better discussed later, the

decrease of the signal of the conjugated double-bound of the carbonic acid from caffeic acid is faster than the disappearance of the phenolics moiety.

In opposition to that trend is the progress of the caffeic acid signals upon roasting in the low molecular weight fraction (lmw). With onset of heating, a balanced and slight decrease of both peaks could be measured. With progress of the roasting, no significant alteration was detected. In Figure 4-19 the comparison can be done more clearly. As the Raman signals are overlapping, the integration of the single peaks is not possible and their height was used to calculate the ratio between the intensity of the signals originated from the conjugated double bound of the carbonic acid (or its ester) and the aromatic system from polyphenol. While in the low molecular weight fraction the relative intensities are quite stable, in the whole coffee brew a notable decrease on this ratio can be seen. These observations allow the attribution of the difference in behavior to the lacking fraction: the melanoidins.
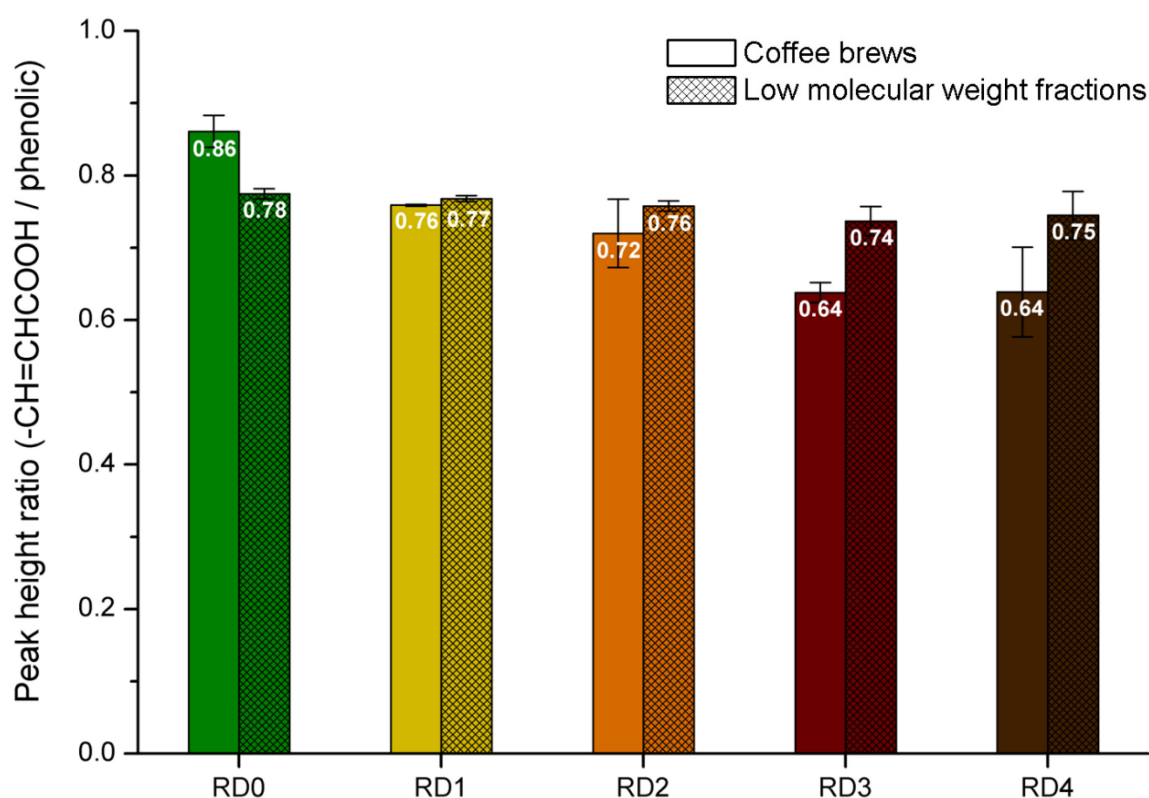
Figure 4-19: Ratio between the peak heights of the conjugated double bond of the carbonic acid and of the phenolics ring, with evolution of the roast degree of the samples. RD0 to RD4 correspond to the increasingly roast degree of the samples.

Taking together all the literature reports about the role of chlorogenic acids in the formation of coffee melanoidins and the results from the present work, we suggest that the

incorporation of polyphenols on the melanoidin backbone happens, at least in part, by a Michael-like nucleophilic addition of amino or sulfhydryl groups of proteins on the conjugated double-bond of the caffeic or chlorogenic acid. The adduct is stabilized by tauto-enol isomery. The proposed mechanism can be seen on Figure 4-20.

R-X = R-NH₂ or R-SH
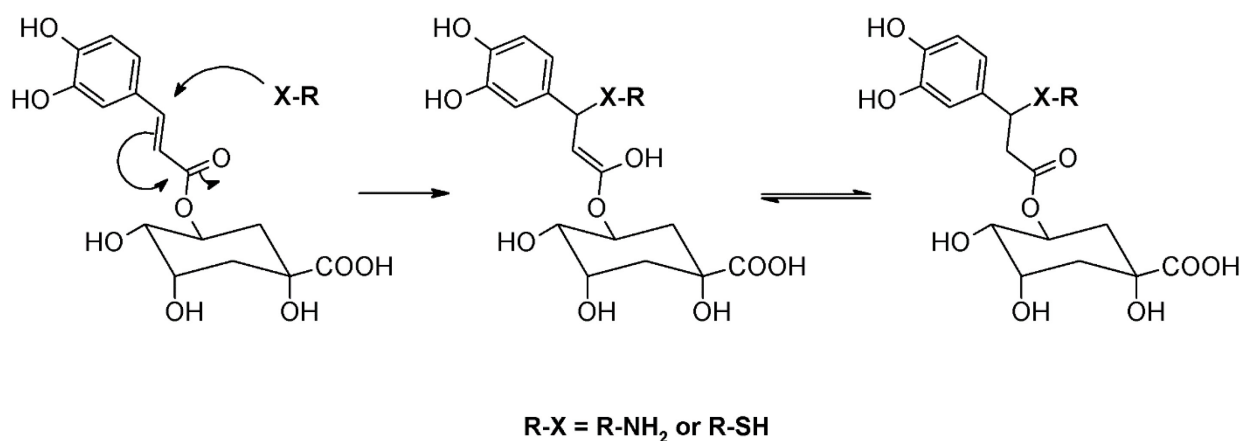
Figure 4-20: Suggested reaction mechanism for the incorporation of chlorogenic acids on melanoidin structure

