COFFEE BREW MELANOIDINS

Structural and Functional Properties of Brown-Colored Coffee Compounds

E. Koen Bekedam

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Abstract

The aim of the work presented in this thesis was the identification of structural and functional properties of coffee brew melanoidins, and their formation mechanisms, that are formed upon roasting of coffee beans. To this end, coffee brew was fractionated on the basis of e.g. molecular weight, charge, and hydrophobicity. The composition of the isolated coffee brew melanoidin populations was determined using a wide range of techniques leading to an improved insight in the structural and functional properties of coffee brew melanoidins.

A new parameter, $K_{\text{mix 405nm}}$, was introduced that allowed the quantification of the melanoidin level in coffee brew fractions. The determined melanoidin levels correlated with both the protein contents and the nonprotein-nitrogen contents, from which it was concluded that proteins become part of melanoidin structures upon roasting. Additionally, it was found that intact chlorogenic acids are incorporated into melanoidin structures via the phenolic acid moiety through nonester-bonds. The extent of chlorogenic acid incorporation correlated with the melanoidin level, indicating that phenolic oxidation contributes to the brown color as well as Maillard reactions. Another finding was that coffee brew melanoidins were shown to expose negative charges at the pH of coffee. Furthermore, arabinogalactan proteins (AGPs) were found to participate in melanoidin formation upon roasting. A 'pure' AGP-melanoidin population could be isolated from coffee brew due to the high specificity of the reagent used for AGP precipitation. Characterization of low molecular weight melanoidins provided strong indications that sucrose is involved in the formation of melanoidins too. Electron spin resonance studies revealed that roasting leads to formation of antioxidative structures in coffee brew melanoidins. This should be due to the formation of novel roasting-induced antioxidative structures and due to the incorporation of chlorogenic acids in melanoidins. Investigation of the effect of the degree of roast on coffee brew melanoidins properties confirmed that proteins and chlorogenic acids are primarily involved in melanoidin formation. Furthermore, arabinogalactans seem to be more involved in melanoidin formation than galactomannans. Additionally, it was found that prolonged roasting especially led to accumulation of HMw coffee brew melanoidins.

Finally, a scheme that describes melanoidin-related formation pathways for coffee beans compounds was introduced. The reaction pathways involved are explained in detail per coffee bean compound.

KEYWORDS

Coffee; brew; melanoidins; Maillard reaction; phenol oxidation; degree of roast; formation mechanisms; arabinogalactan proteins; chlorogenic acid incorporation; antioxidant; charge

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CHAPTER 1

General Introduction

ABSTRACT

In this chapter, an introduction into the topic of coffee brew melanoidins is given, starting with brief information on coffee growing and processing. The composition of green beans, and the compositional changes that occur during roasting are discussed. The characteristics of coffee bean carbohydrates, proteins, and chlorogenic acids are discussed in further detail. Subsequently, the Maillard reaction is reviewed with special attention for the non-volatile, macromolecular, nitrogenous, and brown-colored compounds that are formed during roasting, the melanoidins. Studies dealing with melanoidins are reviewed while making a distinction between melanoidins from model systems and real food systems. Knowledge reported in the literature on coffee brew melanoidins is summarized with special attention for the chemical and functional characteristics.

The last part of this chapter describes why research on coffee brew melanoidins is needed and why research on coffee melanoidins is expected to provide valuable information for both the scientific world as well as for coffee producing industries. Finally, the aim and outline of this thesis are defined.

COFFEE

These days, coffee is reckoned to be the most widely traded commodity in the world after oil. In 2005, the worldwide coffee bean production was more than seven million tons (*1*). The cultivation of coffee beans is gradually spread across the world, with Brazil as the world's largest producer (*1*). The 2 species that are of commercial importance are *Coffea arabica* and *Coffea canephora,* which are known under their trade names Arabica and Robusta coffee beans, respectively.

Coffee beans as we know them are actually the seeds of bright red cherries of the coffee plant (**Figure 1A**). After harvesting of the cherries, the coffee cherries are 'wet' or 'dry' processed resulting in cherries that have a moisture content of about 12%. Subsequently, the outer casing is removed, yielding the green beans (**Figure 1B**). After shipment to coffee processing industries, green beans are roasted till the desired degree of roast (**Figure 1C**). After grinding, the last step is the infusion of the ground and roasted coffee beans with water yielding the well known cup of coffee (**Figure 1D**). Detailed information on growing of coffee and coffee processing can be found in the literature (*2, 3*).

Due to the enormous size of the coffee market, much research has been devoted to this product. Much research focused on growing, roasting, infusion techniques, and consumer perception (*3*). Additionally, the chemical composition and functional properties of coffee beans and brew were investigated thoroughly. The chemical composition of green and roasted coffee beans and changes induced by roasting will be discussed below and references to detailed reviews on each subtopic will be given.

A **B** **C** **D**

Figure 1. Pictures of (**A**) coffee cherries, (**B**) green beans, (**C**) roasted beans, and (**D**) a cup of coffee brew.

GREEN BEANS

The most abundant components in green coffee beans are carbohydrates, proteins, lipids, and chlorogenic acids as can be seen from the compositional data for green Arabica coffee beans (**Table 1**). This compilation provides a resume of the data available and, although these were produced in 1975, only relatively few additional data have been produced since (*2, 3*). These major green coffee bean components will be discussed in detail below except for

Table 1. Compositional Data (% of Dry Matter) for Green and Roasted Arabica Coffee Beans[a]

	Green Beans	Roasted Beans
Minerals	3.0 – 4.2	3.5– 4.5
Caffeine	0.9 – 1.2	~ 1.0
Trigonelline	1.0 – 1.2	0.5 – 1.0
Lipids	12.0 – 18.0	14.5 – 20.0
Chlorogenic acids	5.5 – 8.0	1.2 – 2.3
Aliphatic acids	1.5 – 2.0	1.0 – 1.5
Oligosaccharides	6.0 – 8.0	0.0 – 3.5
Polysaccharides	50.0 – 55.0	24.0 – 39.0
Amino acids	2.0	–
Proteins	11.0 – 13.0	13.0 – 15.0
Melanoidins	–	16.0 – 17.0

[a] Data were extracted from Clarke and Macrea (*2*) and were originally produced by Clifford (*4*).

lipids since I) no papers were found that reported on the involvement of lipids on coffee brew melanoidins formation, and II) lipids are not extracted into coffee brew due to their hydrophobic properties. We refer to the literature for elaborate information on coffee lipids (*3*) and lipid involvement in food browning (*5*).

Chlorogenic Acids in Green Coffee Beans

Chlorogenic acids (CGAs) are the main phenolic compounds in coffee beans and the CGA content might be as high as 8% in green beans (**Table 1**). Several beneficial health effects have been attributed to CGAs which may be largely explained by their potent antioxidant activities (*6*). Furthermore, studies showed that CGAs are absorbed in humans and thus might exert their antioxidative activity in vivo (*6, 7*). Chlorogenic acids are a family of esters of *trans*-cinnamic acids, such as caffeic, ferulic, and *p*-coumaric acid, with quinic acid (*8, 9*). More than 44 different CGAs were already identified in green coffee beans with 5-caffeoylquinic acid (**Figure 2**) being the most prevalent one (*10-12*). The group of Clifford specialized in CGA characterization and they wrote a review on CGAs (*8*).

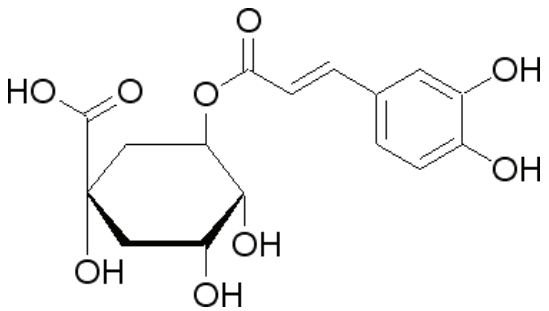

Figure 2. The chemical structure of 5-caffeoylquinic acid.

Amino Acids and Proteins in Green Coffee Beans

The predominant free amino acids in green beans are glutamic acid, proline, alanine, asparagine, and aspartic acid (*13*) which together account for up to 2 w/w% of the green bean (**Table 1**). The protein content in green bean ranges from 8 to 13% (*2*) and these proteins can be divided into water-soluble (50%) and water-insoluble proteins (50%) (*14*). The water-soluble coffee proteins consist for 85% of globulins (*15*), and these globulins were shown to be 11S storage proteins (*16*). In general, it can be said that coffee bean proteins were little investigated despite their involvement in flavor and color formation in the roasting process (*3*).

Carbohydrates in Green Coffee Beans

Green beans contain around 50% carbohydrates and the 4 major carbohydrate sources in green beans are sucrose, arabinogalactan, galactomannan, and cellulose (*3, 17*). A review on coffee bean carbohydrates was provided by Redgwell and Fischer (*17*).

Sucrose

Sucrose is a disaccharide consisting of a glucose and fructose moiety. Sucrose belongs to the oligosaccharides in **Table 1**. Sucrose is the most prevalent free sugar in green beans with sucrose contents of 6–8 % of the green bean dry matter weight (*17*). Sucrose does not contain a reducing group like most carbohydrates do which is due to the fact that glucose is 1→2 linked to fructose. However, sucrose is known to be readily degraded during roasting and its content is minimal in a medium roast coffee (*3*), as can be seen in **Table 1** too.

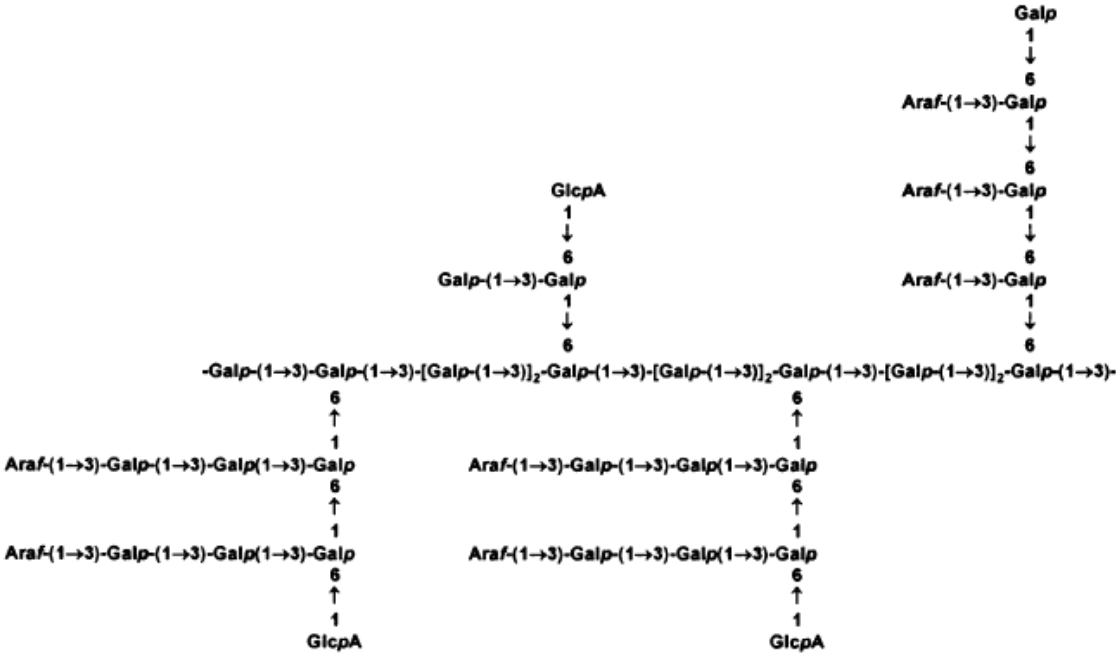

Figure 3. Possible structure of arabinogalactan part of coffee arabinogalactan protein as proposed by Redgwell et al. (*18*).

Arabinogalactans

Arabinogalactans are highly water-soluble coffee bean polysaccharides that consist of β1–3 linked galactosyl residues, substituted at intervals at the O-6 position with various combinations of arabinosyl and galactosyl residues (*3, 17*). These polysaccharides account for roughly one third of the polysaccharides in mature green beans. Additionally, it was reported that these type II arabinogalactans from coffee beans were negatively charged due to the presence of 6–8% glucuronic acid monomers (*18*). A proposal for the structure of coffee bean arabinogalactan was postulated by Redgwell et al. (*18*) and is shown in **Figure 3**. They furthermore reported that coffee arabinogalactans are part of arabinogalactan proteins (AGPs). The AGP content in green beans was around 15% and consisted of approximately 12% protein and 85% arabinogalactan, the latter with an Ara:Gal:GlcA ratio of 3:9:1 (*19*). **Figure 4** shows the Wattle-Blossom model for AGPs in which it can be seen that AGPs are molecules that consist of a protein backbone to which the arabinogalactan moieties are attached (*20*).

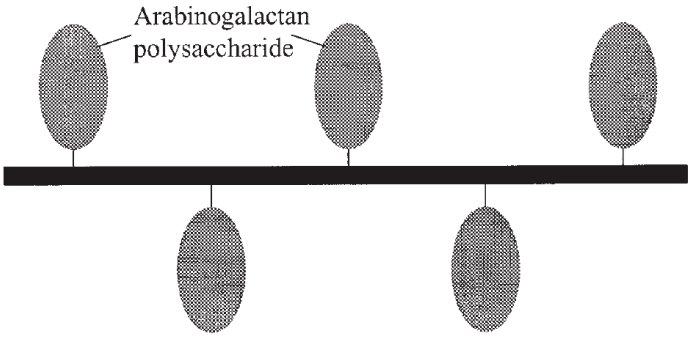

Figure 4. The Wattle-Blossom model for AGPs by Showalter (*20*). The black line indicates the protein backbone (representing ~6 w/w% of AGP), the gray spheroids represent the arabinogalactans (see **Figure 3**) attached to the backbone.

Galactomannans

Galactomannans are the third major carbohydrate population in green coffee beans accounting for roughly half of the polysaccharides in mature green coffee beans. Galactomannans are coffee bean polysaccharides that consist of β1–4 linked mannosyl residues with single galactose units α-linked at O-6 of a mannosyl residue (*17*). Various degrees of substitution are reported in the literature ranging from 130:1 (*21*) to 3:1 (*22*). Oosterveld et al. (*22*) reported on the presence of acetyl groups within galactomannan molecules. Nunes et al. (*23*) later reported that these acetyl groups are linked at the O-2 and O-3 positions of the mannosyl residues. Additionally, it was described that water-soluble galactomannans contained few arabinosyl residues, as side chains (*23*). The structure of galactomannans that can be proposed using data available in literature is shown in **Figure 5**. Galactomannans are far less water-soluble than arabinogalactans, which might be ascribed to the linear character of galactomannans, especially for the galactomannans with a low degree of substitution (*17*).

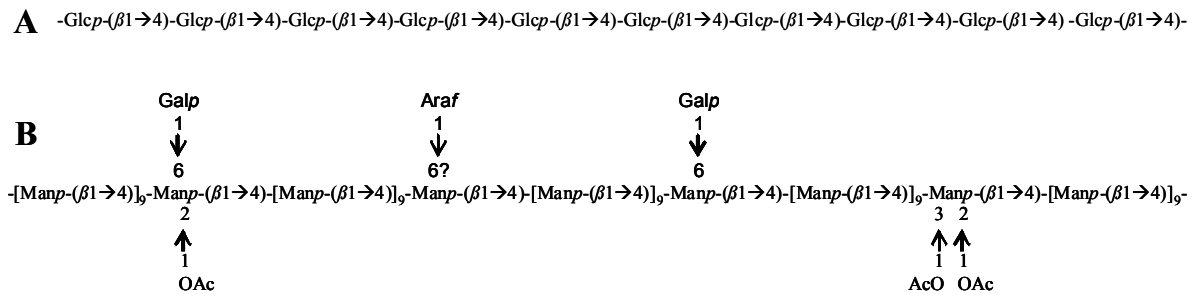

Figure 5. Proposed structure for coffee bean (**A**) cellulose and (**B**) galactomannan (*17, 22, 23*).

Cellulose

Cellulose is the last major polysaccharide in green beans. Cellulose is a linear polysaccharide that consists of β1–4 linked glucosyl residues (**Figure 5**). As a result of the linearity, the solubility of cellulose is extremely low. Furthermore, the β1–4 linked glucosyl linkage is extremely stable and it is therefore that cellulose shows low reactivity during roasting. It is likely because of the limited solubility and low reactivity that there has been no published work on the structure of coffee cellulose (*17*).

ROASTED BEANS

Roasting of green coffee beans is an essential step in the coffee production process as it induces chemical reactions that lead to the formation of the characteristic coffee flavors and melanoidins, the latter being the dark-colored components. The roasting process comprises a 2-stage transformation of the bean: I) evaporation of 10-13% free water, this takes up to 80% of the roasting time, and II) pyrolysis within the beans, with swelling of the beans, and a fairly rapid darkening, followed by the emission of oily smoke and crackling sounds (*2*). Also the dry weight of the coffee beans decreases during roasting, this especially occurs during the pyrolysis stage of roasting and is due to formation of roasting gasses such as carbon dioxide and other volatiles. The ‘degree of roast’ is a parameter for the extent of roasting and is defined as the total weight-loss that occurs upon roasting, including the water evaporation. Alternatively, the extent of roasting is also often expressed by the ‘organic roast loss’, abbreviated as ORL, which is the weight loss of the green bean due to roasting on a dry matter basis. The chemical reactions that take place during the roasting of coffee beans are extremely complex. Compounds present in the green coffee beans degrade, for example polysaccharides and proteins, and subsequently take part in chemical reactions. As a result of all these reactions, the chemical composition of the beans changes drastically during roasting (*2*). Several attempts have been made in review articles to compile compositional tables but none has been entirely successful. This is mainly due to the lack of reliable analytical data, which is particularly due to our poor understanding of the structural properties of the brown-colored roasting products, called melanoidins (*2*). It is not even clear which compounds are included under given categories, e.g. melanoidins, degraded polysaccharides, and proteins. In

other words, it is still unknown whether polysaccharide degradation yields unbound oligosaccharides or that degraded polysaccharide fragments are part of a larger unidentified melanoidin structure. Given these limitations, the compilation produced by Clifford (*4*) would seem to provide a reasonable resume of the data available (**Table 1**). The chemical and/or structural changes that occur on the above described green bean components chlorogenic acids, proteins, and, carbohydrates during roasting are described below.

Chlorogenic Acids in Roasted Coffee Beans

The CGA content decreases continuously upon roasting and may decrease to a level as low as 0.2% for very dark roasted beans (*24*). Using the word 'loss' for the decrease in CGA content might be misleading as loss indicates a complete disruption of the structural properties of a molecule (*25*). However, the observed decrease of unbound CGAs could also be due to incorporation of CGAs in other molecules, like melanoidins. The fate of the disappearing CGAs upon roasting is not totally understood. The reactions that are expected to be involved are acyl migration, hydrolysis, oxidation, fragmentation, polymerization, and association with proteins (*25*). It is suggested that part of the CGAs is converted into flavor compounds upon roasting (*26, 27*), whereas others reported that CGAs might be incorporated in coffee brew melanoidins (*28, 29*). The phenolic acid moiety from CGA is far more prone to oxidative changes than the quinic acid moiety. It is therefore that the phenolic acid likely participates in the chemical reactions during roasting (*30*). Leloup et al. proposed a CGA degradation mechanism based on a mechanistic study they conducted on coffee beans (**Figure 6**) (*26*).

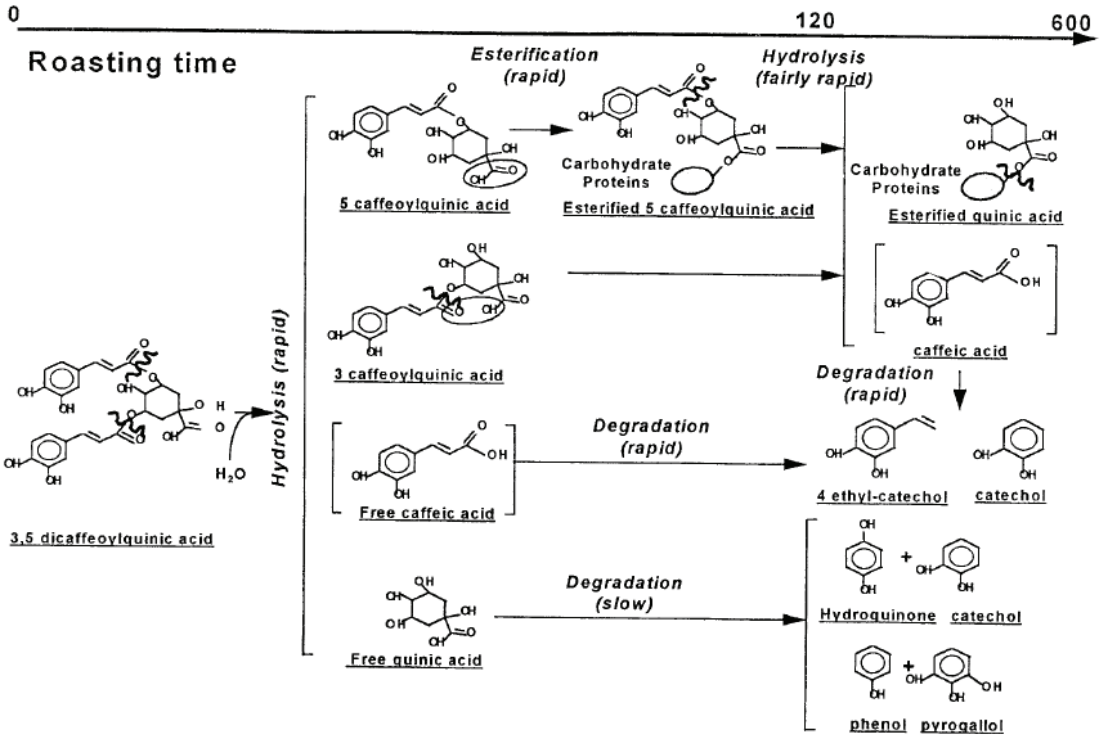

Figure 6. Degradation mechanism of CGAs during roasting, as suggested by Leloup et al. (*26*).

Even though indications were reported, it was not yet discovered through which mechanisms CGAs are actually degraded upon roasting. Thus, it still remains to be found out whether CGAs and/or the CGA building blocks quinic and caffeic acid are really chemically linked to melanoidins upon roasting.

Amino Acids and Proteins in Roasted Coffee Beans

Free amino acids are extremely reactive under roasting conditions, and it is therefore that the amino acid content decreases rapidly upon roasting. After roasting, no or negligible amounts of unbound amino acids are present in the beans (**Table 1**) (*30*). These amino acids do likely participate in the Maillard reaction as they have free amino groups available for reaction. Amino acids are involved in the formation of both flavor and color of coffee (*30*). The protein-bound amino acids are less reactive than free amino acids (*30*), which can be ascribed to the availability of less reactive amino groups. However, reports suggest the contribution of proteins or peptides to the formation of aroma, bitter taste and metal-chelating compounds in coffee (*30*). Rizzi suggested that proteins might play a key role in aroma and melanoidin formation in coffee beans because of the higher reactivity of ε-amino, thiol or methylthio groups, and the higher content of protein compared to amino acids (*31*). Furthermore, Montavon et al. (*32*) investigated the changes in green coffee bean protein profiles during roasting. They also found that coffee bean proteins were both fragmented and polymerized upon roasting. One of their conclusions was that the 11S protein is integrated into melanoidins upon roasting. Also, a reaction scheme was proposed for the autoxidative mechanisms that occur during roasting (**Figure 7**). Additionally, Rawel et al. (*33*) reported on the formation of covalent linkages between chlorogenic acid and proteins upon roasting. In conclusion, even though the mechanisms are poorly understood, these few studies conducted on coffee bean proteins clearly indicate their involvement in flavor and melanoidin formation (*30-33*).

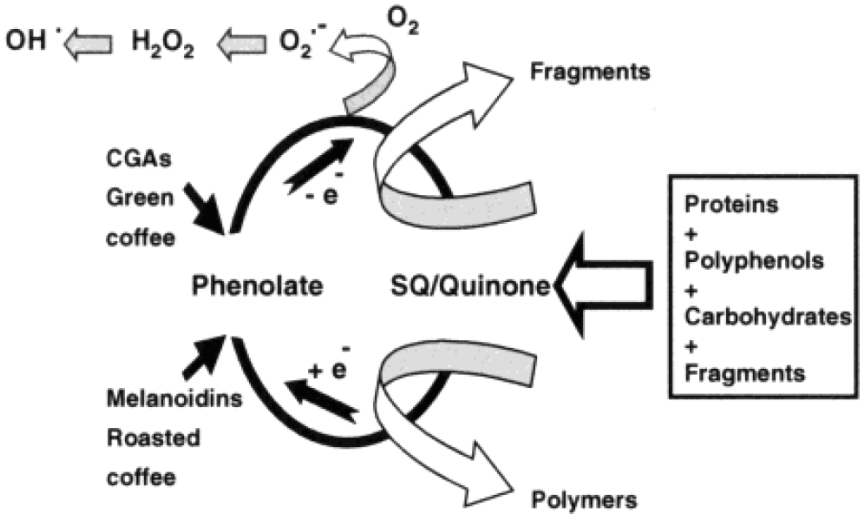

Figure 7. Proposed reaction scheme of autoxidative mechanisms in coffee, by Montavon et al. (*32*).

Carbohydrates in Roasted Coffee Beans

The effect of roasting on carbohydrate properties is relatively well investigated and well-documented in the literature. Sucrose is rapidly converted upon roasting and the content decreases to ~1% of its original content in green beans (*2*). The fact that sucrose lacks a reducing end suggests that I) sucrose is hydrolyzed followed by participation of the released monosaccharides in further chemical reactions, or II) that either the glucose or the fructose moiety undergoes ring opening enabling 'sucrose' to react with other coffee compounds. The exact fate of sucrose is unknown although several reaction mechanisms have been proposed (*3*). The polysaccharides also undergo degradation and modification upon roasting, although in a lesser extent than low molecular weight sugars (*17*). Redgwell et al. (*34*) and later Oosterveld et al. (*35*) reported that up to 40% of the coffee bean polysaccharides were lost during roasting. A scheme for coffee polysaccharide degradation and modification which occurs during roasting was postulated by Oosterveld et al. (*36*) (**Figure 8**).

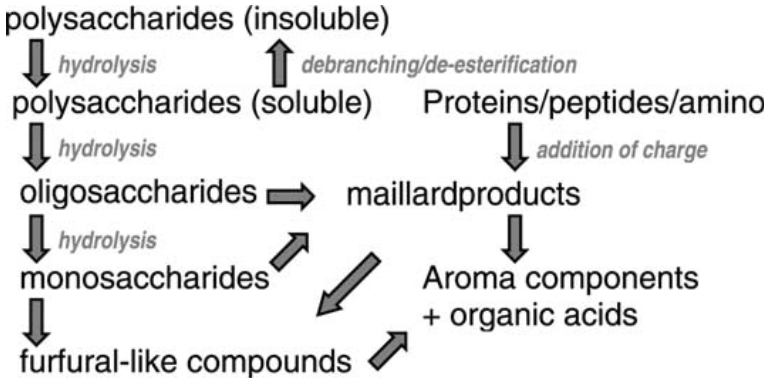

Figure 8. Schematic representation of the polysaccharide conversion reactions which occur during roasting and extraction of coffee beans, by Oosterveld et al. (*36*).

Arabinogalactan is the coffee bean polysaccharide that is most susceptible to conversion upon roasting which is particularly due to the heat-labile arabinosyl residues (*17*). However, the magnitude of the molecular weight decrease of arabinogalactans was more than could be predicted from the loss of arabinosyl residues from the side chain and must therefore have resulted from fission of the galactan backbone (*17*). In addition, Oosterveld et al. reported that the debranching of the arabinose sidechains occurred more rapidly than hydrolysis of the galactan backbone (*17, 35*). Furthermore, Nunes and Coimbra (*37-39*) found similar results for coffee infusions; they reported in a series of publications that the amount of terminally linked arabinosyl residues in arabinogalactans decreased as well as a decrease of the molecular weight. Galactomannan is more resistant towards degradation because of the rather heat stable β1–4 linked mannosyl residues. Oosterveld et al. (*35*) reported on a decrease of the molecular weight of galactomannans in coffee beans upon roasting. Nunes and Coimbra (*37-39*) reported that both the degree of branching and the degree of polymerization of the galactomannans in coffee infusions decreased upon roasting. The water-insoluble

polysaccharide cellulose is hardly degraded and solubilized upon roasting which is mainly due to the extremely stable β1–4 linked glucosyl linkages (*17*).

The formation of the characteristic coffee flavors and dark-colored components during roasting is mainly ascribed to the occurrence of the Maillard reaction upon roasting. To obtain a better insight in the formation of these coffee components, the Maillard reaction will be discussed below.

THE MAILLARD REACTION

The Maillard reaction, which is a non-enzymatic browning reaction, is a complex network of reactions involving carbonyl and amino compounds, such as reducing sugars and amino acids. It is the main reaction responsible for the transformation of precursors into colorants and flavor compounds during food processing (*40*). The Maillard reaction has been named after the French chemist Louis Maillard (*41*) who first described such conversion reactions in 1912 but it was only in 1953 that the first coherent scheme was put forward by Hodge (*42*)

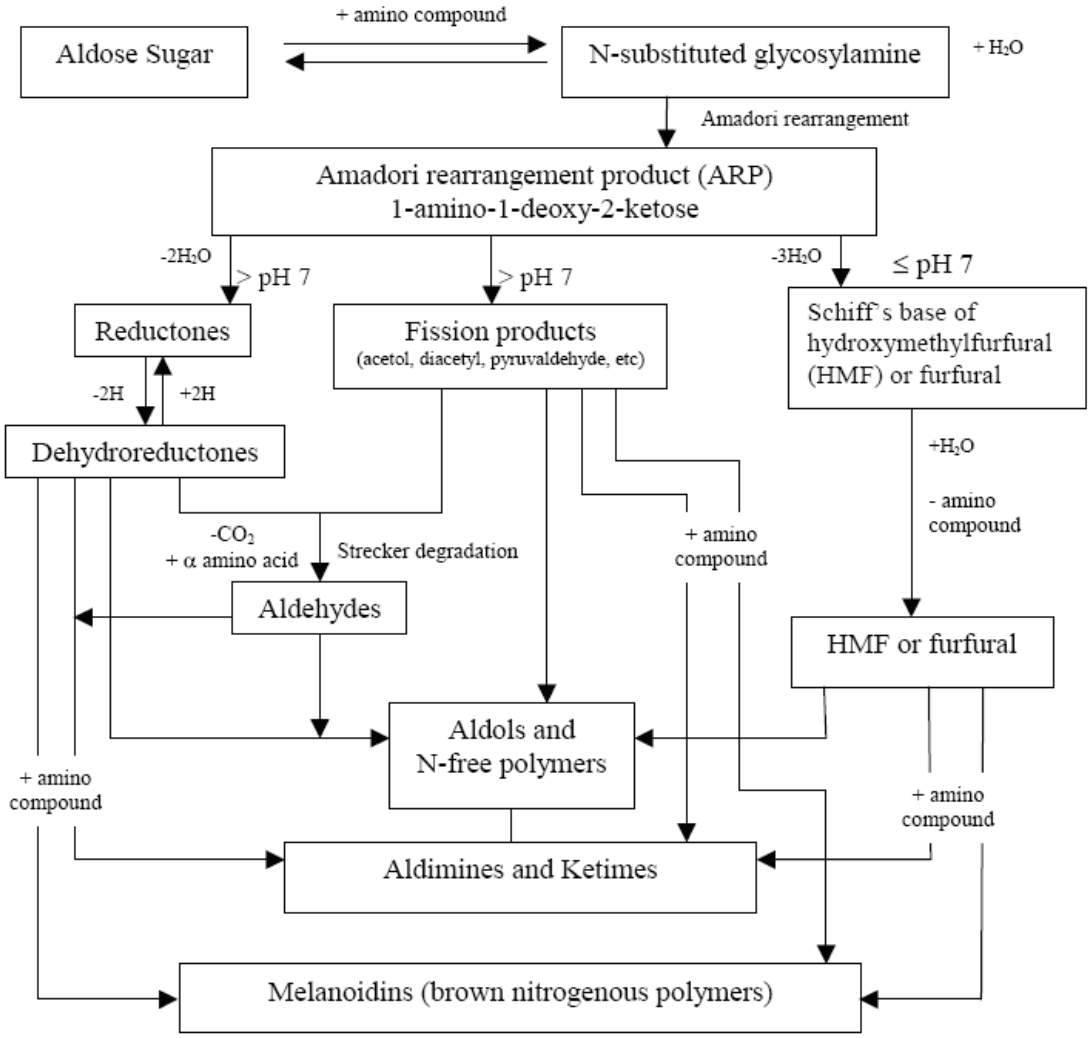

Figure 9. Maillard reaction scheme originally by Hodge (*42*), and adapted by Martins et al. (*43*).

(**Figure 9**). More recently, Tressl et al. (*44*) revealed additional reaction mechanisms, like the retro-aldolization reactions and the formation of C1-C5 fragments. As shown in **Figure 9**, the Maillard reaction comprises a cascade of successive reactions in which each reaction might proceed via different pathways leading to a wide range of Maillard reaction products that might be formed. A series of parameters that significantly affect the Maillard reaction pathway and the final product structure were identified: I) time of heating (*45*), II) temperature (*46, 47*), III) water activity (*46*), IV) pH (*48*), V) buffering capacity (*49*), VI) chemical composition of the system (*46, 50*), VII) moisture content (*51*), VIII) pressure (*52*), and IX) type of heating (*46*).

An article written by Ledl and Schleicher (*53*) gives a good overview on the Maillard reaction up to 1990. More recently, Martins et al. (*43*) gave an overview on the established mechanisms of the Maillard reaction in food. The Maillard reaction can be divided into three different stages: the initial, advanced and final Maillard reaction stage (*54*). The Maillard reaction products can be roughly divided in 2 classes, the volatile components and the brown-colored non-volatile Maillard reaction products (*55*). The volatile Maillard reaction products can be referred to as Maillard flavors and the non-volatile Maillard reaction end products are generally referred to as melanoidins (*43, 56, 57*).

Maillard flavors

In the past, most of the studies on Maillard reaction products were directed towards the flavor class of the Maillard reaction products. The primary aim in this field involved the structural characterization and quantification of the volatiles end-products responsible for aroma formation, and not so much the formation pathways of these Maillard reaction products. A series of review articles provide a rather complete overview on volatiles formed by the Maillard reaction (*58-61*). Furthermore, Buffo and Cardelli-Freire (*62*) and Flament and Bessière-Thomas (*63*) provided overviews on Maillard coffee flavors formed during processing of coffee beans.

Melanoidins

Relatively little work focused on melanoidins formed during the Maillard reaction, compared to the amount of work that focused on Maillard flavors. Melanoidins are generally defined as macromolecular, nitrogenous, brown-colored Maillard reaction end-products (*32, 42, 50, 64*). Melanoidins are formed during home and industrial heat processing of foods, and are widely distributed in our diet (for instance in coffee, cocoa, bread, malt, and honey). Melanoidins are formed by cyclizations, dehydrations, retroaldolizations, rearrangements, isomerizations, and condensations of initial Maillard reaction products (*43*), but none of them have been fully characterized yet. There are 3 procedures that are used to describe or measure the extent of melanoidin formation. First, the amount of melanoidins is determined by difference, which means that the amount of melanoidins is the percentage of compounds that can not be accounted for in a food product (100% – % of known compounds) (*65, 66*).

Second, the brownness can be quantified by humans in order to measure the color potency of Maillard reaction products (*50*). This procedure yields a factor, the color dilution (CD) factor, which indicates the coloring potency of a Maillard reaction product (*50*). Third, the extent of melanoidin formation can also be determined by measuring the absorption at wavelengths higher than 400 nm, e.g. 405 nm (*67, 68*) or 420 nm (*69*). At these wavelengths, almost no or no other naturally occurring food components are known to absorb light, and it is therefore that melanoidins are exclusively measured at these wavelengths. As a result, determination of the absorption at 405 nm is basically a parameter that describes the brownness of a product.

Till date, the chemical structures of melanoidins remain unknown which can be ascribed to the extreme complexity of these compounds. However, three main proposals for the structure of melanoidins have been put forward till now. First, melanoidins were reported to be low molecular weight coloured substances which crosslink with free amino groups in proteins (Arg, Lys) leading to high molecular weight colored structures (*70*). Second, it was proposed that melanoidins are macromolecular structures of repeating units that are built up from polycondensated furan-like and pyrrole-like structures (*71*). Third, it was reported that the melanoidin skeleton is mainly built up from sugar degradation products which are formed in the early stages of the Maillard reaction, polymerized and linked by amino compounds (*72, 73*).

Several researchers are active in the melanoidin field and the groups of Ames, Blank, Coimbra, Fogliano, Hofmann, Kroh, Morales, Obretenov, Tomlinson, Van Boekel, Wedzicha and Yaylayan have made important contributions to the knowledge on melanoidins, with each group having its own specialization. Their studies mainly focused on color formation (*47, 50, 56, 57, 74-79*), radical formation (*80*), flavor interactions (*65, 66, 81, 82*), antioxidative properties (*67, 83-86*), and reaction kinetics (*43, 47, 56, 74, 79, 87-90*).

Melanoidins in Model Systems

Many studies have been conducted using model systems instead of real food products to circumvent complexities caused by matrix properties of food products. Most of these model systems used mixtures of single amino acids and monomeric sugars in aqueous systems.

The model browning reaction between glucose and glycine was extensively investigated, for example, by Maillard himself (*41*), Homma et al. (*30*), Kim and Park (*91*), Kato et al. (*92-95*), Obretenov et al. (*96*), Wedzicha and Vakalis (*97*), Taguchi and Sampei (*98*), Feather and Nelson (*99*), Benzing-Purdie et al. (*100*), Nam and Kim (*101*), Wedzicha and Kaputo (*47, 102*), Cämmerer and Kroh (*73*), Martins and Van Boekel (*77, 78*), and recently by Mundt and Wedzicha (*103*).

Model reactions using other amino acids (*45, 104*), proteins (*50, 75, 105-107*), or amines (*108*), instead of glycine; other monomeric sugars (*73, 109*), retro-aldol sugar degradation compounds (*69*) or di-, oligo-, or polymeric carbohydrates (*45, 110, 111*) instead of glucose were also conducted. Mundt and Wedzicha (*79*) reported a synergistic effect on the rate of browning when the 2 sugar monomers glucose and fructose were heated with glycine. It was

also reported that MR results in a faster color formation and a larger high-molecular-weight fraction was obtained in case only the NH_2^{ε} of lysine is available for the Maillard reaction (*112*). It is clear that the advantage of model systems is that the Maillard reaction is kept as 'simple' as possible. However, there are 2 important reasons why research should not be restricted to model systems. First, the reaction products of these model systems are still very complex (*50*). Second, model systems do not resemble the Maillard reaction in foodstuffs because the compositional complexity of foodstuffs is not taken into account in model reactions, while it is assumed that that other food compounds (e.g. chlorogenic acid, polysaccharides, different types of amino acid and monosaccharide) also play a role in the Maillard reaction (*113-115*). The composition of foodstuffs is far more complicated than any simple model systems. In coffee, for example, lipids, chlorogenic acids, sugars, free amino acids, and proteins are also present. As described above, it is likely that at least some of these components will coincide with Maillard reaction processes. It must be stated though that model systems can provide valuable information, but they should preferably be used in addition to research on the Maillard reaction within complex foodstuffs.

Melanoidins in Food Products

Research on melanoidins flourished in the 8th decade of the previous century and many publications dealing with melanoidins appeared this decennium (**Figure 10**). After a period in which the attention given to melanoidins continuously decreased (1980-1995), people showed a renewed interest in melanoidins which resulted in increasing numbers of publications written on melanoidins in the last decade (**Figure 10**). The publications on melanoidins within the Food Science and Technology Abstracts (FTSA) database can be roughly divided into 2 categories: I) studies using model systems and II) studies using real food products. It was found that 40% of all publications from could be related to food products, leaving 60% for model studies.

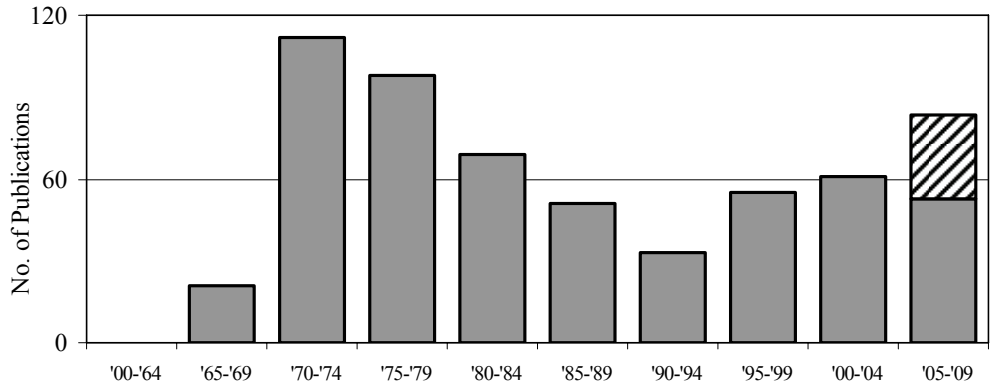

Figure 10. Number of publications with the term "melanoidin" per 5 years found in the "Food Science and Technology Abstracts" database. Gray bars: publications already appeared, striped bar: estimated publications to appear in 2008 and 2009 by extrapolating published papers in 2005-2007.

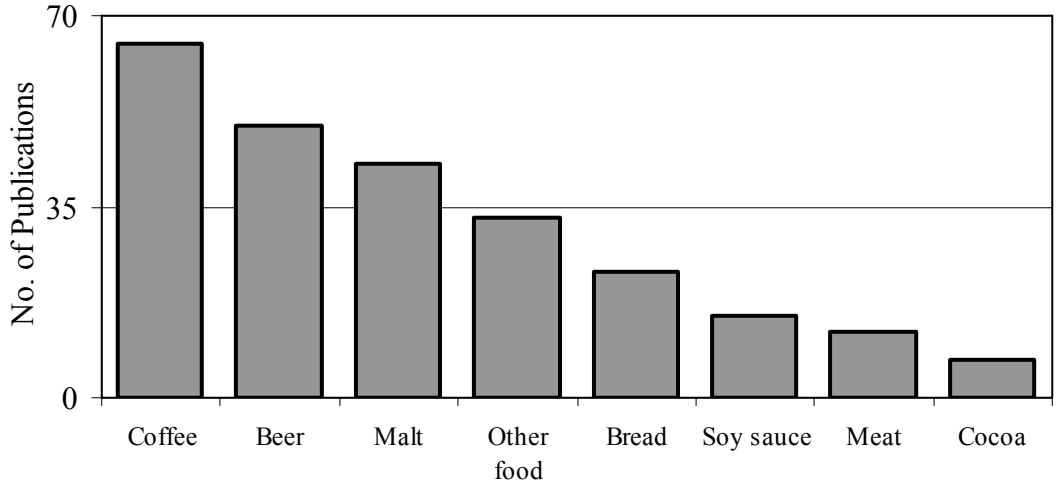

Figure 11. Distribution of foodstuff related publications on melanoidins into several food categories for the period 1900-2007.

A number of chromatographic and electrophoretic attempts have been undertaken to isolate and purify melanoidins from food products, for example, from coffee (*116-118*), soy sauce (*119, 120*), bread (*121*), meat (*122*), malt (*123, 124*), and dark beer (*125*). Most of the publications on foodstuff melanoidins dealt with specific food products. The division of these publications over various food categories is shown in **Figure 11.** The fact that most attention was given to melanoidins in coffee can be attributed to 3 important reasons. First, coffee is a food product that is relatively rich in melanoidins, with levels as high as 28% (*123*). Second, coffee brew melanoidins are water-soluble which makes research less complicated. Third, coffee is of great commercial importance as coffee is consumed by millions of people worldwide every day. Despite the high amount of melanoidins in processed foods, it has as yet not been possible to isolate and characterize a pure melanoidin from food products.

COFFEE BREW MELANOIDINS

Due to the complexity of agricultural raw materials, like coffee beans, the Maillard reaction that takes place during the roasting of coffee beans leads to the formation of a broad range of products. Roasted coffee beans may even contain over 30% of these relatively poorly characterized molecules (*126*). Studies on coffee melanoidins can be divided in studies that focus on the chemical characteristics and studies that focus on functional properties of coffee melanoidins.

With respect to the chemical characteristics of coffee melanoidins, it was suggested that coffee melanoidins might contain protein or protein fragments (*3, 31-33*), that chlorogenic acids are incorporated (*28, 33*), and that polysaccharides or polysaccharide fragments are likely involved in melanoidin formation (*36*), as was discussed already in the section above on roasted coffee beans. Despite these studies, it remains unknown how the chemical structure of coffee melanoidins exactly looks like. It is even unknown whether proteins and/or

polysaccharides are the carrying structures, the backbone, of the brown-colored chromophores in coffee melanoidins. Thus, with respect to coffee melanoidins, quite some observations have been described, but not much is known on the precise chemical structure yet.

Coffee brew melanoidins are said to have quite some functional properties. It is therefore that coffee melanoidins are not only of interest due to their contribution to color formation. It was reported that coffee melanoidins I) are able to bind flavors (*65, 66, 81, 82, 127-129*), II) expose an antioxidative capacity (*67, 68, 84, 86, 130, 131*), III) expose metal-chelating properties (*131, 132*), and IV) are rather reactive in coffee brew (i.e., ageing of coffee) (*113*).

The roasting products in coffee brew, especially its large melanoidin fraction, are said to have both adverse and positive effects on human health. The adverse effects comprise discoloration of dental carious lesions (*133*) as well as the 'stimulating' effect on the intestinal tract (*134*). They are also considered to be carcinogenic as possible relationships between coffee and cancer risk were considered in epidemiological studies. However, a recent review by La Vecchia and Tavani (*135*) showed that no correlation between coffee intake and cancer could be found. Even oppositely, an inverse correlation was found several times between coffee intake and cancer. This latter observation is in line with the recent findings of Alexander who found that melanoidins should be considered as antioxidant dietary fiber which prevents cardiovascular disease and colorectal cancer (*136*). The positive effects of melanoidins on health comprise their preventive effect on certain forms of cancer (*135, 136*), their antioxidative properties (*67*), and it is likely, due to the complex structure, that they serve as prebiotics in vivo too (*136*). Thus, coffee melanoidins have been studied in recent years because of their nutritional, biological, and health implications.

In coffee melanoidin research, it is often seen that an isolated brown-colored coffee brew fraction (e.g. all macromolecular material) was simply denominated as melanoidins without any knowledge on the composition of this fraction. This approach is rather short-sighted as the isolated fraction might contain many more components as well, like protein or polysaccharides which do not have to be part of the melanoidin structure. However, this approach is the only one that can be used as no procedures to exclusively isolate specific melanoidins are known. Summarizing, it can be stated that due to the complexity of roasted coffee beans, the work performed on the non-volatile compounds in coffee has been rather descriptive and much less in chemical detail.

FOCUS OF THIS RESEARCH

The approach used for investigation of the Maillard reaction within this research project differs in two aspects from most other research conducted on melanoidins.

The first aspect is that this research project focused on the formation of melanoidins in a real food product; while many other researchers used model systems. As mentioned before, the advantage of model systems is that the Maillard reaction is kept as 'simple' as possible. However, the two reasons that were mentioned in the previous section already showed why we are of the opinion that studying real food systems should be preferred over model systems.

Briefly, the effect of other food components, which are not included in the model system, on the heat-induced reactions are not taken into account. By using a real food product, a better understanding can be obtained on the actual processes that occur during heat processing of food products. This makes research on melanoidins of course also far more relevant and interesting for industries active in the field of the selected food product.

The second aspect is that we want to focus on the structural and functional properties of the final macromolecular Maillard reaction products, the melanoidins. This is in contrast with many other groups who study the initial phase of the Maillard reaction, which they probably do because the structure of initial Maillard reaction products is somewhat less complicated. After isolation and characterisation of the final melanoidin structures, the next step is the investigation of formation mechanisms involved in melanoidin formation by working back towards the elucidation of Maillard reaction intermediates.

It is clear from the general introduction that investigation of structural and functional properties of melanoidins that are present in real food products, will be extremely complicated. However, novel findings on melanoidin structural and/or functional properties will be of high importance as they describe the actual characteristics as they are in the food product studied. Furthermore, no complications have to be solved concerning the extrapolation of 'model' melanoidin properties to 'in food' melanoidins properties. Novel findings can therefore directly be interpreted to understand, and subsequently control, melanoidin formation in food.

For the course of this research, roasted coffee beans were selected as a model for the heat treatment of other dry products like cacao and breakfast cereals. The reasons for selecting coffee were already mentioned above: coffee melanoidins are water-soluble, coffee contains high levels of melanoidins, and the industrial market for coffee is enormous. The latter reason makes it interesting from a commercial point of view as well.

AIM AND OUTLINE OF THIS THESIS

The aim of the work presented in this thesis is the identification of structural and functional properties of coffee brew melanoidins, and their formation mechanisms, that are formed upon roasting of coffee beans.

In **chapter 2**, we introduce the parameter $K_{\text{mix 405nm}}$ which enabled quantification of melanoidins in coffee brew fractions. Furthermore, protein degradation and incorporation, as well as chlorogenic acid incorporation are addressed in this chapter. **Chapter 3** reports on the anionic charge properties of melanoidins. This chapter furthermore discusses the isolation and characterization of 'pure' AGP-melanoidin complexes. **Chapter 4** deals with chlorogenic acid incorporation in melanoidins. The incorporation mechanisms involved are discussed as well. **Chapter 5** introduces a novel procedure which enables isolation of low molecular coffee brew melanoidin. Isolated melanoidins were characterized. **Chapter 6** describes our findings on antioxidative structures that are formed in coffee melanoidins caused by the

roasting process. In **chapter 7**, we discuss the effect of the extent of roasting on the characteristics of the coffee brew melanoidin properties.

