Elucidation of the components involved in the antioxidant activity of honey

Danielle Miotto, BSc.

Biotechnology

Submitted in partial fulfillment
of the requirements for the degree of

MSc in Biotechnology

Faculty of Biological Sciences, Brock University
St. Catharines, Ontario

Abstract:

Canadian honeys were analyzed for sugar concentration, honey colour, total phenolic content, the level of brown pigments, and antioxidant activity in order to elucidate the main components involved in the antioxidant activity of honey. By employing size-exclusion chromatography in combination with activity-guided fractionation, it was demonstrated that the antioxidant components are of high molecular weight (HMW), brown in colour and absorb at both 280nm and 450nm. The presence of brown HMW antioxidant components prompted an investigation on the influence of heat-treatment on the Maillard reaction and the formation of melanoidins. Heat-treatment of honey resulted in an increase in the level of phenolics in the melanoidin fractions which correlated with an increase in antioxidant activity. The preliminary results of this study suggest for the first time that honey melanoidins underlie the antioxidant activity of unheated and heat-treated honey, and that phenolic constituents are involved in the melanoidin structure and are likely incorporated by covalent or non-covalent interaction.

Table of Content:

List of Tables

List of Figures

List of Abbreviations:

ABTS,	2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid
AEAC,	Ascorbic acid Equivalent Antioxidant Content
AGEs,	Advanced Glycation End-products
AU,	Absorbance Units
AUC,	Area Under the Curve
DP,	Degree of Polymerization
DPPH,	2,2-diphenyl-1-picrylhydrazyl
FRAP,	Ferric Reducing/Antioxidant Potential
GAE,	Gallic Acid Equivalents
HMF,	5-hydroxmethyl-2-furfural
HMW,	High Molecular Weight
kDa,	Kilodalton
LC-ESI-MS,	Liquid Chromatography Electrospray Ionization Mass Spectrometry
MRLPs,	Maillard Reaction-Like Products
MRPs,	Maillard Reaction Products
ORAC,	Oxygen Radical Absorbance Capacity
PAGE,	Polyacrylamide Gel Electrophoresis
RCF,	Relative Centrifugal Force
RSD,	Relative Standard Deviation
SD,	Standard Deviation
SDS,	Sodium Dodecyl Sulfate
SEC,	Size-Exclusion Chromatography
SPE,	Solid Phase Extraction
TE,	Trolox Equivalents
TIC,	Total Ion Current
UV/VIS,	Ultraviolet/Visible

1. Introduction

1.1 Overall goal of study

The emphasis of this study focuses on elucidating the chemical components that contribute to the antioxidant activity of Canadian honeys. Current literature indicates that honey components such as phenolic acids, flavonoids and Maillard reaction products, as well as compounds that influence honey color, underlie this activity. However, when flavonoids or polyphenols were tested alone in *in vitro* systems, they could not account for the total antioxidant activity of honey (Gheldof et al., 2002). Thus, the general consensus is that these compounds are involved in, but are not fully responsible for this activity. Therefore, the search for other antioxidants in honey is required.

There is a growing public interest in antioxidants in food products and their corresponding health benefits. Honey has been shown to possess both antioxidant and antibacterial activities, and these beneficial properties could make it a valuable functional food. Identification of compounds with antioxidant activity, and the elucidation of their mechanism of action, would be of substantial significance in establishing the health-promoting functionalities of honey. A comprehensive study on the antioxidant activity of Canadian honeys is currently lacking. Therefore, the purpose of this study was to 1) determine the antioxidant activity using a large representation of Canadian honeys derived from a diverse set of botanical sources, and 2) to elucidate the chemical components involved in this activity.

As the current literature indicates that the antioxidant activity of honey is due to the combined activity of a wide range of compounds (Gheldof et al., 2002), it was hypothesized that 1) the main antioxidant activity is due to the synergistic action of antioxidant functional groups that belong to different compounds, and of which interact

to form bioactive complexes and 2) that the antioxidant complexes resemble the class of compounds formed in the Maillard reaction, the melanoidins, which have previously been described in food products such as coffee (Borrelli et al., 2002b).