This mechanism could explain the experimental findings about the incorporation of chlorogenic acid in melanoidin structure reported in the last years and conciliate much of the contradictory interpretations found in the literature, as described in sections 2.2.2.1 and 4.3.4. A Michael-like addition of a nucleophilic group from a protein in the conjugated double bound of caffeic acid:

- is a covalent and nonester linkage;
- the incorporation takes place at a nitrogenous structure;
- allows the incorporation of the intact chlorogenic acid molecule;
- explains the absence of the propenyl resonance in the NMR investigations.

On the basis of these observations, the proposed reaction pathway may be one of the reactions responsible for the heat-induced incorporation of chlorogenic acids in coffee melanoidins.

In order to study the reactivity and gain some evidences about the mechanism of polyphenol incorporation, model melanoidins can be prepared in complex systems that simulate realistically the conditions of coffee beans during the roasting. Interesting would be the study of variation of the phenolics acid, as illustrated in Figure 4-21.

Coumaric acid **Caffeic acid** **Ferulic acid** **Sinapic acid**
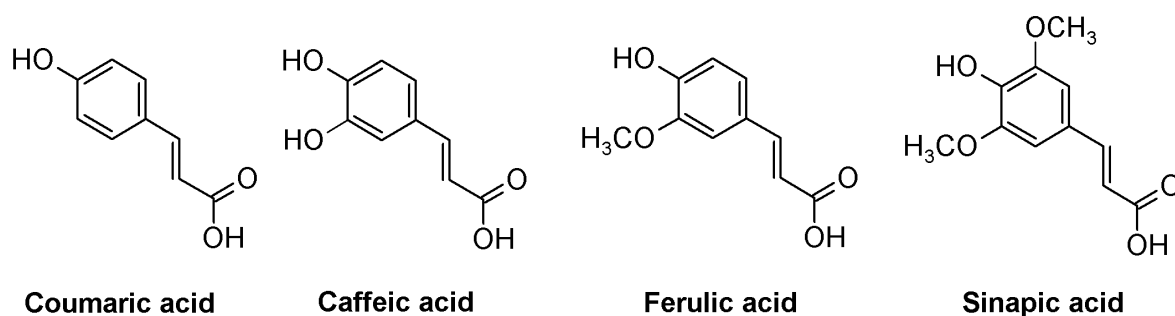
Figure 4-21: Phenolic compounds proposed for the model investigation of the mechanism of incorporation of phenolic compounds in the coffee melanoidin structure, under roasting conditions.

By comparing the behavior of coumaric acid (only one phenolic group in para position), caffeic acid, ferulic acid (meta-methylated derivative of caffeic acid), and sinapic acid (2 meta-methylated phenolics groups, one free phenolic group at para position) in presence of a standard protein, the structural features involved in the incorporation can be closely investigated, as the reactivity of the phenolic groups and of the conjugated double bound can be closely compared, depending on the presence of activating groups in the aromatic ring and disponibility of phenolic groups for the reaction.

4.4 Derivatization of peptides

Potent ACE-inhibitors used in therapy of arterial hypertension, including captopril, enalapril and lisinopril, combine an effective zinc-binding group with a substrate-mimicking structure, characteristics which assure a competitive tight-binding at the active site of ACE, by both coordinating with the catalytic Zn(II) and satisfactorily occupying substrate pockets of the enzyme *(Natesh et al., 2004; Natesh et al., 2003; Hayashi and Camargo, 2005)*. One of the most frequently used zinc-binding moieties in clinically used ACE inhibitors is carboxylate *(Yiotakis and Dive, 2009)*.

The purpose of this section is to compare the inhibitory activity of native peptides and their N^α-carboxymethyl- and N^α-carboxymethyl derivates, products of N-terminal glycation possibly found in food, against ACE.

The glycated peptides studied are not commercially available and were synthesized. Nine peptides (IW, AP, IP, IPP, GP, LL, IA, GA, GL) were chosen based on their relevant inhibitory potential against metallopeptidases, being five of them (IW, GP, AP, IP, IPP) known in the literature for their bioactivity against ACE. All of them correspond to

sequences commonly found in food, especially milk, but also gelatin, seed storage proteins, and a diversity of other alimentary plant and animal protein sources contain known ACE-inhibitory di- and tripeptides encrypted in their primary structures, which can be liberated by proteolysis *(De Leo et al., 2009; Hernández-Ledesma et al., 2011; Murray and FitzGerald, 2007; Fitzgerald and Murray, 2006; Saito, 2008)*

4.4.1 **Nα-carboxyalkylation of peptides by reductive alkylation**

In the present study, the N^{α}-carboxymethyl- and N^{α}-carboxyethyl-derivates of selected peptides were synthesized by reductive alkylation of commercial peptides with glyoxylic acid and pyruvic acid, respectively. The reaction between aldehydes and ketones with primary or secondary amines under reductive conditions is currently a broadly used method for both laboratory and industrial preparation of N-substituted amines *(Krupka and Patera, 2007)*. In fact, it is among the most useful and important methods for the production of mixed secondary amines, which are difficult to prepare by other methods *(Nishimura, 2001)*. Generally, after the condensation and the amine to form a carbinolamine, the loss of a H_2O molecule takes place, to give an imine or Schiff base. The reduction of the imine intermediate to give the aimed amine can occur by action of different reagents, being the use of hydride donating reagents or molecular hydrogen in the presence of a catalyst convenient and broadly applied procedures *(Gomez et al., 2002)*. The formation of the iminium species is reversible, but not the reduction *(Baxter and Reitz, 2004)*. The general pathway of a reductive alkylation is illustrated on Figure 4-22.