Finally, all results are combined and discussed in **chapter 8** and a scheme for coffee brew melanoidin formation mechanisms is proposed. Additionally, the relevance of these findings for coffee producing industries is discussed.

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CHAPTER 2

High Molecular Weight Melanoidins from Coffee Brew

ABSTRACT

The composition of high molecular weight (HMw) coffee melanoidin populations, obtained after ethanol precipitation, was studied. The specific extinction coefficient (K_{mix}) at 280, 325, 405 nm, sugar composition, phenolic group content, nitrogen content, amino acid composition, and non-protein nitrogen (NPN) content were investigated. Results show that most HMw coffee melanoidins are soluble at high ethanol concentrations. The amino acid composition of the HMw fractions was similar, while 17% (w/w) of the nitrogen was NPN, probably originating from degraded amino acids/proteins and now part of melanoidins. A strong correlation between the melanoidin content, the NPN, and protein content was found. It was concluded that proteins are incorporated into the melanoidins and that the degree of chemical modification, for example, by phenolic groups, determines the solubility of melanoidins in ethanol. Although the existence of covalent interaction between melanoidins and polysaccharides were not proven in this study, the findings suggest that especially arabinogalactan is likely involved in melanoidin formation. Finally, phenolic groups were present in the HMw fraction of coffee, and a correlation was found with the melanoidin concentration.

KEYWORDS

Coffee brew; melanoidins; Maillard reaction; amino acid analysis; ethanol precipitation

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INTRODUCTION

The chemical composition of a coffee brew is strongly influenced by the composition of the green bean, the roasting procedure, and the extraction conditions (*1*). Green coffee beans are rich in carbohydrates, proteins, and phenolic compounds, and these compounds are transformed and/or degraded upon roasting (*2-5*). During the roasting process polysaccharides (*6*) and proteins (*7*) are degraded, and the Maillard reaction takes place between amino acids/proteins and reducing sugars, which results in the formation of flavor and colored compounds (*8*). The water-soluble brown colored compounds that are formed are referred to as melanoidins. The formed melanoidins make up a large part of a coffee brew and might be up to 25% (w/w) of the brew's dry matter (*9*).

Melanoidins are of interest not only due to their contribution to color formation but also for their flavor binding properties (*10-14*), antioxidative capacity (*9, 15-17*), metal-chelating properties (*17, 18*), and reactivity in coffee brew (i.e., aging of coffee) (*19*). Melanoidins have been studied in recent years because of their nutritional, biological, and health implications.

Although more knowledge about the structure of melanoidins has been revealed in recent years, the chemical structure of melanoidins has not been elucidated yet. At the moment, there are three main proposals for the structure of melanoidins (*20, 21*): Heyns and Hauber (*22*), and Tressl et al. (*23*) suggest that melanoidins are a polymer built up of repeating units of furans and/or pyrroles, formed during the advanced stages of the Maillard reaction, linked by polycondensation reactions. Hofmann (*24*) detected low-molecular-weight colored substances, which were able to crosslink proteins via ε-amino groups of lysine or arginine to produce high-molecular-weight colored melanoidins. And Kato and Tsuchida (*25*), and more recently the group of Cämmerer (*20, 21*), suggest 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 linked by amino compounds. Although these proposals provide valuable information on what melanoidins might look like, it is important to realize that these suggested melanoidin structures are mostly based on model studies. In food systems, like coffee beans, the composition of melanoidins is likely to be far more complex due to the presence of many more possible reactants. Therefore, it is likely that all the proposed structures for melanoidins can be found in coffee melanoidins, and they may even occur within the same melanoidin complex.

As the molecular structure of melanoidins is largely unknown, these compounds are generically defined as brown, nitrogenous macromolecular material (*9, 26*). The quantity of melanoidins is often determined "by difference", which is the quantity that remains after subtraction of the known compounds (carbohydrates, proteins, caffeine, etc.) from the starting material. Melanoidins are often measured by their absorbance at 405 nm, which is an arbitrary chosen wavelength at which the intensity of the brown color is measured.

The objective of the present investigation was to isolate and characterize different melanoidin populations present in a coffee brew. While the primary focus was on coffee

melanoidins, attention was also given to the quantity and composition of carbohydrates, proteins, non-protein nitrogen (NPN), and phenolics in the coffee brew.

MATERIALS AND METHODS

Materials.

Roasted coffee beans (*Coffea arabica*), originating from Colombia, were provided by a local factory. The degree of roast, which represents the weight reduction upon roasting, was 16.4% (w/w) and was 8.0% (w/w) on a dry matter basis.

Preparation of Coffee Brew.

The roasted beans were ground using a Retsch ZM200 mill equipped with a 0.4-mm sieve and operating at maximum speed (18 000 rpm). Coffee brew was prepared as described by Borrelli et al. (*9*). The coffee brew was obtained from the milled beans by adding 200 g of ground coffee to 1200 g of filtered demineralized water (Millipore Corp, Billerica, MA) at 90 °C. Subsequently, this coffee suspension was kept at 90 °C for 15 min while stirring continuously. The extract was filtered over a Büchner funnel using a S&S 595 filter (Whatman, Maidstone, UK). For characterization purposes, part of the coffee brew and the residue were lyophilized, yielding "Brew" and "Brew Residue", respectively. The major part of the coffee brew was used for further isolation.

Isolation of High Molecular Weight Material from Coffee Brew Using Diafiltration.

High molecular weight material of the coffee brew was obtained by diafiltration using a 0.7 square meter hollow fiber filter with a MW cutoff of 3 kDa (A/G Technology Corp., Needham Heights, MA). The flow was applied by a KBl CD/SF 1200 pump, and the pressure over the system was not allowed to exceed 1 bar. A freshly prepared brew was first concentrated 3 times using the diafiltration system and subsequently diafiltered until the conductivity of the dialysate became lower than 10 μS/cm. For characterization purposes, part of the retentate and the dialysate were lyophilized, yielding "Brew HMw" and "Brew LMw", respectively. The major part of the retentate was used for further isolation.

Isolation of High Molecular Weight Material from Coffee Brew Using a Dialysis Membrane.

Fresh coffee brew, Brew HMw, and Brew LMw were dialyzed using a dialysis membrane (MW cutoff 12-14 kDa, Visking size 9, Medicell International Ltd., London, UK) for 2 days against running tap water and 1 day against demineralized water with two water renewals. The retentates of the coffee brew, the Brew HMw and Brew LMw were lyophilized, yielding "Brew-Dial", "Brew HMw-Dial", and "Brew LMw-Dial", respectively.

Ethanol Precipitation of the Brew HMw.

The Brew HMw obtained directly after diafiltration was subjected to ethanol precipitation. Absolute ethanol (Merck, Darmstadt, Germany) was slowly added to the Brew HMw under continuous stirring until an ethanol concentration of 20% (w/w) was reached. The solution was left for precipitation (8-15 h) at 4 °C. This solution was then centrifuged for 20 min at 18900*g*. The supernatant and the residue were separated, and the residue was washed again using an ethanol concentration of 20% (w/w). The first supernatant was subjected to further ethanol precipitation steps in which the ethanol concentration was increased in steps to 40, 60, and a final concentration of 80% (w/w) following the same procedure. The ethanol, present in the residues of the ethanol precipitation steps at 20, 40, 60, and 80% (w/w) ethanol and in the supernatant of 80% (w/w) ethanol, was removed by flushing under a stream of dry nitrogen at room temperature; nitrogen was used to prevent oxidation of the coffee compounds. Subsequently, the samples were lyophilized, yielding "EP20", "EP40", "EP60", and "EP80" for the coffee fractions that precipitated at 20, 40, 60, and 80% (w/w) ethanol, respectively. The lyophilized supernatant of 80% (w/w) ethanol was coded "ES80".

Defatting of Coffee Samples.

All the lyophilized coffee samples were defatted by Soxlet extraction using a Soxtherm, which was connected to a Multistat system (Gerhardt, Königswinter, Germany). The solvent used for extraction was dichloromethane. After defatting, dichloromethane was evaporated in an oven at 40 °C for approximately 30 min.

Molecular Weight Distribution.

High-performance size-exclusion chromatography was performed on two 300 × 7.8 mm i.d. TSKgel columns in series (G4000 PWXL and G2500 PWXL) (TosoHaas, Stuttgart, Germany), in combination with a PWX-guard column. Elution took place at 40 °C with 0.2 M sodium nitrate at 0.8 mL/min. The eluate was monitored by refractive index detection using a Spectra System RI-150 detector, and the absorbance was measured at 280, 325,

and 405 nm using a Spectra System UV2000. The sample was dissolved in the eluent and was centrifuged prior to injection (100 μL).

Analysis of the Nitrogen Content.

The nitrogen content of various samples was estimated according to the Dumas method using an NA2100 nitrogen and protein analyzer (Carlo Erba Instruments, Milan, Italy) according to the manufacturer's instructions. Methionine was used as a standard.

Sugar Analysis.

The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (*27*) using inositol as an internal standard. The samples were prehydrolyzed with 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C, and the constituent sugars released were analyzed as their alditol acetates. The uronide content was determined by the automated colorimetric *m*-hydroxydiphenyl method (*28, 29*).

Total Phenolic Groups Content.

The total phenolic group content of the coffee samples was determined with the Folin-Ciocalteu reagent. For all coffee samples, a 0.33 or 1 mg/mL solution was prepared. To 1000 μL of the coffee sample solution, 500 μL of Folin-Ciocalteu (Merck, Darmstadt, Germany) reagent was added. After mixing of the sample, 1000 μL of a saturated Na_2CO_3 solution was added and filtered demineralized water was added until the total volume was 10 mL. After mixing, followed by 1 h of reaction, the absorbance of the sample was measured at 725 nm on a UV-mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan). Chlorogenic acid was used as reference phenolic compound.

Spectroscopic Analysis.

The absorption spectra of coffee solutions were determined in the range 200-700 nm. The coffee solutions were prepared (0.1 mg/mL) by dissolving 1 mg of the lyophilized coffee material in 10 mL of filtrated demineralized water. Each solution was prepared just before measurement. Absorption spectra were recorded on a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan).

Specific Extinction Coefficient of Coffee Material at 280, 325, and 405 nm.

A 1.5 mg/mL sample solution was prepared by dissolving 45 mg of lyophilized coffee sample in 30 g of water. Subsequently, various dilutions were prepared from this coffee solution. To minimize errors that do occur during dilution based on volumes, preparation of these coffee solutions was performed by weighing. The quartz cuvette used for absorption measurements was rinsed thoroughly with the coffee dilution prior to measuring the absorption. The absorption at 280, 325, and 405 nm was measured using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). To guarantee linearity, the value of the absorbance at all three wavelengths had to be between 0.1 and 1.3. In practice, this meant that one dilution had to be prepared for measurement of the absorption at 405 nm and another dilution with a concentration around 5 times lower had to be prepared for measurements at 280 and 325 nm. The measurements were conducted in triplicate. The specific extinction coefficient (K_{mix}) was calculated using the law of Lambert–Beer: E (–) = K_{mix} (L g^{-1} cm^{-1}) × concentration (g L^{-1}) × length of light path (cm). The use of the specific extinction coefficient (K) was preferred over the molar extinction coefficient (ε) since the molecular weight of melanoidins is unknown and is probably variable. By using K, the concentration parameter used in the law of Lambert–Beer is expressed in L g^{-1} cm^{-1}, which makes it applicable for coffee.

Amino Acid Analysis.

Samples for amino acid analysis were hydrolyzed using liquid-phase hydrolysis in 6 M HCl at 110 °C for 24 h. Amino acid analyses were performed using a Hewlett-Packard Aminoquant 1090M using an automated two-step precolumn derivatization with two different reagents, *o*-phthalaldehyde for primary and 9-fluorenylmethylchloroformate for secondary amino acids (*30*).

RESULTS AND DISCUSSION

Characteristics of the Brew, Brew HMw, and Brew LMw.

The obtained Brew consisted of 4.4% (w/w) dry matter. The yield of the coffee brew preparation was 20% (w/w) of the roasted beans, which is 23.5% (w/w) of the dry and

defatted roasted bean. This extraction yield is in line with yields reported by Borrelli et al. (17%, w/w) (*9*), Nunes et al. (19%, w/w) (*31*), and Clarke and Vitzthum (24%, w/w) (*32*).

The diafiltration step yielded 16% (w/w) Brew HMw and 82% (w/w) Brew LMw. The Brew HMw is 3.6% (w/w) of the dry and defatted beans (**Table 1**). Nunes and Coimbra (*33*) reported a HMw coffee fraction that was 7.6% (w/w) of the dry and defatted beans, using membrane dialysis (MW cutoff 12-14 kDa). It was expected that the yield of the Brew HMw after diafiltration would be larger than 7.6% (w/w) since the MW cutoff was smaller (3 kDa) compared to the membrane dialysis and should therefore retain more molecules. On the other hand, Hofmann (*34*) suggested that such observed differences might be explained by the fact that coffee compounds react during dialysis, yielding more HMw molecules, resulting in larger amounts of the HMw fraction. To investigate this hypothesis, fresh coffee brew, the Brew HMw, and Brew LMw were also dialyzed using a membrane dialysis. **Table 1** shows that the yield of the Brew-Dial (7.4%, w/w) is in line with the yield reported by Nunes and Coimbra (*33*). The fractions Brew HMw-Dial and Brew LMw-Dial are 3.5 and 3.6% (w/w) of the dry and defatted beans, respectively. It is unlikely that the LMw coffee compounds (Mw < 3 kDa) react and form a high molecular weight (Mw > 12 kDa) fraction that is as large as the Brew HMw. This suggests that the reactivity of the coffee compounds is not responsible for the larger yield and appears that the observed differences should be explained by differences in separation techniques. It is likely that the difference is caused by intrinsic properties of the membranes (cellulose acetate vs. polysulfone) and the separation principle (diffusion vs. diffusion and pressure). Moreover, the dialysis technique strongly affects the yield of the high molecular weight fraction of coffee. The Brew HMw, obtained after diafiltration, was used for further analysis.

Table 1. Yields of High Molecular Weight Coffee Fractions Using Different Dialysis Techniques

	diafiltration		membrane dialysis	
	HMw fraction (%, w/w) brew[a]	HMw fraction (%, w/w) dry[b]	HMw fraction (%, w/w) brew[a]	HMw fraction (%, w/w) dry[b]
Brew	15.5	3.6	31.7	7.4
Brew HMw[c]			95.5	3.5
Brew LMw[c]			18.7	3.6

[a] % (w/w) of the dialyzed coffee fraction. [b] % (w/w) of the dry and defatted coffee beans. [c] The Brew LMw and HMw fraction after diafiltration were dialyzed using membrane dialysis.

Spectroscopic Analysis of the Brew, Brew HMw, and Brew LMw.

Absorption spectra of Brew, Brew HMw, and Brew LMw fractions were recorded (**Figure 1**), and it appeared that coffee Brew shows two absorption maxima, one at 280 nm and one at 325 nm. The absorption maximum at 280 nm can be explained by the presence of proteins, caffeine, chlorogenic acid, and caffeic acid. The absorption maximum at 325 nm can be explained by the presence of chlorogenic acid and caffeic acid. It is generally accepted that melanoidins contain conjugated systems which result in light absorption throughout the whole

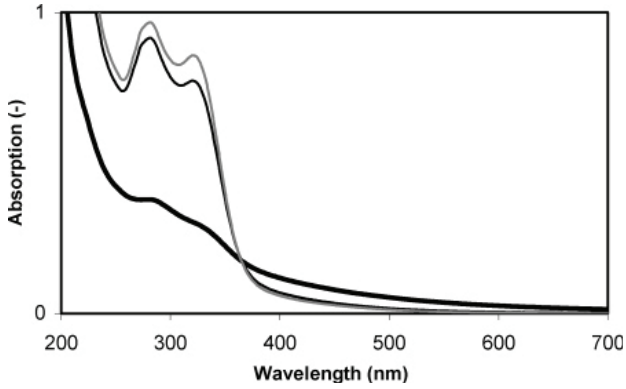

Figure 1. Absorption spectra of 0.1 mg/mL Brew (thin black line), 0.1 mg/mL Brew HMw (thick black line), and 0.1 mg/mL Brew LMw (thin gray line).

Table 2. K_{mix} Values for the Brew, Brew HMw, and Brew LMw[a]

	$K_{mix\ 280nm}$ (L/cm/g)	$K_{mix\ 325nm}$ (L/cm/g)	$K_{mix\ 405nm}$ (L/cm/g)
Brew	9.1	7.7	0.7
Brew HMw	3.9	3.0	1.1
Brew LMw	9.7	8.4	0.6

[a] The average and maximal standard deviation were 0.5 and 1.0% of the Kmix values, respectively.

spectrum. The wavelength selected for measuring melanoidin is most often chosen to be 405 nm (*9*), since other coffee compounds do not absorb light at this wavelength. On the basis of these absorption spectra, it can be stated that measurement of the absorption at 280, 325, and 405 nm provides useful information on the relative amount of melanoidins and other compounds in a specific coffee fraction. To be able to compare absorption values at a specific wavelength of different coffee samples, or to compare absorption values at different wavelengths within one coffee fraction, the absorption of a coffee fraction at a specific wavelength was expressed as *K* values. Since coffee samples used in this study are most likely a mix of various compounds, the specific extinction coefficient was defined as K_{mix}. The value of K_{mix} provides information on the relative amount of melanoidins ($K_{mix\ 405nm}$) and other coffee compounds like proteins ($K_{mix\ 280nm}$) and chlorogenic acid ($K_{mix\ 280,\ 325nm}$) present within a coffee fraction. By using K_{mix}, it becomes possible to determine which coffee fraction has the highest melanoidin concentration, by comparing $K_{mix\ 405nm}$ values. Furthermore, by calculating the $K_{mix\ 280nm}$ to $K_{mix\ 405nm}$ and $K_{mix\ 325nm}$ to $K_{mix\ 405nm}$ ratios, by which the absorption of melanoidins at these wavelengths is taken into account, it is also possible to estimate the relative amount of chlorogenic acid and/or proteins compared to the amount of melanoidins. The K_{mix} values for the Brew, the Brew HMw and Brew LMw are shown in **Table 2**. From the $K_{mix\ 280nm}$ and $K_{mix\ 325nm}$ values it is clear that the most of the compounds responsible for the absorption at 280 and 325 nm end up in the Brew LMw. This can be explained by the fact that coffee compounds like degraded proteins, peptides, chlorogenic acid, and caffeine end up in the low molecular weight fraction. However, the K_{mix}

$_{405nm}$ is the highest for the Brew HMw, suggesting that the Brew HMw is relatively rich in melanoidins. In addition, the Brew HMw also shows absorption maxima at 280 and 325 nm (**Figure 1**), indicating that proteins and/or chlorogenic acid are present in the Brew HMw. The fact that an absorption maximum at 325 nm is present in the Brew HMw suggests that the low molecular weight chlorogenic acid is incorporated into the HMw material, as was also reported in other studies (*33, 35-37*). The question remains whether these compounds are incorporated into the melanoidin complex or not.

Although the highest $K_{\text{mix }405nm}$ is observed for the Brew HMw, it is worth mentioning that a large fraction (71%) of the total amount of melanoidins from the Brew, as calculated from the $K_{\text{mix }405nm}$ values, ends up in the Brew LMw since the Brew LMw represents 82% (w/w) of the Brew. Since the Brew HMw is rich in melanoidins, and since this fraction is free of low molecular weight compounds like caffeine and free chlorogenic acid, it was decided that the Brew HMw was most interesting for further studies on melanoidins.

Molecular Weight Distribution of the Brew, Brew HMw, and Brew LMw.

Initially, the molecular weight distributions of the Brew, Brew HMw, and Brew LMw were investigated by size exclusion chromatography using Sephadex G-25 as column material since this material is frequently used for coffee (*5, 9, 20, 38-41*). However, after extensive research it was found that the method did not yield results that were reproducible enough to use for comparison of different coffee fractions. It is expected that certain coffee compounds interact with the column material and thereby influence subsequent runs, even after thorough regeneration steps. Buffered eluents were used, in order to suppress ionic interactions, which resulted in very poor separation; therefore, this was not an option. It was found that interaction of amino acids with Sephadex as column material was previously reported by Eaker and Porath (*42*). Therefore, it is suggested that the separation of coffee material on Sephadex G-25 is not only based on size exclusion but that some kind of interaction with the column material is also involved. Since results with Sephadex G-25 were not satisfying, other size exclusion techniques were tested.

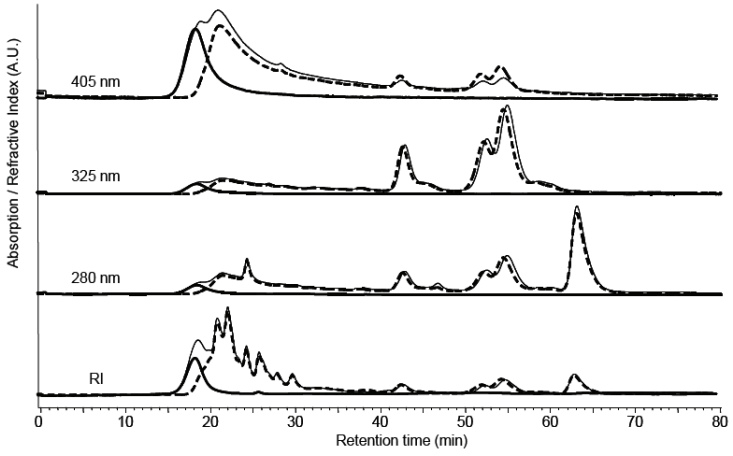

Figure 2. Size exclusion chromatography patterns of the Brew (solid thin line), Brew HMw (solid thick line), and Brew LMw (dashed line) using RI and spectrometric detection.

TSKgel size exclusion columns on an HPLC system were found to give reproducible results, and the elution patterns of the Brew, Brew HMw, and Brew LMw are shown in **Figure 2**. In addition to measuring the absorbance at 280, 325, and 405 nm, the effluent was monitored using a RI detector to be able to also detect carbohydrates. The 405 nm absorbance pattern of the Brew shows that two melanoidin populations were present in the Brew; the first population ended up in the Brew HMw, while the second melanoidin population ended up in the Brew LMw. This is in agreement with findings reported by Hoffmann, who stated that melanoidins can be LMw colored compounds (*24*). The fact that two populations are observed in the elution pattern of the Brew (**Figure 2**) suggests that the molecular weight distribution of melanoidins is not totally heterogeneous. When examining the RI, 280, and 325 nm patterns, it is noteworthy that part of the compounds present in the Brew and the Brew LMw elute after the included volume (~26 min). From the elution behavior of reference compounds, the compounds eluting at 55 and 64 min were identified as chlorogenic acid and caffeine, respectively. From these results, it was concluded that the TSKgel size exclusion columns do not separate molecules based on size only but that some kind of interaction with the column material occurs. Therefore, the HPSEC elution patterns cannot be interpreted solely as molecular weight information. However, the HPSEC method was found to give reproducible results, and the elution patterns do provide an insight into the diversity of the composition of coffee fractions. It was concluded that the Brew HMw was free of low molecular weight compounds like chlorogenic acid and caffeine and contained the melanoidin population with the highest molecular weight present in the Brew.

Ethanol Precipitation of the Brew HMw.

As mentioned above, the Brew HMw was rich in melanoidins and free of LMw compounds that could have disturbed other measurements. Fractionation of this Brew HMw in several fractions was conducted by ethanol precipitation. The reason for choosing ethanol

Table 3. Yield, $K_{mix\ 405nm}$ Value, $K_{mix\ 280,\ 325nm}$ to $K_{mix\ 405nm}$ Ratios, Level of Phenolic Groups, and Phenolic Groups to $K_{mix\ 405nm}$ Ratio of Coffee Fractions[a]

	yield (%, w/w)	$K_{mix\ 405nm}$ (L/cm/g)	ratio $K_{mix\ 280:405nm}$ (-)	ratio $K_{mix\ 325:405nm}$ (-)	Phenolic groups (%, w/w)	phenolic groups/ $K_{mix\ 405nm}$ (% g cm L^{-1})
Brew		0.7			29	41
Brew HMw	100	1.1	3.5	2.8	15	14
Brew LMw		0.6			31	52
EP20	19	0.5	3.1	2.5	6	12
EP40	11	0.7	3.6	2.8	8	11
EP60	28	0.6	6.0	5.0	11	18
EP80	17	1.2	3.7	2.9	17	14
ES80	13	2.5	3.3	2.5	26	10

[a] The average and maximal standard deviation were 0.5 and 1.0% of the K_{mix} values, respectively.

precipitation over preparative gel filtration is based on the observation that coffee compounds like galactomannans and arabinogalactans show different precipitation behavior and can be selectively precipitated by this methodology (*33, 39, 40, 43*); however, in these studies only minor attention was given to melanoidins (*33, 43*). Therefore, we conducted ethanol precipitation with the main focus on melanoidins, while carbohydrates, proteins, and phenolic groups were also investigated. The yields of the HMw coffee fractions and the corresponding K_{mix} values and ratios obtained by ethanol precipitation are shown in **Table 3**. All five fractions from the ethanol precipitation were obtained in significant amounts, the smallest fraction still being larger than 10% (w/w) of the starting material. The recovery of the ethanol precipitation experiments was 87% (w/w), probably because the supernatant of the wash step of the precipitates was not included. The lyophilized EP20 fraction was poorly soluble (~33% (w/w)) in water, while the other fractions were completely soluble in water. Because of the partial solubility of EP20, the determined K_{mix} values will be an underestimation. The $K_{mix\ 405nm}$ increases with increasing ethanol concentration, the ES80 showed a $K_{mix\ 405nm}$ of 2.5, twice as high as the K_{mix} of the Brew HMw. Taking the yield into account, it can be stated that EP80 and ES80 together account for 60% of the absorption at 405 nm present in the Brew HMw, indicating that these two fractions, especially ES80, are rich in melanoidins. The $K_{mix\ 280,\ 325nm}$ values (data not shown) of the ethanol precipitation fractions showed the same trend as $K_{mix\ 405nm}$, the higher the ethanol concentration, the higher the K_{mix} value. This indicates that melanoidins themselves appear to show absorption at 280 and 325 nm. The ratio of the K_{mix} values of 280:325:405 nm is around 3.4:2.6:1 for all fractions, except for EP60, which show a ratio of 6:5:1. An explanation for this might be that this EP60 contains relatively much UV 280 and 325 nm absorbing compounds, which might be due to a higher bound chlorogenic acid content in this fraction.

Molecular Weight Distribution of the Ethanol Precipitation Coffee Fractions.

The fractions obtained from the ethanol precipitation were analyzed by HPSEC with the aim to see whether any differences in elution patterns could be observed. Differences in elution time were observed for the ethanol precipitation fractions, when the effluent was monitored at 405 nm (**Figure 3**). The RI, and absorbance at 280 and 325 nm elution patterns were found to be similar to the absorbance at 405 nm elution pattern. Especially for the EP60, EP80, and ES80 fractions, differences in elution time were observed, EP60 eluting first and ES80 eluting last. The differences might be explained by the fact that there is a difference in molecular weight, with the EP60 having the highest and the ES80 having the lowest molecular weight. On the other hand, it might also be that the differences are caused by molecular properties and not so much by the molecular weight. It was previously shown in this study that the separation of coffee on this SEC column was not only molecular weight based. It is possible that a higher protein or nitrogen content might result in a delay in elution time since the TSKgel column is primarily designed for the analysis of carbohydrates. Whatever the true explanation may be, differences between the fractions were observed. Further analysis of the fractions is required to characterize the fractions on a more molecular basis.

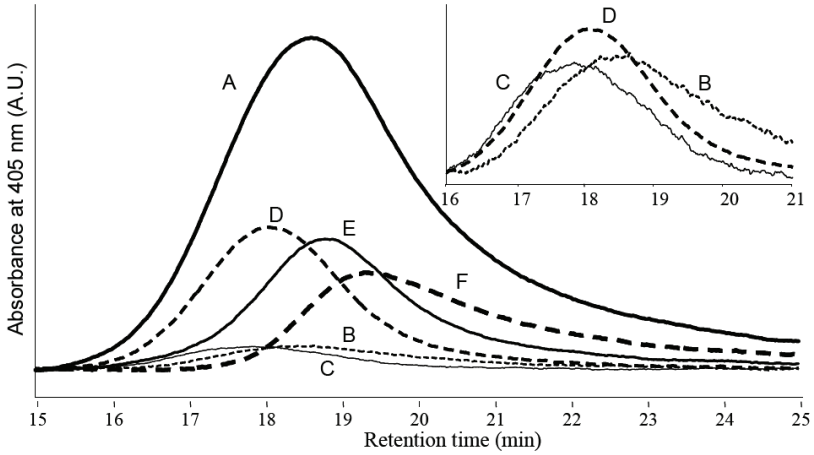

Figure 3. Size exclusion chromatography elution patterns of 0.8 mg/mL Brew HMw (**A**), 0.15 mg/mL EP20 (**B**), 0.08 mg/mL EP40 (**C**), 0.21 mg/mL EP60 (**D**), 0.13 mg/mL EP80 (**E**), and 0.10 mg/mL ES80 (**F**). The inset shows the EP20, EP40 (both 5 times enlarged), and EP60 patterns.

Sugar Analysis of the Coffee Fractions.

The sugar composition of the coffee fractions was determined, and the results are shown in **Table 4**. The quantity as well as the composition of the beans, Brew Residue and Brew, are in accordance with literature (*44*). The sugar content of the Brew HMw was found to be 70% (w/w), and this fraction represents 2.5% (w/w) of the dry and defatted beans. Nunes and Coimbra (*33*) report a sugar content of the Brew HMw of ~30% (w/w), which was 2.4% (w/w) of the dry and defatted beans. This suggests that the amount of carbohydrates retained during diafiltration and membrane dialysis is the same although the overall Brew HMw yield is twice as low for diafiltration. The most abundant sugars in the Brew HMw are mannose and galactose. The Brew LMw contained 16% (w/w) sugars, with also mannose and galactose being the most abundant sugars. It is important to note that 100 g of Brew dry matter

Table 4. Sugar Composition of Coffee Fractions[a]

	Rha (%)	Ara (%)	Xyl (%)	Man (%)	Gal (%)	Glc (%)	uronic acid (%)	total sugar (%)
roasted beans	<1	2	<1	25	11	8	4	50
Brew residue	<1	2	<1	29	11	12	3	57
Brew	<1	2	<1	9	9	2	4	26
Brew HMw	1	6	<1	29	26	1	7	70
Brew LMw	<1	2	<1	5	5	1	3	16
EP20	<1	1	<1	71	6	<1	3	81
EP40	<1	2	<1	53	12	nd	10	77
EP60	<1	5	<1	13	47	<1	8	73
EP80	3	12	<1	6	40	<1	8	70
ES80	4	10	<1	2	10	<1	4	30

[a] % (w/w). nd = not determined.

contained 26 g of carbohydrates, of which 13 g ended up in the Brew LMw. Thus, only half of the carbohydrates in the Brew were polymeric (>3 kDa), while the other half was present as LMw carbohydrates. At low ethanol concentrations (EP20 and EP40), mainly galactomannans precipitate and at high ethanol concentrations mainly arabinogalactans precipitate, as was also reported (*33*). Since the ratio Ara/Gal increased dramatically when the ethanol concentration was increased, it can be stated that the higher the ethanol concentration, the higher the degree of substitution of arabinose on the galactan backbone. The fractions obtained by ethanol precipitation all contained significant amounts of carbohydrates. However, EP20, EP40, EP60, and EP80 contained higher amounts (70+%, w/w) than did ES80, which contained only 30% (w/w) carbohydrates. Obviously, most of the sugar precipitated before 80% ethanol, and highly substituted arabinogalactans were the most abundant sugars present in ES80.

Combining results from **Tables 3** and **4**, it can be stated that arabinogalactans are the most abundant sugars present in the melanoidin-rich fractions (EP80 and ES80). This could be the result of incorporation of arabinogalactans in melanoidins or due to coprecipitation. Since sugars are required for the Maillard reaction to occur and since Oosterveld et al. (*6*) showed that coffee polysaccharides are degraded upon roasting, it is likely that polysaccharides are part of melanoidins. With increasing ethanol concentration, the galactomannan content decreased gradually and the arabinogalactan content increased gradually, as did the melanoidin content. Combining this observation with the fact that Nunes et al. (*45*) also found that, after enzymatic degradation of galactomannans, an arabinogalactan-rich and galactomannan-poor fraction coeluted with the majority of the melanoidins it can be suggested that arabinogalactans are involved in melanoidin formation. Concerning the galactomannans, it was previously shown that galactomannans might be involved in melanoidin formation (*45*). However, the presence of galactomannans at higher ethanol concentrations (7-10% in ES80) was expected since the presence of galactomannans in a 75% ethanol solution of green bean extract, which is melanoidin free, was reported (*39*). Therefore, it is possible that galactomannans are involved in melanoidin formation, although the inverse correlation between melanoidin and galactomannans content suggests that galactomannans are not the most important carbohydrate involved in melanoidin formation.

Phenolic Groups in the Coffee Fractions.

The phenolic group levels of the coffee fractions are shown in **Table 3**. It was found that the Brew and Brew LMw were rich in phenolic groups, 29 and 31% (w/w), respectively. This was no surprise since chlorogenic acid, which is the most abundant “native” phenolic in coffee beans, is present in these fractions (**Figure 2**). The level of 29% (w/w) in the Brew is in agreement with findings reported in literature since green Arabica coffee beans contain around 5-7.5% chlorogenic acid (*2*), resulting a theoretical phenolic groups level of 21-32% in the brew. However, it is important to mention that chlorogenic acid is susceptible to degradation upon roasting. Since the fate of caffeic acid, the phenolic moiety in chlorogenic

acid is unknown, the theoretical phenolic group level as calculated from literature is likely an overestimation.

The phenolic group content of the Brew HMw was found to be 15% (w/w). Since the protein content was rather low in the Brew HMw, the phenolic group content cannot be explained by the presence of phenolic amino acids like tyrosine. The presence of these phenolic groups in the HMw fraction might be a result of binding of coffee phenolics to high molecular weight material and/or formation of phenolic groups from nonphenolics upon roasting.

As mentioned previously, the ratio of the K_{mix} values at 325 nm compared to 405 nm in the EP60 fraction was higher than the ratio of the other ethanol precipitation fractions. It was suggested that the amount of chlorogenic acid or caffeic acid in this fraction was relatively high compared to the melanoidin content. By expressing the amount of phenolic groups as determined by the Folin-Ciocalteu color assay relative to the melanoidin content (**Table 3**), it was found that again the EP60 fraction had the highest ratio of phenolic groups to melanoidins. Both the $K_{mix\ 325nm}$ and the color assay point to an increased presence of phenolic groups in EP60 compared to other ethanol precipitation fractions.

Borrelli et al. (*9*) and Nunes and Coimbra (*33*) showed that the HMw fraction of coffee brews were rich in phenolic groups. On the basis of Curie point pyrolysis, Adams et al. (*46*) suggested that chlorogenic acid might be incorporated into the Brew HMw. Rawel et al. (*37*) proved that chlorogenic acid can be covalently bound to the 11S storage protein of coffee beans. Montavon et al. (*36*) even suggested that the polyphenols might bind to the 11S coffee protein and contribute to the behavior and formation of melanoidins. Our results are in line with the findings of these authors and suggest that Brew HMw contains significant amounts of phenolic groups. Additionally, it was found that the phenolic group content increased with increasing ethanol solubility, just like the melanoidin content. It is worth mentioning that 62% of the total amount of phenolic groups present in Brew HMw ended up in the EP80 and ES80 fractions. Previously, it was mentioned that 60% of the melanoidin content ended up in these EP80 and ES80 fractions. This suggests that phenols are not only present in the Brew HMw but most likely are incorporated in the melanoidin complex.

Nitrogen, Protein, and Amino Acid Content and Composition.

Proteins make up 8.7-12.2% (w/w) of green coffee beans and can be divided into the water-soluble (50%) and the water-insoluble proteins (50%) (*7*). The water-soluble coffee proteins will end up in the brew, and they consist of 85% of globulins (*47*), and these globulins were shown to be 11S storage proteins by Rogers et al. (*48*). The protein content of the coffee fractions was calculated by multiplying the nitrogen by a factor of 5.5 (**Table 5**). Although a nitrogen to protein factor of 6.25 is frequently used in literature (*7, 9, 33*), a factor of 5.5 for the 11S storage protein was preferred since this value was calculated for 11S storage protein from coffee (*48, 49*).

Table 5. Nitrogen and Protein Content According to Dumas and Amino Acid Analysis, and the NPN Content for the Coffee Fractions

	Nitrogen[a] (%, w/w)	Protein[a] (%, w/w)	Protein[b] (%, w/w)	Nitrogen[b] (%, w/w)	NPN[c] (%, w/w)
green beans	nd[d]	nd	12.0	2.18	nd
roasted beans	2.75	15.1[e]	10.1	1.84	0.91
Brew	3.13	17.2[e]	6.4	1.16	1.97
Brew HMw	1.44	7.9	6.0	1.09	0.35
Brew LMw	3.33	18.3[e]	6.0	1.09	2.24
EP20	1.05	5.8	4.4	0.80	0.25
EP40	0.72	4.0	2.7	0.49	0.23
EP60	0.70	3.9	2.4	0.44	0.26
EP80	1.39	7.6	5.6	1.02	0.37
ES80	4.44	24.4	19.6	3.56	0.88

[a] Nitrogen and protein (% N*5.5) content according to Dumas. [b] Protein and nitrogen (% protein/5.5) content according to the amino acid analysis. [c] Non-protein nitrogen. [d] nd = not determined. [e] Overestimation of the protein content due to non-protein LMw material.

The protein content of the green beans, roasted beans, Brew, and Brew LMw are overestimations of the real protein content since no corrections were made for the nitrogen originating from caffeine and trigonelline and other non-protein nitrogen containing compounds. The nitrogen content of the Brew HMw and ethanol precipitation fractions are expected to give more accurate estimations of the protein content since the LMw nitrogen containing compounds are not present in these fractions. However, no distinction could be made between nitrogen originating from amino acids/proteins and non-protein nitrogen (NPN). The protein content of the Brew HMw (8%) is consistent with Nunes and Coimbra (*33*) who report an average protein content of 9.3% (w/w). From the nitrogen content of the ethanol precipitation fractions, it is clear that the higher the ethanol concentration, the higher the nitrogen content and presumably the higher the protein content. However, it should be realized that not all the nitrogen present in the HMw fractions has to be present in intact amino acids or proteins. It is likely that part of the amino acids or proteins are degraded upon roasting and that the nitrogen from these amino acids may end up in pyrrole-like structures within the melanoidin complex.

To be able to make a distinction between nitrogen from intact amino acids and non-amino acid nitrogen, referred to as nonprotein nitrogen (NPN), the protein content was also determined by amino acid analysis, which is known to be an accurate technique for the determination of the protein content in coffee (*7*). Results of the amino acid analysis are shown in **Table 6**. The roasting process lowered the protein content in the beans from 12% in the green beans to 10% in the roasted beans. Because of the roasting process, 21% of the proteins are lost, taking the weight loss of the beans into account. These results are in agreement with literature (*7*). Arginine (-93%, w/w), lysine (-87%), serine (-58%), threonine

Table 6. Amino Acid Composition (Mol %) of Coffee Fractions

	green bean	roasted bean	Brew	LMw	HMw	EP20	EP40	EP60	EP80	ES80
Ala	7	9	8	7	8	7	7	10	9	8
Cys	nd[a]	nd	nd	nd	nd	nd	nd	nd	nd	nd
Asx	10	10	10	9	9	10	11	11	9	8
Glx	18	22	29	30	29	29	29	29	29	29
Phe	4	5	4	4	4	4	3	3	4	4
Gly	11	12	13	12	13	14	15	15	14	12
His	2	2	2	2	1	2	2	2	2	1
Ile	4	5	4	4	4	4	3	3	3	4
Lys	6	1	0	0	1	1	1	0	0	1
Leu	9	11	9	9	9	10	8	7	8	10
Met	1	1	1	1	0	0	0	0	0	0
Pro	6	7	8	8	7	7	7	6	7	7
Arg	4	0	0	0	0	0	0	0	0	0
Ser	5	3	3	3	3	3	3	3	3	3
Thr	4	3	2	3	3	3	3	3	3	4
Val	6	8	5	5	6	6	5	6	6	7
Trp	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Tyr	2	2	2	2	1	1	2	1	1	2
total %[b]	12.0	10.1	6.4	6.0	6.0	4.4	2.7	2.4	5.6	19.6

[a] nd = not determined. [b] Total amount of amino acids in % (w/w).

(-36%), histidine (-35%), and asparagines/aspartic acid (-24%) especially were significantly reduced during roasting. It is remarkable that all hydrophilic amino acids containing amine (Arg, Lys, His) or amide (Asp) side groups showed a reduction of 24% (w/w) or more upon roasting. Obviously, this can be explained by the fact that ε-amino groups on amino acids are reactive and were shown to be involved in nonenzymatic browning reactions during thermal processing of foods (*50-52*). Rizzi (*53*) suggested that proteins play a key role in melanoidin formation because of the high reactivity of ε-amino, thiol, or methylthio groups. The hydrophilic amino acids serine and threonine showed losses of 36% or more upon roasting. In the literature, no information was found on the losses of these amino acids upon roasting. The fact that tyrosine, which also contains a hydroxyl group does not show such a decrease (-7%) might be because the oxygen molecule from the hydroxyl group is stabilized by the phenyl group.

The concentration of proteins in the Brew was found to be 6.4%. Although half of the proteins in green beans are water soluble (*47*), only 13% of the proteins present in the roasted beans are extracted into the Brew. This can be explained by the fact that proteins become less water soluble due to denaturation; that proteins might become less soluble due to chemical reactions occurring during roasting; and/or that proteins (amino acids) are degraded and

converted into aroma compounds. The amino acid composition of the roasted beans and the Brew were quite similar, except for the fact that glutamine/glutamic acid increases from 22 to 29 mol %. The major amino acids in the Brew were glutamine/glutamic acid (29 mol %), glycine (13 mol %), and asparagine/aspartic acid (10 mol %), which corresponds with earlier data (*47*). Both the Brew LMw and HMw consisted of 6% of amino acids or proteins, and the amino acid composition of both fractions was similar to the amino acid composition of the Brew. The fact that the amino acid composition of the Brew LMw and HMw are similar suggests that the same protein was present in both fractions, although degraded to different extents. It was expected that the amino acid content in the Brew LMw would be negligible since the free amino acid content in green beans is only 0.3-0.6% (*54*) and are easily transformed upon roasting due to their reactivity toward the Maillard reaction (*32*). When examining the ethanol precipitation fractions, it is remarkable that the protein content first decreased from 4.4% in EP20 to 2.4% in EP60 and than increased again to 19.6% in ES80. The proteins from coffee beans, albumins and the 11S storage protein (a globulin), are reported not to be soluble in alcohol (*55*). Albumins and globulins readily precipitate at 25% ethanol (*56*). Therefore, it was not expected that half of the proteins from the Brew HMw would be soluble in 80% ethanol. This suggests that the proteins indeed did undergo a chemical reaction upon roasting, thereby changing their physical properties and thereby becoming soluble in alcohol. Incorporation of these proteins into a melanoidin complex could be a likely explanation for this. Another remarkable aspect is that the amino acid composition of the different Brew LMw, HMw, and ethanol precipitation fractions was similar for all fractions. This suggests that the proteins in the fractions are of the same type and that the fact whether they precipitate at a certain ethanol concentration only depends on the type and degree of chemical modification or degradation during roasting. It is hypothesized that proteins that were modified or degraded to a low extent, precipitate at low ethanol concentrations (20%); when they have undergone more chemical modification or degradation they become more alcohol soluble.

Comparison of the Nitrogen and Amino Acid Content.

When the nitrogen levels and the amino acid analysis are compared, it is important to mention that the protein content based on the nitrogen content multiplied by a factor 5.5 is higher for all the HMw fractions when compared to the actual protein content (**Table 5**). Hence, in all HMw fractions nitrogen is present that cannot be ascribed to amino acids/proteins. The amino acid nitrogen and NPN contents of the HMw fractions, Brew HMw and ethanol precipitation fractions are plotted as a function of the total nitrogen content in **Figure 4**. It was calculated that the NPN was more or less a constant percentage (17%) of the total amount of nitrogen. At very low nitrogen contents, there was relatively more nitrogen originating from non-amino acids than from amino acids. Proteins are the most abundant nitrogen-containing HMw compounds in green beans, and amino acids from these proteins are probably the source for this NPN. The relatively large NPN content suggests that one out

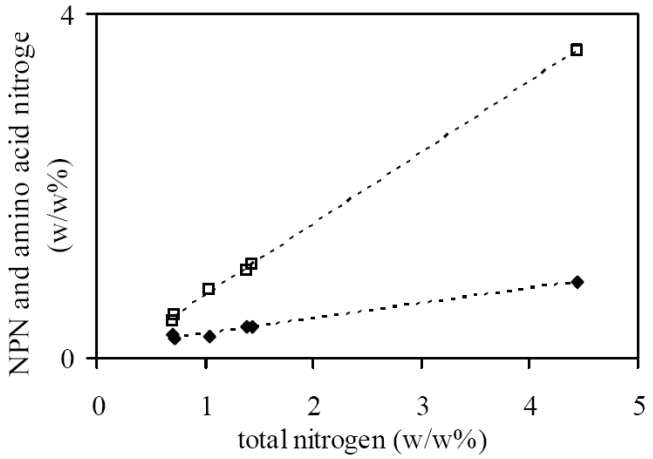

Figure 4. Amount of amino acid nitrogen and NPN from the HMw fractions as a function of the total amount of nitrogen: (open squares) nitrogen from amino acids; (solid diamonds) NPN.

of six nitrogen molecules from amino acids were chemically modified or degraded and remained present in the HMw components while losing their amino acid characteristics. This finding is in line with the observation that 21% of the amino acids was lost upon roasting. From these results, it can be concluded that proteins are greatly affected by the roasting process and that the chemically modified or degraded proteins possess totally different solubility properties.

Plotting the $K_{\text{mix 405nm}}$ values of all the HMw fractions against the corresponding nitrogen contents (figure not shown) showed a clear correlation (R^2 = 0.989) between the nitrogen content and the $K_{\text{mix 405nm}}$ values. One measurement seems to have a divergent $K_{\text{mix 405nm}}$ value; this measurement corresponds to the EP20 fraction that was not completely soluble, explaining the underestimation of the $K_{\text{mix 405nm}}$ value. Since the $K_{\text{mix 405nm}}$ value represents the melanoidin content, there appears to be a correlation between the melanoidin content and the nitrogen content. Thus, the suggestion is made that the nitrogen present in the Brew HMw indeed is incorporated into the melanoidin complex. Since the nitrogen contents from amino acids/proteins and the NPN were proportional to the total nitrogen content, it can be suggested that all the NPN and proteins were incorporated into the melanoidin complex.

In this research, it was shown that ethanol precipitation led to unique melanoidin populations differing in carbohydrate content and composition. The introduction of K_{mix} allowed us to recognize the presence of possible substituents on melanoidins. From these ratios, it was concluded that, in addition to proteins, also other UV absorbing compounds like phenolic groups (chlorogenic acid) are present in the high molecular weight melanoidins. This is further emphasized by the fact that relatively more UV280 absorbance was found for fractions with low protein content. Although the existence of covalent interaction between melanoidins and polysaccharides were not proven in this study, the findings suggest that arabinogalactan is likely involved in melanoidin formation. Furthermore, proteins, which were shown to be partly degraded, seem to be part of melanoidins as well.

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CHAPTER 3

Arabinogalactan Proteins Are Incorporated in Negatively Charged Coffee Brew Melanoidins

ABSTRACT

The charge properties of melanoidins in high molecular weight (HMw) coffee brew fractions, isolated by diafiltration and membrane dialysis, were studied. Ion exchange chromatography experiments with the HMw fractions showed that coffee brew melanoidins were negatively charged whereas these molecules did not expose any positive charge at the pH of coffee brew. Fractions with different ionic charges were isolated and subsequently characterized by means of the specific extinction coefficient ($K_{\text{mix 405nm}}$), sugar composition, phenolic group content, nitrogen content, and the arabinogalactan protein (AGP) specific Yariv gel-diffusion assay. The isolated fractions were different in composition and AGP was found to be present in one of the HMw fractions. The AGP accounted for 6% of the coffee brew dry matter and had a moderate negative charge, probably caused by the presence of uronic acids. As the fraction that precipitated with Yariv was brown ($K_{\text{mix 405nm}}$ = 1.2), compared to a white color in the green bean, it was concluded that these AGPs had undergone Maillard reaction resulting in an AGP-melanoidin complex. The presence of mannose (presumably from galactomannan) indicates the incorporation of galactomannans in the AGP-melanoidin complex. As the uronic acid content in the more negatively charged melanoidin-rich, AGP-poor HMw fractions decreased, it was hypothesized that acidic groups are formed or incorporated during melanoidin formation.

KEYWORDS

Coffee brew; melanoidins; arabinogalactan proteins; Yariv; anion exchange chromatography

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INTRODUCTION

Coffee is a popular beverage that is consumed worldwide by many people every day. In 2004, the total coffee consumption was estimated to be almost 7 billion kilograms of coffee beans (*1*). Many studies have dealt with coffee in relation to growing the crop, its composition, and its effects on health. Also, much research is focused on the formation of aroma, taste, and color during the roasting process. During the roasting of coffee beans, flavors and colored compounds are formed because of the Maillard reaction (*2*) that takes place between carbohydrates or degraded carbohydrates (*3*) and proteins (*4*). Brown-colored compounds are formed, and these compounds are referred to as melanoidins. It has been suggested that, next to the Maillard reaction, autoxidation of polyphenols contributes to the formation of melanoidins as well (*5*). These melanoidins make up to 25% of a coffee brew (*6*).