1.2 What is honey?

Honey is a substance that consists of over 300 components, where fructose, glucose and water constitute the main portion of the honey mass (Jeffrey & Echazarreta, 1996). Other constituents, which are present in much lower amounts, include flavonoids, polyphenolic acids and their derivatives, proteins and enzymes, oligo- and poly-saccharides, pollen, wax (Anklam, 1998) and minerals (Lachman et al., 2007). The diversity and concentration of these components is dependent on the botanical and geographical origin of honey. Moreover, it is also influenced by the variation in climate and availability of plant sources for the honeybees to harvest nectar within a specific region (Anklam, 1998). Therefore, honeys originating from different floral sources differ in their chemical compositions.

In addition to being a valuable food product, honey has been used since ancient times as a medicinal substance. The therapeutic effectiveness of honey, particularly for the treatment of wounds and burns (Molan, 1999), has been demonstrated to be the result of its antibacterial activity (Molan, 1992). Honey showed bacteriostatic and bactericidal activities against a broad range of microorganisms, including both Gram-positive and Gram-negative bacteria (Molan, 1992; Al Somal et al., 1994; Taormina et al., 2001; Mundo et al., 2004; French et al., 2005; Lusby et al., 2005; Brudzynski, 2006), and two antibiotic-resistant bacteria that plague hospitals: methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE) (Natarajan et al., 2001; Cooper et al., 2002). As a result of honey's ability to inhibit the growth of a broad range of micro-organisms, a new application of honey was developed; honey as a therapy for

infected wounds. Honey -based wound dressings have been tested in several clinical trials (Efem, 1988; Subrahmanyam, 1996, 1998, 1999), and have recently been approved by the FDA (U.S. Food and Drug Administration).

In addition to antibacterial activity, honey has also been reported to exhibit several other biological activities such as antioxidant (Gheldof et al., 2002; Blasa et al., 2006; Bertoncelj et al., 2007), antiradical (Baltrušaitytė et al., 2007; Silici et al., 2010), antitumor (Swellam et al., 2003), anti-inflamatory (Tonks et al., 2003) and antibrowning properties (Oszmianski & Lee, 1990; Chen et al., 2000). Although the chemical identity of compounds responsible for these activities require further investigation, some of these biological effects may be attributed to the phenolic compounds.

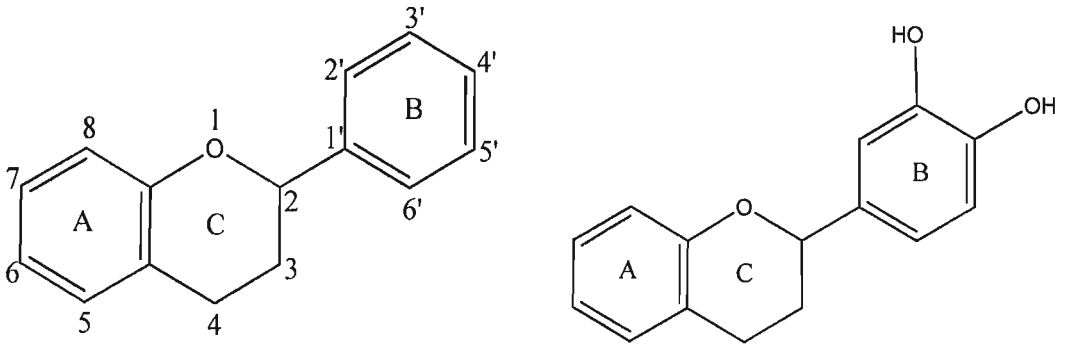
1.3 Components identified in honey having antioxidant activity

In recent decades, much attention has been focused on the beneficial effects of natural antioxidant components in food products, such as coffee (Del Castillo et al., 2002), wine (Alonso et al., 2002), beer (Vinson et al., 2003), olive oil (Lavelli, 2002) and honey (Gheldof & Engeseth, 2002; Vela et al., 2007). Evidence in the literature demonstrates that free radicals contribute to the development of disease such as cardio-vascular disease, neurodegenerative disease, and cancer (Halliwell & Gutteridge, 1999). As a result, the interest in the beneficial effects of antioxidants stems from their ability to protect against the development of disease by scavenging free radicals in the body.