All derivates were obtained with high purity. Except for the alkylation of IW, the heterogeneous hydrogenation using Pd as catalyst was shown to be chemoselective and led to appropriate purities of all peptide derivatives. The chemoselective hydrogenation of C=C from unsatured carbonyl compounds is usually achieved under mild conditions by catalytic hydrogenation *(Nishimura, 2001)*, however aromatic rings often also hydrogenated under Pd-catalized C=C hydrogenation conditions *(Kyriakou et al., 2011)*. In fact, in the present work, the derivatization of tryptophan was shown to be impossible using this method. The indolyl group is highly sensitive to hydrogenolysis. The reduction from tryptophan by treatment with gaseous hydrogen in presence of palladium on charcoal was described, and the extent of tryptophan modification appears to be difficult to reduce *(Mery and Calas, 1988)*. For the conditions where the catalytic hydrogenation does not lead to a chemoselective reduction, the use of hydride reducing agents can be possible *(Abdel-Magid*

and Mehrman, 2006). Sodium cyanoborohydride is considered to be the best known reagent for reductive alkylations *(Baxter and Reitz, 2004)*. Thus, the syntheses of the N^α-carboxymethyl- and N^α-carboxyethyl-derivatives of IW were realized using $NaBH_3CN$ as reducing agent. Sodium cyanoborohydride has shown to be a suitable hydride reagent for the present reductive alkylation of IW, even though the formation of considerable amount of bis-carboxymethylated IW was observed. The occurrence of dialkylation of amines as a side reaction, especially by the use of an aldehyde as carbonyl compound, is well known in the literature *(Baxter and Reitz, 2004; Abdel-Magid and Mehrman, 2006)*. Monoalkylation with aliphatic aldehydes can be stimulated by using either an excess of amine or a 1:1 mixture of aldehyde to amine and ensuring that imine formation is complete prior to addition of reducing agent *(Baxter and Reitz, 2004)*. Future syntheses should take this into account.
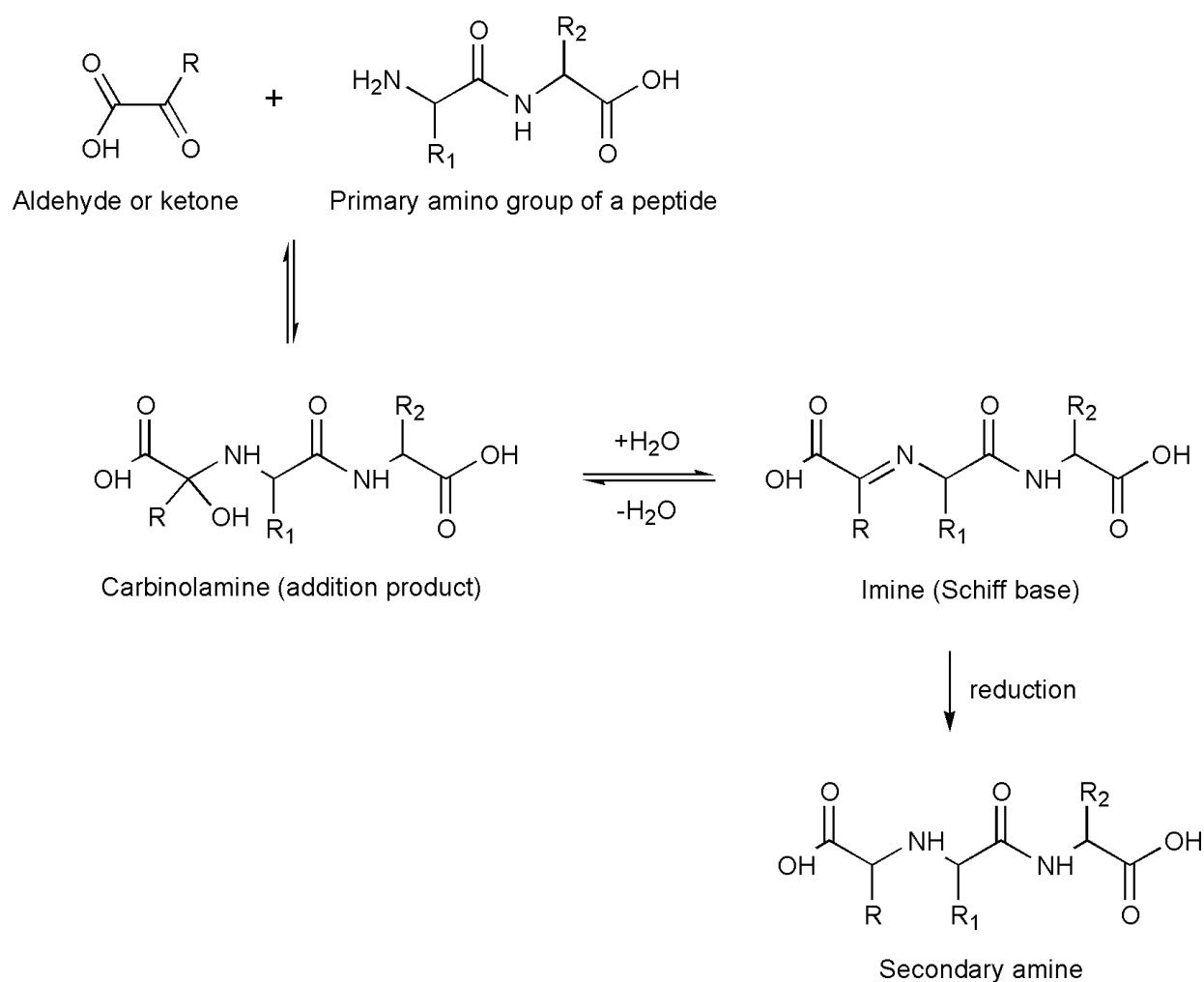
Figure 4-22: General reaction pathway of N-terminal reductive alkylation of dipeptides by aldehydes or ketones R = H, alkyl or aryl substituent. R^1 and R^2 are amino acid side-chains. Adapted from *Nishimura (2001)* and *Abdel-Magid and Mehrman (2006)*.