Melanoidins are not only of interest because of their contribution to color formation but also for their flavor binding properties (*7-13*), antioxidative capacity (*6, 14-16*), metal-chelating properties (*16, 17*), and reactivity in coffee brew (i.e., aging of coffee) (*18*). Thus, melanoidins have been studied in recent years because of their nutritional, biological, and health implications. Melanoidin chemical structures and properties are very complex and largely remain unknown. The complexity of the structure of melanoidins is due to the fact that many green coffee bean constituents play a role in melanoidin formation (*19*). This is also the reason that most research on melanoidins is conducted on melanoidins prepared by model systems. To date, three proposals for the structure of melanoidins have been suggested (*20*). Structural information obtained from melanoidins prepared by model reactions, often heating a reducing sugar and an amino acid, might contribute to understanding what occurs in melanoidin formation in real foods. However, this structural information is quite limited because the Maillard reaction in real foods is far more complicated, since many more possible reactants are present. As the molecular structure of melanoidins is largely unknown, these compounds are generically defined as brown, nitrogenous macromolecular material (*6, 21*). Melanoidins can be detected by measuring their absorbance at 405 nm, which is an arbitrary chosen wavelength at which the intensity of the brown color is measured (*6, 9, 15, 22*).

As many possible structures for melanoidins might occur within coffee, it is interesting to determine whether melanoidins possess charged groups, since this could affect their physical behavior in coffee brew. However, only limited attention has been given to the possible charge aspects of melanoidins from coffee. Tomlinson et al. (*23*) have shown, using capillary zone electrophoresis, that melanoidins generated by the reaction of 5-hydroxymethylfurfural with glycine possess a partially anionic character. More recently, Morales (*24*) showed that high molecular weight coffee material also showed an anionic character. The high molecular weight coffee melanoidins fraction is rich in carbohydrates (*22*), and in green coffee beans, the most abundant carbohydrates are cellulose, galactomannans, and arabinogalactan type II (*25*). Redgwell et al. (*26*) reported that these arabinogalactans type II (arabinogalactan proteins) are acidic in the cell wall of coffee beans.

The objective of the present study was to investigate the charge properties of coffee melanoidins. Coffee brew melanoidin populations were isolated and characterized, and the melanoidin chemical and chromatographic characteristics were investigated with emphasis on the carbohydrate part.

MATERIALS AND METHODS

Materials.

Roasted coffee beans (*Coffea arabica*), originating from Colombia, were provided by a local factory. The degree of roast, which represents the weight reduction upon roasting, was 16.4% (w/w) and was 8.0% (w/w) on a dry matter basis.

Preparation of Coffee Brew.

Green (frozen with liquid nitrogen) and roasted coffee beans were ground and an extract was prepared as described previously by Bekedam et al. (*22*). For characterization purposes, part of the roasted bean brew was lyophilized, yielding "Brew" (**Figure 1**). The major part of the roasted bean brew was used for further isolation. The green bean extract was lyophilized and was not used for further isolation, yielding "green bean extract".

Defatting of Coffee Samples.

The lyophilized coffee samples Brew, Brew HMw1, and Brew LMw were defatted by Soxhlet extraction using a Soxtherm, which was connected to a Multistat system (Gerhardt, Königswinter, Germany). The solvent used for extraction was dichloromethane (Sigma Chemical Co., St. Louis, MO). The extraction procedure comprised boiling in dichloromethane (40 °C) for 15 min followed by refluxing with dichloromethane for 2 h. After defatting, the dichloromethane was evaporated in an oven at 40 °C for approximately 30 min.

Isolation of Two High Molecular Weight Coffee Fractions from Coffee Brew.

The first high molecular weight fraction of the coffee brew was obtained by diafiltration according to the procedure previously described Bekedam et al. (*22*). The retentate and dialysate were lyophilized, yielding a high molecular weight brew fraction ("Brew HMw1") and a fraction with a lower molecular weight ("Brew LMw"), respectively (**Figure 1**).

The second high molecular weight fraction was obtained from defatted Brew LMw by using membrane dialysis. The Brew LMw was dialyzed using a Visking size 9 dialysis membrane with a cutoff of 12-14 kDa

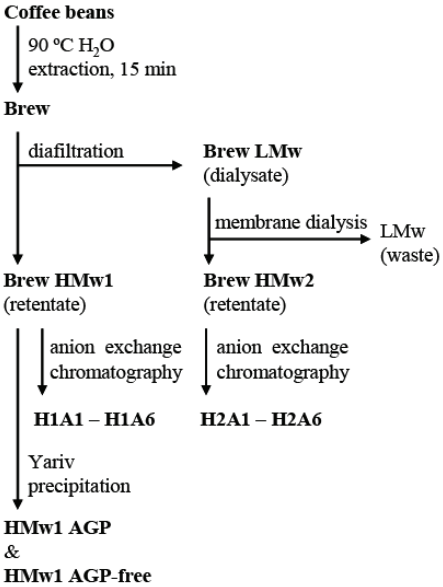

Figure 1. Scheme for the isolation of various melanoidin coffee fractions from roasted Arabica coffee beans.

(Medicell International Ltd., London, United Kingdom) for 2 days against running tap water and 1 day against demineralized water with two water renewals. The retentate of the Brew LMw was lyophilized, yielding a second high molecular weight brew fraction ("Brew HMw2") (**Figure 1**).

Anion Exchange Chromatography of the High Molecular Weight Coffee Fractions.

Anion exchange chromatography was performed on a 5-mL HiTrap Q Fast-Flow column (Amersham Biosciences, Uppsala, Sweden) using an ÄKTA Purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden). The absorbance of the eluate was measured at 280, 325, and 405 nm using the UV-900 detector. Three eluents were used: (A) 5 mM NaOAc, pH 5.1; (B) 2 M NaOAc, pH 5.1; and (C) 2 M NaCl in 5 mM NaOAc, pH 5.1. Samples were dissolved in eluent A (25 mg/mL), and 500 μL was applied onto the column at a flow rate of 4 mL/min. The elution profile consisted of 75 mL isocratic elution with eluent A, in 0.75 mL to 15% eluent B followed by 50 mL isocratic elution, in 0.75 mL to 30% eluent B followed by 50 mL isocratic elution, in 3.5 mL to 100% eluent B followed by 50 mL isocratic elution, in 7.5 mL back to 100% eluent A followed by 35 mL isocratic elution, in 2.5 mL to 50% eluent C followed by 50 mL isocratic elution, in 2.5 mL to 100% eluent C followed by 50 mL isocratic elution. The column was regenerated by elution with 0.5 M NaOH (50 mL), eluent B (100 mL), and eluent A (100 mL).

Cation Exchange Chromatography of the High Molecular Weight Coffee Fractions.

Cation exchange chromatography was performed on a 5-mL HiTrap SP Fast-Flow column (Amersham Biosciences, Uppsala, Sweden) using an ÄKTA Purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden). The absorbance of the eluate was measured at 280, 325, and 405 nm using the UV-900 detector. Two eluents were used: (A) 5 mM NaOAc, pH 5.1, and (B) 1 M NaCl in 5 mM NaOAc, pH 5.1. Samples were dissolved in eluent A (12.5 mg/mL) and 50 μL was applied onto the column at a flow rate of 3 mL/min. The elution profile consisted of 10 mL isocratic elution with eluent A, in 5 mL to 100% eluent B followed by 10 mL isocratic elution, in 5 mL to 100% eluent A. The column was reconditioned by elution with eluent A (25 mL) to prepare the column for the next run.

Preparative Anion Exchange Chromatography of the High Molecular Weight Coffee Fractions.

Preparative anion exchange chromatography was performed on 5 × 5 mL HiTrap Q Fast-Flow columns (Amersham Biosciences, Uppsala, Sweden) connected in series using an ÄKTA Explorer system (Pharmacia Biotech, Uppsala, Sweden), and the absorbance of the eluate was measured at 280, 325, and 405 nm using the UV-900 detector. Brew HMw1 and Brew HMw2 were dissolved in eluent A (4 mg/mL) and were applied (50 mL) onto the columns at a flow rate of 6 mL/min using eluent A. Three eluents were used: (A) 5 mM NaOAc, pH 5.1; (B) 2 M NaOAc, pH 5.1; and (C) 2 M NaCl in 5 mM NaOAc, pH 5.1. The elution profile was 100 mL isocratic elution with eluent A (fraction A1), in 12 mL to 15% eluent B followed by 100 mL isocratic elution (fraction A2), in 12 mL to 30% eluent B followed by 100 mL isocratic elution (fraction A3), in 60 mL to 100% eluent B followed by 150 mL isocratic elution (fraction A4), in 12 mL back to 100% eluent A followed by 60 mL isocratic elution (fraction A4), in 30 mL to 50% eluent C followed by 140 mL isocratic elution (fraction A5), in 30 mL to 100% eluent C followed by 100 mL isocratic elution (fraction A6). The column was regenerated by elution with 0.5 M NaOH, eluent B, and eluent A. The six fractions were dialyzed for 2 days against running tap water and 1 day against demineralized water, with two water renewals. The dialyzed fractions were lyophilized, yielding "H1A1-A6" for Brew HMw1 and "H2A1-A6" for Brew HMw2 (**Figure 1**).

Yariv Assay for Arabinogalactan Proteins.

The Yariv assay was based on the procedure as described by Van Holst and Clarke (*27*). The Yariv phenyl glycoside (1,3,5-tri[4-β-D-glucopyranosyl-oxyphenylazo]2,4,6-trihydroxybenzene) was prepared as described by Yariv et al. (*28*). A 1% (w/v) agarose solution containing 0.15 M NaCl, 0.02% (w/v) sodium azide, and 0.002% (w/v) Yariv reagent was heated to boiling and was subsequently poured onto Petri dishes to give a layer of approximately 3-mm thick. Wells were made (3 mm in width) and 20 μL sample solution was pipetted into a well. The Petri dishes were sealed with Parafilm and were left for 2 days at ambient temperature to allow the colored halo to develop. Lyophilized coffee sample was dissolved in water (2 mg/mL) and was pipetted into a well. Gum Arabic (Sigma Chemical Co., St. Louis, MO) (1, 2, 3, 4 mg/mL), an arabinogalactan (Novo Industries, Bagsværd, Denmark) (2 mg/mL), and a galactomannan (Diamalt GmbH, München, Germany) (2 mg/mL) were used as test polysaccharides. Water was used as blank.

Arabinogalactan Protein (AGP) Isolation.

AGP was isolated from coffee in duplicate on the basis of a procedure described by Immerzeel et al. (*29*). Brew HMw1 (50 mg) was dissolved in 42.5 mL 0.176 M NaCl and was centrifuged at 10000*g* for 25 min at 5

°C. To the supernatant, 7.5 mL of Yariv reagent (10 mg/mL in water) was added and the Yariv-AGP complex was precipitated overnight at 4 °C. Next, the solution was centrifuged at 10000*g* for 25 min at 5 °C, yielding the AGP pellet and the AGP-free supernatant. The pellet was washed three times with 75 mL 0.15 M NaCl and was subsequently dissolved in 50 mL water. Sodium dithionite (1 g) was added to the AGP and AGP-free fractions to decompose the Yariv phenyl glycoside, followed by heating at 50 °C (15 min) until the red color disappeared. The solutions were dialyzed at 4 °C for 2 days against running tap water and 1 day against demineralized water, with two water renewals. The dialyzed AGP and AGP-free fractions were lyophilized, yielding "Brew HMw1 AGP" and "Brew HMw1 AGP-free" (**Figure 1**).

Analysis of Nitrogen Content.

The nitrogen content of various samples was estimated according to the Dumas method using an NA2100 nitrogen and protein analyzer (Carlo Erba Instruments, Milan, Italy) according to the manufacturer's instructions. Methionine was used as a standard.

Molecular Weight Distribution.

High-performance size-exclusion chromatography was performed as described by Bekedam et al. (*22*) on two 300 × 7.8 mm i.d. TSKgel columns in series (G4000 PWXL and G2500 PWXL) (TosoHaas, Stuttgart, Germany), in combination with a PWXL-guard column (TosoHaas, Stuttgart, Germany). Elution took place at 40 °C with 0.2 M sodium nitrate at 0.8 mL/min. The eluate was monitored by refractive index detection using a Spectra System RI-150 detector (Thermo Electron Company, Waltham, MA), and the absorbance was measured at 280, 325, and 405 nm using a Spectra System UV2000 (Thermo Electron Company, Waltham, MA). The sample was dissolved in the eluent and was centrifuged prior to injection (100 μL).

Sugar Analysis.

The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (*30*) using inositol as an internal standard. Briefly, samples were prehydrolyzed with 72% (w/w) H_2SO_4 for 1 h at 30 °C followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C, and the constituent sugars released were analyzed as their alditol acetates. The neutral sugar content was also determined by the phenol sulfuric acid assay according to Dubois et al. (*31*). The uronide content was determined by the automated colorimetric *m*-hydroxydiphenyl method (*32, 33*).

Total Phenolic Groups Content.

The total phenolic groups content of the coffee samples was determined with the Folin-Ciocalteu reagent. For all coffee samples, a 0.33 or 1 mg/mL solution was prepared. To 1 mL of the coffee sample solution, 500 μL of Folin-Ciocalteu (Merck, Darmstadt, Germany) reagent was added. After mixing, 1 mL of a saturated Na_2CO_3 solution was added, and filtered demineralized water was added until the total volume was 10 mL. After mixing, followed by 1 h of reaction, the absorbance of the sample was measured at 725 nm on a UV-mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan). Chlorogenic acid (Sigma Chemical Co., St. Louis, MO) was used as reference phenolic compound.

Specific Extinction Coefficient of Coffee Material at 280, 325, and 405 nm.

Coffee fractions were dissolved in water (1 g/L), and the absorption was determined at 405 nm using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). The coffee solutions were further diluted in case the absorption was higher than 1.3. The specific extinction coefficients $K_{\text{mix 405nm}}$ (L/g/cm) were calculated as was previously described by Bekedam et al. (*22*)**.**

RESULTS AND DISCUSSION

Characterization of the Brew HMw Fractions.

The isolation of the Brew, Brew HMw1, and Brew HMw2 was reported previously (*22*). Brew HMw1 was isolated from Brew by diafiltration (cutoff 3 kDa), and the dialysate of this filtration step was subsequently subjected to membrane dialysis (cutoff 12-14 kDa), yielding Brew HMw2. In this previous research, the Brew and Brew HMw1 were characterized, while no characteristics on Brew HMw2 were given. To be able to compare the characteristics for

Table 1. Yield, $K_{mix\ 405nm}$ Value, Nitrogen and Phenolic Groups Level of Coffee Fractions[a]

	yield[b] (%, w/w)	$K_{mix\ 405nm}$ (L/cm/g)	nitrogen (%, w/w)	phenolic groups[c] (%, w/w)	total sugar (%, w/w)
Brew	100[d]	0.7	3.13	21	26
Brew HMw1	15.5[d]	1.1	1.42	11	69
Brew HMw2	16.2[d]	1.5	2.81	18	43
H1A1	5	0.2	0.93	2	39
H1A2	30	0.3	0.55	4	74
H1A3	16	0.9	1.17	6	57
H1A4	25	1.2	2.75	9	33
H1A5	9	1.4	3.34	10	16
H1A6	10	0.7	2.91	5	10
H2A1	11	0.3	1.15	2	60
H2A2	21	0.9	4.37	8	41
H2A3	9	1.6	4.57	12	20
H2A4	18	2.6	3.34	17	16
H2A5	7	2.7	2.66	12	13
H2A6	5	1.0	2.27	4	7
HMw1 AGP	41	1.2	1.30	7	50
HMw1 AGP-free	45	1.0	1.48	7	56

[a] The standard deviation of the various parameters was 2.5% on average. [b] From starting HMw material. [c] As chlorogenic acid equivalents. [d] From Brew.

Table 2. Sugar Composition (Mol %) of Brew, Brew HMw1, Brew HMw2, HMw1 AGP, and HMw1 AGP-free

	Rha	Ara	Xyl	Man	Gal	Glc	uronic acid	total sugar (%, w/w)
Brew	2	11	0	33	34	6	14	26
Brew HMw1	2	10	1	41	37	1	8	69
Brew HMw2	1	11	0	46	31	1	9	43
HMw1 AGP	0	11	1	9	68	2	9	50
HMw1 AGP-free	3	11	1	54	19	1	10	56

Brew HMw2 with the Brew and Brew HMw1, part of the characteristics on the Brew and Brew HMw1 is shown again in this study. The Brew, Brew HMw1, and Brew HMw2 were analyzed for their composition (**Table 1** and **2**). The yield of Brew HMw1 and Brew HMw2 were both 16% (w/w). Both the HMw fractions were analyzed by size exclusion chromatography, and **Figure 2** shows that Brew HMw1 and HMw2 are two distinct populations in the brew with respect to their molecular weight distribution, with Brew HMw1 having the largest molecular weight (Mw) distribution and Brew HMw2 having the lower Mw

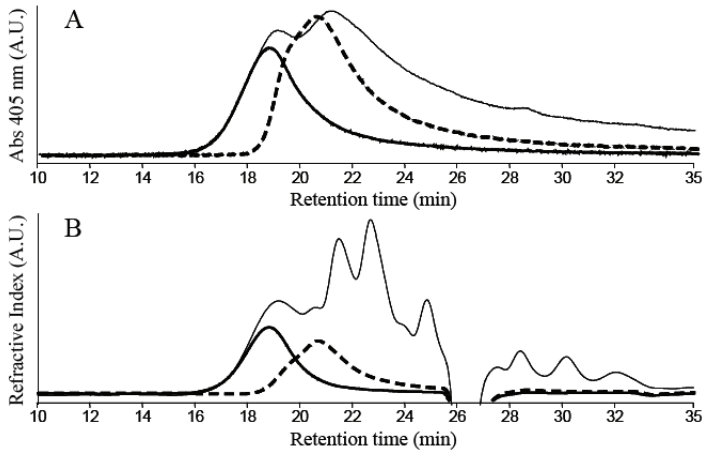

Figure 2. Size exclusion patterns of Brew (solid thin line), Brew HMw1 (solid thick line), and Brew HMw2 (dashed line). **A**: 405-nm absorption patterns; **B**: refractive index patterns.

distribution. The fact that Brew HMw2 elutes slightly earlier compared to the second HMw peak of the Brew is possibly due to an interaction of the polymers with other coffee compounds. The $K_{\text{mix 405nm}}$ value, a parameter for the melanoidin content (*22*), was 1.1 for Brew HMw1 and 1.5 for Brew HMw2, indicating that Brew HMw2 contains 1.4 times more melanoidins than Brew HMw1. Brew HMw1 (24%) and Brew HMw2 (35%) together account for 59% of the melanoidins present in the Brew while they represent 32% of the Brew dry matter. Thus, a large part of the melanoidins was isolated by the procedures used. The nitrogen and phenolic groups contents were also found to be higher for Brew HMw2, being 2 and 1.5 times higher for Brew HMw2 compared to Brew HMw1, respectively. These results suggest that there is a relationship between the melanoidin content and the nitrogen and phenolic groups level in coffee brew, as previously suggested on the basis of ethanol precipitation (*22*). With respect to the sugar content, it is clear that Brew HMw2 contained less sugars (43%) compared to Brew HMw1 (69%). However, the sugar composition was found to be quite similar in these two fractions, although Brew HMw2 contained relatively more mannose and slightly less galactose. The lower sugar content in Brew HMw2 can be explained by the higher protein/nitrogen and phenolic group content. Taken together, two melanoidin–rich high molecular weight fractions were isolated from coffee Brew, and both fractions had a similar sugar composition but differed in sugar, nitrogen level, and phenolic group content. Both fractions were subjected to further analysis.

Ion Exchange Chromatography of Brew Fractions.

Coffee material was analyzed for its charge by ion exchange chromatography. The Brew, Brew HMw1, and Brew HMw2 were applied on a strong cation exchange column using an eluent with a low ionic strength. For all coffee fractions, all the material eluted directly under the conditions chosen and no material was retained on the column (data not shown) as judged on 280, 325, and 405 nm absorption. Since no binding was observed, it was concluded that coffee melanoidins do not expose positively charged groups at the pH of coffee brew.

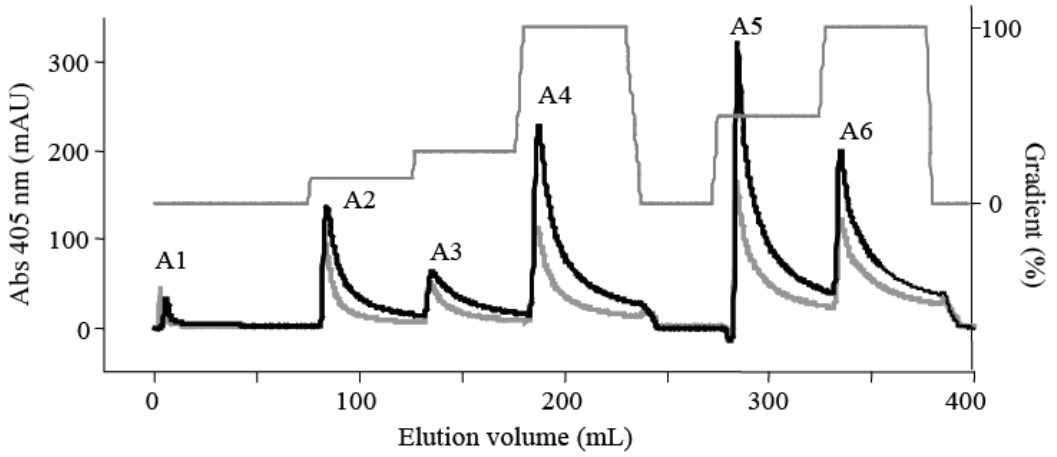

Figure 3. Anion exchange chromatography elution patterns of Brew HMw1 (gray line) and Brew HMw2 (black line).

When coffee fractions were applied on a strong anion exchange column, totally different results were obtained. Brew HMw1 and Brew HMw2 showed strong binding to the anion exchange column and almost no material eluted in the unbound fraction, on the basis of the absorption at 280, 325, and 405 nm. By increasing the ionic strength gradually, no distinct populations could be observed and all material was eluted in a very broad peak (data not shown). However, by increasing stepwise the ionic strength of the eluent, six fractions were obtained and collected for further analysis. The elution patterns at 405 nm of both Brew HMw1 and Brew HMw2 are shown in **Figure 3**. The elution patterns at 280 and 325 nm were quite similar although the peak area at 280 and 325 nm decreased slightly with increasing ionic strength compared to the 405-nm peak areas. Brew HMw1 and Brew HMw2 showed similar elution patterns. From **Figure 3**, it can be concluded that melanoidins in both HMw fractions possessed a negative charge at the pH of coffee brew.

Characterization of Negatively Charged Brew HMw Fractions.

Brew HMw1 and Brew HMw2 were applied on preparative scale on anion exchange columns and the fractions were collected for analysis. The elution patterns obtained were identical to the pattern shown in **Figure 3** (data not shown). Re-elution of the fractions obtained resulted in elution of the fraction at the same ionic strength as that at which it was collected, from which it was concluded that the observed elution behavior was reproducible and that the different fractions have different charges. Characteristics of the fractions obtained are shown in **Tables 1** and **3**. The yield of the unbound fraction of HMw1 and HMw2 was only 6 and 16% (w/w), respectively (**Table 1**), proving that indeed most of the material present in the Brew HMw was negatively charged. On the basis of the 405-nm absorption, only 1% and 3% of the melanoidins eluted in the unbound fraction of HMw1 and HMw2, respectively, from which it was concluded that almost all coffee melanoidins are negatively charged. For both HMw fractions, it was observed that the melanoidin content ($K_{\text{mix 405nm}}$) increased with increasing retention on the anion exchange material (**Table 1**), except for the last fraction A6 in which the melanoidin content decreased again. The nitrogen level was

Table 3. Sugar Composition (w/w %/(Mol %)) of Anion Exchange Chromatography Fractions of Brew HMw1, and Brew HMw2

	Rha	Ara	Xyl	Man	Gal	Glc	uronic acid	total sugar (%, w/w)
H1A1	<1 / (0)	1 / (2)	<1 / (1)	34 / (85)	3 / (7)	1 / (2)	1 / (2)	39
H1A2	2 / (3)	8 / (13)	<1 / (1)	21 / (27)	37 / (48)	1 / (1)	6 / (7)	74
H1A3	2 / (3)	7 / (14)	<1 / (1)	7 / (12)	32 / (56)	<1 / (1)	8 / (13)	57
H1A4	1 / (3)	4 / (14)	<1 / (1)	5 / (14)	15 / (45)	1 / (2)	8 / (21)	33
H1A5	<1 / (3)	2 / (16)	<1 / (3)	3 / (22)	6 / (40)	1 / (4)	2 / (12)	16
H1A6	<1 / (2)	1 / (9)	<1 / (4)	4 / (35)	2 / (20)	1 / (9)	2 / (20)	10
H2A1	1 / (2)	7 / (13)	<1 / (1)	23 / (37)	25 / (42)	1 / (2)	2 / (4)	60
H2A2	1 / (2)	5 / (15)	<1 / (1)	6 / (14)	22 / (54)	1 / (1)	6 / (12)	41
H2A3	<1 / (2)	2 / (13)	<1 / (1)	2 / (12)	8 / (38)	1 / (5)	6 / (28)	20
H2A4	<1 / (2)	2 / (12)	<1 / (3)	2 / (12)	5 / (31)	1 / (6)	6 / (35)	16
H2A5	<1 / (2)	1 / (14)	<1 / (4)	2 / (12)	4 / (29)	1 / (10)	4 / (29)	13
H2A6	<1 / (2)	1 / (12)	1 / (8)	1 / (16)	2 / (22)	2 / (20)	2 / (20)	7

found to be low in the unbound fractions and varied over the fractions A2-A6. The phenolic group content correlated with the melanoidin content. A positive correlation for the nitrogen and phenolic group content with the melanoidins content was seen for Brew HMw1. The phenolic group content and the melanoidin content in Brew HMw2 showed a positive correlation as well, although no correlation between the nitrogen content and the melanoidin content was found. Thus, it seems that phenolic groups are present within the melanoidin complex. From the results obtained by the Folin-Ciocalteu reagent, no statement about the nature of the phenolic groups in melanoidins can be made. Incorporation of low Mw phenolics might be possible, e.g., ester-linked chlorogenic acid. Formation of neoformed phenolic-like compounds out of nonphenolics during the Maillard reaction is possible as well since Tressl et al. (*34*) mentioned the presence of furans and pyrrole-like structures in melanoidins, which might in the close vicinity of hydroxyl groups behave as phenolic groups.

The sugar composition of each anion exchange fraction is shown in **Table 3**. It is clear that the sugar content in the fractions decreased with increasing ionic strength and that mannose-rich polymers (galactomannans) especially were eluted at low ionic strength (A1 and A2). Elution of galactomannans in the unbound fractions may not be surprising because galactomannans from green beans did not bind to an ion exchange column either (*35*). The uronic acid content initially increased with increasing ionic strength (A1→A2→A3) up to maximally 8% (w/w) of the Brew HMw. This increase in uronic acid content might explain the difference in charge in the different fractions (A1→A3). It might even explain the anionic character of the melanoidins, but in that case, the uronic acid containing carbohydrate should be part of the melanoidin complex. For the populations with the highest melanoidin content (A4 and A5), it is clear that the uronic acid content remained constant (A3→A4) and then

decreased (A4→A5). This shows that the anionic character of the material in fractions A5 and A6 cannot be explained by uronic acids only, indicating that another characteristic negative-charged group should also be present to be able to explain the further increase in negative charge. Incorporation of negatively charged low molecular weight coffee compounds (e.g., acids) might be an explanation, as is the possible formation of newly formed acid groups upon roasting.

The recovery of arabinose, galactose, and uronic acids was good (>80%) for both fractions, but the mannose recovery was relatively low, 37 and 31% for Brew HMw1 and Brew HMw2, respectively. Determination of the neutral sugar content of the effluent fractions by the phenol sulfuric acid assay according to Dubois (*31*) showed that the sugars were not lost during the chromatography isolation step. Therefore, it seems that part of the HMw material is lost during dialysis, and as the HMw2 fraction was previously dialyzed thoroughly, it seems that dialysis conditions affect the final yield. In other words, the HMw fraction size will decrease after conducting a second dialysis step using another membrane.

From the results, it can be concluded that coffee melanoidins are negatively charged molecules which are heterogeneous with respect to their polyanionic behavior since melanoidins eluted at different ionic strengths. Furthermore, it was shown that characteristic groups other than uronic acids are likely to be present to account for the total negative charge. The presence of these negative charges on melanoidins probably contributes to the biological and functional properties of melanoidins in coffee brew. For example, covalent or ionic binding of chlorogenic acid with negative-charged groups on melanoidins might explain the antioxidative properties observed for melanoidins (*6, 14-16*). Binding of coffee flavor compounds to melanoidins as suggested by others (*7-13*) might occur via the anionic groups of melanoidins and might affect the sensorial perception of coffee. Also, positively charged metal ions might also bind to the negatively charged melanoidins, resulting in the metal–chelating properties observed by others (*16, 17*). Another option is that melanoidins might contribute to the foaming properties of coffee brews (*36, 37*) since they might consist partly of hydrophobic sections with mainly furan and pyrrole-like structures (*34*), and partly of hydrophilic sections with the negative charged groups, resulting in a surface-active molecule. Overall, the anionic character of melanoidins might be responsible for quite some biological and functional properties that are ascribed to melanoidins in the literature.

Identification of Arabinogalactan Proteins (AGPs) in Coffee Brew.

Arabinogalactan is, together with galactomannan, the most abundant carbohydrate in coffee brew. The presence of acidic AGPs (~15%, w/w) in the cell wall of green coffee beans was reported by Redgwell and co-workers (*26, 38*). Since these acidic coffee bean AGPs are extracted into the coffee brew, it was questioned whether these AGPs are also present in the negatively charged polymer fractions that were isolated, as described above. Therefore, the presence of AGPs in coffee brew fractions was investigated by a Yariv gel-diffusion assay, in

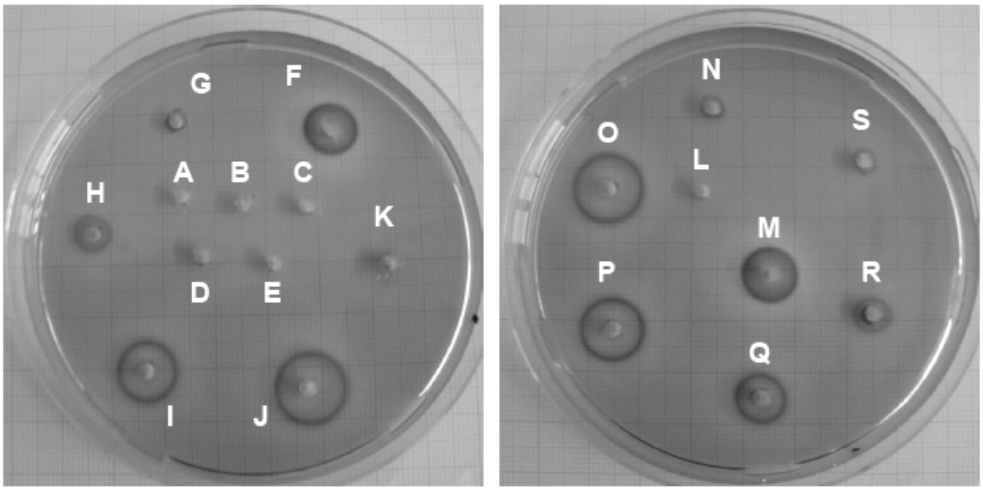

Figure 4. Yariv-gel diffusion assay of water (**A** and **L**), 2 mg/mL arabinogalactan (**B**), galactomannan (**C**), Brew HMw2 (**D**), Brew LMw (**E**), gum arabic (**F** and **M**), green bean extract (**G**), Brew (**H**), Brew HMw1 (**I**), Brew HMw1 AGP (**J**), Brew HMw1 AGP-free (**K**), H1A1 (**N**), H1A2 (**O**), H1A3 (**P**), H1A4 (**Q**), H1A5 (**R**), and H1A6 (**S**).

which the Yariv phenyl glycoside specifically binds to AGPs resulting in a colored halo (**Figure 4**). Water, arabinogalactan, and galactomannan solutions were used as controls. Gum arabic, an AGP-rich compound, was used as a positive control, and the development of a clear halo was observed. A linear correlation between the applied amount of gum arabic and the square of the diameter of the halo was obtained (data not shown) as mentioned by Van Holst and Clarke (*27*). The control samples showed that the Yariv reagent very specifically reacts with AGPs and not with carbohydrates (galactomannan and arabinogalactan) that are not bound to protein. A green coffee bean extract was prepared and applied on the Yariv-plate to determine whether the green coffee bean cell wall AGPs (*26, 38*) are extracted when heated in water at 90 °C. This green bean extract (**Figure 4G**) showed only slight color development in the well area, while no distinct halo could be observed, indicating that AGPs in green beans are not readily extracted. This is in agreement with findings by Redgwell et al. (*26*) who reported that harsh conditions are needed to isolate the majority of the coffee arabinogalactans (8 M KOH and enzymatic degradation of other cell wall components). The Brew (**Figure 4H**) gave a distinct halo, and it can be concluded that the Brew contains AGPs. In addition to the findings reported by Redgwell et al. (*26*), who stated that roasted coffee cell wall material contains AGPs, it is now found that at least part of these AGPs end up in coffee brew as well. Since the Brew showed a halo and since the green bean extract did not show a halo, it can be stated that the roasting process improves the extractability of AGPs. The AGPs present in the green bean are likely entrapped in the cell wall of green coffee beans and the roasting process affects the cell wall in such an extent that they become more readily extractable, resulting in their presence in coffee brew. **Figure 4** also shows that all AGPs from the coffee Brew end up in the Brew HMw1 fraction and that Brew LMw and Brew HMw2 are free of AGPs. Obviously, all AGPs are retained by the diafiltration membrane which was used to isolate

Brew HMw1 from Brew. AGP was isolated to investigate their content in the Brew and to determine whether they are included in the melanoidin formation.

Characterization of the AGP Fraction of Brew HMw1.

The AGPs were isolated from Brew HMw1 by precipitation with the Yariv reagent on a preparative scale. Isolation of the AGPs from Brew HMw1 yielded an AGP (41%, w/w) and AGP-free (45%, w/w) fraction (**Table 1**), and these AGPs make up ~6% of the Brew's dry matter. Both the isolated fractions were recovered as a brown-colored material. Redgwell et al. (*38*) reported that AGPs in green coffee beans were white and gave a colorless clear solution in water. As the Yariv reagent is very specific for AGPs, and as the isolated fraction was brown instead of white, it allowed us to infer that coffee bean AGPs undergo chemical reactions during roasting and become part of the melanoidin complex. This material is referred to as the AGP–melanoidin complex. The composition of the Brew HMw1 AGP and AGP-free fraction was determined and the results are shown in **Tables 1** and **2**. The $K_{\text{mix 405nm}}$ values, the parameter for the melanoidin content, of the AGP and AGP-free fraction were 1.2 and 1.0, respectively, showing that both fractions were rich in melanoidins. The AGP fraction accounted for 45% of the melanoidins present in the Brew HMw1. With respect to the Brew, this AGP fraction (~6%, w/w) accounted for 11% of all the melanoidins present in the Brew. Because of the specificity of the Yariv reagent and the intensity of the washing steps, it was concluded that coprecipitation of melanoidin molecules and compounds other than AGPs is very unlikely. Thus, the isolated AGP fraction is a melanoidin-rich population from coffee brew that consisted of only one type of compounds, namely, the AGP–melanoidin complex.

The nitrogen levels in the AGP and AGP-free fractions were 1.30% and 1.48%, respectively. The nitrogen content of the AGP fraction is quite in line with the literature. Redgwell et al. (*38*) reported a nitrogen content of 1.88% for green coffee bean AGPs. The nitrogen in the melanoidin-rich AGP fraction can probably largely be explained by the presence of intact amino acids from the protein moiety in the AGPs as they occur in green beans. However, degradation of part of these amino acids upon roasting cannot be ruled out.

The phenolic group content for both the AGP and AGP-free fraction was found to be 7%. Thus, phenolic groups were present in the AGP fraction but the phenolic groups were not specifically related to the AGPs or AGP-free fraction. The relative low phenolic group content in the AGP and AGP-free fractions (7%), compared to the Brew HMw1 fraction (11%), might be explained by incomplete dissolving of the fractions or by the loss of compounds upon dialysis.

The sugar content for the AGP (50%, w/w) and AGP-free (56%, w/w) fractions was similar, but these fractions differed with respect to the sugar composition. The AGP-free fraction contained mainly mannose (54 mol %), galactose (19 mol %), arabinose (11 mol %), and uronic acids (10 mol %) indicating that galactomannans were the most abundant sugars in this fraction and that arabinogalactans are present in a lesser extent. The galactose/arabinose ratio in the AGP-free fraction was only 1.7 and part of this galactose is probably part of the galactomannans, therefore, the galactose/arabinose ratio for the arabinogalactans is probably

even lower. The uronic acids are expected to be part of the arabinogalactans as well. On the basis of these findings, it can be concluded that the arabinogalactans in the AGP-free fraction are highly branched polymers. For the AGP fraction, it was found that this fraction contained mainly galactose (68 mol %), arabinose (11 mol %), mannose (9 mol %), and uronic acids (9 mol %), indicating that arabinogalactan was the most abundant sugar, as expected (*38*). Comparing the galactose/arabinose ratio of 6.2 with data found in the literature, it was found that this value was rather high as the AGP fraction of green coffee beans had a value of 3.0 (*38*) and the ratio of gum arabic was 1.8 or lower (*39*). The high galactose/arabinose ratio can be explained by the fact that arabinose is the monosaccharide that is most prone to degradation upon roasting, and losses up to 65% (w/w) were reported (*3*). The presence of 9 mol % mannose in the AGP fraction was unexpected since no mannose was found to be present in AGP from green coffee beans (*38*) and AGP from gum arabic contains only trace amounts of mannose. Since galactomannans do not show precipitation with the Yariv reagent, it allowed us to infer that mannose, probably as galactomannan, is incorporated into the AGP–melanoidin complex.

Redgwell et al. (*26*) reported that green Arabica coffee beans (e.g., var. Yellow Caturra) contained 45% (w/w) carbohydrates, of which 15% (w/w) were from galactose (10%), arabinose (3%), and uronic acids (2%). On the basis of the data that green coffee beans contain ~15% (w/w) AGPs and that 85% (w/w) of these AGPs are carbohydrates (galactose, arabinose, and uronic acids) (*38*), it can be stated that almost all arabinogalactan (85%) in green coffee beans should be present in AGPs. The AGP fraction isolated from green coffee beans by Redgwell et al. (*38*) was positive for the Yariv gel-diffusion assay as well, although a different isolation procedure was applied. It was calculated from the sugar composition and yield of the two Brew HMw fractions that only 60% (w/w) of the arabinose, galactose, and uronic acids ended up in Brew HMw1. From the sugar composition and yield of the two fractions isolated from Brew HMw1, the AGP and AGP-free fraction, it was calculated that only 67% (w/w) of the arabinose, galactose, and uronic acids ended up in the AGP fraction. Overall, only 40% (w/w) of the arabinose, galactose, and uronic acids present in the two HMw fractions ended up in the AGP fraction. The remaining 60% of the arabinose, galactose, and uronic acids were not part of the AGP fraction, while most of the arabinose, galactose, and uronic acids were present in AGPs prior to roasting (*26, 38*). This implies that AGPs present in green coffee beans are chemically modified or degraded during roasting to such an extent that the AGP loses its specific characteristic of binding to the Yariv reagent. It is likely that the protein backbone of the AGPs is degraded by the Maillard reaction, resulting in molecules with a lower Mw. This could then explain the presence of “non-AGP” arabinose, galactose, and uronic acids in the Brew HMw2 fraction. It can be reasoned that the AGPs first react into brown–colored compounds (AGP-melanoidins) which still bind to the Yariv reagent, followed by further reaction in which the AGP–melanoidins lose their specific Yariv binding characteristics. AGPs seem to be very prone to (Maillard) reaction upon roasting of the coffee bean, resulting in brown-colored molecules.

Relation between AGPs and Charge Properties of Melanoidins.

The six anion exchange fractions of Brew HMw1 were 1analyzed by the Yariv gel-diffusion assay to determine which fractions contained most of the AGPs. From **Figure 4**, it is clear that H1A2 contained most of the AGPs and that the AGP content decreased from H1A2 to H1A6. Using the diameter of the halo and the yields of the anion exchange fraction for Brew HMw1, the distribution of the AGPs over the Brew HMw1 anion exchange samples was calculated and is shown in **Figure 5**. The anion exchange fraction H1A2 contained 58% (w/w) of AGPs present in Brew HMw1. Thus, the majority of AGPs had a relatively low charge, probably the result of the uronic acids and possibly negatively charged groups on amino acids. As the AGPs are part of melanoidins, it can be stated that the AGP–melanoidins are slightly negatively charged molecules.

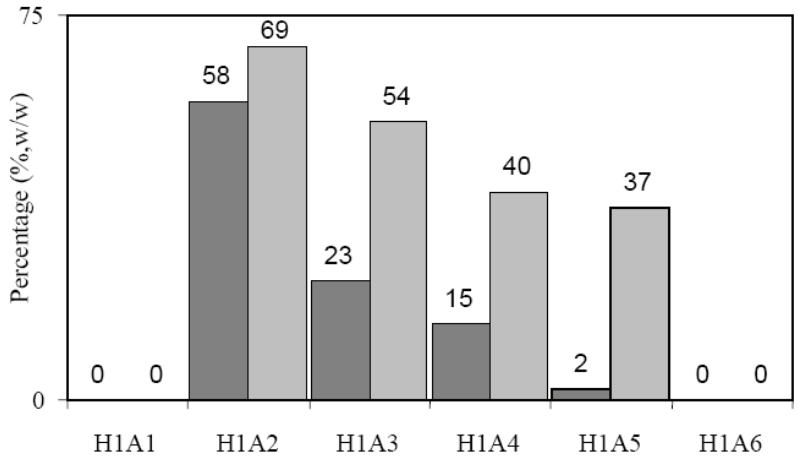

Figure 5. Distribution (%, w/w) of the AGPs over the six Brew HMw1 anion exchange fractions (dark gray columns) and the percentage (%, w/w) of (galactose, arabinose, and uronic acids) that is part of the AGP (light gray columns).

The percentage of galactose + arabinose + uronic acids present in AGP relative to the total galactose + arabinose + uronic acids content was calculated and is shown in **Figure 5**. It is clear that the galactose + arabinose + uronic acids content present in AGPs decreased from H1A2 to H1A5. This might again be explained by chemical modification of the AGPs, resulting in more negatively charged groups being bound/formed leading to less precipitation with the Yariv reagent.

The fractions H1A4 and H1A5 were melanoidin-rich (**Table 1**), had a strong negative charge, and were found to be poor in AGPs. These fractions contained 33% and 16% sugars, respectively, of which mannose was the most abundant monosaccharide (**Table 3**), suggesting that galactomannan is present in these fractions. These two fractions contained less uronic acids (%, w/w) than the fractions with a lower negative charge. It can be reasoned that these melanoidin-rich fractions had to contain negatively charged groups not originating from uronic acids or from amino acids from AGPs. The origin of these negative charged groups remains unknown. A possible explanation could be that other acidic molecules are

incorporated into the melanoidin complex or that newly formed acid groups are formed within the HMw coffee melanoidins upon roasting because of the Maillard reaction.

In this study, it was shown that coffee brew melanoidins were found to be negatively charged molecules, which are heterogeneous with respect to their polyanionic behavior. Arabinogalactan proteins were present in the HMw fraction that was retained by diafiltration, whereas the remainder of the coffee was free of AGP. The AGP had a brown color, as opposed to their white color in green coffee beans, allowing us to conclude that they are incorporated into the melanoidin complex upon roasting. This AGP-melanoidin complex is a distinct melanoidin population that can be specifically isolated from coffee brew. The negative charge of the AGP-melanoidin complex is probably due to the presence of uronic acids, which are known to be bound to arabinogalactans. The uronic acid content in the highly negative, melanoidin-rich fractions could not explain the total negative charge of these fractions. This led to the conclusion that other negatively charged groups are probably also incorporated in HMw melanoidins. Future research should focus on the structural characterization of the AGP-melanoidin complex and also on the fate of AGPs during roasting.

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CHAPTER 4

Incorporation of Chlorogenic Acids in Coffee Brew Melanoidins

ABSTRACT

The incorporation of chlorogenic acids (CGAs) and their subunits quinic and caffeic acids (QA and CA) in coffee brew melanoidins was studied. Fractions with different molecular weights, ionic charges, and ethanol solubilities were isolated from coffee brew. Fractions were saponified, and the released QA and CA were quantified. For all melanoidin fractions, it was found that more QA than CA was released. QA levels correlated with melanoidin levels, indicating that QA is incorporated in melanoidins. The QA content correlated with the ionic charge of the melanoidin populations, suggesting that QA may contribute to the negative charge and consequently is, most likely, not linked via its carboxyl group. The QA level correlated with the phenolic acid group level, as determined by Folin-Ciocalteu, indicating that QA was incorporated to a similar extent as the polyphenolic moiety from CGA. The QA and CA released from brew fractions by enzymes confirmed the incorporation of intact CGAs. Intact CGAs are proposed to be incorporated in melanoidins upon roasting via CA through mainly nonester linkages. This complex can be written as Mel=CA-QA, in which Mel represents the melanoidin backbone, =CA represents CA nonester-linked to the melanoidin backbone, and –QA represents QA ester-linked to CA. Additionally, a total of 12% of QA was identified in coffee brew, whereas only 6% was reported in the literature so far. The relevance of the additional QA on coffee brew stability is discussed.

KEYWORDS

Coffee; melanoidins; phenolic; chlorogenic; quinic; caffeic acid; incorporation

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INTRODUCTION

Coffee is one of the most important crops in the world; its worldwide production was almost seven million tons in 2005 (*1*). Green coffee beans are roasted; this process changes the chemical composition drastically. The Maillard reaction is one of the dominant reactions that occur during roasting. This reaction, in which reducing sugars react in a cascade of reaction steps with amino groups, leads to the formation of desired characteristic flavor and color compounds (*2, 3*). The brown-colored Maillard-reaction end-products are referred to as melanoidins, and they are often defined as brown-colored, high-molecular-weight (Mw), nitrogenous Maillard-reaction end-products (*4*). The melanoidin content in coffee brew is frequently determined by difference and may account for 25% of the dry matter (*5*). The chemical and functional properties of coffee melanoidins are not fully understood, which can be ascribed to the extremely complex chemical composition of these molecules. One of the important functional properties of melanoidins is its antioxidative activity (*6-9*), which is believed to be at least partly due to the incorporation of chlorogenic acids (CGAs) (*10-15*).

Many CGAs are present in coffee beans, and 5-caffeoylquinic acid is by far the most abundant one (*16*). Green Arabica coffee beans contain around 7% of CGA; this level decreases drastically upon roasting to levels as low as 0.2% for dark roasted beans (*17*). The fate of the disappearing CGAs upon roasting is not totally understood. It was suggested that part of the CGAs is converted into flavor compounds upon roasting (*14, 18*), whereas others reported that CGAs might be incorporated in coffee brew melanoidins. Already in 1971, Klöcking et al. identified the release of caffeic acid (CA) and ferulic acid (FA) from high-Mw (HMw) coffee material after alkaline pressure hydrolysis (*13*). More than a decade later, thermal degradation studies indicated the involvement of CGAs in melanoidin formation, because many phenolics were released from HMw coffee material (*10, 12*). Delgado-Andrade et al. (*11*) showed that part of the antioxidative compounds in coffee, which are supposedly phenolics, might be bound ionically to melanoidins. Leloup et al. (*14*) reported that roasted coffee beans contained ester-linked quinic acid (QA) as well as ester-linked CA and FA, which are not present within CGAs. Recently, Takenaka et al. (*19*) and Nunes and Coimbra (*15*) identified and quantified phenolic components that were released from HMw coffee brew fractions after alkaline fusion. As these fragments were indicative for CA and FA, they concluded that phenolic acids from CGAs were covalently linked to HMw coffee material (*15*). In previous research, we found that the melanoidin level correlated with the level of phenolic groups present in coffee fractions which were isolated by using different techniques (*4, 20*). Next to that, it was found that the negative charge on melanoidins could not only be caused by uronic acids in arabinogalactans but that there are most probably other negatively charged groups present as well (*20*). Incorporation of CGAs into melanoidins might explain these observations because the CA or FA moiety from CGAs might explain the observed correlation between melanoidins and phenolic groups level, whereas the QA moiety might provide the negative charge on melanoidins.

The aim of this research was to gain insight into the involvement of CGA in coffee melanoidin formation. To this end, series of coffee brew fractions differing in melanoidin content were investigated for their unbound and ester-bound CGA, QA, CA, and FA levels.

MATERIALS AND METHODS

Materials.

Green and roasted Colombian coffee beans (*Coffea arabica*) were provided by a local factory. The degree of roast was 16.4% (w/w), which corresponds to 8.0% (w/w) on a dry matter basis.

Preparation of Coffee Brew.

Roasted beans were ground, and a brew was prepared as described previously (*4*). The extraction yield was 20%. For characterization purposes, part of the brew was lyophilized, yielding Brew. The major part of the brew was used for further isolation.