An important group of phytochemicals that are involved in the antioxidant activity of honey are the polyphenols (flavonoids and phenolics acids). Numerous polyphenols have been identified in honey: flavonoids such as apigenin, pinocembrin, pinobanksin, kaempferol, quercetin, galangin, chrysin, luteolin, hesperitin and myricetin, and phenolic

acids such as caffeic, ferrulic, ellagic, vanillic, coumaric, chlorogenic, cinnamic and benzoic acids (Gheldof & Engeseth, 2002; Gheldof et al., 2002; Aljadi & Kamaruddin, 2004; Baltrušaitytė et al., 2007).

Variations in the antioxidant activity of honey may, in part, be due to the quantitative and qualitative nature of polyphenols, which, in turn, is highly dependent on the floral source. The reason for this is that the structure of polyphenols plays an important role in determining the antioxidant activity. For example, the number and spatial arrangement of hydroxyl, methoxy and glycosyl groups, particularly on the catechol backbone, are fundamental factors for determination of the final activity (Heim et al., 2002). The mechanism by which polyphenols and their derivatives may function as antioxidants includes acting as free radical scavengers (Salah et al., 1995), metal-chelators (Cao et al., 1997) or hydrogen donating agents (Rice-Evans et al., 1996). Ultimately, the accumulation of polyphenols of a certain structure will influence the final antioxidant activity of honey.



A Base structure of flavonoids **B** 3'4'-catechol (dihydroxy) structure

Figure 1: Structure of flavonoids

In addition to the antioxidant activity, polyphenols also influence honey colour. The conjugated systems of the double bonds, such as those present in polyphenolics (flavonoids and long chain phenolics), among other components such as terpenes and isoprene units found in honey (D`Arcy, 2005; Castro-Vásquez et al., 2008), give rise to a wide range of colours as they are chromophores that absorb visible light. Numerous studies in the literature have indicated that the antioxidant activity of honey varies widely with the floral source, where darker coloured honeys, which reflect the presence and concentration of pigments, were demonstrated to exhibit greater antioxidant activities (Frankel et al., 1998; Gheldof & Engeseth, 2002; Blasa et al., 2006; Bertoncelj et al., 2007). Thus, a strong correlation between honey colour, phenolic content and antioxidant activity has been documented.

In addition to polyphenols, other constituents are known to contribute to honeys antioxidant effect. These include vitamins (C and E), enzymes (catalase, peroxidase and glucose oxidase), carotenoids and products of the Maillard reaction (Gheldof et al., 2002; Aljadi & Kamaruddin, 2004). Many honey components, particularly the flavonoids and phenolic acids, have been shown to contribute significantly to the antioxidant capacity. However, when separated from honey and tested in *in vitro* systems, they produce only a portion of the total antioxidant activity of honey. These results led to the conclusion that several antioxidants may act synergistically in honey (Gheldof et al., 2002). What these compounds are and what type of chemical interactions could underlie this "synergistic action" still remains to be elucidated. Thus, it was my keen interest to elucidate the main antioxidant components in honey.

1.4 Methods used to analyze the antioxidant activity of honey

Due to the lack of an established analytical method for estimation of the antioxidant activity of honey, numerous assays have been tried by different research

groups. Commonly used methodologies for determination of the antioxidant activity of honey include the DPPH (2,2-diphenyl-1-picrylhydrazyl) and ORAC (oxygen radical absorbance capacity) assays, however, less commonly used methods such as FRAP (ferric reducing/antioxidant potential), AEAC (ascorbic acid equivalent antioxidant content), ABTS (2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) and the phosphomolybdenum method have also been employed (Gheldof & Engeseth 2002; Beretta et al., 2005; Labrinea & Georgiou, 2005; Blasa et al., 2006; Baltrušaitytė et al., 2007; Bertoncelj et al., 2007; Vela et al., 2007; Lachman et al., 2010; Saxena et al., 2010; Silici et al., 2010). Some studies cited in the literature used a single antioxidant method (Gheldof & Engeseth 2002; Labrinea & Georgiou, 2005; Blasa et al., 2006; Vela et al., 2007), whereas other studies employed multiple methodologies to estimate honeys antioxidant activity (Beretta et al., 2005; Baltrušaitytė et al., 2007; Bertoncelj et al., 2007; Lachman et al., 2010; Saxena et al., 2010; Silici et al., 2010). The majority of these assays measured an antioxidant property in the reaction mixture that involved free radicals, which was followed by a comparison of the observed effects to a known standard. Regardless of the analytical approach, most of these studies addressed the antioxidant activity in relation to honey colour and phenolic content. As a result, a strong correlation between antioxidant activity and these parameters was established.