The synthetic pathway and purification procedures are described in sections 3.2 and 3.3. The structures of the obtained carboxyalkylated peptides can be seen at Figure 4-23.
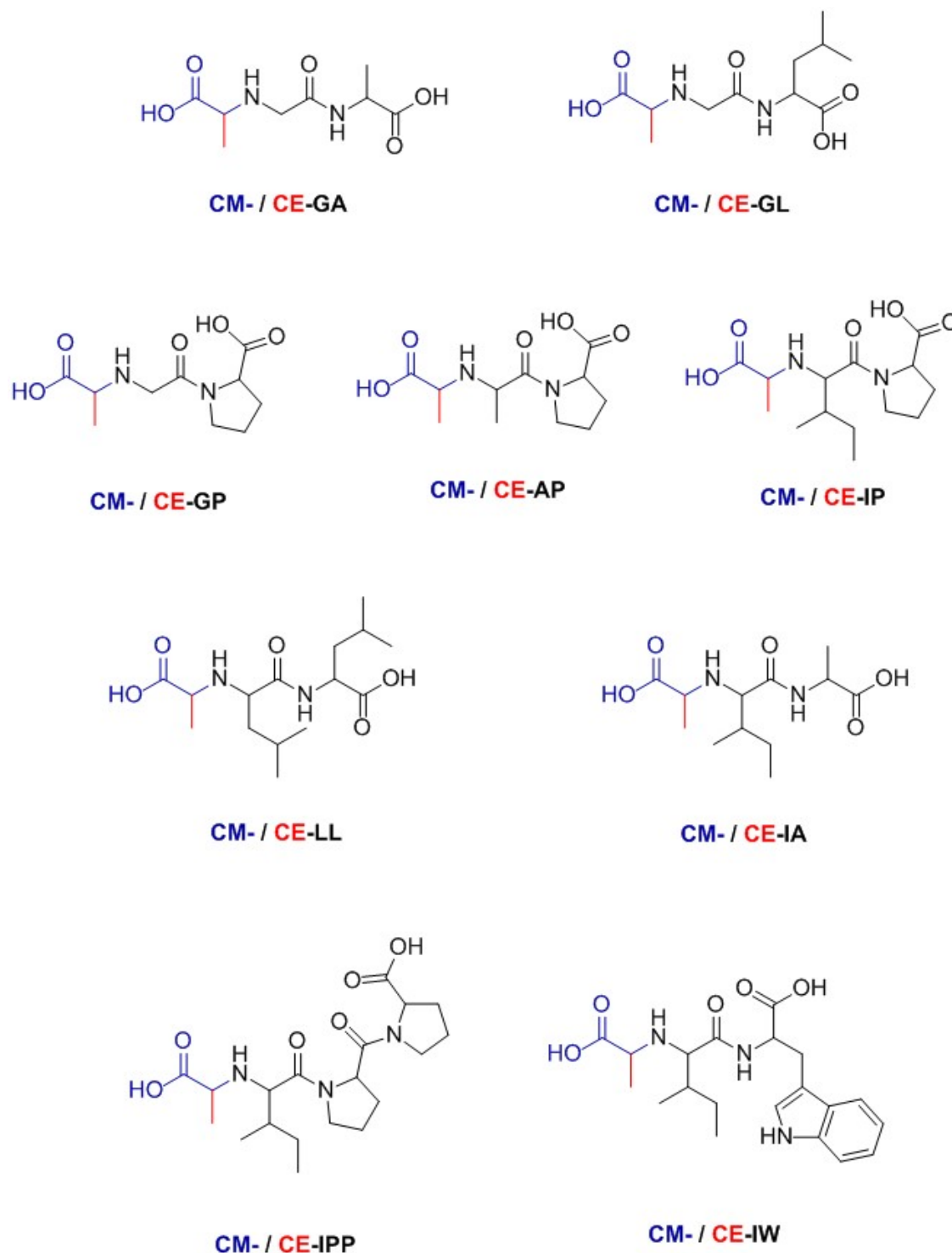
Figure 4-23: Structures of the studied peptides and their synthesized CM- and CE-derivatives.

The yields of the derivatives of IPP (30% for CM-IPP and 4 % for CE-IPP) were markedly lower than of their counterparts, from which at least 40%, mostly around 70% yield was

obtained. This is probably due to steric hindrance, as the peptide IPP has longer substituents. It is known that the rate of the reaction between an amine and a carbonyl group to form the intermediate imine and the rate of the hydrogenation of the imine both decrease with increasing the size of the groups in the neighborhood of the mentioned functions. Therefore, yield and selectivity are strongly dependent upon the steric hindrance of the starting compounds *(Gomez et al., 2002)*. The yield of reductive alkylation is also known to be variable dependent on reagents, solvent, temperature, and H_2 pressure, in addition to the catalyst chosen for the hydrogenation of the imine *(Krupka and Patera, 2007)*. In most hydrogenation reactions, an increase in the hydrogen pressure increases the rate of reaction, reduces reaction time and favors an efficient use of the catalyst *(Nishimura, 2001)*. For the preparation of derivatives of slow reacting peptides, the reaction under pressure is suggested.

It must be noticed that all CE-derivatives are likely to be diastereoisomeric mixtures, as the α-C-atom of the carboxyethyl substituent is chiral, as well as the α-carbons of the amino acids. Fast racemization during palladium-catalyzed hydrogenation is described in the literature *(Murahashi et al., 1983)*. In general, the critical step, which leads to stereodifferentiation, is not the hydrogen transfer, but the adsorption of the reactant on the catalyst, and the formation of the half-hydrogenated state *(Kyriakou et al., 2011; Tungler and Fogassy, 2001)*. Which diastereotopic face of the substrate will preferentially bind to the catalyst surface is defined by steric features and electrostatic interactions *(Heitbaum et al., 2006)*. Higher diastereoisomeric excesses were observed using $NaBH_3CN$ than by catalytic hydrogenation. Equimolar diastereoisomeric mixture would be an advantage in the present study, as is not known which of the diastereoisomers show activity against the enzyme and diastereoisomeric excesses distort the results difficulting comparisons and interpretation on the bioactivity.