Defatting of Coffee Samples.

Lyophilized coffee brew samples were defatted by Soxhlet extraction by using a Soxtherm which was connected to a Multistat system (Gerhardt, Königswinter, Germany), as described previously (*20*). The solvent used for extraction was dichloromethane.

Extraction of 5-Caffeoylquinic Acid, QA, CA, and FA from Green and Roasted Beans.

Green beans (frozen in liquid nitrogen) and roasted beans were ground by using a Retsch ZM200 rotor mill as described previously (*4*). Ground beans were lyophilized; this step was followed by a second milling step by using a Retsch MM2000 ball mill operating at maximum amplitude for 1 min, yielding a powder. A mixture of 6 g of water and 1 g of coffee powder was stirred for 30 min at room temperature. This step was followed by centrifugation for 5 min at 2500*g*. This procedure was repeated five times, and the supernatants were pooled, filtered over a Büchner funnel by using a S&S 595 filter (Schleicher and Schuell, Dassel, Germany), and subsequently lyophilized. Lyophilized green and roasted bean extracts were used for determination of unbound and total 5-caffeoylquinic acid (IUPAC), QA, CA, and FA levels.

Isolation of HMw Coffee Brew Material.

HMw material was obtained from the brew by diafiltration as described previously (*4*). The retentate and dialysate were lyophilized, yielding a HMw fraction and a fraction with a lower Mw (DF-dialysate), respectively (**Figure 1**).

Isolation of Intermediate-Mw Coffee Brew Material.

Intermediate-Mw (IMw) material was obtained from the defatted DF-dialysate sample by membrane dialysis. The DF-dialysate sample was dialyzed by using a Visking size 9 dialysis membrane with a cutoff of 12–14 kDa (Medicell International Ltd., London, U.K.) for 2 days against running tap water and 1 day against demineralized water with two water renewals. The retentate was lyophilized, yielding the IMw fraction (**Figure 1**).

Isolation of Low-Mw Coffee Brew Material.

The low-Mw (LMw) fraction was obtained from the defatted DF-dialysate sample by membrane dialysis. The DF-dialysate sample (500 mL, 100 mg/mL) was dialyzed by using a Visking size 9 dialysis membrane with a cutoff of 12–14 kDa (Medicell International Ltd.) for 3 days at 4 °C against 4.5 L of demineralized water with three water renewals. The dialysates were pooled and lyophilized, yielding the LMw fraction (**Figure 1**).

Arabinogalactan Protein Isolation.

Arabinogalactan proteins (AGP) were isolated from the HMw fraction as described previously (*20*). The obtained material was lyophilized, yielding HMw AGP (**Figure 1**).

Ethanol Precipitation of the HMw Fraction.

The HMw fraction obtained after diafiltration was subjected to ethanol precipitation, as described previously (*4*). Absolute ethanol was added to the HMw solution until the desired concentration was reached. The solution was left for precipitation and was subsequently centrifuged. The supernatant was subjected to further precipitation steps. Coffee fractions that precipitated at 20, 40, 60, and 80% ethanol were coded EP20, EP40, EP60, and EP80, respectively. The supernatant of 80% ethanol was coded ES80.

Preparative Anion-Exchange Chromatography of the HMw and IMw Fractions.

Preparative anion-exchange chromatography was conducted as described previously (*20*). Briefly, HMw and IMw solutions were loaded on a strong anion exchanger. Coffee material was eluted in steps by elution with 5,

300, 600, and 2000 mM NaOAc, and subsequently with 1 and 2 M NaCl, yielding fractions A1, A2, A3, A4, A5, and A6, respectively. The obtained HMw and IMw fractions were coded HMw-A1-HMw-A6 and IMw-A1-IMw-A6, respectively (**Figure 1**).

Sample Preparation for Free and Total 5-Caffeoylquinic Acid, CA, and FA Determination.

For determination of the free 5-caffeoylquinic acid, CA, and FA levels, a sample solution (5 mg/mL in water) was centrifuged and subsequently analyzed by reversed-phase high-performance liquid chromatography (HPLC). For determination of the total CA and FA levels, the sample was saponified by using a procedure that prevented oxidation of phenolics (*21*). To 750 μL of sample solution (12 mg/mL), 750 μL of 2 M NaOH solution containing 2% (w/w) ascorbic acid and 20 mM ethylenediaminetetraacetic acid was added. After incubation for 1 h at 30 °C, the mixture was quenched to pH ≈ 1 with 330 μL of 5 M HCl to precipitate most of the coffee material, preventing precipitation during further analysis. The mixture was stored for 2 h at 4 °C, the precipitate was removed by centrifugation, and the supernatant was analyzed by reversed-phase HPLC. Experiments were performed at least in duplicate.

Sample Preparation for Free and Total QA Determination.

For free QA determination, a sample solution (5 mg/mL in water) was centrifuged and subsequently analyzed by ion-moderated partitioning HPLC or by gas chromatography (GC) after silylation, as described below. For total QA determination, the sample was saponified by using the same procedure as for the determination of the total CA and FA levels, although no ascorbic acid and ethylenediaminetetraacetic acid were added. The saponified solution was analyzed by ion-moderated partitioning HPLC or by GC after silylation. Experiments were performed at least in duplicate.

Enzyme Incubation of Coffee Material.

Brew and HMw and IMw fractions were subjected to enzymatic degradation by using commercial chlorogenate esterase from *Aspergillus japonicus (22, 23),* kindly provided by Kikkoman Corp. (Tokyo, Japan).

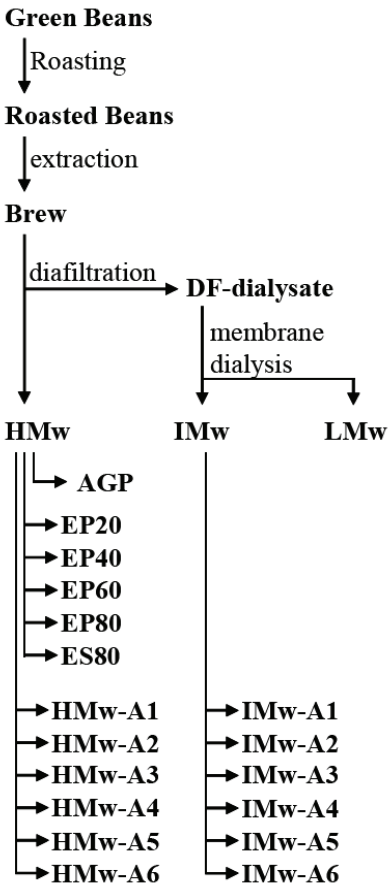

Figure 1. Scheme of the coffee brew isolation procedure (see text for abbreviations).

A sample of 10 mg was dissolved in 500 μL of 25 mM 4-morpholineethanesulfonic acid sodium salt buffer, pH 6. To this solution, 500 μL of 1 mg/mL chlorogenate esterase in 25 mM 4-morpholineethanesulfonic acid sodium salt buffer, pH 6, was added. After overnight incubation at 40 °C, enzymes were inactivated by heating for 10 min at 100 °C. Experiments were performed at least in duplicate. The quantities of released QA, CA, and FA were determined by ion-moderated partitioning and reversed-phase HPLC.

Determination of 5-Caffeoylquinic Acid, CA, and FA by Reversed-Phase HPLC.

For determination of free and total 5-caffeoylquinic acid, CA, and FA levels, untreated and saponified samples (5 mg/mL) were analyzed by reversed-phase HPLC on a 150 × 4.6 mm i.d. XTerra MS C18 3.5 μm column (Waters, Milford, MA) in combination with a 20 × 3.9 mm i.d. XTerra MS C18 3.5 μm guard column (Waters). Elution took place at room temperature (0.5 mL/min) by using (A) 0.1% acetic acid in water and (B) 0.1% acetic acid in methanol. The elution profile after injection (20 μL) was as follows: 1 min isocratic elution with 100% A, in 30 min to 63% B, in 10 min to 100% B, 1 min isocratic elution, in 1 min to 100% A, and 17 min re-equilibration. The absorbance of the eluate was measured at 325 nm by using a Spectra System UV3000 (Thermo Electron Company, Waltham, MA). Aqueous solutions of 5-caffeoylquinic acid, CA, and FA were used as reference compounds for determination of the free 5-caffeoylquinic acid, CA, and FA levels. Saponified CA and FA were used as reference compounds for determination of the total CA and FA levels. The recovery of CA and FA after saponification was 129 ± 2% and 114 ± 2% of the expected value, respectively. Saponification of 5-caffeoylquinic acid resulted in a recovery of 91 ± 2% of the CA. Enzymatic degradation of 5-caffeoylquinic acid resulted in complete degradation of 5-caffeoylquinic acid, and all CA was recovered.

Determination of QA by Ion-Moderated Partitioning HPLC.

For determination of free and total QA levels, untreated and saponified coffee samples (~5 mg/mL) were analyzed for their QA levels based on the procedure described by Zeppa et al. (*24*). High-performance cation-exchange chromatography was performed by using a 300 × 7.8 mm i.d. Aminex HPX 87H column equipped with a cation H+ guard column filled with AG 50W-X4 (Bio-Rad, Hercules, CA). After injection (100 μL), isocratic elution took place with 5 mM sulfuric acid at 0.6 mL/min for 150 min at 40 °C. The eluate was monitored by a Spectra System RI-150 refractive index detector (Thermo Electron Company). The recovery of D(-)-QA (Fluka Chemie GmbH, Buchs, Switzerland) after saponification was 83 ± 5% of the expected value. Saponification of 5-caffeoylquinic acid resulted in a recovery of 75 ± 3% of the QA, which is similar to the recovery of saponified QA. Therefore, untreated QA and saponified QA were used as reference compounds for determination of the free and total QA levels, respectively. Enzymatic degradation of 5-caffeoylquinic acid resulted in a complete recovery of the QA.

Determination of QA by GC after Silylation.

Untreated and saponified coffee samples were silylated on the basis of procedures described by Huang et al. (*25*) and Butts (*26*). A total of 200 μL of the untreated or saponified solution (~5 mg/mL) was transferred into a 1 mL reaction vessel with screw cap (Alltech, Deerfield, IL), subsequently frozen, and lyophilized. Pyridine (3 mL) (Aldrich, Steinheim, Germany) was added to 3 mL of trimethylsilyl 2,2,2-trifluoro-n-(trimethylsilyl)-acetamide containing chlorotrimethylsilane (99:1) (Supelco, Bellefonte, PA). A stirring bone and 600 μL of this solution were added to the lyophilized material. After incubation for 2 h at 125 °C under continuous stirring, the solution was cooled down, and 100 μL of 200 μg/mL dodecane (Merck, Darmstadt, Germany) in hexane was added as internal standard. The solution was mixed and centrifuged, and the supernatant was transferred into a GC vial. A GC Trace gas chromatograph (Thermo Finnigan, Waltham, MA) with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a flame ionization detector was equipped with a 3000 × 0.25 mm i.d. DB-1 column, 0.25 μm film thickness (Agilent Technologies, Santa Clara, CA). The sample (1.5 μL) was injected, and the oven temperature program was as follows: 3 min at 40 °C, in 4.5 min to 130 °C, 5 min at 130 °C, in 19.5 min to 325 °C, and 15 min at 325 °C. QA was used as reference compound.

Total Phenolic Groups Level.

The total phenolic groups contents of the coffee samples were determined with the Folin-Ciocalteu assay as described previously (*4*). The reference compound was 5-caffeoylquinic acid.

Specific Extinction Coefficient of Coffee Material.

The absorption of aqueous sample solutions (1 g/L) was determined at 405 nm by using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). The specific extinction coefficient $K_{\text{mix 405 nm}}$ (L/g/cm) was calculated as described previously (*4*)**.**

RESULTS AND DISCUSSION

5-Caffeoylquinic Acid, QA, CA, and FA in HMw and IMw Fractions.

Brew fractions HMw and IMw were previously shown to be rich in melanoidins and free from LMw molecules (*20*). Therefore, HMw and IMw fractions were the first fractions that were screened for the presence of ester-bound QA, CA, and FA. The untreated HMw elution patterns (**Figure 2**) did not show unexpected peaks, from which it was concluded that this fraction did not indeed contain any free 5-caffeoylquinic acid, QA, CA, or FA (**Table 1**). The saponified HMw fraction elution patterns (**Figure 2**) showed peaks corresponding to 1.7% of QA and 0.5% of CA in the HMw fraction (**Tables 2** and **3**). It was found that a HMw fraction from green beans isolated by using the same conditions did not contain any free or esterbound QA, CA, or FA. The IMw fraction elution patterns were similar to those of the HMw fraction. The trace amounts (<0.1%) of 5-caffeoylquinic acid in the IMw fraction (**Table 1**) might be explained by the fact that the isolation technique involved membrane dialysis, a passive and slow procedure, even though it was conducted for a prolonged time. The IMw fraction was saponified, and the levels of total QA, CA, and FA released are shown in **Table 2**. After correction for 5-caffeoylquinic acid, it was calculated that the IMw fraction contained 3.7% of QA, 0.9% of CA, and 0.1% of FA (**Table 3**). To the best of our knowledge, this is the first

Table 1. Free 5-Caffeoylquinic Acid, QA, CA, and FA Levels in Various Coffee Fractions

	5-caffeoylquinic acid (%, w/w)[a]	QA (%, w/w)[b]	CA (%, w/w)[a]	FA (%, w/w)[a]
Green beans	3.0	0.6	0.0	0.0
Roasted beans	0.7	0.7	0.0	0.0
Brew	2.9	3.0	0.1	0.0
HMw	0.0	0.0	0.0	0.0
IMw	0.1	0.0	0.0	0.0
LMw	4.3	4.6	0.1	0.1

[a] The coefficient of variation was 1% on average. [b] The coefficient of variation was 3% on average.

Table 2. QA, CA, and FA Levels After Saponification in Various Coffee Fractions

	QA (%, w/w)[a]	CA (%, w/w)[b]	FA (%, w/w)[b]
Green beans	3.9	3.3	0.3
Roasted beans	3.4	1.7	0.2
Brew	11.8	4.6	0.6
HMw	1.7	0.5	0.0
IMw	3.7	0.9	0.1
LMw	15.1	6.4	0.8

[a] The coefficient of variation was 3% on average. [b] The coefficient of variation was 1% on average.

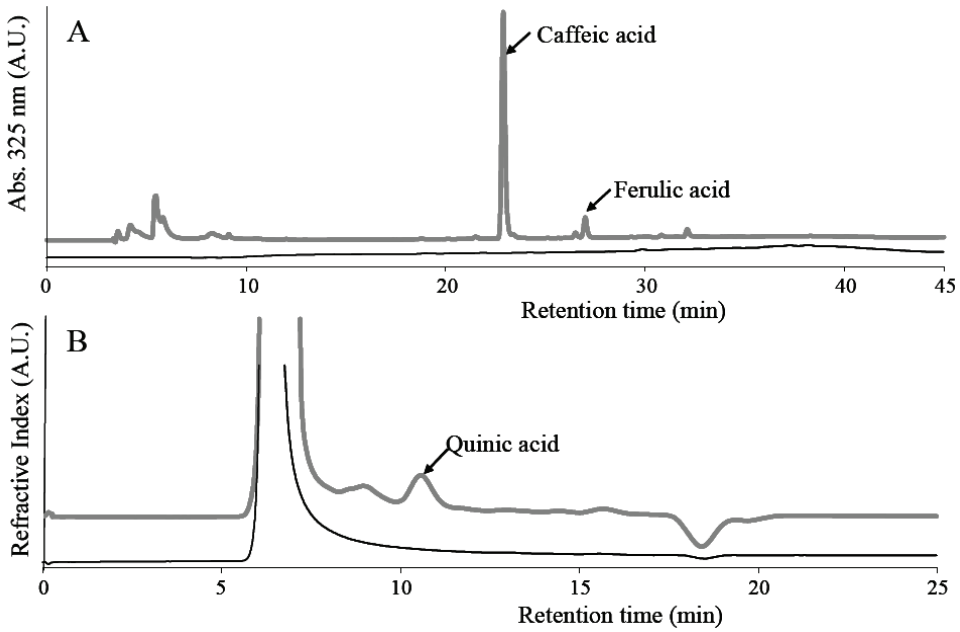

Figure 2. (**A**) Reversed-phase and (**B**) ion-moderated partitioning HPLC elution patterns of HMw (thin black line) and saponified HMw (thick gray line) fractions.

Table 3. Ester-Bound QA, CA, and FA Levels Not from CGAs in Various Coffee Fractions

	$K_{mix\ 405}$ (L/g/cm)	Phenolic groups (%, w/w)[a]	QA (%, w/w)[b]	CA (%, w/w)[b]	FA (%, w/w)[b]
Brew	0.7	29	-	-	-
HMw	1.1	11	1.7	0.5	0.0
IMw	1.5	18	3.6	0.8	0.1
LMw	0.4	23	-	-	-

[a] As 5-caffeoylquinic acid equivalents. [b] Calculated from **Table**s **1** and **2**.

time that release of phenolic acids from HMw and IMw coffee materials by saponification is reported. Previously, Nunes and Coimbra did not detect any phenolic acids after saponification with sodium hydroxide (*15*). One possible explanation for our observations could be the high level of precautions taken in our experiments to prevent oxidation.

On the basis of these findings, it could be concluded that at least part of the CGAs present in green beans is incorporated into HMw and IMw coffee materials as a result of the roasting process. It was found that the ester-linked QA level was around 3-4 times higher than the ester-linked CA level, whereas QA and CA are present in a ratio of around 1:1 in CGAs. This observation might be ascribed to the fact that QA is less prone to oxidative changes during roasting (*27*).

QA, CA, and FA in the AGP-Melanoidin Complex.

Previously, it was shown that AGPs isolated from roasted coffee brew were part of a melanoidin complex (*20*). Because of the specificity of the Yariv isolation procedure, it could be concluded that this fraction was a pure AGP-melanoidin complex. As expected, it was

found that HMw AGPs did not contain any unbound 5-caffeoylquinic acid, QA, CA, or FA. After alkaline hydrolysis, the levels of ester-bound QA, CA, and FA (**Table 4**) were found to be 0.9, 0.2, and 0.02%, respectively. Because this fraction was a pure melanoidin population, and QA and CA were present in this fraction, the conclusion is that these ester-linked acids are incorporated in the melanoidin complex. It is not known how these CGA derivatives are bound within the AGP-melanoidin complex. Both covalent binding of phenolic compounds to sugar (*28*) and protein (*29*) were reported. Additionally, it is known that arabinose from arabinogalactan is quite susceptible to degradation upon roasting (*30, 31*). Therefore, arabinose might be a possible binding site for CGA derivatives. At this moment, it is unclear whether CGA is first split into QA and CA upon roasting and then incorporated in melanoidins or whether intact CGA is incorporated. In the case of intact CGA incorporation, it also remains to be determined whether the intact CGA molecule is connected through the QA or CA moiety to melanoidins.

To the best of our knowledge, this is the first time that it can be decisively concluded that CGAs are involved in melanoidin formation upon roasting of coffee beans. Both building blocks of CGA, QA and CA, are incorporated in melanoidins although at different levels.

Table 4. Ester-Bound QA, CA, and FA Levels Not from CGA in Various Coffee Fractions

	$K_{mix\ 405}$ (L/g/cm)	Phenolic groups (%, w/w) [a]	QA (%, w/w) [b]	CA (%, w/w) [c]	FA (%, w/w) [c]
HMw-AGP	1.1	10	0.92	0.19	0.02
HMw-EP20	0.5	6	1.04	0.40	0.04
HMw-EP40	0.7	8	0.97	0.36	0.03
HMw-EP60	0.6	11	1.52	0.56	0.05
HMw-EP80	1.2	17	2.12	0.63	0.06
HMw-ES80	2.5	26	3.47	0.58	0.05
HMw-A1	0.2	2	0.07	0.03	0.01
HMw-A2	0.3	4	0.58	0.11	0.01
HMw-A3	0.9	6	0.76	0.09	0.01
HMw-A4	1.2	9	0.99	0.07	0.01
HMw-A5	1.4	10	0.98	0.07	0.01
HMw-A6	0.7	5	0.32	0.03	0.00
IMw-A1	0.3	2	0.07	0.11	0.02
IMw-A2	0.9	8	0.85	0.24	0.03
IMw-A3	1.6	12	1.58	0.29	0.04
IMw-A4	2.6	17	2.55	0.29	0.03
IMw-A5	2.7	12	3.25	0.39	0.04
IMw-A6	1.0	4	1.86	0.27	0.03

[a] As 5-caffeoylquinic acid equivalents. [b] The coefficient of variation was 3% on average. [c] The coefficient of variation was 1% on average.

5-Caffeoylquinic Acid, QA, CA, and FA in Ethanol Precipitation Fractions.

HMw Brew was previously fractionated by ethanol precipitation, and it was found that fractions which were soluble at high ethanol concentration had the highest melanoidin levels (*4*). Furthermore, a relation between the phenolic groups level and the melanoidin level was observed (*4*). The ethanol precipitation fractions did not contain any unbound 5-caffeoylquinic acid, QA, CA, or FA, which was expected. The ester-bound QA level varied significantly, ranging from 1% in HMw EP20 to 3.5% in HMw ES80 (**Table 4**). The ester-bound CA and FA levels were quite low and showed a less-pronounced differentiation over the ethanol precipitation fractions; the CA levels were between 0.36 and 0.63%, and the FA levels were between 0.03 and 0.06% (**Table 4**). Such low levels were not expected because these fractions contain many phenolic groups as measured by the Folin-Ciocalteu assay (**Table 4**). Because CGAs are the predominant source of phenolic acids in green beans, it is expected that the phenolic groups in HMw and IMw fractions are due to the incorporation of CA and FA. This CA and FA incorporation occurs in such a way that CA and FA are not ester-linked and might be due to oxidation of these polyphenolics upon roasting (*27*).

On the basis of the results found so far, several options for the CGA incorporation can be defined. First, CGA might be first hydrolyzed, QA might then be separately incorporated through ester linkages, and CA might be incorporated mainly through nonester linkages. Second, intact CQA might be incorporated via the QA moiety through an ester linkage, after which most of the CA is oxidized or degraded. Third, intact CQA might be incorporated via the CA moiety mainly through nonester linkages. The nonester linkage of CA to the melanoidin backbone might be the result of single or multiple condensation reactions. In the case where CA is condensated into the melanoidin complex, it might well be that the conjugated ring structure of CA contributes to the observed brown color of melanoidins. Furthermore, the pronounced distribution of QA suggests that the incorporation of QA from CGAs occurs at specific types of molecules.

5-Caffeoylquinic Acid, QA, CA, and FA in Anion-Exchange Chromatography Fractions.

HMw and IMw brews were previously fractionated by anion-exchange chromatography, and it was found that brew melanoidins are negatively charged (*20*). The ester-linked 5-caffeoylquinic acid, QA, CA, and FA levels in these fractions were of special interest, because the incorporation of these acids might contribute to this negative charge. The levels of the ester-bound QA, CA, and FA, not present as free acids or in intact CGAs, are shown in **Table 4**. It is noticeable that the ester-linked acids levels in all anion-exchange fractions were lower than the levels in the parent fractions, indicating that part of the ester-bound acids was not recovered. Supposedly, this is due to the fact that these acids are linked to melanoidins that have such a strong negative charge that these complexes did not elute from the column. This is evident from the fact that the melanoidin recovery of this fractionation step was not optimal either, being 67% for HMw and 72% for IMw fractions (*20*). Nevertheless, the fractions differing in charge (A1-A6) represent the majority of the melanoidins and were still of special interest, because their esterlinked acid levels might provide information on the

binding mechanism of the CGA incorporation. As can be seen in **Table 4**, similar results were obtained for both the HMw and IMw anion-exchange series. The ester-bound QA level initially increased (A1→A4/A5) and subsequently decreased (A6). This increase confirmed that QA might indeed be, at least partly, responsible for the observed anionic properties of the melanoidins. This automatically implies that the carboxyl group of QA is not involved in CGA incorporation. The low level of esterlinked QA in fraction A6 indicated that a component other than QA is present in this fraction and provides the anionic character. As the melanoidin level in both A6 fractions was low too (*20*), it is expected that another negatively charged nonmelanoidin component is present in these fractions. With respect to the esterlinked CA and FA, observations similar to those for ethanol precipitation fractions from HMw fractions were made (**Table 4**): CA and FA levels were much lower than the QA levels and showed a less-pronounced differentiation over the fractions. Thus, CA and FA levels, again, did not match with QA levels.

These results indicate that the carboxyl group of QA is not primarily involved in CGA incorporation. On the basis of this finding, it can be stated that the second option given for CGA incorporation, that is, intact CGA incorporation via QA, is not the most prevalent reaction. Because CA levels were relatively low as well, it is most likely that intact CGA is incorporated into melanoidins via the CA moiety through nonester linkages, or that CGA is first split, and QA and CA are then separately incorporated.

Free 5-Caffeoylquinic Acid, QA, CA, and FA Levels in Green Beans, Roasted Beans, and Coffee Brew.

The HMw and IMw fractions and subfractions were shown to contain esterlinked CGA derivatives in melanoidin structures. However, it should be realized that HMw and IMw fractions together represented 32% (w/w) of the Brew (*20*), leaving 68% (w/w) not studied so far. Furthermore, the LMw fraction still contained 40% of the melanoidins (*20*). To be able to place the values for HMw and IMw fractions in a broader perspective, the 5-caffeoylquinic acid, QA, CA, and FA levels in green beans, roasted beans, and Brew were determined as well. The reversed-phase HPLC elution pattern of the Brew (**Figure 3A**) showed several peaks, among which the largest peak was identified as 5-caffeoylquinic acid. The molecules causing the other peaks were not identified but were expected to be due to other CGAs (*16, 32*). The 5-caffeoylquinic acid level was 3.0% in green beans, 0.7% in roasted beans, and 2.9% in the Brew (**Table 1**). These results were in agreement with the literature; Perrone et al. (*16*) found 3.5% 5-caffeoylquinic acid in green Arabica beans and 0.7% 5-caffeoylquinic acid in roasted Arabica beans (degree of roast, 16%). No or only trace amounts of free CA and FA were found in green beans, roasted beans, and the Brew, which was also in agreement with the literature (*33*). The free QA level was initially determined by ion-moderated partitioning HPLC, and the elution pattern of the Brew is shown in **Figure 3B**. The observed peak for QA corresponds to a level of 3.0% of QA in the Brew. The free QA level was also determined by GC after silylation because no baseline separation was obtained for QA by using ion-

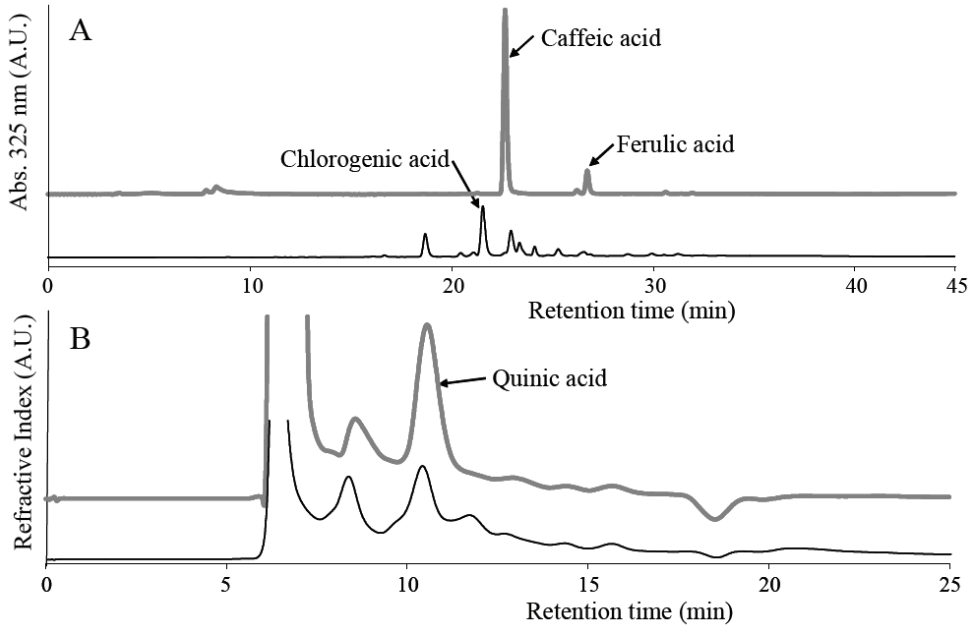

Figure 3. (**A**) Reversed-phase and (**B**) ion-moderated partitioning HPLC elution patterns of Brew (thin black line) and saponified Brew (thick gray line).

moderated partitioning HPLC. The free QA level in the Brew determined after silylation was calculated to be 3.3%. Thus, both techniques gave similar free QA levels, and the QA levels for the beans and the Brew were in agreement with the literature (*14, 34*).

Total QA, CA, and FA Levels in Green Beans, Roasted Beans, and Coffee Brew.

The reversed-phase HPLC elution pattern of the saponified Brew (**Figure 3A**) showed two main peaks which were identified as CA and FA. The peaks observed in the elution pattern of the untreated Brew were not present in the elution pattern of the saponified Brew anymore, confirming that these peaks were indeed from other CGAs. The saponified green beans, roasted beans, and Brew contained 3.3, 1.7, and 4.6% CA and 0.3, 0.2, and 0.6% FA, respectively (**Table 2**). The total QA level was determined by ion-moderated partitioning HPLC, and the elution patterns of the saponified Brew is shown in **Figure 3B**. The total QA level was 3.9% in green beans, 3.4% in roasted beans, and 11.8% in Brew (**Table 2**). For confirmation, the total QA level in the Brew was determined by GC after silylation and was precisely 11.8% as well. For green beans, it stands out that the level of ester-linked QA (3.3%, total - free) was similar to the levels of ester-linked CA and FA (3.6%), indicating that these ester-linked acids were originating from CGAs. The 11.8% total QA was unexpectedly high; such levels have not been reported so far. The distribution of these acids over the various coffee components will be discussed after estimation of the CGAs level.

Enzymatic Degradation of Coffee Brew Material.

The applied saponification procedure did not allow the detection of intact CGA in melanoidins because the internal ester bond in CGA is hydrolyzed as well. It was reasoned that, if intact CGA is incorporated in melanoidins, the internal ester linkage might still be

intact. This binding might be selectively split by enzymes. As enzymes have high substrate specificity, a release of QA, CA, or FA from coffee melanoidin material would indicate that the binding between CGA's nonphenolic and phenolic moieties is still intact. This would then strongly indicate that intact CGA is incorporated in melanoidin structures. To this end, Brew and HMw and IMw fractions were incubated with chlorogenate esterase, and the quantities of QA, CA, and FA released were measured (**Table 5**). Even though the enzyme used accepts few other phenolic acid-based substrates as well, it is still quite specific for CGAs (*22, 23*). However, because CGAs are the only source of CA esters in coffee beans, a release of CA would strongly indicate that intact CGA is incorporated in melanoidins. It was found that the enzyme released 2.3% of QA, 3.8% of CA, and 0.3% of FA from the coffee Brew. The fact that no or negligible amounts of free CGAs were present after enzyme incubation of the Brew showed that CGAs were indeed a substrate that was effectively split. The enzyme incubation released 0.2% of QA, 0.1% of CA, and no FA from the HMw fraction. Thus, 12 and 20% of the ester-linked QA and CA were released, respectively (**Tables 3** and **5**). Enzymatic degradation of the IMw fraction yielded 0.1% of QA and 0.4% of CA, representing 3% of the QA and 44% of the CA that were present in the IMw fraction in ester linkages. The observed release of QA and especially CA from HMw and IMw fractions indicated that the ester linkage between the phenolic and nonphenolic moieties of CGA was still intact, and thereby, that intact CGA was incorporated. Relatively more CA than QA was released, whereas the QA level was larger than the CA level, indicating that the enzyme required ester-bound CA from CGA in its active cleft. This implies that a small part of the incorporated CGAs are linked via QA to the melanoidins.

It was shown by Delgado-Andrade et al. (*11*) that part of the antioxidant activity of melanoidins could be due to components that are ionically bound to melanoidins (*11*); these components might be CGAs. One could argue that the QA and CA released by the saponification procedure were not released because of the cleavage of ester bonds but were ionically bound and were released because of the high ionic strength of the 2 M NaOH solution. The fact that the enzyme treatment, conducted by using a low ionic strength, yielded both QA and CA shows that these molecules were really ester bound.

Table 5. QA, CA, and FA Released After Enzymatic Degradation of Coffee Fractions with Chlorogenate Esterase[a]

	QA (%, w/w)[b]	CA (%, w/w)[c]	FA (%, w/w)[c]
Brew	2.3	3.8	0.3
HMw	0.2	0.1	0.0
IMw	0.1	0.4	0.0

[a] After correction for free acids level. [b] The coefficient of variation was 3% on average. [c] The coefficient of variation was 1% on average.

CGAs in Green Beans and Brew.

To be able to discuss the fate of QA, CA, and FA present in coffee brew, it is necessary to estimate the extent in which these molecules are present in CGAs. Because the precise level of the different CGAs was unknown, estimations were made. The actual CGAs level in green beans was estimated by using the total and free QA, CA, and FA levels (Σ total acids - Σ free acids). This level for QA (3.3%) was similar to the combined CA and FA levels (3.6%), indicating that these ester-linked acids were solely from CGAs. On the basis of these values, the level of CGAs in green beans would be 6.9%, which is in agreement with values reported in the literature (*16*). It is impossible to calculate the CGAs level for the Brew in a similar manner, because a part of the QA, CA, and FA was present in CGAs, and a part was incorporated in melanoidins. Alternatively, as the enzyme treatment degraded almost all CGAs in the Brew, the actual level of CGAs could be estimated by using the level of QA released by the enzymes. The enzyme incubation released 2.3% of QA from the Brew, from which it was calculated that the total CGAs level should be around 4.2% when all CGAs are monocaffeoylquinic acids.

Distribution of QA and CA in Coffee Brew over Various Components.

It was never reported so far that the total QA level in coffee brew could be as high as 11.8%. The total QA in brew is present as free QA, QA in CGAs, QA incorporated in HMw and IMw materials, and quinides. Quinides are QA lactones that are formed during roasting and are present up to levels of 2.0% in roasted beans (*34*). However, the quinide level is rather low in coffee brew because of hydrolysis of the internal ester during the extraction process, leaving quinide levels of 0.2–0.4% in the coffee brew (*35*). HMw and IMw fractions each represented 16% (w/w) of the brew (*20*). By using these isolation yields as well as their ester-linked QA levels (**Table 3**), it was calculated that HMw and IMw fractions together account for 0.9% of QA in coffee brew (percent of brew solids). The 11.8% of total QA in brew is distributed as follows: (I) 3.0–3.3% of free QA, (II) 2.3% of QA from 4.2% CGAs, (III) 0.9% of QA ester-linked to HMw and IMw materials, and (IV) 0.2–0.4% of QA from quinides (*34, 35*). In total, 6.9% of QA in the Brew was accounted for, and the remaining 4.9% should be ester linked to LMw coffee components such as melanoidins.

It was calculated that the ester-linked CA in HMw and IMw fractions together accounted for 0.2% of CA in coffee brew (percent of brew solids). The 4.6% of total CA in brew (**Table 2**) is distributed as follows: (I) 0.1% of free CA, (II) ~2.1% of CA from 4.2% CGAs, and (III) 0.2% of CA ester-linked to HMw and IMw materials (percent of brew solids). In total, 2.4% of CA was accounted for, and the remaining 2.2% should be ester-linked to LMw coffee components such as melanoidins.

Correlation of Melanoidin, Phenolic Groups, and CGA Derivatives Levels.

It was shown that CA, FA, and especially QA were incorporated in HMw and IMw melanoidins through ester linkages. It was of importance to determine whether the level of these acids showed a correlation with the melanoidin level and/or the phenolic groups level. Therefore, the phenolic groups level, the melanoidin level ($K_{\text{mix 405 nm}}$), and the esterlinked

QA, CA, and FA levels were plotted as a function of the HMw ethanol precipitation fractions (**Figure 4A**) and HMw and IMw anion-exchange fractions (**Figure 4B**,**C**). These figures show that the ester-linked QA level correlates with the melanoidin level as well as with the phenolic groups level. Because CGAs are the predominant phenolics in coffee, it is believed that the presence of these phenolics in HMw and IMw fractions is the result of incorporation of CA and FA from CGAs. However, no correlation was observed between the ester-bound CA and FA levels and the phenolic groups level as determined by the Folin-Ciocalteu assay. Therefore, it is expected that CA and FA are responsible for the observed phenolic groups level, and also that these phenolic acids are mainly linked through nonester linkages. Because the ester-linked QA level correlated with the phenolic groups level, it is expected that the nonphenolic and phenolic moieties from CGAs are incorporated together. In other words, these findings indicate the incorporation of intact CGAs in coffee material. If CGAs were hydrolyzed first, the observed correlation between QA and the phenolic groups levels would indicate that both QA and CA or FA are incorporated in a similar extent in various series of fractions. Because QA and CA or FA have different chemical properties, it is rather unlikely that this occurred. Therefore, the proposed incorporation of intact CGAs seems more logical. The fact that the enzyme treatment with chlorogenate esterase released some QA and CA further strengthened the fact that the linkage between QA and CA in incorporated CGAs can survive roasting.

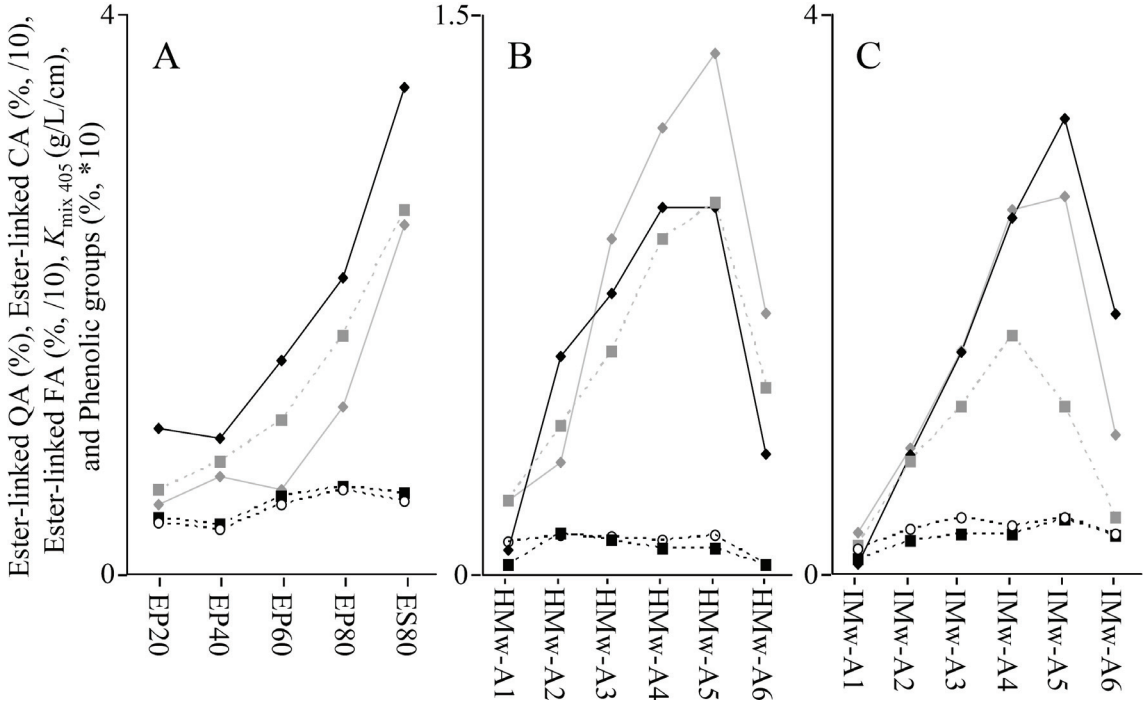

Figure 4. Ester-linked QA levels (black solid line, solid diamonds), ester-linked CA levels (black dashed line, solid squares), ester-linked FA levels (black dashed line, open circles), melanoidin levels (gray solid line, solid diamonds), and phenolic groups levels (gray dashed line, solid squares) in various Brew fractions plotted as a function of (**A**) ethanol solubility, (**B**) negative charge in HMw material, and (**C**) negative charge in IMw material.

On the basis of these results, it can be proposed that the CGA incorporation mechanism comprises incorporation of intact CGA molecules into melanoidins via the CA moiety mainly through nonester linkages upon roasting of coffee beans. This complex can be written as Mel=CA-QA, in which Mel represents the melanoidin backbone, =CA represents CA nonester linked to the melanoidin backbone, and -QA represents QA ester-linked to CA. Within these structures, the nonester-linked CA provides phenolic characteristics, whereas the free carboxyl group from QA contributes to the observed negative charge. However, it is unclear whether the part of QA that is ester-linked to the CA moiety is split off upon further roasting.

Comparison of the Proposed CGA Incorporation Mechanism with the Literature.

Nunes and Coimbra (*15*) recently reported that alkaline fusion released ~3% of phenolics from a HMw coffee material. Their HMw fraction was comparable to the combined HMw and IMw fractions used in this study. The released phenolics by alkaline fusion were degradation products similar to those from CA, strongly indicating that CA was incorporated in HMw coffee material. The level of 3% of phenolics did not match with the relatively low level of CA released by saponification as reported herein. The difference between alkaline fusion and conventional saponification is that alkaline fusion hydrolyzes other linkages than ester linkages too, such as double bonds and ether bindings (*15, 19, 36*). On the basis of this difference, the results of Nunes and Coimbra actually complement the proposed hypothesis. The saponification step only releases ester-linked QA from Mel=CA-QA, whereas nonester-linked CA is hardly split off. The ester-linked QA level of the combined HMw and IMw fractions was 2.6%. Alkaline fusion is capable of releasing CA from Mel=CA because alkaline fusion degrades more types of bindings. If the combined HMw and IMw fractions were subjected to alkaline fusion, a CA level of 2.6% would be expected, which would be as large as the ester-linked QA level. This level is quite similar to the level of 2.7–3.2% that was reported by Nunes and Coimbra. Thus, their findings support the incorporation of CGA via CA through a nonester linkage.

In a mechanistic study on whole coffee beans, Leloup et al. (*14*) also proposed that intact CGA was incorporated in coffee bean material. However, Leloup et al. suggested that CGA was incorporated via the carboxyl group on QA. In their model, the low level of ester-linked CA, relative to the level of ester-linked QA, was ascribed to the fact that CGA incorporation was followed by hydrolysis of the internal CGA, yielding LMw phenols from CA. The results, on which their incorporation mechanism is based, are also in agreement with the incorporation mechanism proposed by us. However, in their study, no attention was paid to, for instance, the phenolic groups level and the nonester-linked CA and FA levels. Results presented in this study and results reported by Nunes and Coimbra point out that the phenolic moiety should be present in the melanoidin structure, and that it is therefore more likely that CGA is incorporated via CA. This is further strengthened by the fact that thermal degradation of HMw coffee material yielded a broad series of phenolic compounds as well (*10, 12*).

Influence of CQA Incorporation on Physico-chemical Properties of Coffee Brew.

It was found that coffee brew contained 12% of QA, about half of which could be ascribed to free QA, quinides, and QA in CGAs. The additional 6% ester-linked QA was not reported so far and might have significant effects on the physico-chemical properties, such as coffee acidification. So far, acidification is ascribed mainly to a release of QA because of hydrolysis of CGAs and quinides upon storage. However, the increase in QA and other LMw acids can not fully account for the increase in acidity (*37, 38*). Now, with the additional 6% ester-linked QA in coffee brew, additional possibilities for coffee acidification are possible. For example, it might be that the ester-linked QA is actually a quinide, or that the ester-linked QA is covalently linked to CA as well as to another molecule via its carboxyl group. In both cases, hydrolysis that occurs upon storage leads to an increase of carboxyl groups and acidity, without affecting the free QA level. Therefore, it might well be that melanoidins are an important contributor to coffee acidification.

Further research, in which the releases of phenolics by saponification and alkaline fusion are compared, might lead to complete understanding of the CGA incorporation mechanism. Furthermore, investigation of the effect of the roasting degree on CGA incorporation in similar coffee brew fractions is expected to provide more insight in the kinetics of CGA incorporation in coffee brew melanoidins.

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CHAPTER 5

Low Molecular Weight Melanoidins in Coffee Brew

ABSTRACT

Analysis of low molecular weight (Mw) coffee brew melanoidins is challenging due to the presence of many non-melanoidin components that complicate analysis. This study focused on the isolation of low Mw coffee brew melanoidins by separation of melanoidins from non-melanoidin components that are present in low Mw coffee brew material. Low Mw coffee fractions differing in polarity were obtained by reversed-phase solid phase extraction and their melanoidin, sugar, nitrogen, caffeine, trigonelline, 5-caffeoylquinic acid, quinic acid and caffeic acid and phenolic groups contents were determined. The sugar composition, the charge properties, and the absorbance at various wavelengths were investigated as well. The majority of the low Mw melanoidins were found to have an apolar character while most non-melanoidins have a polar character. The three isolated melanoidin-rich fractions represented 56% of the low Mw coffee melanoidins and were free from non-melanoidin components. Spectroscopic analysis revealed that the melanoidins isolated showed similar features as high Mw coffee melanoidins. All three melanoidin fractions contained ~3% nitrogen indicating the presence of incorporated amino acids or proteins. Surprisingly, glucose was the main sugar present in these melanoidins and it was reasoned that sucrose is the most likely source for this glucose within the melanoidin structure. It was also found that low Mw melanoidins exposed a negative charge and this negative charge was inversely proportional to the apolar character of the melanoidins. Phenolic groups levels as high as 47% were found, which could be explained by the incorporation of chlorogenic acids in these melanoidins.

KEYWORDS

Coffee; melanoidins; low molecular weight; solid phase extraction; anion exchange chromatography

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INTRODUCTION

Coffee melanoidins are an abundant group of compounds that are formed during the roasting process of coffee beans (*1*). Melanoidins are generally defined as nitrogenous, macromolecular, brown-colored, final Maillard reaction products that absorb light at 405 nm (*1-3*). The chemical structure of melanoidins is largely unknown due to its complex composition. This is evident from the fact that heating of one reducing sugar in the presence of one amino acid already yields numerous Maillard reaction products (*4*). In real food systems, the chemical structure of melanoidins is further complicated due to the presence of a wide range of reactants. In coffee beans, different reducing sugars are present and these sugars can be present in various forms (mono-, di-, oligo-, and polysaccharides), the same holds true for the amino acids (*5*). Additionally, other compounds like chlorogenic acids were shown to be involved in melanoidin formation as well (*6, 7*).

In coffee brew, melanoidins may account for up to 25% of the dry matter (*1*). Most of the research on coffee brew melanoidins has been devoted to macromolecular melanoidins (*8-12*), which account for 59% of all coffee melanoidins (*8*). Even though 41% of the coffee melanoidins have a low molecular weight (Mw), relatively little research has been dedicated to these low Mw melanoidins. This might be ascribed to the presence of many other low Mw compounds (e.g. caffeine, chlorogenic acids, trigonelline, minerals) in coffee brew. To circumvent this complexity, model systems are often used for the investigation of structural properties of low molecular Maillard reaction intermediates (*13-17*). As research in this field is sophisticated, most of it is dedicated towards single type of reaction intermediates and do not cover the whole range of melanoidin formation mechanisms. However, one should be extremely careful with extrapolating results of model systems to complex foodstuffs like coffee beans. As a result, no or not much information is known on the overall structural properties of low molecular coffee melanoidins.

Previously, it was proposed that melanoidins are polymers built up from repeating units of furans and/or pyrroles linked by polycondensation reactions in the advanced stages of the Maillard reaction (*18, 19*). This implies that melanoidins start off as low molecular compounds, which polymerize upon prolonged roasting. Thus, even if one does not consider low molecular brown-colored compounds as melanoidins, it is important to obtain knowledge on these compounds as they might be Maillard reaction intermediates ending up in the final macromolecular melanoidins. The proposed furan/pyrrole-like structure suggests that low molecular melanoidins are apolar compounds.

The aim of this research was to gain insight in the structural properties of low Mw coffee brew melanoidins. To this end, low Mw coffee brew material was fractionated depending on polarity and isolated fractions were characterized.

MATERIALS AND METHODS

Materials.

Roasted Colombian coffee beans (*Coffea arabica*) were provided by a local factory. The degree of roast was 16.4% (w/w), which was 8.0% (w/w) on a dry matter basis. Chemicals were bought from Sigma Aldrich (Sigma Chemical Corp., St. Louis, MO) in the highest purity available.

Preparation of Coffee Brew.

Coffee beans were ground, and a brew was prepared as described previously (*2*). Briefly, 600 g water was added to 100 g milled beans and this mixture was heated for 15 min at 90 °C, followed by filtration to obtain the brew. For characterization purposes, part of the brew was lyophilized, yielding "brew". The major part of the brew was used for further isolation.

Isolation of High and Low Mw Material from Brew.

High and low Mw material were isolated from 2 L freshly prepared coffee brew by membrane dialysis (MW cutoff 12-14 kDa, Visking size 9, Medicell International Ltd, London, UK) against 6 L demineralized water with three water renewals at 4 °C for 4 days. In total, four 6L dialysate-batches were used for dialysis to assure that the retentate was free from LMw material. Small scale dialysis experiments revealed that the first two 6 L dialysate-batches should contain 95% of the LMw material. The last 2 dialysate-batches, which should contain only the last 5% of LMw material, were discarded to minimize possible artifacts induced by prolonged dialysis. Prolonged dialysis was conducted to assure that the HMw fraction would be really free from low Mw material. The retentate and the first two dialysates were lyophilized, yielding a high Mw fraction "HMw" and a low Mw fraction "LMw" (**Figure 1**).