Use of the ORAC assay in recent years, which tests antilipoperoxidant activity, has increased due to its high sensitivity and its ability to minimize cross-reactions between samples and reagents in the reaction mixture (Vela et al., 2007). Using the ORAC assay, the radical scavenging activity of honey is usually analyzed using the peroxyl radical generator, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), and is typically expressed as Trolox equivalent (TE) units (Gheldof & Engeseth, 2002; Beretta et al., 2005). The reason that this method is superior to others is because (1) it involves taking a free radical reaction to completion by monitoring the loss in fluorescence of a fluorescent probe over time, (2) it employs an area-under-the-curve technique to establish antioxidant activity, and (3) because different free radical

generators or oxidants can be used in the reaction mixture to test a different set of parameters (Cao et al., 1997). In spite of this, the DPPH assay is the more widely used methodology as it uses a common laboratory instrument, the spectrophotometer, while the ORAC assay requires a spectrofluorometer.

1.5 Factors that influence the quality of honey

Beekeepers are well aware of the fact that honey darkens upon storage. The rate of darkening has been shown to be dependent on the pH and composition of the honey, as well as the time and temperature at which the honey is stored (Gupta et al., 1992; Gonzales et al., 1999). Although controversy over the main cause of darkening still exists, the darkening of honey has mainly been attributed to the Maillard reaction, fructose caramelization, and the degradation of polyphenols (Lynn et al., 1936; Gonzales et al., 1999; Bulut & Kilic, 2009).

Darkening of honey is generally associated with a loss in quality, which directly impacts the consumer's acceptance of the product. The quality of honey is typically assessed by measuring the accumulation of 5-hydroxmethyl-2-furfural (HMF), by determining the diastase activity or by establishing honey colour (Sancho et al., 1992; Bulut & Kilic, 2009). Depending on the storage and temperature conditions of honey, many authors have associated the loss in quality with an increase in HMF content, a reduction in diastase activity or the darkening of honey colour (Sancho et al., 1992; Castro-Vázquez et al., 2008).

In addition to darkening during storage, the darkening of honey was also noted to be an indicator of honey thermal-treatment (Turkmen et al., 2006). The reason that honey is usually subjected to thermal treatment is to modify its tendency to undergo crystallisation or to destroy contaminating micro-organisms (Tosi et al., 2002). Typically,

honey processing involves heating honey in air-ventilated chambers for 4 to 7 days at 45-50°C, or immersing honey drums in hot water (Fallico et al., 2004). However, upon prolonged thermal treatment of honey at temperatures between 50-70°C, an increase in brown pigment formation occurred that resulted in darker coloured honey which correlated with the increase in antioxidant activity (Turkmen et al., 2006). It was also reported that the increase in brown pigment formation and antioxidant activity was due to the formation of Maillard reaction products, which are known to possess antioxidant activity (Turkmen et al., 2006). Thus, the Maillard reaction, at least in part, appears to be involved in the browning of honey.

1.6 What is the Maillard reaction?

The Maillard reaction is a non-enzymatic browning reaction that was first described by Louis Maillard in 1912. The reaction is usually divided into three stages: the early, intermediate and advanced Maillard reaction (Figure 2). The first stage involves a reaction between the amino group of an amino acid/protein and the carbonyl group of a reducing sugar (Hodge, 1953). The resulting Schiff base undergoes a rearrangement to form the reasonably stable Amadori product, which is a precursor for the formation of a wide range of compounds. These products that are formed in the early stage of the Maillard reaction are colourless and do not absorb UV light (Hodge, 1953).

Reactive α-dicarbonyl compounds have very recently been identified in honey (Weigel et al., 2004; Marceau & Yaylayan, 2009). Most of the studies in the literature focus on methylglyoxal in manuka honey because this compound was recently identified as the principle antibacterial component (Mavric et al., 2008; Adams et al., 2009). A recent study by Adams et al., (2009) demonstrated that methylglyoxal in manuka honey originates from dihydroxyacetone which occurs in high amounts in the nectar of manuka flowers. On the other hand, methylglyoxal and glyoxal naturally occur in the human

body. The increased amount of glyoxal and methylglyoxal, such as in diabetes, leads to protein crosslinking and the formation of very harmful advanced glycation end-products (AGEs) such as pentosidine and N^{ϵ}-[carboxymethyl]-lysine (Singh et al., 2001).