4.5 Preliminary investigations on the inhibitory potential of N^α-carboxyalkyl derivatives of peptides against metalloproteases

This section aimed to investigate the potential of non-enzymatic glycation on the generation of inhibitors of metalloproteases, by introducing a zinc-binding group in a relevant peptide structure. The selectivity of the molecules was evaluated by testing the three zinc-metalloproteases, which were investigated under action of coffee melanoidins (section 4.2).

4.5.1 **Inhibition against ACE**

Solutions of the synthesized N^α-alkylated derivates and the original peptides were tested as inhibitors for angiotensin converting enzyme. Assay details are reported in section 3.7.1.

ACE is an exopeptidase, which cleaves the C-terminal dipeptide (HL) from the decapeptide angiotensin I. Important knowledge about the specificity of substrate and inhibitor binding in the catalytic site of ACE has been gained without any insight from the three dimensional structure of the enzyme *(Natesh et al., 2004)*. A systematic study of inhibitory potency of L-dipeptides showed a range of four orders of magnitude, from VW to PG. Analyzing these results, it was found that ligands possessing C-terminal aromatic or prolyl residues have high affinity for the catalytic binding pockets of ACE, while dicarboxylic amino acid cannot bind efficiently. In N-terminus, branched-chain amino acids, valine and leucine promotes stronger interactions as other residues *(Ondetti and Cushman, 1984)*. The peptides studied in this work are variations over these motifs.

As can be seen on Table 4-4, the glycation of the studied dipeptides increased enormously the inhibitory potential of the molecules against ACE.

Table 4-4: In vitro IC_{50} values of peptides and their N^α-carboxymethyl- and N^α-carboxyethyl-derivatives against ACE.

Peptide	IC_{50} (µM)		
	native	N^α-carboxymethyl-	N^α-carboxyethyl-
GA	1907 ± 61	863 ± 95	103 ± 7
GL	2785 ± 204	834 ± 32	41.0 ± 1.7
GP	371 ± 44	138 ± 25	15.6 ± 3.2
AP	37.5 ± 6.7	3.69 ± 0.66	0.27 ± 0.16
IP	241 ± 32	46.2 ± 15.6	10.1 ± 0.1
IA	1023 ± 160	30.6 ± 0.8	0.26 ± 0.05
LL	n.d.	760 ± 57	50.0 ± 14.1
IW	1.71 ± 0.12	4.31 ± 0.48	0.41 ± 0.01
IPP	3.42 ± 0.45	434 ± 54	203 ± 14

n.d. = not determinable; means ± SD, n = 2-3.

In all cases, besides for IW, the CM-derivates showed IC_{50} values 2 to over 30 times lower than the original peptides. The CE-derivatization was even more effective, leading to molecules almost 4000 times more active than the underivatized peptides. In fact, a general advantage of the carboxyethyl derivatization, in comparison with the carboxymethyl functionalization, can be observed. Similar tendency was reported by *Patchett et al. (1980)*, who synthesized a series of substituted N-carboxymethyl-dipeptides, which inhibited ACE in nanomolar levels. Among the reported substances, CM-AP and CE-AP can be seen. Using a slightly diverging in vitro assay, these authors described IC_{50} of 2.4 µM for CM-AP and 0.09 µM for CE-AP, which are in line with the inhibitory potentials measured in the present work. They also found the ACE inhibition of the derivates to be independent of zinc concentration, ruling out the inhibition by removing Zn(II) from active site. The competitive tight-binding inhibitor ACE inhibitor captopril is a substrate-derived molecule. Its peptidomimetic structure, which simulates the peptide Ala-Pro, interacts with the active site Zn(II) ion by direct coordination through a thiol metal-binding group, deep inside the channel at the active site *(Natesh et al., 2004)*.

As schematically represented in Figure 4-24, the subsite S_1 is a relevant affinity-giving position. It can be supposed that the additional methyl group from CE-derivatives, in comparison with CM-derivatives, interacts in this region, leading to an energetic advantage for the binding, even if not so markedly as the phenylalanine residues of the represented drugs.

A very important observation is the positive effect of proline residues at the carboxyl terminus of the dipeptide. GP and their derivates were more active as their counterparts from GA and GL. Also between native IA and IP a positive effect of C-terminal proline can be seen. Proline and hydroxyproline at the C-terminus of peptides are known to increase the inhibitory potency against ACE (Ondetti and Cushman, 1984). However, although this trend could be extrapolated to the derivates of the glycyl-peptides, the effect of the carboxyalkylation over IA is much more intensive as the observed for IP, resulting in more active carboxyalkylated IAs, than the respective IP derivates. In this case, energetic compensations in the active site, other than the interactions of proline, must have been decisive.
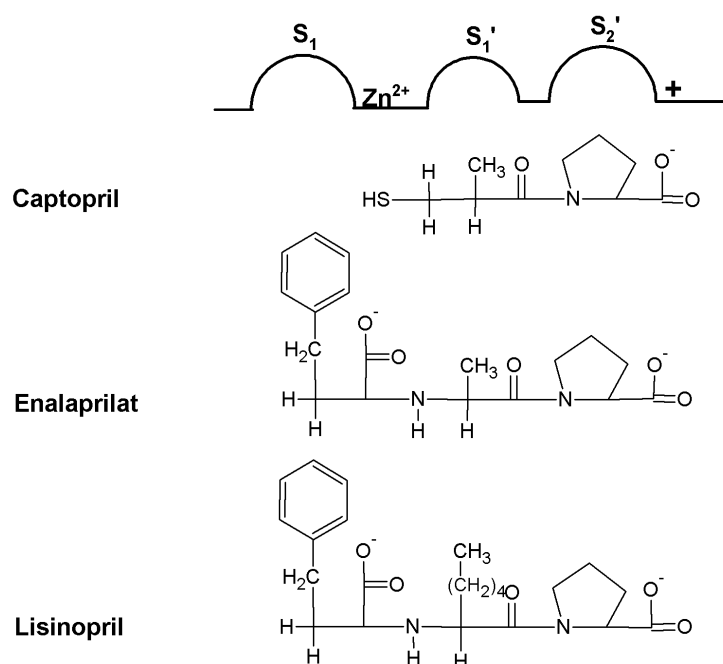
Figure 4-24: Schematic illustration of the substrate subsites of ACE, binding to the three important inhibitors: captopril, enalapril and lisinopril, showing important features for a competent enzyme blockage. Extracted from *Natesh et al. (2004)*.