Defatting of Coffee Samples.

Lyophilized coffee brew samples were defatted by Soxhlet extraction using a Soxtherm, which was connected to a Multistat system (Gerhardt, Königswinter, Germany), as described previously (*8*). The solvent used for extraction was dichloromethane.

Solid Phase Extraction of the Low Mw Fraction.

Defatted LMw material was fractionated by reversed-phase (RP) solid phase extraction (SPE) using a 5 g Sep-Pak Vac 20cc C18 cartridge (Waters, Milford, MA). The RP material in the cartridges conditioned prior to usage by rinsing with 50 mL methanol and subsequently with 50 mL Millipore water. Aqueous sample solution (10 mL, 10 mg/mL) was loaded on the cartridge and material that did not bind to the RP matrix was washed from the cartridge by 100 mL demineralized water. Subsequently, bound material was washed from the column in steps using 100 mL 10% aqueous methanol, 100 mL 20% aqueous methanol, 100 mL 40% aqueous methanol, 100 mL 60% aqueous methanol, 100 mL 100% methanol, and 100 mL water. This procedure was repeated 10 times, after which 1.1 g LMw material was fractionated. For each methanol concentration, the effluents were collected, pooled, and methanol was evaporated using a rotary evaporator operating at 40 °C. The fractions were subsequently lyophilized yielding SPE-0, SPE-10, SPE-20, SPE-40, SPE-60, and SPE-100, for the fractions that eluted at 0, 10, 20, 40, 60, and 100% methanol, respectively (**Figure 1**).

Determination of the Content of Various Low Mw Coffee Components.

The level of 5-caffeoylquinic acid (5-CQA), caffeic acid (CA), caffeine, and trigonelline were determined using reversed-phase (RP) high performance liquid chromatography (HPLC). Aqueous sample solution (1 mg/mL) was centrifuged and the supernatant was analyzed using a 150 x 4.6 mm i.d. XTerra® MS C18 3.5 µm column (Waters, Milford, MA) with a 20 x 3.9 mm i.d. XTerra® MS C18 3.5 µm guard column (Waters) as

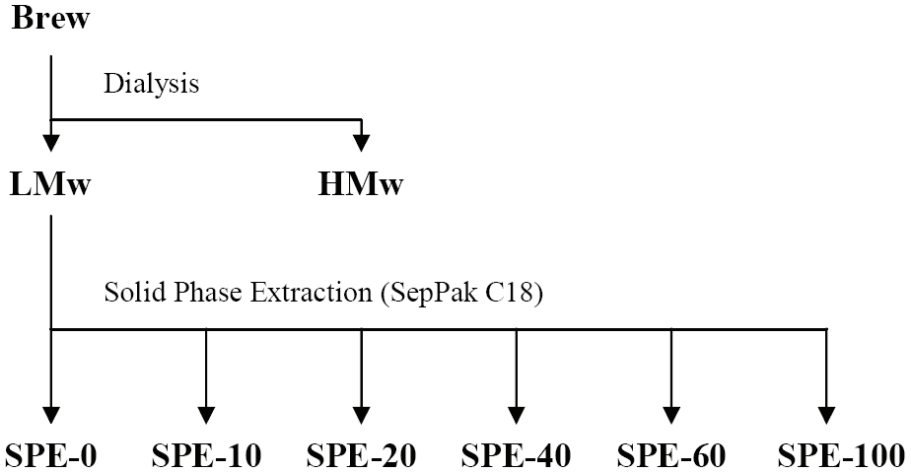

Figure 1. Scheme for the isolation of various LMw melanoidin coffee fractions from Brew.

described previously (*7*). The absorbance of the eluate was measured at 280, 325, and 405 nm using a Spectra System UV3000 (Thermo Electron Company, Waltham, MA). The level of trigonelline and caffeine was determined by their absorbance at 210 and 280 nm, respectively, while the level of 5-CQA and CA was determined by their absorbance at 325 nm. The reference compounds, bought from Sigma Aldrich, were dissolved in Millipore water: caffeine (100 μg/mL), trigonelline (100 μg/mL), 5-CQA (100 μg/mL), and CA (25 μg/mL). Experiments were performed at least in duplicate. The coefficient of variation was 1% on average for all experiments using reversed-phase chromatography.

Determination of Quinic Acid by Ion-Moderated Partitioning HPLC.

The level of quinic acid (QA) was determined using ion-moderated partitioning HPLC. Aqueous sample solution (1 mg/mL) was centrifuged and the supernatant was analyzed using a 300 x 7.8 mm i.d. Aminex HPX 87H column equipped with a cation H+ guard column filled with AG® 50W-X4 (Bio-Rad, Hercules, CA) using the procedure described previously (*7*). An aqueous QA solution was used as reference compound and experiments were performed at least in duplicate. The coefficient of variation was 3% on average for all experiments using ion-moderated partitioning chromatography.

Sample Preparation for Total CA Determination.

For determination of the total CA level, samples were saponified prior to analysis using the procedure described previously (*7*). Analysis was performed by RP-HPLC as described above. Saponified CA was used as reference compounds and experiments were performed at least in duplicate.

Sample Preparation for Total QA Determination.

For determination of the total QA level, samples were saponified prior to analysis using the procedure described previously (*7*). Analysis was performed by ion-moderated partitioning HPLC as described above. Saponified QA was used as reference compound and experiments were performed at least in duplicate.

Anion Exchange Chromatography of Low Mw Fractions.

Anion exchange chromatography (AEC) was performed based on a procedure described previously (*8*). A 1 mL HiTrap Q Fast-Flow column (Amersham Biosciences, Uppsala, Sweden) was connected to an ÄKTA Purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden). The absorbance of the eluate was measured at 280, 325, and 405 nm using the UV-900 detector. Three eluents were used: A) 5 mM NaOAc, pH 5.1; B) 2 M NaOAc, pH 5.1; and C) 2 M NaCl in 5 mM NaOAc, pH 5.1. Sample was dissolved in Millipore water (20 mg/mL) and 500 µL was applied onto the column with a flow rate of 1 mL/min. The elution profile consisted of: isocratic elution with 15 mL eluent A, in 0.15 mL to 7.5% eluent B followed by 15 mL isocratic elution, in 0.15 mL to 30% eluent B followed by 15 mL isocratic elution, in 0.70 mL to 100% eluent B followed by 15 mL isocratic elution, in 1.5 mL back to 100% eluent A followed by 10 mL isocratic elution, in 0.5 mL to 50% eluent C followed by 15 mL isocratic elution, in 0.5 mL to 100% eluent C followed by 15 mL isocratic elution. After each run, the column was regenerated by elution with 0.5 M NaOH (20 mL), eluent B (20 mL) and eluent A (20 mL).

Preparative Anion Exchange Chromatography of SPE-40.

Anion exchange chromatography was conducted using a 5 mL HiTrap Q Fast-Flow column (Amersham Biosciences) connected to an ÄKTA Purifier system (Amersham Pharmacia Biotech). The absorbance of the eluate was measured at 280, 325, and 405 nm using the UV-900 detector. Dry SPE-40 was dissolved in Millipore water (350 μg/mL) and 30 mL was injected onto the column with a flow rate of 5 m mL/min. Material not interacting with the column was collected by isocratic elution with 100 mL 5 mM NH_4Ac, pH 5.1. Subsequently, the eluent was changed in 5 mL to 4 M NH_4Ac, pH 5.1 followed by 300 mL isocratic elution while collecting the effluent containing negatively charged components. The column was regenerated by elution with 2 M NaCl (100 mL), 0.5 M NaOH (100 mL), 4 M NH_4Ac, pH 5.1 (100 mL), and 5 mM NH_4Ac, pH 5.1 (100 mL). The fractions collected were subjected to rotary evaporation at 40 °C and freeze drying, yielding "SPE-40 AEC–" and "SPE-40 AEC+" for the material that eluted at 5 mM and 4 M NH_4Ac, respectively.

Total Phenolic Groups Level.

The total phenolic groups content of the coffee samples were determined with the Folin-Ciocalteau assay as described previously (*2*). The used reference compound was 5-CQA.

Analysis of Nitrogen Content.

The nitrogen content of various samples was estimated according to the Dumas method using an NA2100 nitrogen and protein analyzer (Carlo Erba Instruments, Milan, Italy) according to the manufacturer's instructions. Methionine was used as a standard.

Sugar Analysis.

The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (*20*) using inositol as an internal standard. Briefly, samples were pre-hydrolyzed with 72% (w/w) H_2SO_4 for 1 h at 30 ºC, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 ºC and the constituent sugars released were analyzed as their alditol acetates. The total neutral sugar content was also determined by the phenol sulfuric acid assay according to Dubois et al. (*21*). The uronide content was determined by the automated colorimetric *m*-hydroxydiphenyl method (*22, 23*).

Specific extinction coefficient of coffee material at 280, 325, and 405 nm.

Coffee fractions were dissolved in water (1 g/L) and the absorption was determined at 280, 325, and 405 nm using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). The coffee solutions were further diluted in case the absorption was higher than 1.3. The specific extinction coefficients K_{mix} (L/g/cm) were calculated as was previously described by Bekedam et al. (*2*).

RESULTS AND DISCUSSION

Yield and Melanoidin Levels of Coffee Brew Fractions.

The coffee brew extraction procedure yielded 19% dry matter in the brew (**Table 1**), which was in line with previous results (*2*). The membrane dialysis procedure yielded 37% HMw material which was somewhat higher than the 32% that was expected (*2*). The majority of the brew was found to be low molecular weight material and 51% of the brews dry matter was recovered in the LMw fraction. The fact that the recovery of the dialysis procedure was 88% is caused by discarding the last 2 water renewals, which contained part of the low molecular weight brew material. The $K_{mix\ 405nm}$ value is an indication for the melanoidin content (*2*) as will be explained below and was 0.4 for LMw and 1.3 for HMw (**Table 1**), showing that the melanoidin content was low in the LMw material and high in the HMw material. The low melanoidin content confirmed that quite some non-melanoidin material was present in the LMw fraction. The percentage melanoidins in a fraction was calculated using both the isolation yield and the $K_{mix\ 405nm}$ values: $[\text{yield}*K_{mix\ 405nm}]_{fraction}/[K_{mix\ 405nm}]_{basis}$. For example, the percentage melanoidins from brew ending up in the HMw fraction was calculated by

Table 1. Yield, K_{mix} Values, Percentage Melanoidins, and K_{mix} Ratios of Brew, HMw, LMw, and SPE-0 to SPE-100

	Yield (%, w/w)[a]	$K_{mix\ 405nm}$ (L/cm/g)	$K_{mix\ 325nm}$ (L/cm/g)	$K_{mix\ 280nm}$ (L/cm/g)	Melanoidin (%)[a]	$K_{mix\ 325:405nm}$ ratio (-)	$K_{mix\ 280:405nm}$ ratio (-)
Brew	19[b]	0.7	8	9	100[c]	11	13
HMw	37[c]	1.3	5	6	69[c]	4	5
LMw	51[c]	0.4	9	10	29[c]	23	25
SPE-0	60	0.1	5	4	15	50	40
SPE-10	11	0.4	24	17	11	60	43
SPE-20	8	0.6	10	22	12	17	37
SPE-40	11	0.9	16	28	25	18	31
SPE-60	4	3.4	14	18	34	4	5
SPE-100	1	1.8	4	6	5	2	3

[a] Percentage from LMw. [b] Percentage from beans. [c] Percentage from Brew.

37*1.3/0.7 = 69%. Likewise, it was calculated that 29% of the melanoidins present in the coffee brew ended up in the LMw fraction.

The LMw material was fractionated by RP-SPE with the aim to separate the presumed apolar melanoidins from the more polar compounds like sugars, caffeine, chlorogenic acid, trigonelline, and minerals (**Figure 1**). The majority of the LMw material was highly polar and did not show any interaction with the SPE material. This SPE-0 fraction, being 60% of the applied LMw material, had a $K_{\text{mix 405nm}}$ value of only 0.1, showing that the melanoidin content in this fraction was very low. By this first extraction step, it seems that the majority of the low Mw compounds are obtained while only 15% of the LMw melanoidins are co-extracted. The material in fractions SPE-10 and SPE-20 was slightly less polar than the SPE-0 material as it showed some interaction with the SPE cartridge. These SPE-10 and SPE-20 fractions represented 11% and 8% of the dry matter of LMw. The $K_{\text{mix 405nm}}$ values were still relatively low, being 0.4 for SPE-10 and 0.6 for SPE-20, and these fractions together accounted for 23% of the low Mw coffee melanoidins. The remaining fractions SPE-40, SPE-60, and SPE-100 had relatively high $K_{\text{mix 405nm}}$ values, being 0.9, 3.4, and 1.8, respectively. Together, they accounted for the majority (64%) of the low Mw melanoidins, while these fractions represented only 15% of the dry matter of LMw material. Obviously, low Mw melanoidins seem to be quite apolar and the RP-SPE isolation procedure allowed isolation of most of the melanoidins from the multi-component, low Mw coffee brew material into a fraction with a high melanoidin concentration.

K_{mix} Ratios of the Low Mw Coffee Fractions.

Low Mw coffee components show different light absorbing properties at specific wavelengths. For example, caffeine absorbs light at 280 nm, while chlorogenic acids absorb light at 280 and 325 nm. Melanoidins absorb light throughout the whole wavelength spectrum. They can be exclusively detected at 405 nm as no other coffee components are known to absorb light at this wavelength. The absorption at a specific wavelength, as expressed by the K_{mix} value, gives an indication of the components that might be present in specific coffee fraction. The $K_{\text{mix 325:405nm}}$ and $K_{\text{mix 280:405nm}}$ ratios give an indication of the relative purity of the melanoidins in a fraction (*2*). Relatively pure melanoidin populations will show high $K_{\text{mix 405nm}}$ values and low K_{mix} ratios, as can be seen for the HMw material (**Table 1**). The opposite, low $K_{\text{mix 405nm}}$ values and high K_{mix} ratios indicate that there are many non-melanoidin components present relative to the amount of melanoidins (*2*). However, it should be realized that constituents that do not absorb light at these wavelengths, like carbohydrates and minerals, are not taken into account in this ratio. The high $K_{\text{mix 325:405nm}}$ ratios for SPE-0 and SPE-20 (**Table 1**) indicated higher chlorogenic acids levels than melanoidin levels. The fact that the K_{mix} values at all three wavelengths were low for SPE-0, indicates the presence of many non-light absorbing compounds like carbohydrates and minerals. The presence of these compounds in this fraction was also expected as carbohydrate and minerals are rather polar compounds. The SPE-20 and SPE-40 show very high $K_{\text{mix 280:405nm}}$ ratios (**Table 1**), whereas the $K_{\text{mix 325:405nm}}$ ratios were rather low, which indicates that

there might be relatively much caffeine present in these fractions. The SPE-60 and SPE-100 fractions have $K_{\text{mix 325:405nm}}$ and $K_{\text{mix 280:405nm}}$ ratios of 4 and 5 for SPE-60 and 2 and 3 for SPE-100 (**Table 1**). These values are comparable with the K_{mix} ratios of high Mw melanoidins, indicating that these fractions consist of relatively pure melanoidins. Overall, the $K_{\text{mix 405nm}}$ values and the K_{mix} ratios gave clear indications on which coffee fractions are enriched in melanoidins and which fractions still contain a lot of other coffee components. The melanoidin-rich fraction SPE-40 was expected to be polluted with a non-melanoidin component like caffeine, while the melanoidin-rich fractions SPE-60 and SPE-100 are expected to be free of non-melanoidin components.

Total, Caffeine-, Trigonelline-, and Melanoidin-Nitrogen Content of the Coffee Brew Fractions.

The most abundant nitrogen sources in green coffee beans are free amino acids, protein, caffeine, and trigonelline. The ~0.5% free amino acids in green coffees are largely transformed upon roasting and negligible amounts are present in roasted coffee (*6, 24*). Free amino acids take part in the Maillard reaction, resulting in components that contribute to the flavor and color of coffee brew (*24*). Proteins make up 9-12% of green coffee beans (*6*) and they might become incorporated into coffee melanoidins upon roasting (*2, 6, 25*). Green coffees contain about 1% trigonelline, of which 50-80% is degraded upon roasting (*26*). Major trigonelline degradation products are volatiles such as pyridine and pyrazines, and nonvolatiles such as nicotinic acid and *N*-methylpyridinium ions (*26-28*). Caffeine is a heat stable coffee component which makes up ~1.1% in green coffees (*26*). Nitrogen present

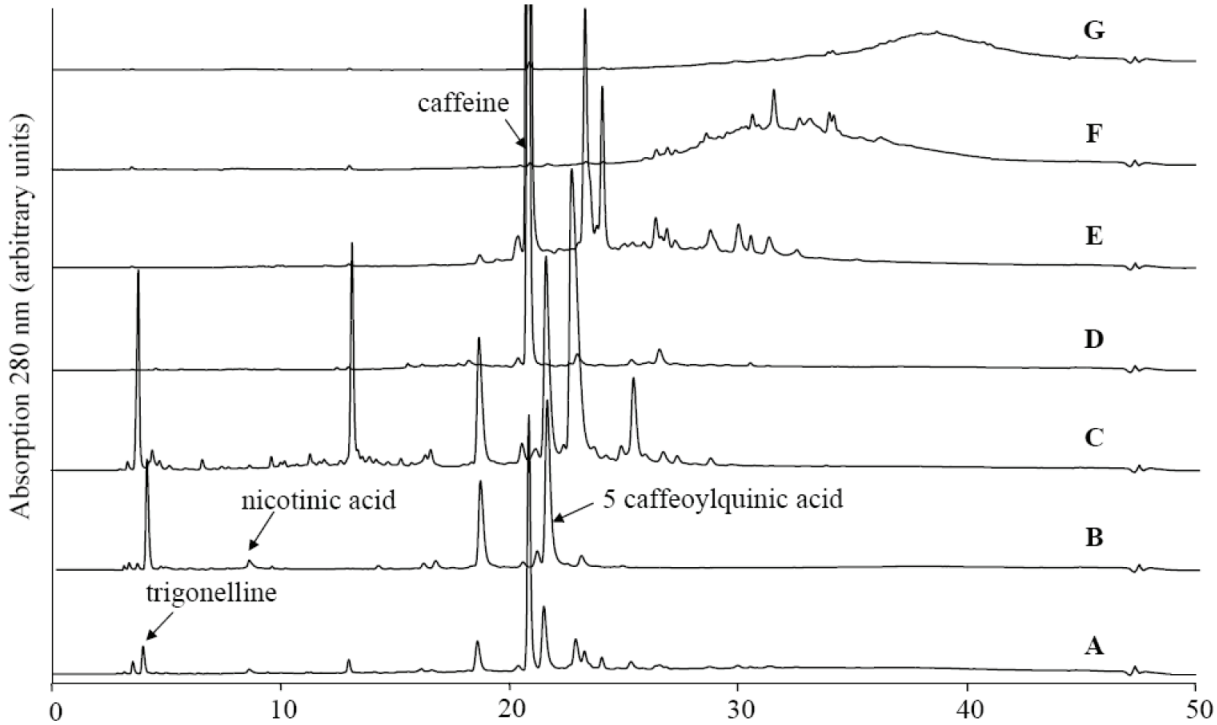

Figure 2. Reversed phase chromatography elution patterns of LMw (**A**), SPE-0 (**B**), SPE-10 (**C**), SPE-20 (**D**), SPE-40 (**E**), SPE-60 (**F**), and SPE-100 (**G**).

in the low Mw moiety of coffee brew can be partly accounted for by nitrogen from caffeine and trigonelline. The remainder should be accounted for by nitrogen present in melanoidin structures, this nitrogen is probably derived from amino acids and peptides.

Reversed-phase HPLC was conducted to investigate the distribution of melanoidins and non-melanoidins, like trigonelline and caffeine, over the various SPE fractions (**Figure 2**). The RP elution patterns of the most polar SPE-0, SPE-10, and SPE-20 fractions, which had low melanoidin levels, (**Figure 2B,C,D**) showed many peaks of which trigonelline, caffeine, 5-CQA, and CA were identified. The elution pattern of the melanoidin-rich SPE-40 fraction (**Figure 2E**) also showed several peaks, of which the main peak was identified as caffeine (R_t = 35 min). The other peaks were not identified, but they were likely due to chlorogenic acids. Additionally, a broad bump can be observed under the sharp peaks between 32 and 55 min of elution. The 405 nm elution pattern (not shown) showed the same bump at the same place, while the sharp peaks were absent. Based on these findings, it can be stated that the observed bump should be caused by low Mw melanoidins. The RP elution patterns of the two most apolar SPE fractions (**Figure 2F,G**) showed even more pronounced bumps, with SPE-60 showing a few minor peaks as well. These bumps and peaks were also visible at 405 nm and are likely caused by low Mw melanoidins. Thus, the RP elution patterns showed that both SPE-60 and SPE-100 were essentially free from non-melanoidin contaminants. The other melanoidin-rich fraction, SPE-40, still appeared to contain some non-melanoidin components, which is in line with the K_{mix} values and ratios.

The total nitrogen, caffeine nitrogen, trigonelline nitrogen, and calculated melanoidin nitrogen (N_{Mel}) levels are shown in **Table 2**. It stands out that all fractions contained nitrogenous compounds since all fractions contained at least 1% total nitrogen. The SPE-0 fraction contained all trigonelline from coffee brew; the trigonelline content in this fraction was 7%, which corresponded to 0.7% nitrogen. The SPE-20 and SPE-40 contained high

Table 2. Total Nitrogen, Caffeine Nitrogen, Trigonelline Nitrogen, and Melanoidin Nitrogen Content of Brew, HMw, LMw, and SPE-0 to SPE-100

	Total nitrogen (%, w/w)	Caffeine nitrogen[a] (%, w/w)	Trigonelline nitrogen[a] (%, w/w)	Melanoidin nitrogen (%, w/w)
Brew	2.8	1.1	0.3	na
HMw	2.1	0.0	0.0	na
LMw	3.4	1.8	0.4	1.2
SPE-0	1.1	0.0	0.7	0.4
SPE-10	2.3	0.0	0.0	2.3
SPE-20	10.7	8.4	0.0	2.3
SPE-40	9.5	7.2	0.0	2.3
SPE-60	3.4	0.0	0.0	3.4
SPE-100	3.4	0.0	0.0	3.4

[a] Percentage nitrogen is 28.9% in caffeine and 10.2% in trigonelline. na = not available due to presence of proteins.

caffeine levels, which were already predicted on the basis of the K_{mix} ratios. The SPE-20 and SPE-40 fractions contained 29% and 25% caffeine, corresponding to 8.4 and 7.2% caffeine nitrogen, respectively. The high caffeine content is, at least partly, the reason for the observed high K_{mix} ratio for SPE-40. Since both amino acids and proteins undergo chemical reaction upon roasting, yielding flavor and melanoidins, all non-caffeine, non-trigonelline nitrogen is likely to be incorporated in melanoidins. In **Table 2**, the N_{Mel} level was calculated by subtracting the caffeine and trigonelline nitrogen from the total nitrogen level. The N_{Mel} levels were 2.3%, 3.4%, and 3.4% for the melanoidin-rich fractions SPE-40, SPE-60, and SPE-100, respectively (**Table 2**). For SPE-40, the percentage of melanoidin-nitrogen is underestimated due to the presence of 25% caffeine in this fraction. Removal of caffeine would lead to a higher N_{Mel} content because the same amount of melanoidin-nitrogen would then be present in 25% less material. The calculated N_{Mel} level would then be 3.0%, which is 1.3 times higher than 2.3%. Thus, the melanoidins in SPE-40 to SPE-100 have quite similar N_{Mel} contents, indicating that the melanoidins present in these 3 fractions possess similar nitrogen levels while the polarity is different.

Phenolic Groups and Chlorogenic Acid Derivatives Level in the Coffee Brew Fractions.

It was previously shown that HMw coffee melanoidins contained phenolic groups, which was ascribed to incorporation of chlorogenic acids upon roasting (*7*). The low Mw SPE fractions had different phenolic groups levels and chlorogenic acid levels compared with each other (**Table 3**). It was found that 5-CQA, CA, and QA ended up in SPE-0 and SPE-10, while the other SPE fractions were free of these components. The fractions SPE-20 and SPE-40 showed many large and unidentified peaks in their corresponding RP elution patterns (**Figure 2D,E**). Even though no 5-CQA was found to be present in SPE-40, it not unlikely that the observed peaks are due to the presence of other chlorogenic acids that are more polar, like dicaffeolylquinic acids (*29*). Therefore, it is difficult to draw conclusions on the phenolic

Table 3. Phenolic groups, 5-CQA, Free and Total QA and CA Content in Brew, HMw, LMw, and SPE-0 to SPE-100

	Phenolic groups[a] (%, w/w)	5-CQA (%, w/w)	Free QA (%, w/w)	Total QA (%, w/w)	Free CA (%, w/w)	Total CA (%, w/w)
Brew	23	2.8	2.6	11.2	0.0	4.3
HMw	17	0.2	0.0	3.5	0.0	1.1
LMw	23	4.4	5.2	11.9	0.0	5.7
SPE-0	11	5.6	7.5	21.4	0.0	3.7
SPE-10	47	8.5	0.2	20.2	0.1	13.9
SPE-20	33	0.2	0.0	9.2	0.0	3.0
SPE-40	53	0.0	0.0	10.1	0.0	8.5
SPE-60	41	0.0	0.0	4.9	0.0	1.6
SPE-100	47	0.0	0.0	0.4	0.0	0.2

[a] As 5-CQA equivalents.

properties of the melanoidins present in the melanoidin-rich SPE-40 fraction. However, it was shown that SPE-60 and SPE-100 were free from non-melanoidin components as they did not show any non-melanoidin peak in their corresponding RP elution pattern (**Figure 2F,G**). Since the Folin-Ciocalteau phenolic groups levels of these latter two fractions were as high as 47%, it can be concluded that the low Mw melanoidin complexes should contain high levels of phenolic groups within their structures. As saponification of these latter two fractions yielded both QA and CA, it can be stated that chlorogenic acids are likely incorporated upon roasting in low Mw coffee melanoidins. This incorporation of chlorogenic acids in coffee melanoidins was previously found for high Mw coffee melanoidins as well (*7*). The total CA levels of the melanoidin-rich fractions SPE-40 to SPE-100 were 6–224 times lower than the phenolic groups level as determined by the Folin-Ciocalteau assay. This indicates that a large part of the phenolics within melanoidins is not caused by the presence of ester-linked CA, but rather by nonester-linked CA. Very recently, Frank et al. (*30*) reported on the formation of apolar, nonester-linked caffeic acid oligomers during heating of CA. The nonester-linked CA molecules in melanoidins, as reported by us, are expected to have similar structural properties as those identified by Frank et al. (*30*). This nonester-linked CA to coffee melanoidins is in line with findings previously reported for HMw coffee melanoidins (*7*). The ferulic acid levels (data not shown) were found to be negligible. The level of QA released by saponification was higher than the level of CA, as was previously found as well for other coffee melanoidins (*7*). The last remarkable point was that the ratio of phenolic groups over total QA level increased steadily with decreasing polarity of the low Mw material. This indicates that low Mw melanoidins contain relatively less incorporated QA compared to CA when the apolar character of the melanoidins is higher. This increase in apolar character can be explained by the fact that QA is a polar molecule, which is especially due to its carboxyl group. Thus, the less QA is present, the lower the effect of this QA on the overall polarity, which results in a more apolar melanoidin structure.

Sugar Composition of the Coffee Brew Fractions.

The isolated fractions were analyzed for their sugar content and composition and results are shown in **Table 4**. The majority of the carbohydrates from coffee brew were probably polysaccharides since 83% of the coffee brew carbohydrates ended up in the high Mw fraction, and only 17% ended up in the low Mw fraction. With respect to the SPE fractions, it was found that all fractions contained carbohydrates, which content ranged between 4% and 13%. It was calculated that 83% of all low Mw sugars ended up in the SPE-0 (56%), SPE-10 (18%), and SPE-20 (9%) fractions. Thus, 17% of the sugars did not end up in these polar to moderately apolar fractions, which was unexpected since carbohydrates are polar compounds and should not bind to the reversed phase media. The fact that 17% of the low Mw sugars was retained on the SPE cartridge even after elution with 20% aqueous methanol suggests that these sugars are part of apolar molecules. These findings strongly implicate that intact sugar moieties are present in apolar low Mw melanoidins after roasting of coffee beans.

Table 4. Sugar Composition (Mol %) of Brew, HMw, LMw, and SPE-0 to SPE-100

	Rha	Ara	Man	Gal	Glc	Uronic acid	Total sugars (%, w/w)
Brew	2	13	34	34	5	12	26
HMw	2	11	42	36	1	8	55
LMw	2	21	10	31	14	22	8
SPE-0	2	21	8	32	14	23	7
SPE-10	3	24	15	31	10	17	13
SPE-20	3	24	12	28	9	24	8
SPE-40	3	17	5	21	32	22	8
SPE-60	2	13	7	17	41	20	10
SPE-100	2	10	4	18	46	20	4

The presence of sugar moieties in high Mw melanoidins was reported previously by various groups (*8, 10, 31, 32*). However, no studies reported on the presence of sugar fragments in low Mw coffee melanoidins. Presumably, carbohydrates with a DP ≥ 2 take part in the Maillard reaction resulting in a Maillard reaction product with apolar characteristics and a carbohydrate moiety. The sugar that took part in the Maillard reaction is of course not recognizable as sugar anymore. The non-reducing sugar moiety from the carbohydrate is then attached via a glycosidic linkage to the brown-colored and apolar Maillard reaction product, the melanoidin.

When looking to the sugar composition of the melanoidin-rich fractions SPE-40, SPE-60, and SPE-100 fractions (**Table 4**), it stands out that glucose makes up almost half of all sugars present in these fractions. As glucose-based carbohydrates were not expected to bind to the SPE cartridge and as glucose is especially present in melanoidin-rich fractions, it can be concluded that this glucose is likely incorporated within the low Mw melanoidins structures. In green coffee beans, there are three glucose containing carbohydrates that could serve as source for glucose-containing melanoidins. The first possible source for glucose is sucrose, which is a disaccharide built up from glucose and fructose and which is the principal low Mw sugar (6-9%) in green coffee (*33*). Upon roasting, sucrose is degraded rapidly and its content after roasting has decreased ~100 times, even though sucrose has no reducing end (*33*). Cellulose is the second glucose source present in green coffees. Cellulose is a polysaccharide consisting of β-(1→4) linked glucan accounting for 6-8% of green coffees (*33*). The β-(1→4) linked glucose is highly stable and therefore, cellulose remains largely undegraded even at prolonged roasting (*34*). The third possible source for glucose is xyloglucan, which was reported to be present in green beans (*35*). As xyloglucan consists of β-(1→4) linked glucose monomers, like cellulose, it should be stable and remain largely undegraded upon roasting as well. It was reported that the glucose content dropped initially during roasting by around 20% (*36*), this was ascribed to the sucrose degradation and not so much to the cellulose or xyloglucan degradation. Combining the facts that i) next to all sucrose is degraded upon

roasting, ii) the β-(1→4) glucose-glucose linkage is heat stable, and iii) only part of the glucose is degraded upon roasting, it can be stated that sucrose is by far the most likely candidate that provides glucose for low Mw, intact glucose-containing melanoidins. Additionally, it can be stated that ring opening of the fructose moiety from sucrose likely occurs prior to further chemical degradation leading to brown color development, otherwise it would not be possible to recover intact glucose from melanoidins.

Charge Properties of the Coffee Brew Fractions.

It was previously shown that high Mw coffee melanoidins expose a negative charge (*8*). Now, low Mw coffee material was investigated for its charge properties. It was found that low Mw coffee material that absorbs light at 405 nm showed interaction with the anion exchange column as well (**Figure 3**). These 405 nm light absorbing compounds eluted at various ionic strengths of the elution buffer. Therefore, it was concluded that LMw coffee melanoidins expose a negative charge and that they are heterogeneous with respect to this negative charge. HMw and LMw melanoidins show differences in their negative charge properties. First, the LMw melanoidins were less negatively charged than the HMw melanoidins: the peak corresponding to uncharged melanoidins (**Figure 3, A1**) was higher for LMw than for LMw; and the peaks corresponding to negatively charged melanoidin (**Figure 3, A2-A6**) were lower for LMw than for HMw. Second, HMw melanoidins were on average more negatively charged than LMw melanoidins which is clear from the increase in HMw melanoidin peak area with increasing ionic strength of the eluent (**Figure 3, A1→A4**), whereas the LMw melanoidins showed a decrease in peak area. Overall, it could be concluded that LMw melanoidins expose less negative charge than HMw melanoidins do.

The charge properties of the melanoidins in the SPE fractions over the anion exchange chromatography peaks A1 to A6 are shown in Figure 4. From this figure, it is clear that the most polar melanoidins, present in SPE-0, expose the highest negative charge and these

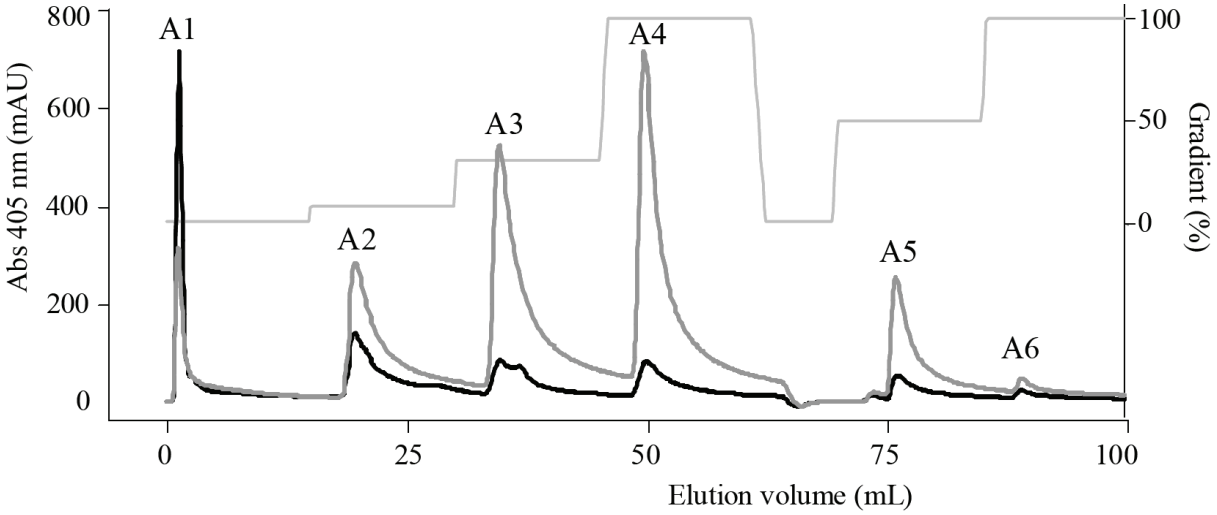

Figure 3. Anion exchange chromatography elution patterns of HMw (gray trace) and LMw (black trace) coffee material.

melanoidins also show the widest distribution of this negative charge over the fractions A1 to A6. It was found that the negative charge on melanoidins decreased with increasing apolarity. In SPE-100, 80% of the melanoidins were found to be neutrally charged versus only 1% in SPE-0. Thus, the apolarity is inversely proportional to the negative charge properties of low Mw coffee melanoidins.

Chlorogenic acids incorporation mechanism.

According to Heyns and Hauber and later Tressl et al. (*18, 19*), it should be expected that low Mw coffee melanoidins evolve towards more apolar compounds upon roasting. This implies that low Mw melanoidins should evolve towards less negatively charged molecules. Even though results are not conclusive, it might be speculated that low Mw melanoidin formation involves: I) Incorporation of the whole chlorogenic acid into the melanoidin structure. The CA moiety provides the phenolic and apolar characteristics, while the QA moiety provides more polar characteristics and it contributes to the negative charge. II) QA is split off upon further roasting, leading to more apolar and more neutrally charged low Mw melanoidins. The CA moiety remains present providing apolarity and causes the high phenolic groups level of around 50%.

This explanation is in line with previous studies which showed I) that rather apolar, phenolic CA oligomers were formed during heating of CA (*30*), and II) that chlorogenic acids are most probably incorporated to melanoidins via the CA moiety through nonester linkages (*7*).

Isolation of One Pure Low Mw Melanoidin Population.

One of the aims of this research was the development of a rapid procedure for the isolation of the majority of the low Mw melanoidins from the multi-component and complex low Mw coffee brew material. Therefore, it would be advantageous to combine melanoidins in SPE-40 (25%) and melanoidins in SPE-60 and SPE-100 (39%). This combined fraction would then account for 64% of the low Mw melanoidins, in less than 16% of the low Mw material. However, the non-melanoidin components in SPE-40 had to be removed to obtain pure melanoidins and therefore, an additional isolation step was needed. Since caffeine is neutrally charged and since 62% of the melanoidins in SPE-40 are negatively charged (**Figure 4**), anion exchange chromatography was performed to separate melanoidins from non-melanoidin components. The material that did not bind (SPE-40 AEC–, non-melanoidins) and the material that eluted at high ionic strength (SPE-40 AEC+, melanoidins) were analyzed by RP-HPLC (data not shown). The RP elution pattern of SPE-40 AEC– showed all peaks that were present in the elution pattern of SPE-40 (**Figure 2E**). The RP elution pattern of melanoidin fraction SPE-40 AEC+ only showed a broad bump corresponding to the melanoidins and no distinct peaks. With respect to the distribution of the melanoidins over the two fractions, it was found that 66% of the melanoidins from SPE-40 ended up in the melanoidin fraction SPE-40 AEC+. The $K_{\text{mix } 405:325}$ and $K_{\text{mix } 405:280}$ ratios for SPE-40 AEC+ were determined to be 6 in both cases. These values are comparable to the K_{mix} ratios for high Mw melanoidins and

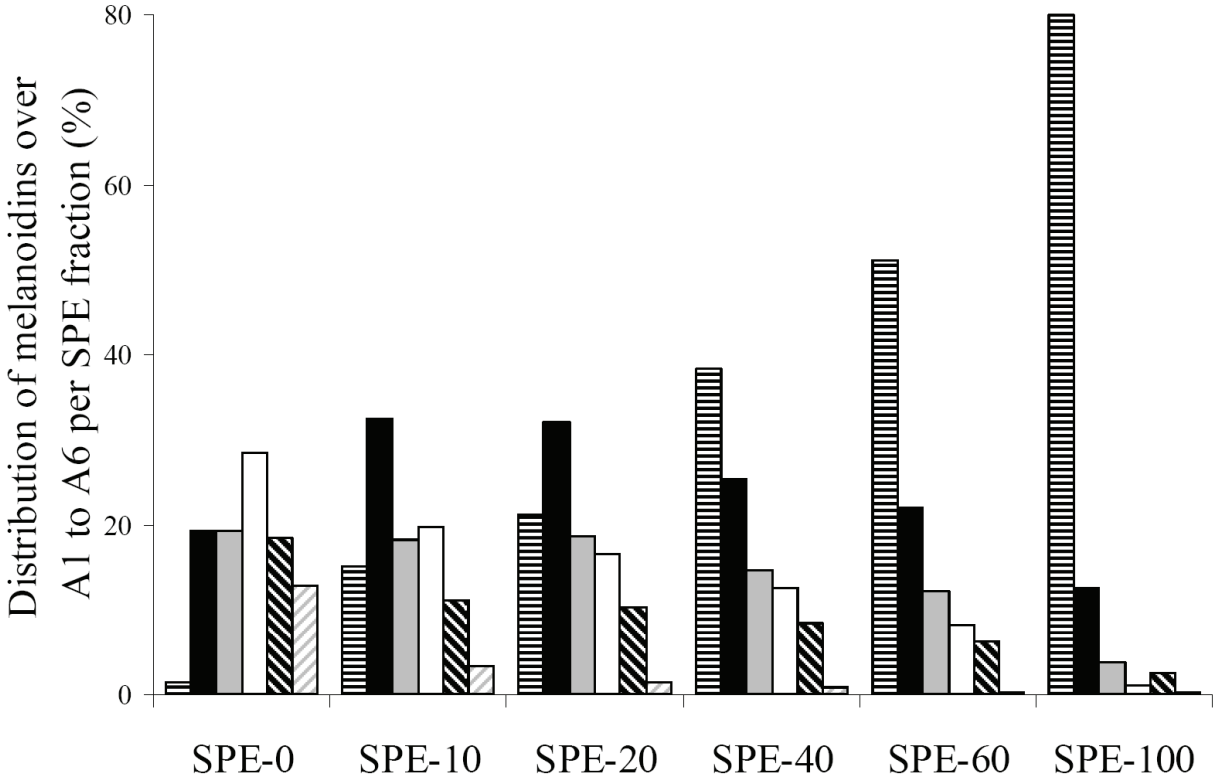

Figure 4. Distribution of melanoidins in the SPE fractions over the anion exchange chromatography peaks A1-A6 (A1: ▤**,** A2: ■, A3: ▩, A4: □, A5: ▨, and A6: ▨).

were also in line with the ratios found for SPE-60 and SPE-100 (**Table 1**). Thus, both the RP elution pattern and the K_{mix} ratios show that the additional purification step performed on SPE-40 results in a "pure" melanoidin fraction. The combination of both RP-SPE and an additional quick purification step on SPE-40 allow the isolation of 56% of the melanoidins into a "pure" melanoidins fraction that was less than 13% (w/w) from the crude low Mw coffee material. The results presented herein open possibilities for further research of individual low Mw "pure" fractions as well as giving us the availability of a quick method, when combining these fractions, to screen total melanoidin levels in crude mixtures.

In this study, it was shown that most of the low Mw coffee melanoidins had an apolar character and could be successfully separated from non-melanoidin coffee components. The presence of intact glucose as main sugar in fractions rich in melanoidins indicated that sucrose is likely involved in melanoidin formation. The low Mw coffee melanoidins exposed a negatively charge and incorporation of chlorogenic acids likely caused the high level of phenolic groups in low Mw melanoidins.

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CHAPTER 6

ESR-Studies on the Formation of Roasting-Induced Antioxidative Structures in Coffee Brews at Different Degrees of Roast

ABSTRACT

Antioxidative properties of coffee brew fractions were studied using electron spin resonance spectroscopy using TEMPO (2,2,6,6-tetramethyl-1-piperidin-1-oxyl) and Fremy's salt (nitrosodisulphonate) as stabilized radicals. TEMPO was scavenged by antioxidants formed during roasting and not by chlorogenic acid, whereas Fremy's salt was scavenged by all antioxidants tested including chlorogenic acid. The stabilized radical TEMPO allowed the exclusive measurement of roasting-induced antioxidants. The roasting-induced antioxidant activity of coffee brews increased with increasing degree of roast, and most of these antioxidants were formed during the initial roasting stage. The majority of these roasting-induced antioxidants were present in the high molecular weight fractions, indicating that formation of these antioxidants preferably occurs at specific high molecular structures, likely being arabinogalactan and/or protein moieties which might be part of the melanoidin complex. It was found that chlorogenic acids most probably do not loose their antioxidant activity and phenolic characteristics upon incorporation in coffee melanoidins. The parameter fast reacting antioxidants (FRA) was introduced as an alternative for the antioxidative potential. FRA levels showed that coffee fractions rich in roasting-induced antioxidants exposed their antioxidant activity relatively slowly, which must be a consequence of its complex structure. Finally, the melanoidin content and the roasting-induced antioxidant activity showed a positive and linear correlation for the coffee brew fractions showing that roasting-induced antioxidants are present within melanoidins. This is the first time that formation of roasting-induced antioxidants could be directly correlated with the extent of Maillard reaction and melanoidin formation in a complex product like coffee.

KEYWORDS

Coffee; melanoidins; antioxidant activity; ESR; Maillard reaction; polyphenolics

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INTRODUCTION

It is known for a long time that both green and roasted coffee beans and coffee brews contain compounds that exert antioxidant activity (*1-8*). The presence of large amounts of chlorogenic acids, a polyphenolic compound, in coffee contributes significantly to this antioxidant activity (*4, 9-11*). Upon roasting, the chlorogenic acids level decreases from 5–8% in green Arabica beans to levels as low as 0.2% for dark roasted coffees (*12*). The fate of this disappearing chlorogenic acids is not totally clear although several explanations can be found in literature, including acyl migration, hydrolysis, oxidation, fragmentation, polymerization, and association with denatured/degraded proteins (*12*). More specifically, it was reported that chlorogenic acids could be converted into flavor compounds upon roasting (*13, 14*) or that they might be incorporated in coffee melanoidins (*15, 16*). Heinrich and Baltes showed that Curie point pyrolysis of coffee melanoidins yielded phenolic degradation products (*16*). Adams et al. indicated that chlorogenic acids might be involved in melanoidin formation (*15*). More recently, it was shown that caffeic acid (*17*) and quinic acid, the building stones of chlorogenic acids, are chemically incorporated in melanoidins upon roasting (*18*).

Melanoidins form an abundant group of complex molecules in coffee brew (*8*). Melanoidins are generally referred to as high molecular weight (Mw), nitrogenous, brown-colored Maillard reaction end-products. Despite all efforts, the molecular structure of melanoidins is still unknown due to the extreme complexity of these molecules. One of the functional properties of coffee melanoidins is its antioxidant activity (*5, 19*). This antioxidant activity could be due to incorporated chlorogenic acids in melanoidins. Additionally, the Maillard reaction may also result in the formation of new antioxidative structures in coffee melanoidins. The latter was shown by Nicoli et al. (*19*), who showed that coffee brews exhibited oxygen scavenging properties compared to a brew prepared from green coffee beans. Furthermore, Charurin et al. (*20*), and Cämmerer and Kroh (*4*) showed that a model reaction between sugars and amino compounds resulted in the formation of antioxidative compounds.

A problem which is faced when the antioxidative properties of coffee are studied, is the difficulty to distinguish polyphenolic antioxidants from antioxidants formed by the Maillard reaction. This problem is most pronounced when Maillard reaction antioxidants are studied since the high levels of polyphenolic antioxidants are expected to dominate total antioxidant activity (*21*). Therefore, there is a need for a detection method that specifically measures non-phenolic antioxidants in coffee. An electron spin resonance (ESR) spectroscopy method using stabilized radicals seems to be capable of making this distinction (*4*). ESR is a rapidly evolving technique that is highly suitable for measuring antioxidant activity (*22*). The usage of different stabilized radicals allows differentiating between antioxidants that differ in structural properties. A wide range of antioxidants can be detected when nitrosodisulphonate (Fremy's salt) is used as stabilized radical, whereas 2,2,6,6-tetramethyl-1-piperidin-1-oxyl (TEMPO) could be scavenged by model system Maillard reaction products while it was not or only negligible sensitive towards antioxidants with a phenolic nature (*4*). Thus, usage of

TEMPO should allow the detection of antioxidative structures in coffee brew melanoidins formed by the Maillard reaction itself, the roasting-induced antioxidants, while incorporated chlorogenic acids should not be detected.

The objective of the present study was to investigate the total antioxidant and roasting-induced antioxidant properties of coffee brew melanoidins. Coffee brew melanoidin populations, isolated from coffee brews with 4 degrees of roast, were investigated for their antioxidative properties.

MATERIALS AND METHODS

Materials.

Green and roasted Colombian coffee beans (*Coffea arabica*) were provided by a local factory. The degree of roast, which is the total weight loss upon roasting, of the light, medium, and dark roasted beans was 14.7%, 16.4%, and 19.2% (w/w), respectively; and was 6.1%, 8.0%, and 11.1% (w/w) on a dry matter basis, respectively. The color of the beans light, medium, and dark roasted beans were 60, 50, and 40, respectively, according to the color test Neuhaus (CTN). The denomination "GB", "LR", "MR", or "DR" was added to the fraction name to indicate that the fraction was isolated from green, light roasted, medium roasted, or dark roasted coffee beans, respectively.

Preparation of Coffee Brew.

Green (frozen with liquid nitrogen) and roasted coffee beans were ground and a brew was prepared as described previously (*23*). Briefly, coffee brew was obtained by adding 200 g of ground coffee beans to 1200 g of filtered and demineralized water (Millipore Corp, Billerica, MA) at 90 °C. Subsequently, this coffee suspension was kept at 90 °C for 15 min while stirring continuously. The extract was filtered over a Büchner funnel using a S&S 595 filter (Whatman, Maidstone, UK). For characterization purposes, part of the brew was lyophilized, yielding "brew". The major part of the brew was used for further isolation.

Defatting of Coffee Samples.

Lyophilized coffee brew samples were defatted by Soxhlet extraction using a Soxtherm, which was connected to a Multistat system (Gerhardt, Königswinter, Germany), as described previously (*24*). The solvent used for extraction was dichloromethane.

Isolation of High Molecular Weight Coffee Material.

High Mw material was obtained from brew by diafiltration (cut off 3 kDa) according to the procedure previously described (*23*). The retentate and dialysate were lyophilized, yielding a high molecular weight fraction ("HMw") and a fraction with a lower molecular weight ("DF-dialysate"), respectively (**Figure 1**).

Isolation of Intermediate and Low Molecular Weight Coffee Material.

Intermediate and low Mw material was obtained from defatted DF-dialysate sample by membrane dialysis based on a procedure previously described (*24*). The DF-dialysate sample (500 mL, 100 mg/ml) was dialyzed using a Visking size 9 dialysis membrane with a cutoff of 12-14 kDa (Medicell International Ltd, London, UK) for 3 days at 4 ºC against 5 L demineralized water with four water renewals. The retentate was lyophilized, yielding the intermediate Mw fraction "IMw" (**Figure 1**). The first 2 dialysate fractions were pooled and lyophilized, yielding the low Mw fraction ("LMw") (**Figure 1**), whereas the last 3 dialysate fractions were discarded.

Ethanol precipitation of the Brew HMw.