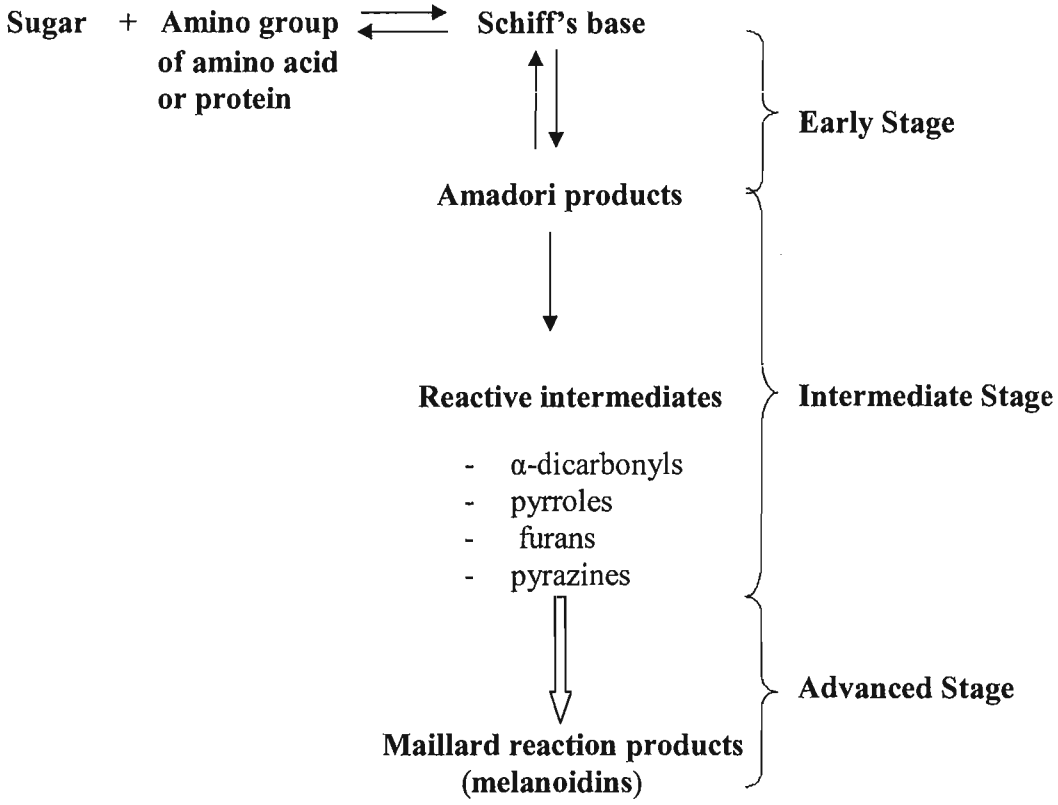


Figure 2: General scheme of the Maillard reaction (Martins et al., 2000; van Boekel, 2006).

Melanoidins, which are carbohydrate-based, high molecular weight, coloured macromolecules, are formed in the advanced stages of the Maillard reaction. These final brown products have been demonstrated in thermally processed foods such as coffee (Borrelli et al., 2002b; Bekedam et al., 2006; Gniechwitz et al., 2008b), bread crust (Borrelli et al., 2003), beer (Kuntcheva & Obretenov, 1996), sugarcane molasses,

(Chandra et al., 2008) and vinegar (Xu et al., 2007). More importantly, melanoidins have been shown to exhibit several biological activities such as antioxidant (Borrelli et al., 2002b; Delgado-Andrade et al., 2005), antibacterial (Rufián-Henares & Morales 2007b; Rufián-Henares & de la Cueva, 2009), antihypertensive (Rufián-Henares & Morales, 2007a), and prebiotic properties (Borrelli, & Fogliano, 2005). Although the Maillard reaction is known to contribute to several negative qualities such as changes in aroma and flavour (Castro-Vásquez, et al., 2008), the interest in melanoidins is mainly due to their relevant biological activities. Thus, in recent years, studies have focused on the effects of melanoidins on the human body, as well as on their nutritional values.