Comparing AP and GP, we can notice a positive effect of the longer alkyl substitute, possibly indicating a more competent interaction with S1'. Nevertheless, despite the branched side-chain, IP and its derivates were less active as their AP counterparts. In this case, the alteration may have involved other changes additional to the occlusion of P_1'. Bulky substituents in P_1' and P_2' are known to give domains specificity. As both N- and C-domains cleave the synthetic substrate used in the study with distinct affinity, the measured activity of the enzyme is dependent from which substrate is occluded.

The most successful modification could be observed with IA and its derivate CE-IA, whose IC_{50} decreased from 1023 µM (IA), to 31 µM (CM-IA) until 0.3 µM (CE-IA). The improvement of two orders of magnitude by changing the carboxymethyl group, for the carboxyethyl- substituent was observed only for this peptide. Probably, other forces, either than the increased interaction with S_1, take place. As observed in Figure 4-25, a possible explanation for the remarkable/striking increase in the inhibitory activity could be the perfect bilateral symmetry of the molecule. In this case, the probability of a successful interaction is increased, and more molecules will be bound, decreasing the concentration necessary for the catalytic impairment.
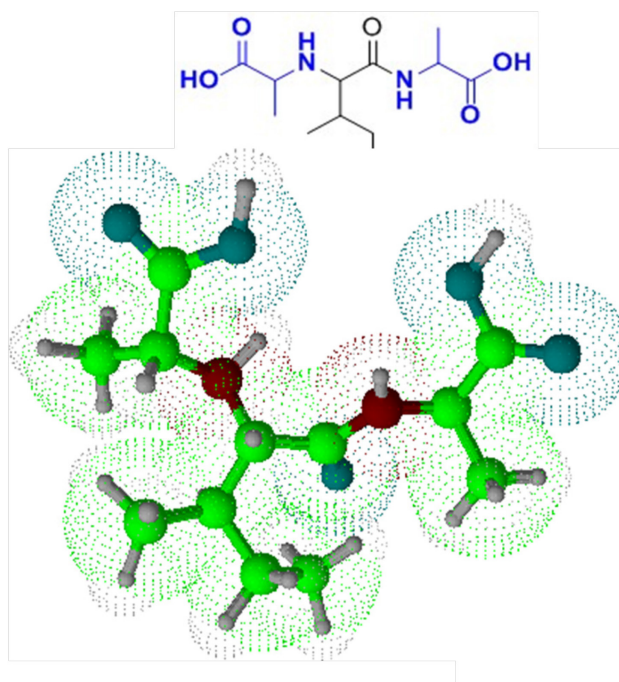
Figure 4-25: Carboxyethyl-Ile-Ala structure, showing bilateral symmetry.

But also the derivatization of LL induced an inhibitory potential not observed for the original peptide. The IC_{50} from LL was too high to be determined, while the CM-derivative inhibited the target enzyme already in μM concentrations (760 μM). The CE- derivatization increased this potential in another order of magnitude, reaching 50μM.

On the other hand, CM-IW showed lower inhibitory potential against ACE as its parent peptide, despite the introduction of a new zinc binding group. Although classic ACE inhibition rely on the ability of the inhibitors to establish coordination with the Zn(II) at the catalytic site *(Hayashi and Camargo, 2005)*, it has been known for a long time that the binding zinc ion alone is not sufficient to produce efficient inhibitors, but, in concert with other interactions, high inhibitory activity can be achieved *(Ondetti and Cushman, 1984)*. Having in mind that IW is one of the most potent peptides against this enzyme, it can be assumed that the interactions in the active site could not be further optimized trough glycation, and molecular modifications displaced the original fittings in the subsites. The glycated molecules might show distinct patterns of interactions with the enzyme as the native peptide, in such a way that glycated IW lacks in some stabilizing intermolecular forces involving the peptide backbone, which are otherwise present in native IW bounding.

Interestingly, the glycation of the studied tripeptide promoted also the inverse effect as the glycation of almost all studied dipeptides. CM- and CE-IPP possess 125 und 60 times

higher IC_{50}-values as the original peptide, respectively. Probably, similarly as discussed for CM-IW, the intermolecular interactions between enzyme and inhibitor happens in a different pattern as with IPP and the introduction of the new group did not contribute for the bioactivity. It is noteworthy that the CE- derivative is still more active as the CM- molecule, suggesting that interaction of this side-chain is crucial for the stabilization of the enzyme-inhibitor complex.

These observations show that the glycation of dipeptides is a very effective tool to improve their inhibitory potential against ACE. The enhanced bioactivity could be explained by a more efficient fitting of the glycated molecules into the catalytic centre of the enzyme, comparing to the original peptides, but no ultimate affirmations about the molecular interaction can be done without a more comprehensive kinetic study.

4.5.2 **Inhibition against other zinc metalloproteases**

Glycation also caused a positive effect on the inhibitory potential of peptides against *Clostridium histolyticum* collagenase, even though the values were less impressive than the data observed for inhibition of ACE. The IC_{50} values measured in vitro according to section 3.7.3 can be seen in Table 4-5.

Table 4-5: In vitro IC_{50} values of peptides and their N^{α}-carboxymethyl- and N^{α}-carboxyethyl-derivatives against ChC.