The HMw fraction obtained after diafiltration was subjected to ethanol precipitation, as described previously (*23*). Absolute ethanol was added to the HMw solution until the desired concentration. The solution was left for precipitation and was subsequently centrifuged. The supernatant was subjected to further precipitation steps. Coffee fractions that precipitated at 20, 40, 60, and 80% ethanol were coded "EP20", "EP40", "EP60", and "EP80", respectively (**Figure 1**). The supernatant of 80% ethanol was coded "ES80" (**Figure 1**).

Reversed-Phase Solid Phase Extraction of the Low Mw Coffee Material.

Defatted LMw fractions were fractionated by reversed-phase solid phase extraction as described previously (*25*). Briefly, 10 mL aqueous LMw fraction (10 mg/mL) was applied on a 5 g Sep-Pak Vac 20cc C18 cartridge

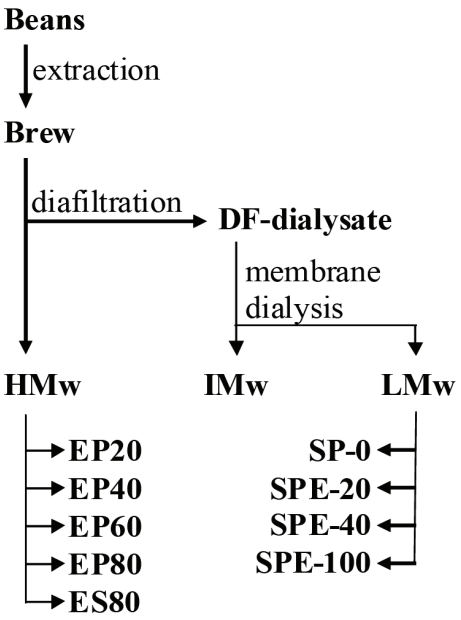

Figure 1. Isolation scheme of various melanoidin coffee brew populations from roasted Arabica coffee beans.

(Waters, Milford, MA). Elution was conducted in four steps: I) 50 mL water, II) 50 mL 20% (v/v) aqueous methanol, III) 50 mL 40% (v/v) aqueous methanol, and IV) 50 mL 100% methanol. This procedure was conducted in twenty-fold, fractions were pooled, and methanol was evaporated by a rotating evaporator. The fractions were lyophilized, yielding "SPE-0", "SPE-20", "SPE-40", and "SPE-100" for the fractions that eluted at 0, 20, 40, and 100% methanol (**Figure 1**), respectively.

Determination of 5-Caffeoylquinic Acid by Reversed-Phase Chromatography.

The 5-caffeoylquinic acid level was determined as described previously (*18*). Briefly, coffee sample was analyzed by reversed-phase high-performance liquid chromatography (HPLC) on an XTerra® MS C18 column in combination with a XTerra® MS C18 guard column (Waters) using 0.1% acetic acid in water and 0.1% acetic acid in methanol as eluents. The absorbance of the eluate was measured at 325 nm using a Spectra System UV3000 (Thermo Electron Company, Waltham, MA). The sample was dissolved in the eluent (1 mg/ml) and was centrifuged prior to injection. Measurements were conducted at least in duplicate. The average coefficient of variation was 1%.

Total Phenolic Groups Content.

The total phenolic groups content of the coffee samples were determined with the Folin-Ciocalteu assay as described previously (*23*). The used reference compound was 5-caffeoylquinic acid. Measurements were conducted at least in duplicate. The average coefficient of variation was 2%.

Specific Extinction Coefficient of Coffee Material.

The absorption of aqueous sample solutions (1 g/L) was determined at 405 nm using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, JP). The specific extinction coefficient $K_{\text{mix 405nm}}$ (L/g/cm) was calculated as described previously (*23*)**.**

Determination of the Antioxidant Activity by ESR Spectroscopy.

ESR spectroscopic measurements were performed on a Miniscope MS 100 spectrometer (Magnettech, Berlin, Germany) with Fremy's salt (Sigma Chemical Co., St. Louis, MO) and TEMPO (Sigma) as stabilized radicals. For investigation with Fremy's salt, Fremy's salt (1 mM) was dissolved in 50 mM phosphate buffer, pH 7.4. Aliquots of coffee sample (100 µL) were allowed to react with an equal volume of an aqueous 1 mM Fremy's salt solution. ESR spectra were recorded every 35 seconds for 10 minutes after which the reaction speed had stabilized. For investigation with TEMPO, TEMPO (10 mM) was dissolved in methanol, and subsequently diluted to 1 mM with water. Aliquots of coffee sample (300 µL) were allowed to react with 100 µL 1 mM TEMPO solution. ESR spectra were obtained after 120 min, by which the reaction speed had stabilized. To guarantee linearity, the sample concentration was chosen in such a way that more than 10% and less than 90% of

the radicals were scavenged. In practice, sample concentration was between 0.25–1.5 mg/mL and between 1–40 mg/mL for measurements with Fremy's salt and TEMPO, respectively. Unless mentioned otherwise, both Fremy's salt and TEMPO antioxidant activity were calculated as Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma) equivalents. The Fremy's salt antioxidant activity was calculated using the equation: ([% Fremy's salt scavenged by sample]$_{t=10min}$/[µg/mL sample])/([% Fremy's salt scavenged by Trolox]$_{t=10min}$/[µg/mL Trolox])*1000‰. The TEMPO antioxidant activity was calculated using the equation: ([% TEMPO scavenged by sample]$_{t=120min}$/[mg/mL sample])/([% TEMPO scavenged by Trolox]$_{t=120min}$/[mg/mL Trolox])*1000‰. Microwave power and signal phase were set at 10 dB and 0 º, respectively. Modulation amplitude, centre field and sweep width were set at 1.5, 3397, and 71G, respectively. Measurements were conducted at least in duplicate. The average coefficient of variation was 3% and 5% for measurements with Fremy's salt and TEMPO, respectively. Trolox, chlorogenic acid (5-caffeoylquinic acid) (Sigma), and ascorbic acid (Serva, Heidelberg, DE) were used as reference compounds.

RESULTS AND DISCUSSION

Scavenging Specificities of Fremy's Salt and TEMPO.

It was previously reported that Fremy's salt reacts rather unspecific as it is scavenged by different types of antioxidants, including the polyphenolic chlorogenic acid (*4*). In this research, this was confirmed since Fremy's salt was indeed scavenged by chlorogenic acid, as well as by Trolox and ascorbic acid, with similar reaction velocities. The stabilized radical TEMPO was effectively scavenged by Trolox and ascorbic acid, but it was not scavenged by chlorogenic acid, being in line with earlier findings (*4*). Thus, by using both Fremy's salt and TEMPO as stabilized radicals, a distinction could be made between phenolic and non-phenolic antioxidants (*4*). It is desirable to make this distinction when antioxidant properties of coffees are studied since coffee brew is expected to exhibit antioxidant activity due to the presence of chlorogenic acids as well as roasting-induced structures that are formed upon roasting.

At first, kinetic ESR experiments with Fremy's salt were conducted for 2 hours to explore the antioxidant properties of coffee brews in time. It was found that Fremy's salt radicals were

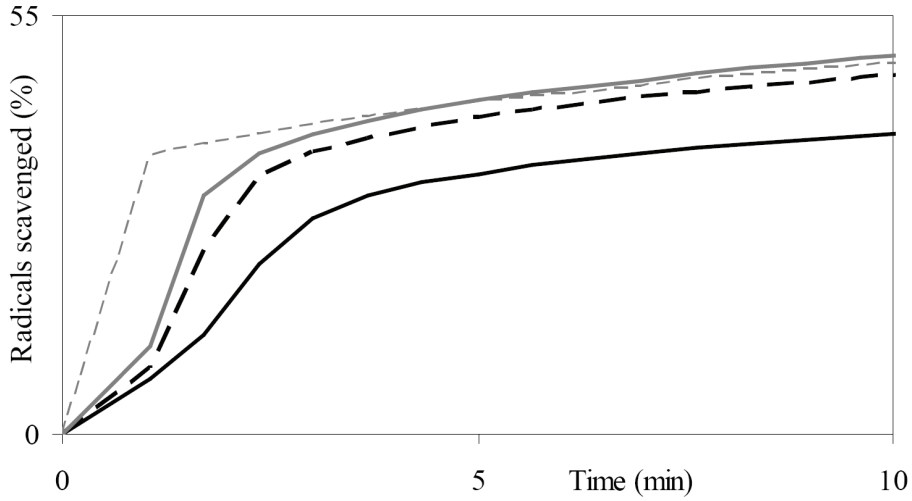

Figure 2. Reaction kinetics of Fremy's salt scavenging by coffee brews (150 µg/mL) made from green (grey dashed line), light (grey solid line), medium (black dashed line), and dark (black solid line) roasted beans.

rapidly consumed by coffee brew antioxidants during the first minutes (**Figure 2**). After that, the radical consumption leveled off and no major differences between different coffee samples were observed. As the first ten minutes provided the most valuable information, it was decided to conduct kinetic ESR measurements with Fremy's salt for ten minutes for all coffee samples. Kinetic ESR experiments with TEMPO (not shown) were also conducted for two hours and these measurements revealed that coffee antioxidants scavenged the radical TEMPO much slower. The radical consumption only leveled off after more than an hour and therefore it was decided to maintain the timeframe of two hours for ESR measurements with TEMPO.

Antioxidant Properties of Coffee Brews with Varying Degree of Roast.

The Fremy's salt and TEMPO antioxidant activity levels, the phenolic groups' level and the 5-caffeoylquinic acid levels for coffee brew from beans with different degrees of roast are shown in **Figure 3**. The green bean brew had a Fremy's salt antioxidant activity level of 23% and a phenolic groups level of 24%, both expressed as 5-caffeoylquinic acid equivalents. The actual 5-caffeoylquinic acid content was 16% instead of 23%. This difference was ascribed to the fact that 5-caffeoylquinic acid was the only chlorogenic acid determined by HPLC while other chlorogenic acids are also present in green beans (*26*). Likewise, Perrone et al. reported that 5-caffeoylquinic acid was around 60% of the total chlorogenic acids present in green Arabica beans (*27*). Additionally, a small part of the phenolics might originate from other phenolic components, like proteins. It was estimated that phenolic amino acids in coffee brew corresponded to ~0.4% 5-caffeoylquinic acid equivalents (*23*). Still, the majority of the

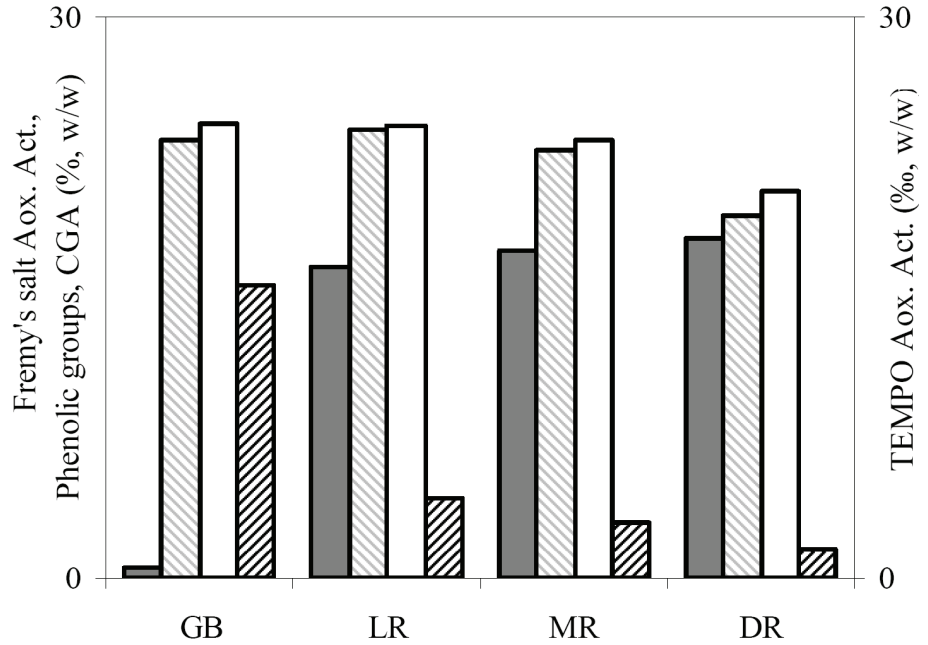

Figure 3. The TEMPO (Trolox eq., grey bars) and Fremy's salt (5-caffeoylquinic acid eq., grey striped bars) antioxidant activity levels, the phenolic groups level by Folin-Ciocalteu (5-caffeoylquinic acid eq., white bars), and chlorogenic acids level by HPLC (5-caffeoylquinic acid eq., black striped bars) of coffee brews made from green (GB), light (LR), medium (MR), and dark (DR) roasted beans.

antioxidant activity and phenolics in green bean brew could be ascribed to chlorogenic acids.

Upon roasting, the 5-caffeoylquinic acid content decreased from 16% in green bean brew to levels as low as 2% in dark roast brew. This drop in chlorogenic acid level was expected as chlorogenic acid degradation is known to occur upon roasting (*12*). While the chlorogenic acids level drastically decreased, the Fremy's salt antioxidant activity only slightly decreased from 24% in light roast brew to 19% in dark roast brew (**Figure 3**). These relative stable Fremy's salt antioxidant activity levels were in line with literature too (*4, 21*) but were also rather remarkable since the chlorogenic acid level had decreased so drastically. These findings imply that the antioxidant activity from chlorogenic acids is not destroyed upon roasting but survives these extreme heating conditions, even though the chemical structure alters. It is expected that the chlorogenic acids are incorporated in melanoidins without losing their antioxidative properties. Alternatively, it might also be that formation of new roasting-induced antioxidants compensate for the loss of antioxidant activity due to chlorogenic acids degradation. The latter is not likely though because of two reasons. First, it will be shown below that the contribution of newly formed roasting-induced antioxidants to the total (Fremy's salt) antioxidant activity is rather limited. Second, it was found that the phenolic groups levels as determined by the Folin-Ciocalteu assay, showed the same trend and had about the same values as the Fremy's salt antioxidant activity levels, with phenolic groups levels of 24% for light roast brew to 21% for dark roast brew (**Figure 3**). The latter implies that phenolic groups dominate the Fremy's salt antioxidant activity in coffee brews and that the relatively constant antioxidant activity should be ascribed mainly to the presence of phenolics. Therefore, it is expected that chlorogenic acids do not loose their antioxidant activity or phenolic characteristics upon incorporation in coffee melanoidins during roasting. This moiety is likely not present as free chlorogenic acids but might be bound to other molecules via ionic bonds (*1*), via ester bonds (*17, 18*), and possibly via other types of bonds such as ether linkages (*18*).

With respect to the TEMPO antioxidant activity levels (**Figure 3**), it stands out that these levels were totally different from Fremy's salt antioxidant activity levels. The TEMPO antioxidant activity level was very low, not even 1‰, for green bean brew. This was expected since the radical TEMPO should only be scavenged by roasting-induced antioxidants (*4*) and these structures were of course not present in green coffee beans. The TEMPO antioxidant activity level of the brew prepared from light roasted beans was 16‰. The steep increase from less than 1‰ in green bean coffee to 16‰ in light roast coffee brew showed that roasting led to the formation of relatively many roasting-induced antioxidants. A similar increase was observed by Nicoli et al. (*19*), who determined the antioxidant activity of Maillard reaction products in coffee by measuring their oxygen scavenging properties. The TEMPO antioxidant activity increased when coffee beans were roasted more intensely (**Figure 3**) indicating that prolonged roasting leads to the formation of more roasting-induced antioxidants. It was shown that TEMPO indeed is capable of exclusively measuring roasting-induced antioxidants in a complex foodstuff like coffee brew. Furthermore, these roasting-

Table 1. Fremy's Salt and TEMPO Antioxidant Activity Levels (‰, w/w) of Coffees with Varying Degrees of Roast[a,b]

	Green bean		Light roast		Medium roast		Dark roast	
	Fremy's	TEMPO	Fremy's	TEMPO	Fremy's	TEMPO	Fremy's	TEMPO
Brew	170	< 1	174	16	166	17	141	18
HMw	5	0	21	19	30	31	36	41
IMw	167	< 1	113	10	92	11	85	11
LMw	207	0	204	3	196	3	155	3

[a] Antioxidant activity levels were measured as Trolox equivalents. [b] The average coefficient of variation was 3% and 5% for the Fremy's salt and the TEMPO antioxidant activities, respectively.

induced antioxidants were especially formed during the initial stage of roasting even though somewhat more roasting-induced antioxidants are formed upon prolonged roasting.

When comparing both the Fremy's salt and TEMPO antioxidant activity levels, it stands out that the levels with TEMPO were much lower than the levels with Fremy's salt (**Table 1**) even though the timeframe for scavenging with the radical TEMPO was 12 times longer. For example, for brew from medium roasted coffee beans, the TEMPO antioxidant activity level was found to be only 10% of the Fremy's salt antioxidant activity level. These results showed that the contribution of the newly formed roasting-induced antioxidants upon roasting to the overall antioxidant activity is rather limited and that phenolic antioxidants dominate the overall antioxidant activity of coffee brews.

With respect to the kinetics, it was observed that coffee brews with different degrees of roast showed differences in reaction kinetics (**Figure 2**). For the brew of green beans, the percentage of radicals scavenged increased rapidly within the first minutes, showing that the antioxidants present in green beans scavenge radicals readily. When looking to the brews from roasted beans, it stands out that the reaction velocity within this first minute decreased with increasing degree of roast. This suggested that the converted or formed antioxidants present in roasted coffee brews have a lower mobility than green coffee antioxidants or that these antioxidants do not react as readily as do the antioxidants present in the brew from green beans.

Antioxidant Activity of High, Intermediate, and Low Mw Coffee Fractions.

It can be seen in **Table 1** that the LMw fractions from coffee brew had the highest Fremy's salt antioxidant activities and that the HMw fractions had the lowest values. The fact that most of the Fremy's salt antioxidants of the green bean brew were recovered in the LMw fraction was expected since chlorogenic acids are low Mw molecules. This observation was also in line with literature (*6*). However, the roasting process seems to cause a change in distribution of this Fremy's salt antioxidant activity. Upon roasting, the activity of the HMw fraction increased from 5 to 36‰, while the activity of the IMw and LMw fractions decreased from 167 to 85‰, and from 207 to 155‰, respectively. These findings can be explained by the fact that LMw and/or IMw antioxidative molecules like chlorogenic acids undergo

reaction and might be incorporated into HMw material during prolonged roasting, as was reported previously (*18*).

With respect to the TEMPO antioxidant activities, the high Mw coffee brew fractions were opposite to the Fremy's salt antioxidant activities since the TEMPO antioxidant activities of the HMw fractions were now higher, instead of lower, compared to the IMw and LMw fractions (**Table 1**). These findings suggest that formation of roasting-induced antioxidants preferably occurs at specific types of molecules that are present in HMw material, which consists out of galactomannans, arabinogalactan proteins, protein, and melanoidins (*23*). The increase in roasting-induced antioxidants in HMw upon further roasting is far more pronounced than was observed for the brews. When the yields of the fractions are taken into account (**Figure 4**), it becomes even more evident that high Mw material contributes most to the observed roasting-induced antioxidants in coffee brews and that the contribution of this HMw material to the total roasting-induced antioxidants present in the brew continuously increases upon prolonged roasting. The observed Fremy's salt antioxidant activities, representing the total antioxidant level, for the HMw coffee fractions are similar to the TEMPO antioxidant activities indicating that a significant part of the total antioxidant activity is caused by roasting-induced antioxidative structures. However, contribution of phenolic antioxidants to high Mw antioxidant activity should not be excluded or underestimated since the Fremy's salt antioxidant activity levels would have been higher when the timeframe for these measurements were two hours as for the TEMPO measurements as well. Thus, the observed antioxidant activity in high Mw coffee material should be caused by both incorporated phenolics (*18*) and by newly formed roasting-induced antioxidative structures.

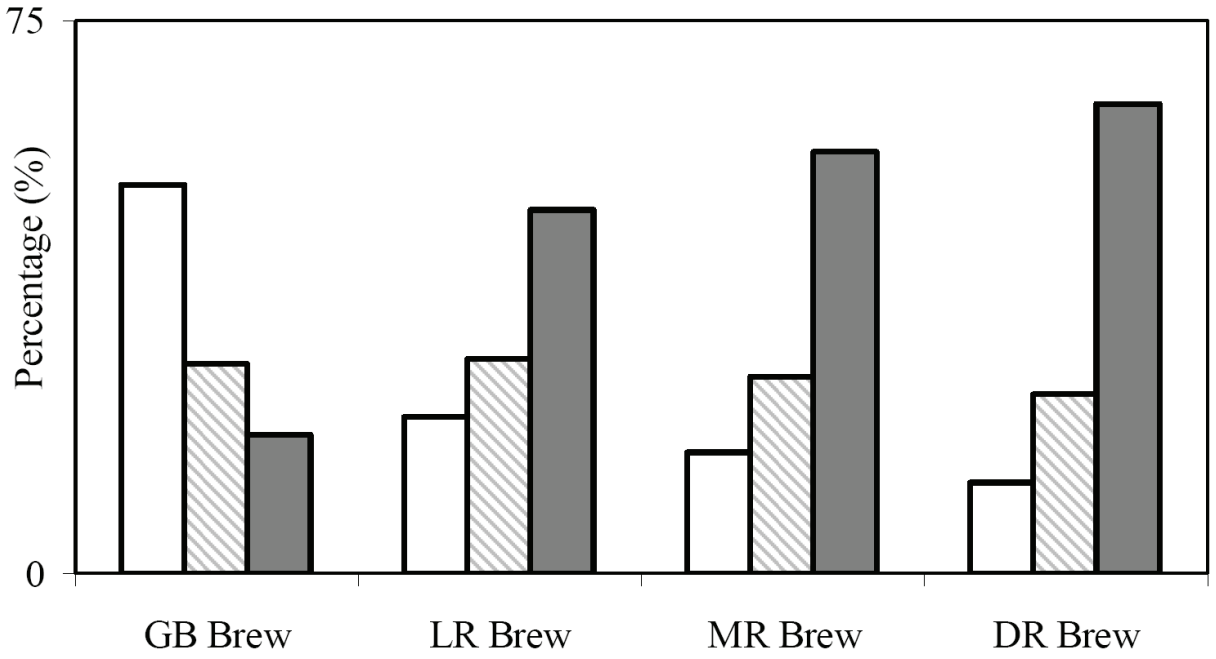

Figure 4. Distribution of TEMPO antioxidant activity from coffee brews with varying degrees of roast over LMw (white bars), IMw (striped bars), and HMw (grey bars). (GB, LR, MR, and DR are green, light, medium, and dark roasted beans, respectively).

Antioxidative Potential of Coffee Brew Fractions.

It is well-known that antioxidants can differ with respect to their reaction kinetics, some antioxidants react slowly whereas other antioxidants are fast-reacting, like many phenols (*28*). A parameter that provides insight in the reaction kinetics of antioxidants is the antioxidative potential, which is the initial reaction velocity by which the radical is scavenged by the antioxidant (*22*). Due to the complex composition of coffee and coffee fractions, which may contain different antioxidants in different concentrations, it is extremely difficult if not impossible to obtain comparable antioxidative potential values. However, differences in reaction kinetics can be observed for different coffee brews (**Figure 2**). In this figure, it can be seen that the radical scavenging curve for green bean brew showed a very steep increase during the first seconds. This was not the case for the curves of roasted coffees, of which the scavenging curve became more and more S-shaped upon prolonged roasting (**Figure 2**). Differences in reaction kinetics can also be observed for coffee brew fractions, even though the determined antioxidative potential can be rather similar (**Figure 5**). From the latter figure, it is evident that the brew reaches an equilibrium phase faster than the HMw fraction. Initially (0-5 min), the reaction velocity for brew was higher than for HMw. However, the reaction velocity for the brew leveled off after 5 minutes while the reaction velocity for HMw decreased much less. This resulted in a higher reaction velocity for the HMw fraction than for the brew between 5 and 10 minutes. Obviously, antioxidants in HMw coffee material expose their antioxidant activity over a wider range of time. This clearly showed that the antioxidants present in different coffee brew fractions might have different properties. An alternative for the antioxidative potential, that also provides insight in the reaction kinetics, was introduced and was defined as the percentage "Fast Reacting Antioxidants" or abbreviated "FRA". This value was calculated using trend line of Fremy's salt scavenging data between 5 and 10 minutes of reaction (**Figure 5**). The percentage FRA was calculated by: (value trend

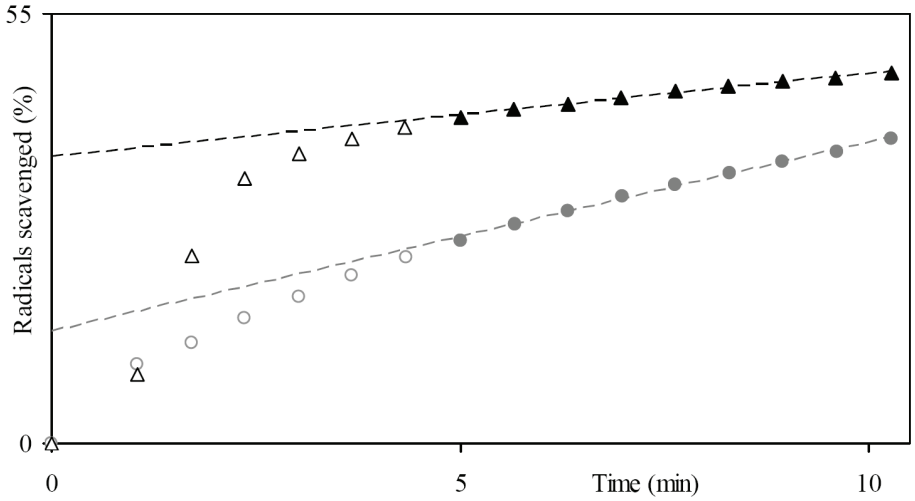

Figure 5. Reaction kinetics of Fremy's salt scavenging by medium roast HMw (750 µg/mL, grey circles) and brew (150 µg/mL, black triangles). Trend line equations using solid data points: 2.4 × t + 14.2 for HMw (grey) and 1.1 × t + 36.7 for brew (black).

line)$_{t=0min}$/(value trend line)$_{t=10min}$ × 100%. This approach was found to yield reproducible results as the average coefficient of variation was only 3% of the value and results for various coffee fractions are shown in **Table 2**. The percentage FRA for the chlorogenic acid rich, melanoidin free green bean brew was 80%, which was similar to the 82% obtained for the reference compound chlorogenic acid. The FRA values for the brews from roasted beans were somewhat lower, which must be ascribed to the presence of antioxidants that react slower. The FRA levels were lowest in the HMw fractions, with FRA levels as low as 30% for the HMw fraction from light roasted beans. As the FRA levels were lowest for the HMw fractions and as their TEMPO antioxidant activity levels were highest for these fractions, it can be reasoned that roasting-induced antioxidants are antioxidants that react slow initially, but keep on scavenging radicals for a relatively long time. The presence of "slow" but highly efficient roasting-induced antioxidants that represent the antioxidant reservoir of food products was also mentioned by Nicoli et al. (*29*). This phenomenon of slow reacting antioxidants might be explained by the fact that roasting-induced antioxidants likely possess structural characteristics that are not as optimal as the structural characteristics of well-known and fast-acting antioxidants. Additionally, the initial reaction velocity of the roasting-induced antioxidants might also be slowed down by a limited mobility of the HMw molecule and by steric hindrance by the non-antioxidant part.

Table 2. Fast Reacting Antioxidants (FRA) Levels of Coffees with Varying Degrees of Roast[a]

	Green Bean FRA level (%)	Light Roast FRA level (%)	Medium Roast FRA level (%)	Dark Roast FRA level (%)
Brew	80	77	78	76
HMw	51	30	37	39
IMw	77	51	34	35
LMw	79	77	77	77

[a] The average coefficient of variation was 3%.

Antioxidant Activity of Ethanol Precipitation Fractions from Medium Roast HMw Material.

The medium roast HMw fraction was separated by ethanol precipitation as described previously (*23*). It was previously found that galactomannans already precipitated at low ethanol concentrations and were mainly present in the fractions EP20 and EP40 (*23*). Proteins and arabinogalactans were soluble at these low ethanol concentrations and were mainly recovered in the fractions EP80 and ES80 (*23*). Now, the antioxidant properties of these HMw fractions were determined (**Table 3**). It was found that the TEMPO antioxidant activity increased with increasing ethanol solubility. The fact that EP80 and ES80 had the highest TEMPO antioxidant activity levels implies that roasting-induced antioxidants are preferably formed at structures that are rich in arabinogalactans and/or proteins. As arabinogalactans proteins (AGPs) are involved in melanoidin formation (*24*) and as arabinose is susceptible to

Table 3. Fremy's Salt Antioxidant Activity, TEMPO Antioxidant activity, and Fast Reacting Antioxidant (FRA) Levels of Medium Roast High Mw Ethanol Precipitation Fractions[a,b]

	Antioxidant activity		
	Fremy's (‰, w/w)	TEMPO (‰, w/w)	FRA level (%)
HMw	30	31	37
EP20	18	12	39
EP40	21	11	38
EP60	22	11	37
EP80	27	19	43
ES80	49	33	46

[a] Antioxidant activity levels were measured as Trolox equivalents. [b] The average coefficient of variation was 3% and 5% for the Fremy's salt and the TEMPO antioxidant activities, respectively.

degradation upon roasting (*30*), it is to be expected that arabinose from AGPs is involved in the formation of roasting-induced antioxidants upon roasting. Furthermore, melanoidins that are rich in galactomannans may also be involved in the formation of roasting-induced antioxidants since the fractions rich in galactomannans, EP20 and EP40, still had TEMPO antioxidant activity values of 12 and 11‰, respectively. The formation of roasting-induced antioxidants on galactomannan-melanoidin complexes seems plausible since galactomannans were shown to be involved in the Maillard reaction upon roasting of coffee beans (*31*).

The level of Fremy's salt antioxidant activity was only slightly (1.4–2 times) higher than for TEMPO (**Table 1**). This indicates that the roasting-induced antioxidants are the main contributor to the overall antioxidant activity of HMw material from roasted coffee brew. It should be mentioned though that the Fremy's salt antioxidant activity levels would have been somewhat higher when the timeframe of these measurements would have been lengthened to two hours as well. The FRA level was highest for the two fractions containing the most ethanol soluble material (EP80 and ES80). This is probably again due to the presence of relatively more, fast reacting, polyphenolic compounds in these fractions, as shown previously (*23*).

Antioxidant Activity of Solid Phase Extraction Fractions from Medium Roast LMw Material.

Previously, low Mw coffee material was fractionated on the basis of polarity using reversed-phase solid phase extraction (*25*). It was found that most of the non-melanoidins, e.g. minerals, sugars, and chlorogenic acids, had a rather polar character and ended up in the fractions SPE-0 and SPE-20. Oppositely, most of the melanoidins were quite apolar and ended up in the fractions SPE-40 and SPE-100. This allowed separation of most of the melanoidins from the majority of the non-melanoidin components using reversed-phase solid phase extraction. Since the isolated apolar fractions were rich in melanoidins, it was expected that the roasting-induced antioxidant activity would be especially high in these apolar fractions. **Figure 6** shows that the TEMPO antioxidant activity was indeed high for fractions

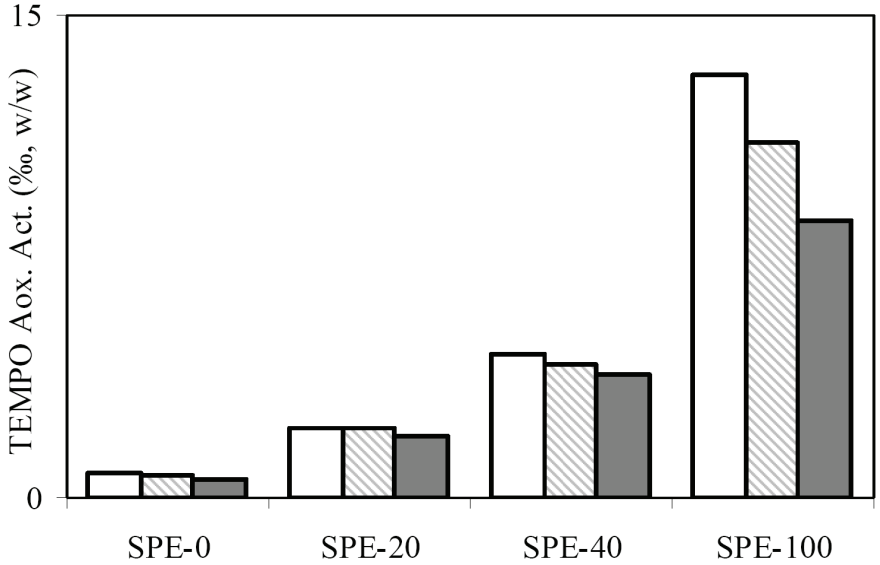

Figure 6. The TEMPO antioxidant activity levels in low Mw coffee brew fractions isolated by reversed-phase solid phase extraction. The white, striped, and grey bars represent the light, medium, and dark roasted coffee brew fractions, respectively.

that had an apolar character (SPE-40 and SPE-100). This proved that also low Mw Maillard reaction products possess a relative high TEMPO antioxidant activity. Noteworthy is that the TEMPO antioxidant activity in these apolar fractions decreased with increasing degree of roast. This suggested that the formed low Mw roasting-induced antioxidants react and might be incorporated in HMw molecules upon prolonged roasting. The results of the Fremy's salt antioxidant activity and FRA levels are shown in **Table 4**. The Fremy's salt activity level in SPE-0 was relatively low which was ascribed to the presence of many non-antioxidative compounds like sugars and minerals, as was shown previously (*25*). The little Fremy's salt antioxidative activity in SPE-0 should be due to polyphenols since the TEMPO antioxidant activity levels were low (**Figure 6**) and the FRA levels (76-80%) were high and similar to the FRA level of chlorogenic acid (82%) (**Table 4**). The SPE-20 fractions showed the highest activity levels due to the presence of high concentrations of chlorogenic acids (*25*) which

Table 4. Fremy's Salt Antioxidant Activity and Fast Reacting Antioxidant (FRA) Levels of Low Mw Coffee Brew Fractions with Varying Degrees of Roast[a,b]

	Light roast		Medium roast		Dark roast	
	Fremy's (‰, w/w)	FRA level (%)	Fremy's (‰, w/w)	FRA level (%)	Fremy's (‰, w/w)	FRA level (%)
LMw	204	77	196	77	155	77
SPE-0	119	80	103	79	70	76
SPE-20	404	79	380	78	350	81
SPE-40	301	79	335	81	285	78
SPE-100	108	70	132	67	155	71

[a] Antioxidant activity levels were measured as Trolox equivalents. [b] The average coefficient of variation was 3% for the Fremy's salt antioxidant activities.

resulted in high FRA levels as well. Some of the chlorogenic acids might have end up in the SPE-40 fraction, resulting in a relatively high Fremy's salt antioxidant activity and high FRA levels. Another explanation might be that caffeine scavenges Fremy's salt too, as caffeine is also capable of scavenging hydroxyl radicals (*32*). The SPE-100 had low Fremy's salt antioxidant activities and low FRA levels, which is in line with the presence of relatively many roasting-induced antioxidants.

Relation between Antioxidant Activity and Melanoidins.

To the best of our knowledge, a positive and linear correlation between antioxidant activity and color was only found in model systems and in foods where the Maillard reaction was the sole or prevalent event (*10, 33*). In coffee, this correlation is not clear because phenolic compounds play an important role in antioxidant activity as well (*10, 11*). In this research, it was shown that the TEMPO antioxidant activity provides insight in the extent of roasting-induced antioxidants formed upon roasting of coffee beans, while polyphenolics are not detected. The melanoidin level can be quantified by determination of the brownness, which is represented by the parameter $K_{\text{mix 405}}$ (*23*). As both the TEMPO antioxidant activity and $K_{\text{mix 405}}$ values are related to the extent of Maillard reaction, it was expected that there would be a positive correlation between these two parameters. The $K_{\text{mix 405}}$ values of the coffee brews, HMw fractions, ethanol precipitation fractions, and solid phase extraction fractions are plotted as a function of the TEMPO antioxidant activity in **Figure 7**. Starting with the coffee brews, it is clear that the light, medium, and dark roast brews showed a positive and linear correlation (R^2= 0.97) between the $K_{\text{mix 405}}$ value and TEMPO antioxidant activity. A similar positive and

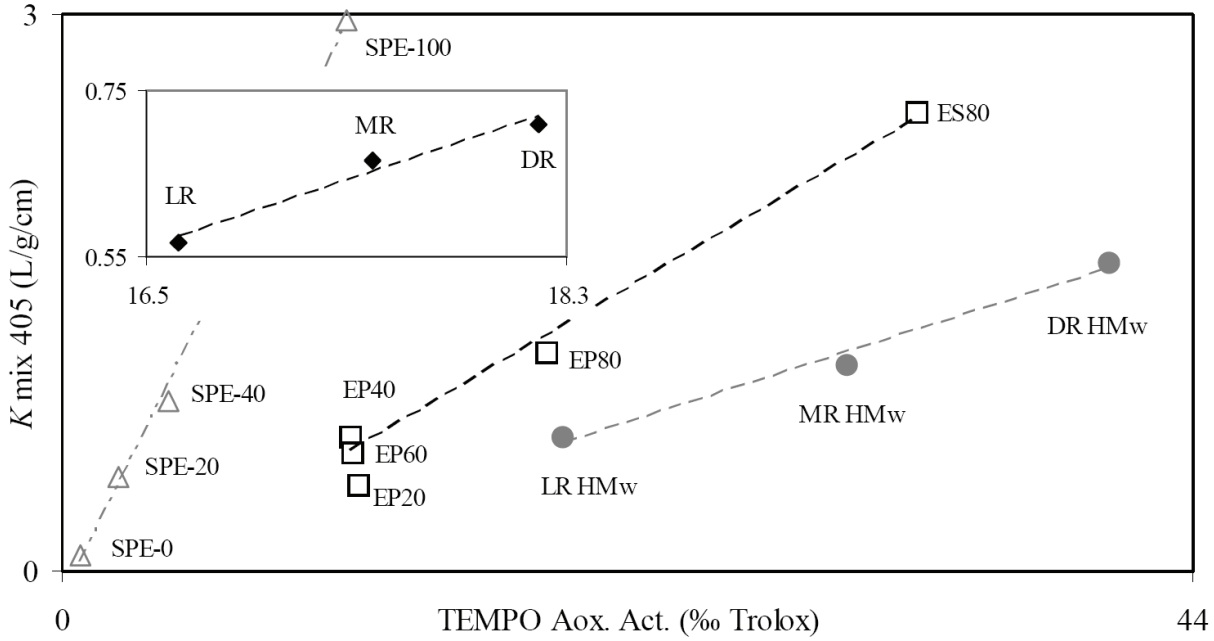

Figure 7. The $K_{\text{mix 405}}$ values of light (LR), medium (MR), and dark (DR) roast coffee fractions plotted as a function of the TEMPO antioxidant activity: HMw fractions (circles), ethanol precipitation fractions (squares), MR solid phase extraction fractions (triangles), and whole brews (inlay, diamonds).

linear correlation was found for the light, medium, and dark roast HMw fractions (R^2= 0.98). It should be noticed that the samples that were isolated at different stages or under different conditions, do not result in one single line in which the $K_{mix\ 405}$ correlates with the antioxidant activity. This might be due to a loss of antioxidant activity during each isolation step while the recovery of the $K_{mix\ 405}$ level was good. Therefore, only samples isolated under similar conditions should be compared. The IMw and LMw fractions were not plotted since all these fractions had similar TEMPO antioxidant activity and $K_{mix\ 405}$ values at different degrees of roast which left little space for observing linearity. However, the solid phase extraction fractions isolated from LMw showed difference in TEMPO antioxidant activity and $K_{mix\ 405}$ values, the values for these fractions are plotted in **Figure 7** as well. Also for these low Mw sub-fractions, a positive and linear correlation (R^2= 0.99) was found. Finally, the ethanol precipitation sub-fractions of the medium roast HMw fraction showed a positive and linear correlation (R^2= 0.99) between the $K_{mix\ 405}$ value and TEMPO antioxidant activity. The fraction EP20 was excluded due to its limited solubility, causing a underestimation of $K_{mix\ 405}$ (*23*). A positive and linear correlation was found for the coffee brews, HMw fractions, and the sub-fractions of both the HMw material and LMw material. From these results, it could be concluded that there is indeed a positive and linear correlation between the $K_{mix\ 405}$ values and TEMPO antioxidant activity for all series, proving that the extent of roasting-induced antioxidant formation is directly linked to the extent of melanoidin formation. From the latter, it can be concluded that the roasting-induced antioxidants formed upon roasting of coffee beans are present within coffee melanoidins. To the best of our knowledge, this is the first time that a positive and linear correlation between roasting-induced antioxidants and melanoidin level was reported in a complex product like coffee.

General discussion.

At present, not much information is available on the bioavailability of coffee melanoidin antioxidants. Rufián-Henrares and Morales (*34*) reported on the degradability of coffee melanoidins by human digestive enzymes present in the GI-tract and its effect on the antioxidant activity. Gniechwitz et al. recently reported on the degradability of coffee brew material that was rich in dietary fiber and melanoidins by human fecal microbiota (*35*). However, research on digestibility and bioavailability of coffee melanoidins is still in its infancy. In general, there is a consensus that melanoidins, of any source, have a low digestibility and bioavailability (*36*). There is also evidence that melanoidins are not digested in the upper part of the GI-tract and are mainly recovered in the feces (*37*). As most of the roasting-induced antioxidants are present in high Mw melanoidins and as melanoidins are complex structures, these molecules are probably not digested and not taken up in the blood stream at all. As a result, it is then likely that coffee melanoidins will pass the GI-tract and that the roasting-induced antioxidants will show antioxidant activity in the intestine if these antioxidants survive gastric conditions. Coffee melanoidins, containing both phenolic antioxidants (*18*) and roasting-induced antioxidants, might then provide protection against a wide range of radicals throughout the GI-tract. It was calculated that high Mw melanoidins

present in one cup of coffee (150 mL) will show antioxidant activity against the radical TEMPO equivalent to 30 mg of Trolox, a derivative of vitamin E. This is twice as much as the daily recommended intake (DRI) for vitamin E (*38*), showing that coffee melanoidins may significantly contribute to the desired daily antioxidant consumption. Of course, much research needs to be conducted to elucidate the reaction mechanism and radical specificity of antioxidants formed in coffee beans upon roasting.

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CHAPTER 7

Roasting Effects on Formation Mechanisms of Coffee Brew Melanoidins

ABSTRACT

The effects of the roasting degree on coffee brew melanoidin properties and formation mechanisms were studied. Coffee brew fractions differing in molecular weight (Mw) were isolated from green, light, medium, and dark roasted coffee beans. Isolated fractions were characterized for their melanoidin, nitrogen, protein, phenolic groups', chlorogenic acid, quinic acid, caffeic acid, and sugar content. It was found for all fractions that the melanoidin level correlated with the nitrogen and protein content. The melanoidin content correlated with the phenolic groups' level and the ester-linked quinic acid level as well. It was concluded that proteins and chlorogenic acids are primarily involved in melanoidin formation. Furthermore, arabinogalactans seem to be more involved in melanoidin formation than galactomannans. Initial roasting to light roasted beans resulted in the formation of twice as much intermediate Mw (IMw) melanoidins than high Mw (HMw) melanoidins. Indications were found that the prevailing IMw melanoidin formation was due to both Maillard reactions and chlorogenic acid incorporation reactions between the reactive chlorogenic acids, sucrose and amino acids/protein fragments. Additionally, it was found that prolonged roasting especially led to accumulation of HMw coffee brew melanoidins. Proteins were solubilized upon roasting resulting in accumulation of melanoidins in HMw material. Furthermore, formation or attachment of chromophores through the arabinose moiety of arabinogalactan proteins (AGP) was hypothesized to occur as well. Finally, it was concluded that galactomannan is continuously incorporated in AGP–melanoidins upon roasting. Overall, it was concluded that chlorogenic acids play an important role in coffee brew melanoidin formation.

KEYWORDS

Coffee; brew; melanoidins; degree of roast; formation mechanisms

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INTRODUCTION

Coffee is a popular beverage that is consumed worldwide by many people every day. The total coffee consumption is estimated to be almost 7 billion kilograms of coffee beans annually (*1*). The roasting process of coffee beans leads to the formation of the characteristic coffee aroma and the dark-colored compounds. These flavor compounds are formed mainly as the result of the Maillard reaction (*2*) that takes place between carbohydrates or degraded carbohydrates (*3*) and proteins (*4*). These dark-colored compounds are referred to as melanoidins and they make up to 25% of the dry matter in coffee brew (*5*). The chemical structure of coffee melanoidins is extremely complex and is still largely unknown. This complexity is due to the fact that many green coffee bean components might play a role in melanoidin formation (*6*). For example, it was shown that next to common Maillard reactions, incorporation of chlorogenic acid plays an important role in coffee brew melanoidin formation as well (*7*). Because complete knowledge on melanoidin structures is lacking, melanoidins are generally defined as brown, nitrogenous macromolecular compounds that absorb light at 405 nm (*5, 8-11*). The introduction of the parameter $K_{\text{mix 405nm}}$, which is the specific extinction coefficient at 405 nm, allowed quantification of the melanoidin level in a coffee fraction (*11*).

Melanoidins are not only of interest because of their contribution to the color of coffee brew but also for their flavor binding properties (*9, 12-16*), antioxidative capacity (*5, 10, 17, 18*), metal-chelating properties (*18, 19*), and reactivity in coffee brew (i.e., aging of coffee) (*20*). It is also therefore that a renewed interest in melanoidin structure can be observed in literature. Recent research focused on the involvement of chlorogenic acids (*7, 21*), galactomannans (*21*), arabinogalactan proteins (*22*) in melanoidin formation as well as on digestibility (*23, 24*), ionic charge properties (*22*), molecular size properties (*25*), and acidifying properties (*7*) of melanoidins. Even though this renewed interest in coffee melanoidins led to an improved understanding of melanoidin structures, the mechanisms involved in coffee melanoidin formation are not or at most poorly understood. The most logical approach to obtain insight in melanoidin formation mechanisms is probably the comparison of coffees prepared from beans with varying degrees of roast. Light roasted coffees should contain relatively many intermediate melanoidins, whereas dark roasted coffees should contain more melanoidins that have evolved towards the final melanoidin structures. In literature, quite some information can be found on effects of the degree of roasting on coffee composition and coffee properties (*10, 12, 17, 26-28*). In some studies, this comparison was even performed to obtain information on the melanoidin structure (*21*). However, no attempts to understand underlying melanoidin formation mechanisms were reported.

The objective of this study was to explore this gap in literature by investigation of the effect of roasting degree on melanoidin development in coffee brews. To this end, coffee brews were prepared from beans with varying degrees of roast, then melanoidin populations differing in molecular size were isolated, and melanoidin chemical and chromatographic characteristics were investigated.

MATERIALS AND METHODS

Materials.

Green and roasted Colombian coffee beans (*Coffea arabica*) were provided by a local factory. The degree of roast of the light, medium, and dark roasted beans was 14.7%, 16.4%, and 19.2% (w/w), respectively; which was 6.1%, 8.0%, and 11.1% (w/w) on a dry matter basis, respectively. The roasting degree of a coffee fraction is denominated as "Green" or "GB" for green beans, "Light" or "LR" for light roasted beans, "Medium" or "MR" for medium roasted beans, and "Dark" or "DR" for dark roasted beans. Chemicals were bought from Sigma Aldrich (Sigma Chemical Corp., St. Louis, MO) in the highest purity available.

Preparation of Coffee Brew.

Green (frozen with liquid nitrogen) and roasted coffee beans were ground and a brew was extracted at 90 °C for 15 min as described previously (**Figure 1**) (*11*). For characterization purposes, part of the brew was lyophilized, yielding "brew". The major part of the brew was used for further isolation.

Isolation of High Mw Coffee Brew Material.

High Mw material was obtained from the brew by diafiltration as described previously (*11*). The retentate and dialysate were lyophilized, yielding a high Mw fraction ("HMw") and a fraction with a lower Mw ("DF-dialysate"), respectively (**Figure 1**).

Isolation of Intermediate and Low Mw Coffee Brew Material.

Intermediate and low Mw material was obtained from defatted DF-dialysate sample by membrane dialysis. Aqueous DF-dialysate solutions (0.5 L, 100 g/L) were dialyzed using a Visking size 9 dialysis membrane with a cutoff of 12-14 kDa (Medicell International Ltd, London, UK) for 3 days at 4 ºC against 5 L demineralized water with four water renewals. The first 2 dialysate fractions were pooled and lyophilized, yielding the low Mw fraction ("LMw") (**Figure 1**), whereas the last 3 dialysate fractions were discarded. The retentate was lyophilized yielding the intermediate Mw fraction ("IMw") (**Figure 1**).

Arabinogalactan Protein Isolation.

Arabinogalactan protein was isolated from high Mw coffee material in duplicate using the Yariv reagent as was described previously (*22*). The dialyzed arabinogalactan protein containing fraction was lyophilized, yielding "AGP" (**Figure 1**).

Defatting of Coffee Samples.

Lyophilized coffee brew samples were defatted by Soxhlet extraction using a Soxtherm, which was connected to a Multistat system (Gerhardt, Königswinter, Germany), as described previously (*22*). The solvent used for extraction was dichloromethane.

Determination of the Unbound and Total Caffeic Acid and the 5-Caffeoylquinic Acid Content.