1.7 Structure of melanoidins obtained from model and food systems

The mechanism of formation and the chemical structure of these brown melanoidins are currently unknown, however many attempts have been made to elucidate the structure. The majority of the studies on melanoidin formation have involved simplified model systems, which contain a limited number of substrates. These systems typically involve a reaction between a reducing sugar (mono- or oligosaccharides) and an amino acid or protein (Cämmerer & Kroh, 1995; Brands et al., 2002; Hofmann, 1998a), or a system involving intermediates such as α-dicarbonyls (Kroh et al., 2008). In addition, the effect of various reaction conditions such as time, pH and temperature on the rate of melanoidin synthesis was also investigated. Depending on the amount and nature of the substrates that may take part in the Maillard reaction of these systems, three different melanoidin structures were discerned (1) polymers involving repeating units of furans or pyrroles (Tressl et al., 1998), (2) carbohydrate-based melanoidin skeletons consisting of polymerized sugar degradation products that are formed by aldol-type condensation and/or intact carbohydrate structures (Cämmerer & Kroh, 1995; Cämmerer et al., 2002); or (3) protein-based melanoidin skeletons which involve protein crosslinks by low molecular weight coloured compounds (Hofmann, 1998b).

In foods, melanoidin formation is complicated by the number and type of reactants that may take part in the Maillard reaction. Numerous attempts have been made to elucidate the structure of melanoidins found in foods such as coffee (Borrelli et al., 2002b; Nunes & Coimbra, 2007; Gniechwitz et al., 2008b), beer (Kuntcheva & Obretenov, 1996) and bakery products (Lindenmeier et al., 2002; Borrelli, et al., 2003). Since food products naturally contain a more diverse set of reactants, and thus a greater number of potential interactions, the melanoidins characterized in foods differed from those elucidated from simple model systems consisting of limited reactants. Despite the structural differences, the food melanoidins were demonstrated to exhibit similar physicochemical properties to those elucidated from model systems, such as being characterized as partially hydrophobic (Gniechwitz et al., 2008b), high molecular weight substances with anionic character (Morales, 2002; Nunes & Coimbra, 2007), that possess reducing properties (Homma et al., 1997), and are capable of chelating metal ions (Borrelli et al., 2002a; Nunes & Coimbra, 2007).

1.8 Antioxidant activity of Maillard reaction products

The relationship between antioxidant activity and Maillard reaction products (MRPs) has been extensively studied in model systems and in some thermally processed foods (Manzocco et al., 2001; Chen & Kitts, 2008). Although a positive correlation has been demonstrated between MRPs and antioxidant activity, there is some controversy over the nature of the compounds involved. Recent research has shown conflicting results regarding the size of the Maillard reaction compounds responsible for the antioxidant activity. Some studies have suggested that the antioxidant activity is due to the low and intermediate molecular weight MRPs (Del Castillo et al., 2002; Delgado-Andrade et al., 2005; Morales & Babbel, 2002), however, other studies have indicated that the high molecular weight MRPs exhibit greater antioxidant activities than those of low molecular weight (Jing & Kitts, 2004; Monti et al., 1999). Moreover, the chemical nature of the melanoidin (Tressl et al., 1998) as well as the type of linkage (covalent or non-covalent)

they have with other compounds (Delgado-Andrade et al., 2005; Gniechwitz et al., 2008b), are also controversial.

1.9 Methods used to detect and isolate Maillard reaction products

Due to the heterogenous mixture of chemical compounds that are formed in the Maillard reaction, specifically in foods, there is no official method to measure MRP content. The brown colour development in foods and model systems is typically achieved by measuring the absorbance at an arbitrarily selected wavelength between 420-480nm (Manzocco et al., 2001). However, these values only reflect the colour and do not consider the concentration of reactants and products due to the unknown extinction coefficients. Thus, this indirect measurement is a non-destructive way to quickly evaluate the brown colour development of a complex food system.

In addition to monitoring changes in colour, the presence of melanoidins can be speculated by obtaining wavelength spectra. As described by Hofmann (1998b), melanoidins exhibit a characteristic UV/VIS profile, which includes featureless end absorption and an increase in intensity with a decrease in the wavelength. Several studies have speculated on the presence of melanoidins in foods such as coffee (Bekedam et al., 2006) and vinegar (Xu et al., 2007), based on the characteristic UV/VIS profile by measuring absorbance in the 200-700nm range. Because melanoidins are heterogenous in nature, the absorbance spectra are generally observed as a plateau starting from the UV region and extending towards the visible region due to the presence of many different chemical species.