Peptide	IC$_{50}$ (µM)		
	native	N^{α}**-carboxymethyl-**	N^{α}**-carboxyethyl-**
IA	n.d. (12% at 5 mM)	4300 ± 640	4150 ± 380
LL	n.d.(16% at 5 mM)	2880 ± 1200	4120 ± 180
IW	n.d. (12% at 1 mM)	n.d. (0% at 5 mM)	n.d. (0% at 5 mM)
GL	n.d. (0% at 5 mM)	2790 ± 950	3010 ± 1390
GP	n.d. (0% at 5 mM)	3100 ± 130	2350 ± 640
AP	n.d. (0% at 5 mM)	2546 ± 459	1952 ± 846
IP	n.d. (10% at 5 mM)	2441 ± 1878	1484 ± 349
IPP	n.d. (11% at 5 mM)	1320 ± 537	n.d. (38% at 5 mM)

n.d. = not determinable.

No studied peptide showed a measurable inhibition against ChC. Nevertheless, an improved inhibitory potential of the peptides through glycation could be observed. Except for the IW derivates and CEIPP, all carboxyalkylated derivatives presented a weak, but significant, inhibitory potential. The weak inhibitory potentials can be possibly explained by the lack of interaction of the studied molecules in the hydrophobic subsites S_2' and S_3', which were shown to be important for ligand affinity *(Gupta, 2007)*.

The IC_{50} values did not differ between the CM- and CE- derivatives. Also among the different peptide basic structures, the values are in the same range. This observation points to the absence of hydrophobic binding pockets around the catalytic cation, and that a substitution in this region does not confer any advantage.

Some of the studied peptides were chosen as possible cleavage sequence within the natural substrate of MMP-1. Therefore, the inhibition potential of the glycated peptides against human matrix metalloproteases was studied. The results of the assay are listed in Table 4-6.

Table 4-6: In vitro IC_{50} values of peptides and their N^{α}-carboxymethyl- and N^{α}-carboxyethyl-derivatives against MMP-1.

Peptide	IC_{50} (µM)		
	native	**N^{α}-carboxymethyl-**	**N^{α}-carboxyethyl-**
IA	n.d. (0% at 5 mM)	1340 ± 110	2210 ± 2100
LL	n.d. (0% at 5 mM)	720 ± 210	1510 ± 1180
IW	n.d. (39% at 1 mM)	n.d. (4% at 5 mM)	n.d. (26% at 5 mM)
GA	n.d. (0% at 5 mM)	n.d. (0% at 5 mM)	n.d. (0% at 5 mM)
GL	n.d. (0% at 5 mM)	n.d. (0% at 5 mM)	n.d. (16% at 5 mM)
GP	n.d. (0% at 5 mM)	n.d. (0% at 5 mM)	n.d. (0% at 5 mM)
AP	n.d. (0% at 10 mM)	n.d. (17% at 5 mM)	n.d. (0% at 5 mM)
IP	n.d. (0% at 10 mM)	n.d. (15% at 5 mM)	n.d. (21% at 5 mM)
IPP	n.d. (0% at 10 mM)	n.d. (0% at 5 mM)	n.d. (0% at 5 mM)

n.d. = not determinable.

In this experiment, no inhibitory potential of peptides and their derivatives was measurable, with two exceptions, namely carboxyalkylated IA and LL, which caused a weak to moderate inhibition. IA and LL are the sequences liberated by the MMP-1 by cleavage of triple-helical collagen *(Whittaker et al., 1999)*. This means that these peptides fit perfectly into the active site of the enzyme. Probably, the modification increased the affinity of the molecules to the active site, serving as anchor and keeping them in the catalytic centre. The general inefficiency observed support the affirmation that an efficient MMP-inhibition needs more than interaction in active centre, as discussed for the melanoidins in section 4.2.3. The glycated peptides, due to their small structure, cannot supply the allosteric binding requirements, although they are able to complexate zinc and inhibit other zinc-peptidases. Apparently, competent ligands of this family of enzymes, which are selected to split macromolecules and are endogenously inhibited by proteins of 22–28 kDa, interact also with non-catalytic sites.

In conclusion, the present results demonstrate that specific inhibition against ACE could be obtained by non-enzymatic glycation of peptides.

5 Summary

With the present work, it was shown for the first time that Maillard reaction products are selective inhibitors for matrix metalloproteases.

In the first part of the study it was demonstrated that high molecular melanoidins, formed during roasting of coffee, have a specific and effective inhibitory potential against human matrix metalloproteases (MMPs), which correlated with the roast degree of the samples. While no inhibitory activity of the high molecular fraction obtained from green coffee beans (mRD0), at concentrations up to 2.5 mg/mL, could be measured against the studied MMPs 1, 2 and 9, a continuous increase on the inhibitory potential of the melanoidin fraction was observed upon roasting. For the melanoidins from the final roasted product (mRD4), IC_{50} values of 0.461 ± 0.006 mg/mL against MMP-1, 0.224 ± 0.040 mg/mL against MMP-2; and 0.728 ± 0.014 mg/mL against MMP-9 were measured. These results indicate a direct effect of the roasting process on the formation of MMP-inhibiting structures within the melanoidin complex. Noteworthy is the fact that zinc addition, in concentrations up to 50μM, did not restore the MMP activity in the presence of melanoidins, suggesting more specific molecular interactions between the inhibitor and the active center of the enzymes other than single metal sequestration. Considering that a cup of coffee brew supplies 200 mg of mRD4, the ingestion of 2 to 3 cups of coffee promotes concentrations of 0.2-0.3 mg/mL of the non-absorbable high molecular weight melanoidins in the colonic lumen. This concentration is in the range of the IC_{50} values of mRD4 against the studied MMPs, suggesting that even conventional drinking habits can result in significant inhibition of intestinal MMPs.

The reported thermal-induced inhibitory potential of coffee melanoidins towards MMPs is suggested to result from the functionalization of the macromolecular fraction, both by the formation of efficient zinc-binding moieties through the Maillard reaction, such as carboxyalkyl side chains or pyridones, and the incorporation of compounds of low molecular weight present in the green beans, principally hydroxycinnamic acids.

Contrary to the process-induced inhibitory activity observed for the high molecular weight fraction, the low molecular weight melanoidin fraction (lmw melanoidin, molecular weight below 10 kDa) showed an impressive inhibitory effect already for the sample isolated from non-roasted coffee beans, which was, however, attenuated upon roasting. The thermal

induced decrease in the inhibitory potential of this fraction can possibly be due to depletion of chelating moieties in the lmw melanoidin, most probably caused by the incorporation of free polyphenols, naturally present in high concentrations in coffee beans, in the high molecular weight melanoidin fraction with progress of roasting.