For determination of the unbound caffeic acid (CA) and 5-caffeoylquinic acid (5-CQA) contents, aqueous sample solution was centrifuged and the supernatant was subjected to analysis. For determination of the total CA content, samples were saponified in the presence of ascorbic acid and ethylenediaminetetraacetic acid prior to analysis as described previously (*7*). Aqueous and saponified samples were analyzed by reversed-phase

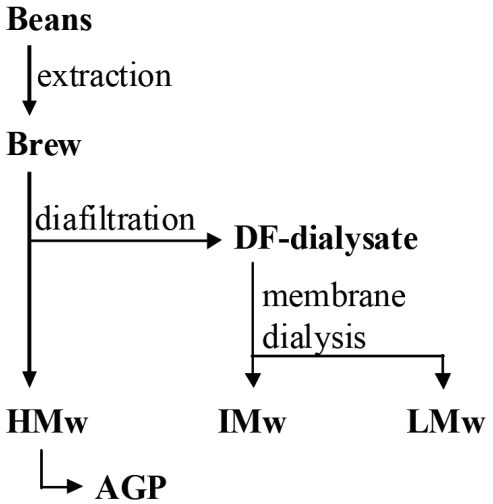

Figure 1. Scheme for the isolation of various coffee fractions.

chromatography using a 150 × 4.6 mm i.d. XTerra® MS C18 3.5 µm column in combination with a 20 × 3.9 mm i.d. XTerra® MS C18 3.5 µm guard column (Waters, Milford, MA) using the procedure described previously (*7*). Aqueous 5-CQA and CA solutions were used as reference for the 5-CQA and unbound CA contents, respectively. Saponified CA solutions were used as reference for the total CA contents. Experiments were performed at least in duplicate The average coefficient of variation was 1%.

Determination of the Unbound and Total Quinic Acid Content.

For the determination of the unbound quinic acid (QA) content, aqueous sample solution was centrifuged and the supernatant was subjected to analysis. For the determination of the total QA content, samples were saponified prior to analysis as described previously (*7*). Aqueous and saponified samples were analyzed by ion-moderated partitioning chromatography using a 300 × 7.8 mm i.d. Aminex HPX 87H column equipped with a cation H+ guard column filled with AG® 50W–X4 (Bio-Rad, Hercules, CA) using the procedure described previously (*7*). Aqueous and saponified QA solutions were used as reference for the unbound and total QA contents, respectively. Experiments were performed at least in duplicate. The average coefficient of variation was 3%.

Analysis of Nitrogen Content.

The nitrogen content of various samples was estimated according to the Dumas method using an NA2100 nitrogen and protein analyzer (Carlo Erba Instruments, Milan, Italy) according to the manufacturer's instructions. Methionine was used as a standard and the average coefficient of variation was 2%.

Sugar Analysis.

The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (*29*) using inositol as an internal standard. Briefly, samples were prehydrolyzed with 72% (w/w) H_2SO_4 for 1 h at 30 ºC, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 ºC and the constituent sugars released were analyzed as their alditol acetates. The uronide content was determined by the automated colorimetric *m*-hydroxydiphenyl method (*30, 31*).

Total Phenolic Groups Level.

The total phenolic groups content of the coffee samples were determined with the Folin-Ciocalteau assay as described previously (*11*). The used reference compound was 5-CQA and the average coefficient of variation was 2%.

Amino acid analysis.

Samples for amino acid analysis were hydrolyzed using liquid phase hydrolysis in 6 M HCl at 110 °C for 24 h. Amino acid analyses were performed using a Hewlett Packard Aminoquant 1090M using an automated two-step pre-column derivatization with two different reagents, *o*-phthalaldehyde for primary and 9-fluorenylmethylchloroformate for secondary amino acids (*32*).

Specific extinction coefficient of coffee material at 280, 325, and 405 nm.

The absorption of aqueous coffee fractions (1 g/L) was determined at 280, 325, and 405 nm using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). The solutions were diluted in case the absorption was higher than 1.3. The specific extinction coefficients K_{mix} (L/g/cm) at different wavelengths and the K_{mix} ratios were calculated as previously described by Bekedam et al. (*11*)**.** The average coefficient of variation was 1% of the K_{mix} values.

RESULTS AND DISCUSSION

Effect of Roasting on Isolation Yields of Coffee Brew Fractions.

All brews that were extracted from roasted beans had a yield of 16%, on the basis of green beans (**Table 1**). It is known that the extractability of some coffee bean compounds, for example galactomannans, might increase upon roasting (*3*) and that the extractability of other coffee bean compounds like amino acid containing components might decrease upon roasting (*33*). Combining these roasting effects with the similar extraction yields, these results indicate that the amount of material that becomes soluble and the amount of material that becomes insoluble should be the same.

Table 1. Yields of the Isolated Coffee Brew Fractions

	Brew[a] (%, w/w)	HMw[b] (%, w/w)	IMw[b] (%, w/w)	LMw[b] (%, w/w)	Recovery[c] (%, w/w)	AGP[a] (%, w/w)
Green	18.9	17.6	5.2	62	85	0.72
Light	15.9	14.8	19.5	54	88	0.93
Medium	16.0	16.7	22.5	51	90	0.96
Dark	16.0	17.2	24.7	46	88	0.69

[a] On green bean basis. [b] On brew DM basis. [c] Combined HMw, IMw, and LMw yield.

The yields of the brews that were extracted from roasted coffee beans with different degrees of roast were similar (**Table 1**). This allowed the comparison of HMw, IMw, and LMw yields when expressed on the basis of the corresponding brew instead of green beans (**Table 1**). The recovery of the isolation procedure of HMw, IMw, and LMw out of the brews was on average still 88% and the loss of some material was due to discarding part of the dialysate after membrane dialysis. The roasting of green beans to light roasted beans led to a decrease in HMw yield from 18% to 15% (**Table 1**). This decrease can be explained by protein denaturation and aggregation which occurs during roasting. Upon prolonged roasting from light to dark roasted beans, the yields of both the HMw and IMw fractions increased. This increase in yield might be due to chemical reactions, like condensation reactions, that lead to transformation of LMw compounds into IMw and HMw compounds, likely melanoidins (*34*). Additionally, it could also be that specific HMw coffee bean compounds, like galactomannans, are more readily extracted after prolonged roasting (*3*). In the latter situation, other coffee brew compounds have to become insoluble upon prolonged roasting as the brew extraction yield remained constant upon prolonged roasting. The IMw fraction was only 5% for the green coffee brew. The yield of this IMw fraction increased rapidly to 20% in light roast and finally up to 25% in dark roast. This steep increase in yield of IMw material implies degradation of coffee bean macromolecules to IMw molecules upon roasting. Alternatively, LMw components might transform into to IMw molecules as well.

Roasting Effects on Melanoidin Levels in Various Brew Fractions.

The melanoidin level is represented by the $K_{\text{mix 405nm}}$ value (*11*) and the melanoidin level of coffee fractions is shown in **Table 2**. The $K_{\text{mix 405nm}}$ value for green coffee was expected to be zero since no roasting products are present. However, due to turbidity, which was only observed for green coffee material, a value of 0.14 for green brew was obtained. Roasting led to a marked increase of melanoidin quantity in coffee brews. The $K_{\text{mix 405nm}}$ value increased to 0.57 for light roasted brew, followed by a further increase to 0.67 and 0.71 for medium and dark roasted brew, respectively. These results confirm that formation of water-soluble melanoidins occurred continuously upon prolonged roasting of coffee beans.

When looking to the HMw, IMw, and LMw fractions of the coffee brews (**Table 2**), it stands out that the melanoidin level in the IMw and LMw fractions remained more or less

Table 2. Melanoidin Levels ($K_{mix\ 405nm}$) in Various Coffee Fractions[a]

	Brew (L/g/cm)	HMw (L/g/cm)	IMw (L/g/cm)	LMw (L/g/cm)	AGP (L/g/cm)
Green	0.14	0.25	0.24	0.05	nd
Light	0.57	0.74	1.05	0.31	0.73
Medium	0.67	1.11	1.08	0.36	1.07
Dark	0.71	1.66	1.03	0.32	1.59

[a] On the basis of the fraction itself. nd = not determined.

constant whereas the melanoidin level in the HMw fractions increased upon prolonged roasting. This shows that the density of brown–colored structures only increased in HMw material upon roasting. This suggest that IMw material consist of highly reactive compounds that took part in the Maillard reaction during the first moments of roasting. Oppositely, HMw material should then consist of somewhat less reactive compounds that react slower but more continuously throughout the roasting process leading to a continuous formation of HMw melanoidins during roasting. Alternatively, IMw melanoidins might act as intermediate melanoidin structures which evolve towards HMw melanoidins upon prolonged roasting.

It should be realized though that the yields of the fractions varied upon roasting (**Table 1**) which could mask the total quantity of melanoidins in a fraction and its contribution to the whole brew. Therefore, the $K_{mix\ 405nm}$ value was also expressed on the basis of the brews (**Figure 2**). This allowed comparison of fractions with different molecular weights and comparison of fractions with different degrees of roast. These results show that, in absolute terms, coffee brew melanoidins accumulate in both the intermediate and high molecular weight material upon prolonged roasting. In light roasted brew, the contribution of melanoidin in HMw material is much smaller than the contribution of the melanoidins in the IMW

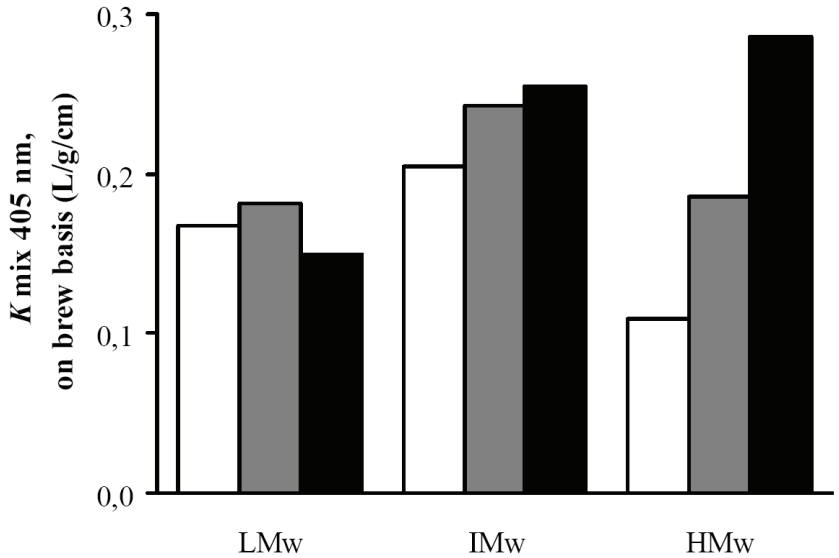

Figure 2. Melanoidin levels of HMw, IMw and LMw material from light (white bars), medium (gray bars), and dark (black bars) roasted coffees expressed on the basis of the corresponding brews.

material. However, the contribution of HMw melanoidins increases far more rapidly than the contribution of IMw melanoidins with continued roasting. As a result, the contribution of HMw in dark roasted brew is larger than for IMw. This is due to the more pronounced increase of both the melanoidin level and fraction size of HMw material upon roasting. Investigation of these fractions for their chemical composition is described below and will provide more insight in melanoidin formation mechanisms.

Effect of Roasting on the Nitrogen and Total Amino Acid Content in Coffee Brew Fractions.

It was shown in previous research that the nitrogen level was closely related to the melanoidin level (*11*). The nitrogen levels of various coffee fractions are shown in **Table 3**. The roasted brews all had nitrogen levels of about 3%, and the nitrogen content did not show correlation with the melanoidin level that increased upon prolonged roasting (**Table 2**). This lack in correlation is due to many nitrogenous LMw compounds like caffeine and therefore, the nitrogen level provided no information on melanoidin structures in whole brews and LMw fractions. Nitrogen contents of 11.3% and 7.4% in green coffee HMw and IMw indicated the presence of 14% proteins in green bean brew when using a nitrogen-to-protein factor of 5.5 (**Table 3**) (*11*). Amino acid analysis revealed total amino acid levels corresponding to 69% protein in HMw and 41% protein in IMw (**Table 4**). Even though amino acid or protein fragments could be part of melanoidin structures, amino acids detected in HMw and IMw fractions were denominated, for clarity, as proteins in this study. In green bean brew, 84% of the amino acids is present as proteins since green coffee LMw contained only 1.8% amino acids (**Table 4**).

Roasting of green to light roast beans led to lower nitrogen and protein levels in light roast HMw material than in green bean HMw material (**Tables 3** and **4**). It was calculated that the total amount of proteins was 18 times lower for light roasted than for green bean HMw material. Furthermore, the calculated total amount of protein/amino acids remained constant in IMw and LMw material upon roasting from green to light roasted beans. In total, 66% of the proteinous material in green coffee brew was not recovered in light roasted brew. This enormous loss must be due to participation of amino acids in Maillard-like reactions that occur during roasting and also due to protein denaturation during roasting leading to limited protein extractability. Comparison of nitrogen and protein losses revealed that 25% of the

Table 3. Nitrogen Content of Various Coffee Fractions[a]

	Brew (%, w/w)	HMw (%, w/w)	IMw (%, w/w)	LMw (%, w/w)	AGP (%, w/w)
Green	4.3	11.3	7.4	2.3	1.2
Light	3.0	1.2	2.7	3.6	0.8
Medium	2.9	1.4	2.6	3.7	1.0
Dark	3.0	1.7	2.4	3.8	1.5

[a] On the basis of the fraction's dry matter itself.

Table 4. Amino Acid Composition (Mol%) and Content of Various Coffee Fractions

	Ala	Arg	Asx	Glx	Gly	His	Ile	Lys	Leu	Met	Phe	Pro	Ser	Thr	Tyr	Val	Total (%, w/w)[a]
Brew Green	6	4	9	24	10	2	4	5	8	1	4	6	6	4	2	5	16.9
Brew Light	7	0	9	32	13	1	3	0	8	0	4	8	4	4	2	5	6.8
Brew Medium	7	0	8	30	13	2	4	0	9	1	4	9	3	3	2	5	6.0
Brew Dark	8	0	8	33	12	1	4	0	9	1	4	8	2	2	2	6	6.7
HMw Green	6	5	9	23	10	2	4	5	9	1	5	5	5	3	2	6	68.6
HMw Light	7	0	9	30	13	1	3	1	9	0	4	6	5	4	2	6	5.5
HMw Medium	8	0	8	29	13	2	4	1	9	1	4	7	3	3	2	6	5.7
HMw Dark	8	0	8	30	14	1	4	1	9	0	4	8	2	3	2	6	7.0
IMw Green	5	4	7	25	10	2	4	4	7	1	2	9	8	6	1	5	40.9
IMw Light	7	0	8	30	13	2	4	0	9	1	4	7	4	4	2	5	12.9
IMw Medium	8	0	8	26	13	2	4	1	10	1	4	9	3	3	2	6	11.9
IMw Dark	8	0	8	28	12	2	4	1	11	1	4	8	2	3	2	6	10.8
LMw Green	12	2	12	28	7	1	2	2	3	2	3	6	5	2	2	3	1.8
LMw Light	7	0	10	39	12	1	3	0	6	1	3	8	3	2	2	3	3.4
LMw Medium	7	0	9	34	12	0	4	0	8	1	3	11	3	2	2	4	3.9
LMw Dark	7	0	9	42	11	1	3	0	7	1	3	7	2	2	2	3	5.0
AGP Green	12	2	9	13	11	1	4	4	11	0	4	5	8	7	1	8	6.4
AGP Light	11	0	9	27	15	1	3	0	7	0	3	6	7	4	1	6	2.2
AGP Medium	10	0	9	26	15	0	4	0	8	0	4	6	7	4	1	6	2.9
AGP Dark	10	0	10	27	16	0	4	0	8	0	4	6	4	3	2	6	4.8

[a] on the basis of the fraction's dry matter itself.

total amino acid loss in HMw is due to participation of amino acids in Maillard-like reactions and that 75% should be due to limited solubility of denatured proteins.

When light roasted coffee beans are further roasted up to dark roasted beans, it stands out that both the nitrogen and protein level increased in HMw material but decreased in IMw material upon prolonged roasting (**Table 3** and **4**). The total amount of amino acids/proteins present in each fraction was calculated on the basis of the brews as well in the similar way as done for the amount of melanoidins (**Figure 2**). These calculations showed that the amount of amino acids/proteins increased by a factor 1.3 for LMw, 1.1 for IMw, and 1.5 for HMw material when roasting from light to dark roasted beans. Thus, the amount of amino acids/proteins increases for all molecular sizes upon roasting, which indicates that prolonged roasting results in solubilization of denatured and possibly degraded proteins. This improved extractability of proteinous coffee bean material might be caused by chemical modifications of the protein, like chlorogenic acid incorporation, resulting in more protein in HMw coffee material. Additionally, degradation of proteins into smaller fragments should occur also because the total amount in the LMw fraction increased as well.

With respect to melanoidins, it can be seen that both the melanoidin level (**Table 2, Figure 2**) and the protein and nitrogen contents (**Figure 3, Table 3** and **4**) increased upon prolonged roasting in HMw coffee brew material. The melanoidin and nitrogen levels remained more or less constant in the IMw fractions upon prolonged roasting (**Table 2** and **3**) while both the total amount of melanoidins (**Figure 2**) and total amount of nitrogen (**Figure 3**) increased upon prolonged roasting. These findings indicate once again that the nitrogen and the melanoidin level are closely related and that nitrogenous compounds should be directly involved in melanoidin formation. It is remarkable though that the nitrogen to melanoidin ratio was higher for all roasted IMw fractions than for all roasted HMw fractions. A possible explanation for this could be that IMw material contains relatively many protein-based

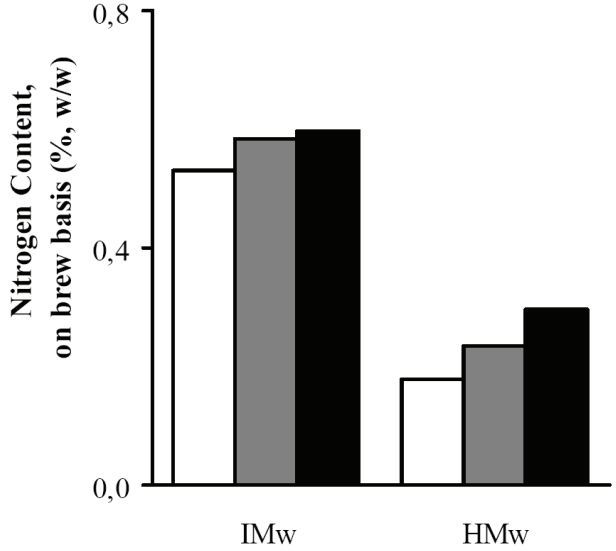

Figure 3. Nitrogen contents of HMw and IMw material from light (white bars), medium (gray bars), and dark (black bars) roasted coffees expressed on the basis of the corresponding brews.

melanoidin structures (**Table 4**) while HMw material contains more carbohydrate-like melanoidin structures as will be discussed below.

Effects of Roasting on the Amino Acid Composition in Various Brew Fractions.

Next to the total amino acid level, the amino acid composition of the coffee fractions was determined as well (**Table 4**). Only few differences in amino acid composition were found for coffee fractions that differed in molecular weight or degree of roast. One of these differences was found in the molar percentage of glutamine/glutamic acid (Glx) between green and light roasted coffee fractions. This increase in Glx upon roasting might be the result of a relatively larger decrease of other amino acids or, alternatively, be due to extraction of different types of proteins. Furthermore, roasting led to a loss of arginine, lysine, serine, and threonine, which was in agreement with the literature (*11*). The fact that the amino acid composition of all HMw, IMw, and LMw coffee fractions were rather similar indicates that the same proteins are present in each fraction, although degraded and/or modified in different extents (*11*). These results showed that the changes in amino acid composition especially occur during the initial stage of roasting and that prolonged roasting did not affect the composition a lot. These findings indicate that coffee bean proteinous material is quite reactive and that most of the reactions involving amino acids that lead to formation of melanoidin occur during the initial roasting stage.

Effect of Roasting on the Phenolic Groups and the 5-CQA Levels in Various Brew Fractions.

The phenolic groups' level by Folin-Ciocalteu and the 5-CQA contents of the coffee fractions are shown in **Table 5** and **Table 6**, respectively. At first sight, the quite constant level of phenolic groups in the coffee brews with varying degree of roast indicated that coffee bean phenolics are quite unreactive (**Table 5**). However, the level of 5-CQA, the most abundant chlorogenic acid and most abundant phenolic compound in green beans, decreased drastically from 16% in green coffee to less than 2% in dark roasted coffee brew (**Table 6**). Additionally, it was found that the phenolic group level in the HMw fractions, which were all four free from unbound 5-CQA, increased gradually from a level as low as 3% in green coffee HMw up to 18% in dark roasted HMw. These findings were in line with literature (*35*) and showed that coffee bean phenolics are quite reactive. They also indicated that incorporation of phenolic compounds in high Mw coffee material is a reaction that occurs continuously upon roasting.

Table 5. Phenolic Groups Level of Various Coffee Fractions[a]

	Brew (%, w/w)	HMw (%, w/w)	IMw (%, w/w)	LMw (%, w/w)	AGP (%, w/w)
Green	24	3	21	24	2
Light	24	9	21	24	6
Medium	23	13	19	23	10
Dark	21	18	18	19	14

[a] As chlorogenic acid equivalents, on the basis of the fraction's dry matter itself.

Table 6. 5-Caffeoylquinic Acid Level of Various Coffee Fractions[a]

	Brew (%, w/w)	HMw (%, w/w)	IMw (%, w/w)	LMw (%, w/w)	AGP (%, w/w)
Green	15.7	0.0	9.7	18.2	0.0
Light	4.2	0.0	0.8	5.8	0.0
Medium	2.9	0.0	0.4	4.3	0.0
Dark	1.5	0.0	0.2	2.3	0.0

[a] On the basis of the fraction's dry matter itself.

It was found that the green coffee IMw fraction contained 21% phenolic groups (**Table 5**), while a level similar to the low level of green coffee HMw was expected. This level was probably mainly caused by chlorogenic acids as 10% 5-CQA was identified (**Table 5**). These chlorogenic acids were likely ionically bound to the 41% proteins that were present in this fraction (**Table 4**). Fortunately, this presumed ionic interaction is not very strong because these ionically bound chlorogenic acids were released and detected by the reversed-phase chromatography procedure used. Upon roasting, the 5-CQA content in IMw material rapidly decreased to levels as low as 0.2% in dark roasted IMw material (**Table 6**) while the phenolic groups' level remained rather constant at 21% in light roasted IMw. This level slightly decreased upon prolonged heating to 18% in dark roasted IMw. The phenolic groups' level in the LMw fractions had high phenolic groups level, ranging from 19% to 24%. The phenolic groups' level was much higher than the 5-CQA level in the roasted LMw fractions. This showed that the phenolic groups in roasted LMw material should be partly ascribed to chlorogenic acids (**Table 6**) as well as to chlorogenic acid degradation products (*7*).

Expression of the phenolic groups' level on the basis of the brew instead of the fraction itself eliminates the effect of fraction sizes which then shows the total amount of phenolics present in each fraction relative to the brew (**Figure 4**). Now, it is even more evident that the amount of phenolic groups in HMw material steadily increased upon roasting (**Figure 4**). Additionally, it was found that the pronounced increase in IMw yield concealed that the total quantity of phenolic groups in IMw material increased upon roasting as well (**Figure 4**). Thus, the total amount of phenolic groups increased in both the high and intermediate Mw material upon roasting. As a result, the total quantity of phenolics in low Mw material decreased upon roasting (**Figure 4**). It was previously shown that chlorogenic acids are incorporated in melanoidins upon roasting (*7*). The results presented in **Figure 4** indicate that roasting leads to a continuous transfer of green bean low Mw phenolics, i.e. chlorogenic acids, into intermediate and especially high Mw coffee material, which are likely melanoidins.

Comparing results for HMw and IMw fraction, it was found that the ratio between the amount of phenolic groups and the melanoidin level (**Tables 2** and **5**) was higher for IMw material than HMw material, at all degrees of roast. Similar observations were made for the ratio between the nitrogen to melanoidin level, which suggests that the phenolics are preferably linked to nitrogenous structures.

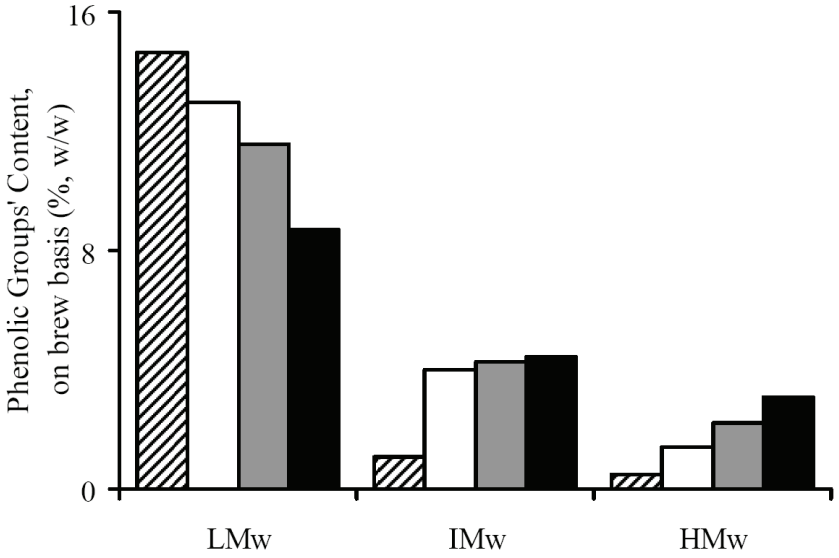

Figure 4. Melanoidin levels of HMw, IMw and LMw material from green coffee (striped bars), light (white bars), medium (gray bars), and dark (black bars) roasted coffees expressed on the basis of the corresponding brews.

Roasting Effects on Unbound QA and CA in Various Brew Fractions.

It was found that the coffee brews contained quite some unbound QA and almost no unbound CA (**Table 7**). All unbound CA and QA present in the roasted coffee brews was recovered in the low Mw fractions as the HMw and IMw fractions were free from CA and QA. The unbound 5-CQA, QA, and CA levels for medium roasted coffee fractions were similar to the levels obtained previously (*7*). The CA and QA levels in coffee brews decreased upon roasting (**Table 7**), indicating that both components are reactive under roasting conditions. Thus, the HMw and IMw fractions were free from unbound 5-CQA, QA, and CA. As saponification hydrolyzes ester-bonds, it can be stated that all QA and CA measured after saponification should be ester-linked to HMw and IMw material.

Roasting Effects on Chlorogenic Acid Incorporation in Various Brew Fractions.

The total QA and CA levels in the coffee brew fractions are shown in **Table 8**. The roasted coffees had extremely high total QA levels. The medium roasted brew had a total QA level similar to the level previously reported (*7*). The total QA level decreased from 12.7% in light

Table 7. Unbound Quinic and Caffeic Acid Levels (%, w/w) of Various Coffee Fractions[a]

	Brew		HMw		IMw		LMw		AGP	
	QA	CA	QA	CA	QA	CA	QA	CA	QA	CA
Green	nd	0.1	0.0	0.1	0.1	0.0	nd	0.1	0.0	0.0
Light	3.4	0.1	0.0	0.0	0.0	0.0	5.7	0.1	0.0	0.0
Medium	3.0	0.1	0.0	0.0	0.0	0.0	5.2	0.1	0.0	0.0
Dark	2.6	0.0	0.0	0.0	0.0	0.0	4.7	0.1	0.0	0.0

[a] On the basis of the fraction's dry matter itself. nd = not determined.

roasted brew to 9.7% in dark roasted brew. This loss of 24% total QA can be due to incorporation of QA into unextractable coffee bean material. Alternatively, this loss might be also due to chemical degradation of QA, or due to incorporation via other bonds than ester-linkages. The total CA level in the coffee brews decreased even more rapidly upon roasting than the total QA level, which can be ascribed to a higher susceptibility of CA to oxidative changes upon roasting (*36*).

The green coffee HMw material contained 0.1% total QA and 0.1% total CA levels, which showed that green coffee HMw material contained negligible amounts of ester-linked QA and CA (**Table 8**). The ester-linked QA and CA content in HMw material initially increased steeply to 1.5% and 0.5% in light roast HMw, respectively (**Table 8**). Subsequently, the ester-linked QA content increased gradually upon further roasting up to 2.1% in dark roasted HMw, whereas the ester-linked CA contents remained rather stable at ~0.5% upon further roasting (**Table 8**). These results indicate that the previously proven incorporation of chlorogenic acids (*7*) is a process that occurs continuously upon roasting into HMw material. Both ester-linked QA and the phenolic groups' levels increased while the ester-linked CA level was lower and rather stable upon prolonged roasting (**Table 5** and **8**). Therefore, these results further strengthen the proposed incorporation of chlorogenic acids in melanoidins via CA through a nonester linkage (*7*).

When looking to total QA and CA levels in the IMw fractions, two remarkable points stand out (**Table 8**). First, the total QA and CA levels are much higher for IMw than for HMw fractions, again indicating that compounds in the IMw fractions are more prone to reaction with CGAs. Second, it was found, after corrections for the yield, that the total amount of ester-linked QA and CA level in IMw material decreased upon prolonged roasting. These findings suggest that ester-linked QA and CA or structures to which QA and CA are ester-linked, present in IMw material, are degraded or transferred to HMw material upon prolonged roasting.

Table 8. Total Quinic and Caffeic Acid Levels (%, w/w) of Various Coffee Fractions[a]

	Brew		HMw		IMw		LMw		AGP	
	QA	CA	QA	CA	QA	CA	QA	CA	QA	CA
Green	nd	10.7	0.1	0.1	7.3	8.1	nd	12.7	0.0	0.0
Light	12.7	6.0	1.5	0.5	5.2	2.0	20.4	7.7	0.8	0.2
Medium	11.2	4.7	1.8	0.6	4.4	1.4	17.2	6.4	1.0	0.2
Dark	9.7	3.0	2.1	0.5	3.5	1.1	15.9	4.1	1.3	0.2

[a] On the basis of the fraction's dry matter itself. nd = not determined.

Effect of Roasting on the Sugar Content and Composition of Various Brew Fractions.

The sugar content in coffee brews slightly increased upon roasting (**Table 9**), indicating an improved extractability of polysaccharides upon roasting. The two main polysaccharides populations in coffee brews are galactomannans and arabinogalactans, the latter are predominantly present in AGPs in green beans (*22, 37*). The increase of mannose shows that

especially galactomannans become better extractable upon prolonged roasting (**Table 9**). Arabinose, from arabinogalactans, is known to be susceptible to degradation upon roasting (*3*), and this was seen in **Table 9** as well.

The sugar content in the carbohydrate-rich HMw fractions slightly decreased from 78% in light roasted HMw to 73% in dark roasted HMw. This should be due to sugar degradation and/or to the additional protein and chlorogenic acid moieties present in HMw material upon prolonged roasting (**Table 4-8**). It was calculated that the total amount of polysaccharides in HMw material slightly increased (+8%) when beans were roasted from light to dark roast. This was due to solubilization of galactomannan (+50% mannose) while a large part of the arabinose (-30%) was degraded. The IMw fractions contained less polysaccharides than the HMw fractions. However, it was calculated that the total amount of carbohydrates in IMw material increased significantly (+50%) upon roasting from light to dark roasted beans. This was again mainly due to an increase in galactomannan (+100% mannose). The loss of arabinose was only 3%, indicating that arabinose in IMw was not as prone to degradation as arabinose in HMw material. Alternatively, it could also be that HMw arabinogalactans

Table 9. Sugar Composition (Mol%) and Content of Various Coffee Fractions[a]

	Rha	Ara	Man	Gal	Glc	Uronic acid	Total sugars (%, w/w)
Brew Green	1	7	13	11	62	6	24
Brew Light	2	16	30	35	7	10	26
Brew Medium	2	15	35	34	5	9	28
Brew Dark	1	11	43	33	4	8	30
HMw Green	5	25	18	42	2	8	26
HMw Light	3	14	33	43	1	6	78
HMw Medium	2	11	40	40	1	6	75
HMw Dark	2	9	46	37	1	5	73
IMw Green	1	5	34	11	42	7	14
IMw Light	2	14	41	33	3	7	50
IMw Medium	2	13	45	32	2	6	53
IMw Dark	1	9	55	28	2	5	60
LMw Green	0	1	12	4	78	5	24
LMw Light	2	20	11	25	26	16	9
LMw Medium	3	24	9	31	16	17	8
LMw Dark	2	21	9	37	14	17	8
AGP Green	3	31	1	56	3	6	60
AGP Light	1	16	3	72	2	6	70
AGP Medium	1	13	7	71	2	6	68
AGP Dark	1	11	11	68	3	6	61

[a] On the basis of the fraction's dry matter itself.

degraded to IMw molecules. Obviously, many macromolecular coffee bean galactomannans were degraded into IMw galactomannans upon roasting. The Ga/Ara ratio increased for both HMw and IMw fractions upon roasting (**Table 9**), indicating that part of the arabinose in both HMw and IMw had been degraded during roasting. Combination of the latter with the fact that the amount of arabinose in HMw (-30%) decreased much more than arabinose in IMw (-3%), it can be concluded that HMw arabinogalactans are probably degraded into IMw molecules upon roasting. Thus, both arabinogalactan and galactomannan seem to be degraded into lower Mw molecules upon roasting.

With respect to the melanoidin levels, the results of the sugar composition and sugar content of HMw and IMw material do not provide firm proof that polysaccharides are directly involved in melanoidin formation. For HMw material, the melanoidin level (+160%) increased much more than the galactomannan level (+50% mannose) upon prolonged roasting. For IMw material, the melanoidin level showed a lower increase (+24%) than the galactomannan level (+100%) upon prolonged roasting. Nunes and Coimbra identified brown-colored structures at the reducing end of galactomannans (*27*). However, no information was given on the contribution of these structures to the overall color of coffee, which is likely limited as each macromolecular coffee galactomannan molecule contains only one reducing sugars. Furthermore, results presented in this study show that the increase in galactomannan can not be correlated to the increase in melanoidin and therefore, the results imply that galactomannans are not the main polysaccharide involved in melanoidin formation. With respect to arabinose, it can be suggested that arabinose, from AGPs, is involved in melanoidin formation as the HMw fractions showed the largest increase in melanoidins (+160%) and showed the largest decrease in arabinose degradation (-30%). The IMw fractions only showed a slight increase in melanoidins (+24%) and only a slight decrease in arabinose degradation (-3%). This inverse correlation between arabinose degradation and melanoidin formation suggests that chromophores might be formed from or attached to the arabinose moiety from AGPs upon roasting.

Roasting Effects on Arabinogalactan Proteins in Coffee Melanoidins.

It was previously shown that arabinogalactan proteins (AGPs) become part of melanoidin structures upon roasting, yielding the AGP– melanoidins (*22*). The Yariv reagent was used to isolate AGPs from HMw coffee material to be able to investigate the changes in properties of AGP–melanoidins upon roasting. **Table 1** shows the AGP isolation yields on a green bean basis to be able to compare extraction yields. These yields were 22%, 40%, 36%, and 25% (w/w) on the basis of green coffee HMw, light roast HMw, medium roast HMw, and dark roast HMw material, respectively. The initial increase in AGP yield from 0.72 to 0.93% (**Table 1**) is likely due to disruption of the cell wall leading to an improved AGP extractability. The AGP yield subsequently decreased upon prolonged roasting which might be ascribed to modifications of the AGP molecules (*7, 22*) leading to a lowered affinity to precipitate with the Yariv reagent.

The AGP–melanoidins had quite some similar properties and showed quite some similar changes upon roasting as the whole HMw fractions did. First, the melanoidin level increased upon prolonged roasting as was observed for HMw as well (**Table 2**). Because the AGP fractions are a substantial part of the HMw fractions, it can be concluded that AGP–melanoidins contribute substantially to the increase in melanoidin level of HMw material. Second, also the nitrogen and the protein content increased upon roasting as was observed for HMw material (**Table 3** and **4**). The nitrogen in green bean AGP was quite similar to the 1.88% reported by Redgwell et al. (*37*). The increase in nitrogen and protein content upon roasting indicated a higher level of proteinous material in AGP–melanoidins which could be due to degradation of arabinogalactan by which the relative nitrogen content increases. Alternatively, this could also be due to incorporation of additional proteinous material into AGP–melanoidin. Third, the AGP fraction also did not contain unbound 5-CQA, QA, or CA (**Table 6** and **7**) while the phenolic groups' level increased upon roasting (**Table 5**). Fourth, the last observation was that the ester-linked QA level increased while the ester-linked CA level remained constant upon prolonged roasting (**Table 8**). From these results it can be concluded that chlorogenic acids are incorporated in the AGP–melanoidin structures upon roasting. This incorporation probably occurs at the proteinous moiety of AGP–melanoidin structures or at the arabinose molecules which are degraded upon roasting.

However, the sugar composition of the AGP and HMw fractions were dissimilar. The AGP fractions mainly consisted of arabinogalactan which was clear from the high arabinose and galactose contents. The galactose to arabinose was 1.8 in green bean AGP and increased upon roasting to 6.2 in dark roasted AGP–melanoidins (**Table 9**). This indicated that arabinose in AGP must be quite reactive. Combining the gradual decrease in arabinose and gradual increase in melanoidin level also suggests that arabinose is likely involved in melanoidin formation. The sugar composition also revealed that mannose increased from 1 to 11 mol% in AGP–melanoidins upon roasting (**Table 9**). As galactomannan is the only source of mannose in coffee beans it can be decisively concluded from these results that galactomannan must be incorporated into AGP–melanoidins upon roasting, as was previously suggested (*22*).

General Discussion on Melanoidin Formation.

The compositional properties of coffee brew fractions, varying in degree of roast and molecular weight, were discussed separately per characteristic above. Combination of the investigated parameters could provide additional insight in coffee brew melanoidin formation mechanisms involved.

One of the two most striking melanoidin-related observations was that roasting from green to light roast led to twice as many IMw melanoidins compared to HMw melanoidins (**Figure 2**). This suggests that especially compounds present in the light roast IMw fraction are suitable for initial melanoidin formation. The other striking observation was that prolonged roasting (light to dark roast) especially led to melanoidin formation in HMw material (+160%), compared to +24% in IMw material (**Figure 2**). This suggests that components present in, or migrating to the HMw fraction are good precursors for melanoidin formation on

the longer run. Identical observations were made for nitrogen and phenolic groups' contents: much higher contents in light roast IMw than HMw, while these contents increased much more in HMw material upon prolonged roasting to dark roast beans (**Figure 3** and **4**).

It is hypothesized that initial melanoidin formation involves both Maillard and CGA-incorporation reactions between sucrose and CGAs on the one side and amino acids and/or protein fragments on the other side leading to LMw and IMw melanoidins (**Figure 2**). This hypothesis is strengthened by the facts i) that the sucrose in green beans is rapidly degraded upon roasting (*38*), and ii) that covalent bonds between CGA and coffee bean proteins were reported (*39*), and that iii) it was previously reported that low Mw melanoidins contained incorporated CGAs and also intact glucose with the latter most probably originating from sucrose (*25*). It was already mentioned that galactomannans, present in quite large quantities in IMw material, are probably not involved in reactions that dominate melanoidin formation. Altogether, this proposed hypothesis can explain why the majority of the initially formed melanoidins have a low to intermediate molecular weight.

The observation that prolonged roasting especially led to formation of melanoidins in HMw material might be explained by two reaction mechanisms. First, it was already mentioned above that denatured and aggregated coffee bean proteins became soluble again upon prolonged roasting due to chemical modifications. Part of this protein has likely been modified by Maillard reactions or by CGA incorporation by which these proteins become soluble and brown, leading to an increased melanoidin level in HMw material upon prolonged roasting. Additionally, the fact that the protein level in AGP–melanoidins more than doubled upon prolonged roasting (**Table 4**) indicated that nonAGP proteinous material may have been incorporated in AGP–melanoidins. Otherwise, half the amount of arabinogalactans had to be split off from the AGP molecules to achieve doubling of the protein content. Whichever of the 2 above mentioned reactions prevails, the increase in proteinous material alone can not fully account for the increase in HMw melanoidins. This is due to the fact, even for dark roasted brew, that the protein content remains higher in IMw material than in HMw material while the melanoidin level is significantly higher in HMw material. Proteins alone can therefore not be responsible for the increase in melanoidin level.

The other explanation for HMw melanoidin formation involves the arabinose from AGP. It was observed that the arabinose content in the HMw and AGP fractions gradually decreased upon roasting (**Table 9**) while the melanoidin level increased upon roasting (**Table 2**). It might well be that Maillard reaction-like or CGA–incorporation-like reactions occur at or are bound to these degraded arabinose molecules resulting in a gradual increase in brown color, QA content, and phenolic groups' levels upon roasting. Too much modifications on these AGP-melanoidins lead to a lower affinity of the AGP to precipitate with the Yariv reagent explaining the presence of arabinogalactan in the AGPfree moiety from HMw (*22*). Degradation of AGP is a continuous process that occurs too upon roasting as an increase of arabinogalactan in the IMw fractions was observed (**Table 9**).

Summarizing, it can be concluded that this study led to a number of important new insights in mechanisms involved in coffee brew melanoidin formation. However, it can not be decisively concluded which reaction mechanisms are leading in coffee brew melanoidin formation. The authors of this study are of the opinion that chlorogenic acid incorporation plays a far more important role, especially the effect that it has on solubility, in coffee melanoidin formation than expected till now. The authors therefore propose to investigate melanoidins prepared by relatively complex model systems, containing carbohydrates (sucrose, arabinogalactans, and/or galactomannans) and proteinous material (amino acids and/or proteins). These model reactions should be conducted in the presence or absence of chlorogenic acid moieties (5-CQA, QA, and/or CA) with the aim to investigate the effect that chlorogenic acids have on coffee brew melanoidin formation.

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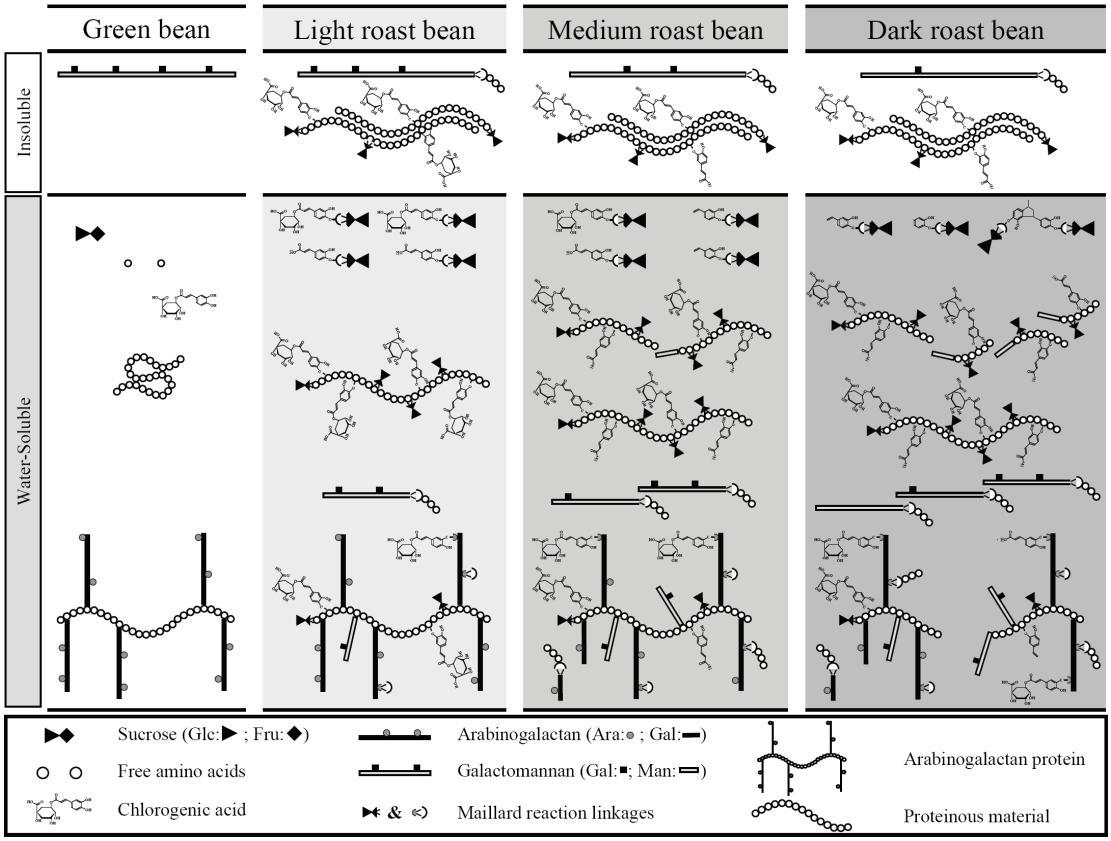
CHAPTER 8

General Discussion

ABSTRACT

The aim of this final chapter is to propose reaction pathways involved in coffee brew melanoidin formation by combining all findings from the literature and this thesis. A scheme that describes melanoidin-related formation pathways for coffee beans compounds was developed and is shown below. The reaction pathways involved are explained in detail per coffee bean compound. Finally, the relevance for coffee producing industries is discussed.



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INTRODUCTION

This PhD study aimed at characterization of structural and functional properties of brown-colored compounds that are formed in coffee many food products during heat-processing. These compounds are generally referred to as melanoidins. The food product that was selected for this study was coffee brew since coffee brew contains high levels of water-soluble melanoidins. Additionally, coffee is a relevant product from a commercial point of view as it is consumed daily by millions of people all over the world. The final goal of this study was to formulate an overview of reaction mechanisms that are involved in coffee brew melanoidin formation. The latter was achieved by combining the melanoidin-related information available in the literature with the structural properties of melanoidins that were investigated in this research.

NONENZYMATIC FOOD BROWNING

In this study, the authors focused on the characterization of the brown-colored compounds in coffee brew. It was assumed at the start of the project that the Maillard reaction was the main contributor to brown-color formation. Indeed, many confirmations were found that indicated that the Maillard reaction was at least one of the prevalent reactions occurring during heating. However, other nonenzymatic food-browning reactions might contribute to the dark color of coffee melanoidins as well (*1*).

First, the nonenzymatic oxidation of phenols was reported to yield brown colored structures too (*1*). Green coffee beans contain high levels of phenolic compounds which is due to the chlorogenic acids. The chlorogenic acids are gradually degraded during the roasting process of coffee beans (*2*). In this project, it was found that part of these chlorogenic acids were incorporated via the phenolic acid moiety through nonester bonds to coffee melanoidin structures. As a result, it seems reasonable to conclude that the dark color of coffee is also caused by oxidation of chlorogenic acids.

Second, nonenzymatic food-browning might also occur by caramelization reactions. Caramelization is the name for a cascade of heat-induced chemical reactions between sugars leading to brown-colored compounds. However, the authors expect that caramelization reactions play a limited role in food-browning of coffee beans upon roasting when compared to the Maillard reaction. This is expected since it was reported that the Maillard reaction yields much more intense colors than caramelization (*3*). Furthermore, caramelization reactions normally involve the heating of pure monosaccharides for quite long lengths of time (*4*). Coffee beans contain many more reactive components, like amino groups and phenolics, which compete with monomeric sugar-sugar caramelization reactions. Additionally, coffee beans are heated for a relatively short length of time when comparing to caramelization reaction. In the last place, the correlation that was observed between the melanoidin and proteinous material levels in the previous chapters indicates that Maillard reaction is more prevalent than caramelization reactions.

Third, lipid oxidation can lead to the formation of brown-colored compounds in food products (*1, 5*). Lipid oxidation and the Maillard reaction might even be intertwined (*1*). However, it is not expected that lipid oxidation is strongly involved in coffee brew melanoidin formation because the lipid content in the beans does not decrease upon roasting (*6*). It is expected that substantial involvement of lipids in coffee brew melanoidin formation would automatically lead to a decrease in lipid content due to roasting, which was not the case. Furthermore, incorporation of lipids in coffee melanoidins would probably yield melanoidins that are less water-soluble due to the apolar fatty acid tail. In that case, these coffee melanoidins will likely not end up in the brew but in the residue of the brewing procedure. As a result, these water-insoluble melanoidins are not consumed in coffee brew by humans and are therefore not that interesting to investigate.

Summarizing, it is concluded that the Maillard reaction and phenol oxidation are the prevalent chemical reactions involved in the food-browning of coffee beans upon roasting. We do not dare to speculate which of these two reactions contributes most to the brown color development of the coffee beans. However, we do expect that incorporation of chlorogenic acids in coffee brew melanoidin structures, by phenolic oxidation, determines to a large extent the functional properties of coffee brew melanoidins. Some examples of these functional properties are antioxidative properties, charge properties, and extractability.

COFFEE BREW MELANOIDIN FORMATION MECHANISMS

The results presented in this study showed that the structure of coffee brew melanoidins is extremely complex. The findings point towards the conclusion that “no melanoidin molecule is alike”. Therefore, it is more realistic and useful at this moment to investigate general structural properties of melanoidins than to pursue revealing the exact chemical structure of a melanoidin molecule. The previous chapters already described the identified general structural properties of coffee brew melanoidins. Now, these general structural properties, together with the information available in the literature, were used to consider degradation pathways of coffee compounds towards melanoidin-like structures.

The reaction mechanisms that were proposed to be involved in coffee melanoidin formation, were combined into one scheme as shown in **Figure 1**. Formulation of such a scheme was a rather complex task as different reaction pathways will have to coexist without making another pathway impossible. Even more complicated, different reaction pathways will likely require certain other reaction pathways to be able to synthesize melanoidins as those found in coffee brew. Furthermore, this proposed reaction scheme should be in agreement with findings reported in this thesis or published elsewhere in the literature. **Figure 1** will be discussed in detail per green bean component in the following sections. It should be emphasized that neither the size nor the concentration of the compounds shown are identical to the precise coffee bean situation. Additionally, other reactions that are not identified so far might play a role as well. Only coffee bean compounds that played a role in melanoidin formation are discussed. The insoluble galactomannans, at least insoluble in green beans, will

be discussed first as they serve as a nice introduction into the reaction scheme. Subsequently, the water-soluble compounds will be discussed, starting with the low molecular compounds followed by the more macromolecular compounds.