The activities of the zinc-containing metallopeptidases angiotensin converting enzyme (ACE) and *Clostridium histolyticum* collagenase (ChC) were not affected by melanoidins, reinforcing the suggestion of a specific inhibition mechanism based on a molecular interaction between the melanoidins and the catalytic centre of the MMPs rather than simple zinc chelation. Considering the preference of matrix metalloproteases for complex and voluminous substrates, able to occupy simultaneously pockets inside the catalytic centre and exosites, classical enzyme-inhibitor binding of the melanoidins in the active centre of the MMPs is supposed: parts of the melanoidin molecules, with high affinity to zinc, may interact with the zinc ion in the active center, and other parts of the melanoidin molecule can maintain the whole structure into the active pocket of the enzyme via non-covalent interactions. However, an allosteric inhibition type cannot be excluded.

Investigations concerning the structure of coffee melanoidins were performed. Using gel-permeation chromatography (GPC), the disappearance of the chromatographic peaks, originally present in the high molecular weight fraction of green coffee beans, with onset of the thermal treatment, was observed. Only one broad melanoidin peak was detected in the light roasted sample. With progress of roasting, a gradual increase in the apparent mean molecular mass of the coffee melanoidins was noticed, changing from 14 kDa (mRD1) to 17 kDa (mRD2), 21 kDa (mRD3), and 28 kDa (mRD4). This observation corroborates results of the literature, which suggest that extremely-high-molecular-weight coffee melanoidins (>100 kDa) may not be real, but made up of non-covalent complexes of melanoidins with molecular masses around 3–22 kDa and high-molecular-weight carriers.

A progressive increase in the absorptivity of the melanoidin isolate at $\lambda=280$ nm and 405 nm, due to the incorporation of chromophores and unsaturated systems to the melanoidin, was found. Via elemental analysis, a continuous increase in the C/N ratio of melanoidins upon roasting was also observed, suggesting the formation of a high molecular melanoidin structure to happen most probably by the coupling of carbon-rich compounds such as carbohydrates, polyphenols and their degradation products, to a nitrogen-rich protein skeleton. The increase in the C/N-ratio correlated with the increase in the apparent

polyphenol content, which changed from 0.02 mg/mg chlorogenic acid equivalents in the high molecular weight fraction of green beans (mRD0) to 0.10 mg/mg, in the medium roasted sample (mRD2), achieving 0.11 mg/mg in the melanoidins obtained from final espresso roasted beans (mRD4). The observed increase in the total phenols content in the high molecular weight fraction is most probably due to covalent incorporation of chlorogenic acids into the melanoidin structure. This results support the findings of the current publications, which suppose the melanoidins as proteinous backbones where carbohydrates, sugar degradation products and polyphenols are progressively incorporated.

A new mechanism of polyphenol incorporation on the melanoidin structure has been suggested, based on evidences of the Raman spectroscopy. A Michael-like nucleophilic addition of amino or sulfhydryl groups of proteins on the conjugated double-bond of the caffeic or chlorogenic acid, could explain not only the findings of the present work, but also reconciliate discrepant interpretations of experimental evidence recently reported.

In the second part of the present work, the inhibitory potential of carboxyalkyl derivatives of selected peptides against zinc-containing proteases was studied. The N^{α}-carboxymethyl- and carboxyethyl- peptide derivatives were obtained via reductive alkylation in good yields and adequate purity. N^{α}-carboxyalkylation of peptides proved to be an efficient tool to improve the specific binding affinity of dipeptides to angiotensin converting enzyme. The carboxymethylated derivatives of GA, GL, GP, AP, IP, IA showed 2 to 30times stronger inhibitory potentials against ACE than the original peptides. The carboxyethyl derivatization was, for all studied peptides, more effective than the carboxymethylation, and improved in almost 4000 times the inhibitory potential of IA, which showed IC_{50}-values of 1023 µM in the native form, 30.6 µM as CM-IA, and 0.26µM as CE-IA.

Against the other studied metalloproteases, the increase in the inhibitory potential due to the derivatization was also present, although less impressive. The derivatized peptides were able to reduce the *in vitro* activity of ChC, showing IC_{50} values in mM concentrations, while the original peptides did not show any relevant inhibition. No explicit difference between the inhibitory potentials of CM- and CE- could be measured. We suggest that the lack of big hydrophobic substituents can have been responsible for the poor affinity to the enzyme.

The inhibition against the MMP-1 was only measurable for the derivatives of the peptides IA and LL, which represent the sequence liberated by the MMP-1 during the hydrolysis of

native triple-helical collagen. We supposed that this enzyme is adapted to the binding of bulky substrates and the small peptides and their derivatives studied are not able to supply all necessary binding motifs for a competent activity inhibition.

In conclusion, as melanoidins and carboxymethyl and carboxyethyl groups are major products of the Maillard reaction, and are present in considerable amounts in an average diet, a potential positive impact of non-enzymatic glycated food in health should not be underestimated. The heat-induced browning is not only a source of sensorial stimuli, but can maybe also represent a daily input of food-borne protective substances.

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Posters

MISSAGIA DE MARCO, L., WOLTER, I., HENLE, T., Studies on the inhibition of matrix metalloproteinases by non-enzymatically glycated peptides. *35. Deutscher Lebensmittelchemikertag*, 18-20.09.06 in Dresden, Germany.

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Presentation

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Erklärung

Die vorliegende Arbeit wurde an der Professur für Lebensmittelchemie der Technischen Universität Dresden unter Anleitung von Prof. Dr. Thomas Henle angefertigt.

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Ich erkenne die Promotionsordnung der Fakultät Mathematik und Naturwissenschaften der Technischen Universität Dresden vom 23.02.2011 in der geänderten Fassung vom 17.06.2014 an. Weiterhin versichere ich, dass bisher kein erfolgloses früheres Promotionsverfahren stattgefunden hat.

Ort, Datum