Galactomannans

Galactomannans are polysaccharides that consist of a mannan backbone with single substituted galactose residues. Green bean galactomannans are poorly water-soluble due to the linear character of the molecule and the architecture of the coffee bean. Therefore, green bean galactomannans are depicted in **Figure 1** as "Insoluble". The degree of galactose substitution on galactomannans decreases upon roasting (*7-9*), which leads to even more linear molecules that are even less water-soluble. This debranching is visualized in **Figure 1** by a gradual reduction of the amount of galactose units on the mannan backbone. Additionally, it was reported that the degree of polymerization decreases upon roasting (*7-9*). This reduction in molecular weight should lead to an improved water-solubility of galactomannan fragments. This is what is found in this research in **chapter 7** as well: the amount of galactomannans in both HMw and especially IMw coffee fractions increased drastically upon roasting. This Mw reduction, and thus improved water-solubility, is shown in **Figure 1** by a gradual increase in shorter mannan chains that are present in the "Water-Soluble" part of the scheme. These shorter mannan chains can still have a quite high or intermediate Mw. This is opposed to galactomannan molecules which did not undergo sufficient weight reduction and remained "Insoluble" due to the very high Mw. It was recently reported that galactomannans can be involved in melanoidin formation as they were found to carry brown-colored Maillard-like amino structures at their reducing end (*10*). This observation made by Nunes and Coimbra (*10*) is shown in **Figure 1** by a linkage of a proteinous fragment which is linked through a Maillard reaction linkage to the reducing end of galactomannan. These Maillard reactions on galactomannan should of course occur on both water-soluble and insoluble galactomannans, as is shown in **Figure 1**. One should however realize that the overall contribution of these galactomannan-based melanoidins is probably rather limited as each galactomannan molecule of course only contains 1 reducing end. This is in agreement with our finding (**chapter 2**) that the galactomannan-rich macromolecular coffee material, which precipitated at 20% and 40% aqueous ethanol (i.e. EP20 and EP40) was only moderately brown-colored. The last reaction pathway identified for galactomannans is their incorporation in melanoidin structures that are formed on arabinogalactan proteins (AGPs), as was discussed in the **chapter 3** and **7**. The galactomannan moiety increased gradually upon roasting up to 6% (w/w) of the AGP-melanoidin complexes. Whether these galactomannans are bound to the protein or the arabinogalactan moiety of AGPs and whether galactomannan is linked directly or though spacer molecules, like chlorogenic acid or an amino compound, is still unknown. The authors are of the opinion that galactomannan is most likely directly linked to the protein moiety of AGPs. However, this aspect remains to be investigated.

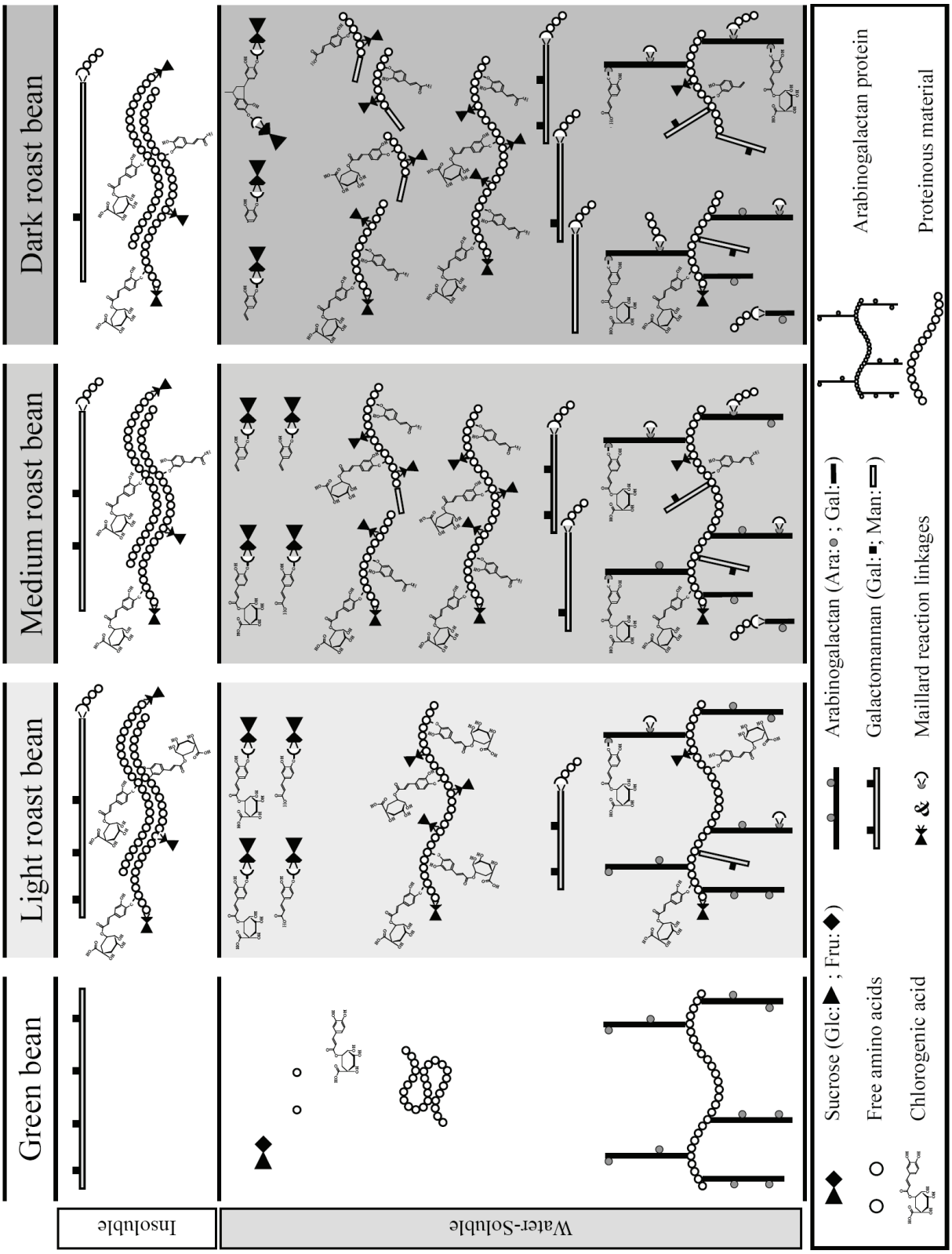

Figure 1. Proposed reaction scheme for coffee brew melanoidin formation that occurs during roasting of coffee beans (not to scale).

Sucrose

It was already mentioned in **chapter 1** that the content sucrose decreases rapidly upon roasting. The degradation pathway for sucrose is not clear, it might be that sucrose is first hydrolyzed, followed by participation in chemical reactions. Alternatively, findings discussed in **chapter 5** indicated that sucrose can also directly participate in chemical reactions though the fructose moiety, leaving the glucose moiety intact. This process should then first involve a caramelization-like reaction in which the fructose moiety is dehydrated. This may lead to ring-opening of the fructose by which aldehyde groups become exposed that might subsequently participate in Maillard-like reactions. Independent of the precise degradation pathways, it can be stated that sucrose degrades rapidly upon roasting and this sucrose will presumably be involved in the formation of the first melanoidin-like structures. This is depicted in **Figure 1** by exclusively introducing sucrose-based Maillard reaction linkages upon initial roasting from green to light roasted beans. This sucrose is linked via fructose as it was found that intact glucose from sucrose could be released from low molecular weight melanoidins (**chapter 5**).

In the Maillard reaction, sucrose can be linked to free amino acids, to nonAGP protein, and AGP proteins (**Figure 1**). When linked to proteins, it should be linked to the *N*-terminal amino group or to the available *ε*-amino groups from e.g. lysine. Additionally, it is possible that glucose is split off from the 'sucrose' that has undergone Maillard-like reactions. Such options are not depicted though in **Figure 1**.

Free Amino Acids

It was already mentioned in **chapter 1** that the free amino acids degrade rapidly upon roasting, which is due to their amino groups. Similarly to sucrose, it can be stated that free amino acids degrade rapidly and will therefore be involved in the formation of the first melanoidin-like structures. This is indicated in **Figure 1** by only showing new Maillard reaction structures that involve reaction of free amino acids upon initial roasting from green to light roasted beans. Upon prolonged roasting, no additional new Maillard reaction structures are shown that involve free amino acids.

We suggest that the majority of the free amino acids will react with degradation products from sucrose or from the arabinose moiety of AGP as these sugars are present in much larger quantities than free amino acids. This leads to the formation of low molecular weight Maillard reaction products (**chapter 5**) which are shown in the upper part of the "water-soluble" segment of the "light roasted bean" in **Figure 1**. Additionally, it is thought that the arabinose moiety of AGP can undergo ring-opening by a caramelization-like reaction (**chapter 7**), as suggested for the fructose moiety of sucrose as well. The aldehyde formed is subsequently available for Maillard-like polymerization reactions with an amino acid. This may lead to AGP-Maillard reaction products (**chapter 7**) which are shown in the lower part of the "water-soluble" segment of the "light roasted bean" in **Figure 1**.

Next to Maillard-like reactions with reducing sugars, it is also reported that chlorogenic acids may react with proteins, thus with amino compounds (*11, 12*). We found strong indications that chlorogenic acids were linked to proteinous material in **chapter 4.** Furthermore, results showed that low molecular weight melanoidins were formed that were rich in both amino and phenolic compounds (**chapter 5**). It is therefore that free amino acids must be capable of reaction both with sugars and with chlorogenic acids. This leads to the formation of low molecular weight Maillard reaction products (**chapter 5**) containing incorporated chlorogenic acids as is shown in the upper part of the "water-soluble" segment of the "light roasted bean" in **Figure 1**.

Chlorogenic acids

Chlorogenic acids (CGAs) are, like sucrose and amino acids, degraded upon roasting. It was shown in **chapter 4** that these CGAs are incorporated in coffee melanoidins. CGA degradation occurs throughout the whole roasting process (**chapter 7**) (*13*). As a result, CGAs can be incorporated into melanoidin structures during the whole roasting process. This gradual incorporation of CGAs is depicted in **Figure 1** by increasing the number of CGA molecules in AGP molecules from 3 CGA units in light roast, to 4 in medium roast, and finally to 5 in dark roast.

The internal ester-linkage in CGA is not degraded prior to incorporation in melanoidins (**chapter 4**). Intact CGA is predominantly incorporated via the phenolic acid moiety, being mainly caffeic acid (CA), and not via the quinic acid (QA) moiety. This newly formed linkage between the CA moiety of CGA and the melanoidin backbone occurred mostly through nonester bonds (**chapter 4**). This incorporation of CGAs into melanoidins via CA through nonester linkages is indicated in **Figure 1** by linking CGA via one of the hydroxyl groups of CA to a sugar or amino moiety. The authors do not claim to know how CA is actually nonester-linked, but one of the options is via one of the hydroxyl groups.

Incorporation of intact CGAs may be followed by a release of QA upon prolonged roasting since I) almost no QA was ester-linked in low molecular weight melanoidins (**chapter 5**), and II) the phenolic groups level was higher than the ester-linked QA levels in both IMw and HMw fractions (**chapter 4**). Therefore, it can be seen in **Figure 1** that incorporation of intact CGA in melanoidin structures can be followed by a release of QA. This loss of QA from intact CGA that was incorporated in melanoidins is shown in **Figure 1** as well.

In the literature, it was reported by Leloup (*14*) and Frank et al. (*15*) that CA can be converted into 4-vinylcatechol upon roasting. It seems reasonable to suggest that this also hold true for CA that is incorporated in melanoidins. Therefore, it can be seen in **Figure 1** that incorporated CA can be converted into 4-vinylcatechol-like structures. Based on the recent paper by Frank et al. (*15*), it can be suggested that 2 of these 4-vinylcatechol-containing melanoidin structures might subsequently condensate. This condensation of two 4-vinylcatechol-containing melanoidin structures can be observed for prolonged roasting from medium to dark roasted beans in **Figure 1**.

The last remark concerning CGAs in melanoidin formation deals with the observed anionic charge properties of melanoidins (**chapter 3**). The carboxyl group on the QA moiety from CGA contributes to the anionic properties of melanoidins under the proposed incorporation mechanism for CGAs. In the case that QA is released upon prolonged roasting, the carboxyl group from CA becomes exposed and contributes to the anionic charge properties of the melanoidins. This carboxyl group is only removed when incorporated CA is converted into 4-vinylcatechol-like structures. In that case, the melanoidins should be less negatively charged. In **Figure 1**, it is indicated that the amount of incorporated carboxyl groups from CGAs increases upon prolonged roasting in AGP melanoidins, being in line with the increase in negative charge in HMw material upon prolonged roasting.

Proteins

Green beans proteins are quite water-soluble (**chapter 7**). Part of these proteins is present in arabinogalactan proteins (*16, 17*) and part is present as 'unbound' proteins, like the 11S storage protein (*18*). The ratio between AGP proteins and unbound proteins in green coffee was around 1 to 50 (**chapter 7**), showing that most proteins were unbound. The unbound proteins will be discussed in this section while the AGPs are discussed in the next section. Upon roasting, unbound proteins denature and aggregate resulting in a reduction in extractability (**chapter 7**). **Figure 1** suggests that the native proteins in green beans are rather "water-soluble". The denaturation and aggregation of these proteins is shown by the unfolded and aligned proteins in the "Insoluble" segment of **Figure 1**. Next to this denaturation and aggregation, the proteins undergo chemical reactions via their reactive groups as well. These proteins become resolubilized when the degree of chemical modification on proteins, by Maillard-like and CGA-incorporation-like reactions, is high enough. The difference between 'insoluble' and 'resolubilized' denatured proteins is shown in **Figure 1** by their difference in degree of 'decoration' with sucrose or CGA molecules. Upon further roasting, it was found that more and more proteinous material ended up in the coffee brew (**chapter 7**). This could be ascribed to depolymerization of proteins into lower Mw fragments upon roasting which then automatically led to an increase in the degree of chemical modifications as well (**chapter 7**). This process of proteinous material accumulation in coffee brew caused by depolymerization of proteins into lower Mw fragments is shown in **Figure 1** too. Protein fragments might also be linked to e.g. arabinogalactan or galactomannans.

Arabinogalactan protein

Arabinogalactan proteins (AGPs) are water-soluble macromolecules. However, not all AGPs were extracted into the brew prepared from green beans probably due to entanglement by the cell wall components. AGPs were the first group of compounds of which we could decisively conclude that they are involved in melanoidin formation (**chapter 3**). There are quite some chemical reactions that may occur on AGP molecules upon roasting.

It was already mentioned that galactomannans and proteins might degrade upon roasting into smaller fragments. This degradation might just as well happen with the arabinogalactan and protein moieties in AGP, as is indicated by the presence of arabinogalactan in intermediate Mw fractions after roasting (**chapter 3** and **7**). This degradation of the protein and arabinogalactan moieties present in AGPs is shown in **Figure 1** as well.

Next to degradation of AGP molecules yielding smaller molecules, AGPs can also be 'decorated' by incorporation reactions in which several coffee compounds are attached to the AGP molecules. First, after ring-opening by caramelization-like reactions yielding aldehydes, sucrose might undergo Maillard-like reactions during the initial roasting stage with the protein moiety of AGP. In a similar way, it can be reasoned that the arabinose moiety from AGP undergoes ring-opening and is transformed into an aldehyde-containing structures by caramelization-like reactions. This is in line with the findings that predominately the amount of arabinose is reduced upon roasting (*19, 20*). This conversion reaction then yields aldehyde structures in the AGP molecules that are capable of reacting with free amino acids during the initial stage of roasting as well. Furthermore, the roasting process yields degraded protein and sugar (e.g. galactomannan) fragments that might be incorporated in the AGP structure upon prolonged roasting. Degraded protein fragments are thought to be attached via Maillard-like reaction to the reducing galactose residue that is formed by degradation of arabinogalactan as discussed above (**Figure 1**). Additionally, the protein fragments might be linked through arabinose molecules on AGPs, as discussed above (**Figure 1**). The galactomannan fragments are thought to be attached to the protein moiety of AGPs via Maillard-like linkages. The most likely possibility for these linkages is through *N*-terminal amino groups that become available by degradation of AGP's protein moiety (**Figure 1**). However, attachment of galactomannan to side chains of amino acids may also occur, especially during the early stage of roasting. This option is also depicted in **Figure 1**. It is expected that galactomannans are mainly linked to *ε*-amino groups during initial roasting while linkage to *N*-terminal amino groups is more abundant upon prolonged roasting.

Next to these protein and sugars, CGAs might be incorporated throughout the whole roasting process as well, as was already discussed in the CGA section (**Figure 1**). These incorporated CGAs might undergo a release of QA (**Figure 1**), and possibly transformation to 4-vinylcatechol (**Figure 1**). These incorporated CGAs contribute to the observed phenolic properties and the negative charge of melanoidins.

It was above already suggested that coffee bean compounds, like CGAs, are linked to the arabinose moieties from AGPs. This was suggested since a gradual availability of chemical reactive binding places is required to be able to gradually incorporate CGAs into AGP-melanoidin structures upon roasting. Linkage to the protein is not likely as the amino acid composition did not alter significantly (**chapter 7**). With respect to the arabinose, it was found that its level decreased while the level of incorporated CGAs increased (**chapter 7**). This may suggest that the arabinose moiety from AGP undergoes ring-opening, a dehydration reaction similar to caramelization reactions, which yields rather reactive aldehyde structures

that might participate in subsequent reactions with e.g. CGAs. The susceptibility of arabinose towards degradation upon roasting was reported more often (*19, 20*). Although this suggested option for incorporation seems pretty likely, no decisive proof could be found.

The Yariv reagent used for AGP isolation is very specific for the structure of AGPs. Why the whole AGP molecule precipitates with Yariv, while the separate AGP building blocks arabinogalactan or protein alone do not precipitate with Yariv is not clear. On the basis of this specificity, it was expected and found that AGPs do not precipitate with the Yariv anymore when the structure of AGP is changed too much by degradation and/or 'decoration' reactions. Thus, precipitation of AGP structures will depend on the degree of 'decoration'/degradation. The 'decoration' and degradation of AGP structures with e.g. CGAs was already discussed above and is shown in **Figure 1**. Whether AGPs do or do not precipitate with Yariv, can depend solely on the degree of "decoration", i.e. in case of no or limited degradation of the AGP structure itself. In that case, the AGP macromolecular structure remains intact but is 'decorated' to such an extent that is can not precipitate with Yariv anymore. This is in line with findings that indicated the presence of macromolecular arabinogalactan material in the high Mw fractions that does not precipitate with Yariv (**chapter 3 and 7**). Degradation of the protein backbone will yield smaller sized AGP structures that do not precipitate with Yariv. These smaller sized AGP structures might then be recovered in IMw fractions, as was observed in **chapter 3** and **7** and indeed did not precipitate with Yariv (**chapter 3**). These smaller sized AGP structures, can just like the originally sized AGPs, be "decorated" with CGAs, sucrose, galactomannan fragments, and protein fragments. For clarity reasons, no distinction was made in **Figure 1** between AGP structures that do react with the Yariv reagent and those that do not.

INDUSTRIAL PERSPECTIVE

This PhD thesis deals with melanoidin formation in a real food product, namely coffee. Therefore, the findings presented in this thesis can be translated to possible consequences for coffee producing industries. These consequences for coffee producing industries can be divided into 2 parts: the physicochemical effects and the health promoting effects.

Coffee melanoidins affecting physicochemical properties

The structural and functional properties from coffee melanoidins that were identified in this study can be translated to characteristics that may be of importance for coffee products. Coffee acidification is one of these characteristics. The fact that liquid, commercially brewed, coffee drinks get more acidic upon storage is only partly understood till now. Coffee brew melanoidins were shown to contain incorporated chlorogenic acids that might contribute to both the perceived and the real acidity of coffee. In total, coffee brew contained 12% quinic acid, of which 6% is likely incorporated in melanoidins (**chapter 4**). Therefore, coffee brew melanoidins might very well contribute to this coffee acidification as quite some carboxyl groups, from incorporated chlorogenic acids, are present in coffee melanoidins. It remains to

be investigated though how this incorporation of carboxyl groups from chlorogenic acids can be steered.

Additionally, the negative charges present within coffee melanoidin structures might affect the interaction of these melanoidins with other compounds. Milk, which is regularly present in coffee brew products, like in cappuccino, is given as an example. The milk whey protein *β*-lactoglobulin (pI = 5.2-5.4) will be slightly positively charged at the pH of coffee and these positively charged proteins and negatively charged melanoidins could interact and possibly precipitate. Such possible effects should be taken into account when developing a novel food product consisting of both coffee and milk, like ready-to-drink coffee products.

Another aspect in which coffee brew melanoidins could be involved is foaming properties. Nowadays, coffee foaming is a hot topic in the coffee world. In this study, it was found that polymeric coffee melanoidins likely contain both hydrophilic and hydrophobic patches. The hydrophilic patches are caused by the proteinous moieties and negatively charged groups, while the hydrophobic patches are the result of polycondensation reactions by the Maillard reaction, as was also found for low molecular melanoidins. The combination of both hydrophilic and hydrophobic patches likely provide high foamability and foam stability to coffee. As roasting effects the melanoidin properties, it is expected that the roasting process will also influence the foaming properties of coffee brew.

Coffee melanoidins as healthy components

It is well known that consumers show more and more interest in health promoting properties of food products. Nowadays, one can find many food products with a health claim in the supermarket. Coffee may be one of them due to the presence coffee brew melanoidins!! Findings in this study show that coffee brew melanoidins have an extremely complex structure which must be due to degradation reactions, “decoration” reactions, and condensation reactions. This complexity will probably result in a limited breakdown by human digestion enzymes, as was very recently confirmed (*21*). As a result, coffee melanoidins will likely reach the lumen of the GI-tract where they might be consumed/degraded by microbiota (*22, 23*). Therefore, it seems reasonable to consider ascribing prebiotic functionalities to coffee melanoidins. These considerations may serve as starting point for further research on coffee brew melanoidins

Additionally, it was found in this thesis that large amounts of novel antioxidative structures are formed in coffee melanoidin structures upon roasting, the properties of which are similar to vitamin E. Fortunately, these novel melanoidin antioxidants expose their activity progressively in time (**chapter 6**). Thus, these Maillard reaction antioxidants in coffee melanoidins are present in large amounts in coffee and they may expose their scavenging properties gradually throughout the GI-tract.

CONCLUDING REMARKS ON COFFEE BREW MELANOIDIN FORMATION

Altogether, it can be concluded that investigation of coffee brew melanoidin formation pathways remains extremely complex. This is due to the fact that many coffee components are involved in the formation of coffee brew melanoidins. Furthermore, the size of coffee brew melanoidins might decrease due to degradation of carrier molecules or might increase due to chemical linkage of low Mw melanoidin structures as well. Additionally, the balance between soluble and insoluble coffee bean compounds is also dynamic as some compounds become extractable upon roasting while other compounds are not present anymore in brew after prolonged roasting due to conversion into roasting gasses. All these aspects together cause that reaction pathways involved in coffee brew melanoidin formation are extremely complex and poorly understood. Therefore, the proposed reaction scheme describing coffee brew melanoidin formation pathways is a major step forwards in understanding the melanoidin formation processes. This reaction scheme provides a useful tool to summarize all knowledge that is available in the literature so far. It is clear that other reactions than Maillard reactions are also involved in coffee brew melanoidin formation.

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Summary

An essential step in the coffee production process is the roasting process as it induces chemical reactions that lead to the formation of the characteristic coffee flavor and brown-colored components. These brown-colored roasting products are called melanoidins and they make up to 28% of coffee brew dry matter. Even though they represent such a large part of coffee brew, the chemical structure of coffee brew melanoidins remains largely unknown, which is due to their extremely complex structure. This complexity is the result of the cascade of chemical reactions that take place during roasting of the coffee beans. Even though structural information on coffee brew melanoidins is limited, being rather descriptive and not in chemical detail, several functional properties were ascribed to melanoidins. Elaborate knowledge on the structural characteristics of coffee brew melanoidins is expected to lead to an improved understanding of their functional properties.

The aim of this PhD thesis was therefore to identify structural and functional properties of coffee brew melanoidins that are formed upon roasting of coffee beans, as well as their formation mechanisms.

In chapter 1, a general introduction into the topic of coffee brew melanoidins is presented. The composition of green beans, and the compositional changes that occur during roasting are discussed with special attention for coffee bean carbohydrates, proteins, and chlorogenic acids. The Maillard reaction and its reaction products, the melanoidins, are reviewed while making a distinction between melanoidins from model systems and real food systems. Knowledge reported in the literature on coffee brew melanoidins is summarized with special attention for their chemical and functional characteristics. Finally, the need for this research project and the aim of this PhD project are discussed

In chapter 2, a new parameter is introduced that enables the quantification of the melanoidin level in coffee brew fractions. This parameter is the $K_{\text{mix 405nm}}$, which is the specific extinction coefficient at 405 nm. High molecular weight (HMw) coffee brew melanoidins were fractionated on the basis of ethanol solubility. Most of the melanoidins were found to be soluble at high ethanol concentrations. Analysis revealed that all fractions had a rather similar amino acid composition and that 17% of the nitrogen was present as nonprotein-nitrogen (NPN), which probably originates from degraded amino acids/proteins. A strong correlation between the melanoidin, the protein, the NPN, and the phenolic groups content was found. It could be concluded that proteins are incorporated into melanoidins and that the degree of chemical modification, for example, by phenolic groups, determines the solubility of melanoidins in ethanol.

In chapter 3, the charge properties of HMw coffee brew melanoidins were investigated by ion exchange chromatography. It was shown that coffee brew melanoidins are negatively

charged at the pH of coffee brew. Fractionation and characterization of HMw material on the basis of ionic charge revealed that the negative charge could be ascribed only partly to the presence of uronic acids. It was discovered that arabinogalactan proteins (AGP) are present in roasted coffee brew. This AGP accounted for 6% of the coffee brew dry matter and had a brown color compared to a white color prior to roasting, from which it was concluded that AGPs participate in Maillard reactions during roasting. They become part of coffee brew melanoidin complexes; the AGP-melanoidins. The presence of mannose indicates the incorporation of galactomannans in the AGP-melanoidin complex. The rather low uronic acid content in the more negatively charged, melanoidin-rich fractions led to the hypothesis that acidic groups are formed or incorporated within melanoidin structures upon roasting.

In chapter 4, the incorporation of chlorogenic acids (CGAs) into melanoidins was investigated. In various series of coffee brew fractions, correlations were observed between the phenolic groups, the quinic acid (QA), and the melanoidin levels. The QA level was correlated with the ionic charge of the melanoidin populations, suggesting that QA contributes to the negative charge and consequently is not linked via its carboxyl group. It was concluded that the coffee bean roasting process induces the incorporation of intact CGAs molecules into melanoidin structures. These CGAs are likely incorporated into melanoidins via the caffeic acid (CA) moiety, mainly through nonester linkages. Additionally, a total of 12% of QA was identified in coffee brew, whereas only 6% was reported in the literature so far. The effect of the additional QA on coffee brew stability is discussed: this additional QA present in melanoidin structures is expected to significantly contribute to coffee acidification.

In chapter 5, the characteristics of low molecular weight (Mw) coffee brew melanoidins were investigated. This was challenging due to the presence of many low Mw non-melanoidin components that complicated analysis. Low Mw coffee fractions were isolated on the basis of polarity using reversed-phase solid phase extraction. Analysis revealed that the majority of the low Mw melanoidins are rather apolar while most non-melanoidins are polar components. The three isolated melanoidin-rich fractions represented 56% of the low Mw coffee melanoidins and were free from non-melanoidin components. Spectroscopic analysis showed that these melanoidins had similar features as HMw coffee melanoidins. All three melanoidin fractions contained ~3% nitrogen indicating the presence of incorporated amino acids or proteins. Surprisingly, glucose was the main sugar present in these melanoidins and it was reasoned that sucrose is the most likely source. It was also found that low Mw melanoidins exposed a negative charge and this negative charge was inversely proportional to the apolar character of the melanoidins. The phenolic groups levels were found to be very high, which could be explained by the incorporation of chlorogenic acids in these melanoidins.

In chapter 6, the antioxidative properties of coffee brew fractions were studied using stabilized radicals in combination with electron spin resonance spectroscopy. The stabilized radical Fremy's salt was scavenged by all antioxidants tested, including chlorogenic acids, and provides an indication for the total antioxidant activity. The stabilized radical TEMPO allowed the selective measurement of roasting-induced antioxidative structures as it was not

or negligibly scavenged by chlorogenic acid. The roasting-induced antioxidant activity of coffee brews increased with increasing degree of roast. These roasting-induced antioxidative structures were mainly formed during the initial roasting stage and the majority was formed at macromolecular material. This indicates that formation of these antioxidative structures preferably occurs at specific high molecular weight compounds, likely being arabinogalactan and/or protein moieties which are part of the melanoidin complex. Furthermore, CGAs that are incorporated in melanoidin structures seem to still expose their original antioxidant activity and phenolic characteristics. Thus, the process of CGA incorporation does not seem to affect its functional characteristics. The parameter 'fast reacting antioxidants' (FRA) was introduced as an alternative for the antioxidative potential in coffee fractions. FRA levels showed that coffee fractions rich in roasting-induced antioxidants exposed their antioxidant activity relatively slowly, which must be a consequence of its structure. The melanoidin level and the content of roasting-induced antioxidative structures of the coffee brew fractions showed a positive and linear correlation, indicating that roasting-induced antioxidants should be present within the coffee brew melanoidin structures. This is the first time that formation of roasting-induced antioxidative structures could be directly correlated with the extent of Maillard reaction and melanoidin formation in a complex product like coffee.

In chapter 7, the effects of the degree of roasting of coffee beans on coffee brew melanoidin properties and melanoidin formation mechanisms were studied. Coffee brew fractions differing in Mw were isolated from green, light, medium, and dark roasted coffee beans. Analysis showed that the melanoidin level correlated with the nitrogen, protein, phenolic groups, and the ester-linked quinic acid level. It was concluded that proteins and chlorogenic acids are primarily involved in melanoidin formation. Furthermore, arabinogalactans seem to be more involved in melanoidin formation than galactomannans. Initial roasting from green beans to light roasted beans resulted in the formation of twice as much intermediate Mw (IMw) melanoidins than HMw melanoidins. Indications were found that the prevailing IMw melanoidin formation was due to both Maillard reactions and phenolic oxidation reactions between the reactive chlorogenic acids, sucrose and amino acids/protein fragments. Additionally, it was found that prolonged roasting especially led to the accumulation of HMw coffee brew melanoidins. Proteins were solubilized upon roasting resulting in accumulation of proteinous material in HMw material. Furthermore, formation or attachment of chromophores through the arabinose moiety of arabinogalactan proteins (AGP) is hypothesized to occur as well. Finally, it was concluded that galactomannan must be gradually incorporated in AGP–melanoidins upon roasting. Overall, it was concluded that chlorogenic acids play an important role in coffee brew melanoidin formation.

The last chapter of this thesis, chapter 8, discusses the reaction pathways that are proposed to be involved in coffee brew melanoidin formation. This was conducted by combining all findings from the literature and findings from our own research. A scheme that describes identified melanoidin-related formation pathways for coffee beans compounds is presented. The proposed reaction pathways are explained in detail per coffee bean compound. Finally,

the relevance of the findings presented in this PhD study for coffee producing industries is discussed.

Samenvatting

Het roosteren van koffiebonen is een essentiële stap in het productieproces van koffie. Tijdens dit proces, waarbij groene bonen worden geroosterd tot gebrande bonen, treedt er een cascade van chemische reacties op wat leidt tot de vorming van bruingekleurde componenten en van het karakteristieke koffiearoma. Deze bruingekleurde componenten worden melanoidines genoemd en deze zijn een belangrijke groep componenten in koffie daar zij ongeveer 28% van de drogestof in koffiedrank uitmaken. De chemische structuur van deze bruingekleurde componenten is nog grotendeels onbekend wat toegeschreven kan worden aan de extreme complexiteit van de melanoidine structuur. Deze complexiteit is het resultaat van het feit dat de vele groene koffieboon componenten, elk in meerdere of mindere mate, op veel verschillende manieren kunnen deelnemen aan chemische reacties tijdens het roosteren. Ondanks de beperkte kennis van de structurele eigenschappen van koffie-melanoidines wordt er toch een aantal functionele eigenschappen aan deze moleculen toegeschreven zoals bijvoorbeeld antioxidant activiteit. Een diepgaander kennis van de koffie-melanoidine structuur is noodzakelijk om tot een beter inzicht te komen van de functionele eigenschappen van koffie-melanoidines.

Het doel van dit promotieonderzoek was het in kaart brengen van structurele en functionele eigenschappen van koffie-melanoidines die gevormd worden tijdens het roosterproces, en tevens het in kaart brengen van de daarbij betrokken zijnde reactiemechanismen,.

In hoofdstuk 1 wordt een introductie in de wereld van koffie-melanoidines gegeven, beginnende bij de beschrijving van de samenstelling van groene koffiebonen. Vervolgens is het effect van het roosterproces op deze samenstelling beschreven waarbij nadruk op koolhydraten, eiwitten en chlorogeenzuren is gelegd. Een overzicht van de Maillard reactie en de daarbij gevormde reactieproducten is beschreven waarbij er een onderscheid is gemaakt tussen melanoidines uit model systemen en melanoidines uit echte levensmiddelen. Een samenvatting van reeds bekende gegevens over koffie-melanoidines is gegeven waarbij de nadruk ligt op de chemische en functionele eigenschappen van koffie-melanoidines. Dit hoofdstuk wordt afgesloten met een verantwoording aangaande dit onderzoek en de daaruit vloeiende doelstellingen.

In hoofdstuk 2 zijn de eerste onderzoeksresultaten over koffie-melanoidines beschreven. Er wordt een nieuwe parameter geïntroduceerd waarmee het melanoidine gehalte in koffie fracties kan worden bepaald. Deze parameter is de specifieke extinctie coëfficiënt bij 405 nm, welke als $K_{\text{mix 405nm}}$ afgekort is. Hoogmoleculaire koffie-melanoidines werden gefractioneerd op basis van hun oplosbaarheid in ethanol waarbij fracties werden geïsoleerd die sterk in samenstelling van elkaar verschilden. De meeste melanoidines bleken oplosbaar bij hoge ethanol concentraties. Uit analyses bleek dat alle geïsoleerde fracties een vergelijkbare

aminozuur samenstelling hadden en dat, naast het stikstof dat gedeeltelijk aanwezig was in aminozuur structuren, maar liefst 17% van het aanwezige stikstof aanwezig was in niet-aminozuur structuren. Dit niet-aminozuur stikstof is waarschijnlijk afkomstig van gedegradeerde aminozuren en/of eiwitten. Een sterke correlatie was aanwezig tussen het melanoidine niveau, het eiwit gehalte, het niet-aminozuur gehalte en het gehalte van de fenolische groepen. Er kon worden geconcludeerd dat eiwitten zijn ingebouwd in de structuur van melanoidines en dat de mate van chemische modificatie, met bijvoorbeeld fenolische groepen, de oplosbaarheid van melanoidines in ethanol bepaalt.

In hoofdstuk 3 werden de ladingseigenschappen van hoogmoleculaire koffie-melanoidines bestudeerd met behulp van een ionwisselaar. Aangetoond werd dat melanoidines in koffie een negatieve lading hebben bij de zuurtegraad (pH ≈ 5.1) van koffie. Fractionering en karakterisering van hoogmoleculair koffie materiaal op basis van ionogene lading toonde aan dat deze negatieve lading niet geheel kan worden toegeschreven aan de aanwezigheid van suikers met een carboxylgroep, de zogenaamde uronzuren. Ook werd ontdekt dat er “arabinogalactan proteins”, afgekort als AGPs, in koffiedrank zitten. Deze AGPs zijn moleculen die bestaan uit een eiwitketen waar meerdere arabinogalactaan zijketens aan gebonden zitten. Deze AGPs waren 6% van de drogestof in koffiedrank en deze hadden een bruine kleur. Aangezien de AGPs in groene koffieboon nog wit waren kon er worden geconcludeerd dat AGPs deelnemen aan chemische, waarschijnlijk Maillard-achtige reacties tijdens het roosteren van koffiebonen. Hierbij worden deze AGPs geïncorporeerd in koffie-melanoidine complexen en worden daarom AGP-melanoidines genoemd. Aangezien in de literatuur nog geen melding was gemaakt van het isoleren van een zuivere melanoidine populatie uit koffie, kon deze isolatieprocedure voor koffie-melanoidines als nieuw en uniek worden benoemd. De aanwezigheid van mannose impliceerde dat galactomannanen worden ingebouwd in het AGP-melanoidine complex tijdens het roosteren. Het relatief lage gehalte van uronzuren in de meest negatief geladen en melanoidine-rijke fracties leidde tot de hypothese dat zure groepen worden gevormd of worden ingebouwd in melanoidine structuren tijdens het roosteren.

Hoofdstuk 4 beschrijft onze bevindingen over de incorporatie van chlorogeenzuren (chlorogenic acid, CGA) in koffie-melanoidines. Er werd gevonden dat CGA inderdaad wordt ingebouwd tijdens het roosteren van koffiebonen. In meerdere series van koffiedrank fracties werd een correlatie gevonden tussen het niveau van fenolische groepen, van veresterd chinazuur (quinic acid, QA) en van melanoidines. Het gehalte veresterd QA correleerde met de lading van de melanoidine populaties, wat suggereerde dat QA bijdraagt aan de negatieve lading van melanoidines en dat QA daardoor niet veresterd kan zijn aan het melanoidine complex via de carboxyl groep. Gebaseerd op deze waarnemingen kon definitief worden geconcludeerd dat roosteren van koffiebonen resulteert in de incorporatie van intacte CGA moleculen in koffie-melanoidine structuren. Deze CGAs worden waarschijnlijk via de fenolische component, caffeïnezuur (caffeic acid, CA), ingebouwd in het melanoidine via een niet-ester binding. Daarnaast beschrijft dit hoofdstuk dat er in totaal 12% QA aanwezig was in

koffiedrank, terwijl maar 6% bekend was in de literatuur tot op heden. De verwachting is dat deze 6% extra QA een significante bijdrage levert aan verzuring van koffie.

In hoofdstuk 5 werden de eigenschappen van de laagmoleculaire melanoidines in koffie onderzocht. Dit was een uitdagend onderwerp aangezien de grote hoeveelheid laagmoleculaire niet-melanoidine koffie componenten de analyse van laagmoleculaire melanoidines bemoeilijkt. Laagmoleculair koffie materiaal werd gefractioneerd op basis van polariteit door gebruik te maken van 'reversed-phase solid phase extraction'. Karakterisering van de geïsoleerde fracties toonde aan dat het merendeel van de laagmoleculaire melanoidines apolaire eigenschappen heeft terwijl de meeste niet-melanoidines polair zijn. Drie fracties die rijk waren in melanoidines, en die geen niet-melanoidines bevatten, waren samen verantwoordelijk voor 56% van alle laagmoleculaire koffie-melanoidines. Spectroscopische analyse toonde aan dat deze laagmoleculaire melanoidines overeenkomsten vertoonden met hoogmoleculaire koffie-melanoidines. Daarnaast impliceerde het gehalte van ~3% stikstof in alle drie de melanoidinerijke fracties dat aminozuren of eiwitfragmenten zijn ingebouwd in deze laagmoleculaire melanoidines. Het was opvallend dat glucose de meest voorkomende suiker in deze laagmoleculaire melanoidines was, dit glucose is hoogst waarschijnlijk afkomstig uit sacharose. Ook bleek dat laagmoleculaire melanoidines een negatieve lading hebben en dat deze negatieve lading omgekeerd evenredig was aan de apolariteit. Het gehalte aan fenolische groepen was hoog, dit kan worden toegeschreven aan incorporatie van CGAs in deze laagmoleculaire koffie-melanoidines.

In hoofdstuk 6 zijn de antioxidatieve eigenschappen van koffie-melanoidines onderzocht waarbij gebruik werd gemaakt van gestabiliseerde radicalen in combinatie met 'electron spin resonance' spectroscopie. In koffie kunnen 2 typen antioxidanten worden onderscheiden: i) de fenolische chlorogeenzuren en ii) de antioxidanten die nieuw gevormd worden tijdens het roosterproces. Het is tot op heden niet of nauwelijks mogelijk geweest om een onderscheid te maken tussen deze typen antioxidanten in koffie. Vooral het kwantificeren van 'rooster'-antioxidanten is gecompliceerd aangezien de fenolische antioxidanten domineren. De verwachting was dat de methode die beschreven is in dit hoofdstuk dit onderscheid wel kon maken. Het gestabiliseerde radicaal Fremy's zout reageerde met alle antioxidanten die getest werden, waaronder chlorogeenzuur. Door de aspecificiteit van Fremy's zout is de mate waarin dit radicaal reageert een indicatie voor de totale antioxidant activiteit van een koffie fractie. Het gestabiliseerde radicaal TEMPO reageerde niet of nauwelijks met fenolische antioxidanten, waaronder het veel in koffie voorkomende chlorogeenzuur. TEMPO reageerde wel met andere typen antioxidanten zoals bijvoorbeeld Trolox, een water-oplosbare variant van Vitamine E. Door deze selectiviteit geeft de mate waarin TEMPO reageert een indicatie voor de hoeveelheid antioxidanten die gevormd worden tijdens het roosteren. Het bleek dat deze methode inderdaad in staat was om specifiek 'rooster'-antioxidanten in koffie te meten en er werd gevonden dat de hoeveelheid 'rooster'-antioxidanten toeneemt met een toenemende mate van roosteren van de koffiebonen. Deze 'rooster'-antioxidanten werden voornamelijk gevormd gedurende de initiële fase van het roosteren. De meeste van deze

antioxidanten werden gevormd aan macromoleculair koffiemateriaal wat impliceert dat de vorming van 'rooster'-antioxidanten plaatsvindt aan specifieke componenten aanwezig in macromoleculair koffie materiaal, zoals arabinogalactanen en/of eiwit structuren aanwezig in koffie-melanoidines. Daarnaast lijkt het dat chlorogeenzuren hun antioxidant activiteit en fenolische eigenschappen niet verliezen als zij worden ingebouwd in melanoidine structuren. Daarom kan er worden gesteld dat het incorporatie reactiemechanisme niet de functionele eigenschappen van CGA beïnvloed. Een alternatieve en nieuwe parameter voor het antioxidatieve potentieel werd geïntroduceerd, namelijk de parameter 'snel reagerende antioxidanten' (fast reacting antioxidants, FRA). De FRA niveaus toonden aan dat de antioxidant activiteit van koffiefracties die rijk waren in 'rooster'-antioxidanten, relatief langzaam vrijkwam. Dit was vermoedelijk het gevolg van de structuur van deze 'rooster'-antioxidanten. Gevonden werd dat het niveau van melanoidines en van 'rooster'-antioxidanten een sterke positieve en lineaire correlatie vertoonden, wat aanduidde dat de 'rooster'-antioxidanten aanwezig moeten zijn in de koffie-melanoidine structuren. Dit was de eerste keer dat de vorming van antioxidanten die worden gevormd als gevolg van een verhittingsstap, direct kon worden gerelateerd aan de mate van Maillard reacties en melanoidine vorming in een complex levensmiddel als koffie.

In hoofdstuk 7 zijn onze bevindingen over de effecten van de mate van roosteren van de koffiebonen op de structurele en functionele eigenschappen van koffie-melanoidines besproken. De verandering in de structurele eigenschappen van koffie-melanoidines, die optreden als gevolg van langer roosteren, zijn vertaald naar mogelijke reactie mechanismen die betrokken zijn bij de koffie-melanoidine vormingsreacties. Koffiedrank werd bereid uit gemalen groene bonen, licht, medium en donker gebrande bonen. Uit deze koffiedranken werden vervolgens fracties geïsoleerd die verschilden in moleculaire grootte. Analyse toonde aan dat het melanoidine niveau in de koffie fracties correleerde met het gehalte aan stikstof, eiwit, fenolische groepen, en met het gehalte aan estergebonden QA. Er kon worden geconcludeerd dat vooral de eiwitten en de CGAs sterk betrokken lijken te zijn bij de vorming van koffie-melanoidines. Daarnaast impliceerden de resultaten dat arabinogalactanen waarschijnlijk meer betrokken zijn bij koffie-melanoidine vorming dan galactomannanen. Gedurende de eerste fase van het roosteren, van groene naar licht gebrande bonen, werden er veel meer melanoidines met een intermediair moleculair gewicht (IMw) gevormd dan met een hoog moleculair gewicht (HMw). Indicaties duidden erop dat de initiële overheersing van IMw melanoidines over HMw melanoidines het gevolg was van Maillard reacties en fenolische oxidaties tussen CGAs, sacharose, en aminozuren/eiwit fragmenten. Het verder roosteren van de bonen tot een donkere branding leidde echter vooral tot een ophoping van koffie-melanoidines met een hoog moleculair gewicht. De koffieboon eiwitten, die denatureerden en aggregeerden gedurende de initiële rooster fase, werden meer oplosbaar gedurende verder roosteren en daardoor nam het percentage eiwit in het hoogmoleculaire koffie materiaal steeds verder toe. De toename van de hoeveelheid melanoidines correleerde met de toename van het gehalte aan eiwit in het hoogmoleculaire materiaal. Daarnaast wordt

in dit hoofdstuk besproken dat gekleurde structuren waarschijnlijk ook worden gevormd aan of chemisch worden gebonden aan de arabinose eenheden van arabinogalactaan proteins (AGPs). De laatste conclusie in dit hoofdstuk is dat galactomannanen gradueel worden geïncorporeerd in AGP-melanoidines gedurende het rooster proces. Alles tezamen werd geconcludeerd dat chlorogeenzuren een zeer grote rol spelen bij de vorming van koffie-melanoidines.

Het laatste hoofdstuk van dit proefschrift, hoofdstuk 8, beschrijft de reactieroutes die waarschijnlijk betrokken zijn bij de vorming van koffie-melanoidines tijdens het roosteren van koffiebonen. Hiertoe werd de informatie over koffie-melanoidines dat beschikbaar was in de literatuur en dat gedurende ons onderzoek werd gevonden, gecombineerd tot een geheel. Dit leidde tot een reactieschema waarin geïdentificeerde melanoidine-gerelateerde vormingsreacties per koffiecomponent worden beschreven. De voorgestelde vormingsreacties zijn in detail per koffiecomponent besproken. Als laatste is de relevantie van de conclusies uit dit proefschrift voor de koffieproducerende industrieën besproken.

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Koen

Curriculum Vitae

Emiel Koen Bekedam werd geboren op 8 december 1979 in Zwolle. In 1998 behaalde hij zijn Atheneum diploma aan het Carolus Clusius College in Zwolle. In datzelfde jaar begon hij aan de opleiding Levensmiddelentechnologie aan de Wageningen Universiteit en volgde daar de specialisatie Levensmiddelenchemie. Hiervoor voerde hij een afstudeeronderzoek uit bij de leerstoelgroep Levensmiddelenchemie. Tevens voerde hij een afstudeeronderzoek uit in opdracht van Solvay Pharmaceuticals bij de leerstoelgroep Homogene Katalyse aan de Universiteit van Amsterdam. Zijn afstudeerstages deed hij aan de Rutgers University in de Verenigde Staten en bij Purac in Gorinchem. In september 2004 studeerde hij af, waarna hij direct als promovendus in dienst trad bij de leerstoelgroep Levensmiddelenchemie van de Wageningen Universiteit. Hier voerde hij het in dit proefschrift beschreven werk uit. Vanaf juli 2008 is Koen werkzaam als senior product scientist bij Mars Nederland in Veghel.

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Kuil, M.; Bekedam, E. K.; Visser, G. M.; van den Hoogenband, A.; Terpstra, J. W.; Kamer, P. C. J.; van Leeuwen, P. W. N. M.; van Strijdonck, G. P. F. Mild copper-catalyzed N-arylation of azaheterocycles with aryl halides. *Tetrahedron Letters* **2005,** 46, (14), 2405-2409.

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Overview of Completed PhD Training Activities

Discipline specific activities:

Courses:

Chemistry and Biochemistry of Antioxidants, FEBS-VLAG (Wageningen, 2004)
International Advanced Course Food Enzymology, VLAG (Wageningen , 2005)
Summer Course Glycosciences, VLAG (Wageningen, 2006)
Reaction Kinetics in Food Science, VLAG (Wageningen, 2006)

Conferences:

Plant Cell Wall Polysaccharides in Food Science, Symposium (Belgium, 2004)
Separation & Characterization of Natural and Synthetic Macromolecules (Amsterdam, 2005)
The Maillard Reaction in Food and Medicine, Cost Action 927 – IMARS (Italy, 2006)
Association for Science and Information on Coffee, 21st conference (France, 2006)
Association for Science and Information on Coffee, 22nd conference (Brazil, 2008)

General courses:

VLAG PhD week, VLAG (Bilthoven, 2004)
Professional Communication Strategies, WGS (Wageningen, 2007)
Philosophy and Ethics of Food Science & Technology, VLAG (Wageningen, 2007)
Career Assessment, WGS (Wageningen, 2007)

Additional activities:

Preparation of the PhD research proposal (2004)
Food Chemistry PhD trip (Japan, 2004)
Food Chemistry PhD trip (Belgium, England, and France, 2006)
Short Term Scientific Mission at the Technical University of Berlin (Germany, 2007)
Food Chemistry Seminars (2004-2008)
Food Chemistry Colloquia (2004-2008)

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