Due to the fact that brown colour development in foods can result from the formation of a wide range of products originating from different pathways, it is extremely

difficult to classify these brown products. In theory, the distinction between the different classes of browning such as (1) enzymatic browning, and (2) nonenzymatic browning which includes caramelization, or when amino-containing compounds are involved, the Maillard reaction is clear. However in practice, it is very difficult to differentiate whether the brown products formed are melanins, which are produced from enzymatic browning, or melanoidins, which are formed in the final stage of the Maillard reaction (Smaniotto et al., 2009). Thus, the final brown products most likely consist of a heterogenous mixture of compounds derived from various pathways that exhibit similar features. To differentiate between them, more sophisticated methodologies must be employed.

Despite all efforts, the chemical structure of melanoidins in food products is still largely unknown. In recent literature, much attention has been focused on the isolation and characterization of coffee melanoidins where numerous methodological approaches have been employed. A common method for the isolation of high molecular weight coffee melanoidins is to use dialysis (Nunes & Coimbra, 2007; Gniechwitz et al., 2008a), diafiltration (Bekedam et al., 2006), or ultrafiltration (Delgado-Andrade et al., 2005; Gniechwitz et al., 2008b) by using membranes with a molecular weight cut-off limit ranging from 2 kDa to over 100 kDa. An alternative approach has been to separate coffee melanoidins comprised of different polysaccharide compositions based on their solubility in ethanol (Bekedam et al., 2006; Nunes & Coimbra, 2007). Further purification of melanoidins was generally required as homogenous mixtures were not obtained. Therefore, separation procedures such as anion-exchange chromatography, copper-affinity chromatography, sequential chromatography on Sephadex LH-20, and hydrophobic interaction chromatography on Octylsepharose were used separately or in combination for purification (for review, Nunes & Coimbra, 2010). As a result of the complexity of the melanoidin structure, it is a complex process to isolate and chemically characterize an individual melanoidin compound.

2 Materials and Methods:

Sodium fluorescein and gallic acid monohydrate were purchased from Sigma-Aldrich. 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Aldrich. Folin-ciocalteu phenols reagent was purchased from Sigma. Black-sided, special optics clear bottom plates (part # 3615) were obtained from Corning.

2.1 Honey Samples

Honey samples were obtained from Canadian beekeepers (Table 1). The honeys were given numbers prior to the experiment and were stored at room temperature in a dark, dry, place throughout the study.

2.2 Heat-treatment of honeys

Honeys were heat-treated at 121°C for 30 minutes (Steris Autoclave, Amesco Century, Erie, PA, USA). Following heat-treatment, the honeys were diluted to 50% (w/v) with sterile, distilled water and centrifuged at 5000 x g (Relative Centrifugal Force) for 20 minutes (Eppendorf 5804 R, Eppendorf, Canada).

2.3 Honey colour

Honey colour was measured using the net absorbance method (A_{560}-A_{720}nm) as described by Vela et al., (2007). Briefly, honey was diluted to a 50% (w/v) solution with sterile, distilled water. The net absorbance was determined using a spectrophotometer

(Ultrospec 3100 Pro) and distilled water as a reference. The results were expressed as absorbance units (AU).

Table 1: Honey samples from various floral sources used in study

Assigned Honey Number	Plant Source
08-1	66% clover blend
08-2	Mixed polyfloral
08-3	Manuka (New Zealand)
08-4	Alfalfa
08-5	Dandelion
08-6	Canola
08-7	Clover
08-8	NA
08-9	Borage
76	Buckwheat
77	Buckwheat
92	Wildflower
105	Tasmanian leatherwood
112	Leafy Spurge
114	Sunflower
116	Buckwheat
118	Leafy Spurge
119	Canola
123	Sunflower
149	Buckwheat
177	Buckwheat
148	Manuka (New Zealand)

NA: not available

2.4 Brown pigment determination

The content of Maillard reaction products is typically achieved by measuring the absorbance at a wavelength between 420-480nm as demonstrated in both model (Manzocco et al., 2001) and food systems (Turkmen et al., 2006). In the present study, the degree of honey browning was determined for a 50% (w/v) honey solution by taking the net difference (A_{450}-A_{720}nm) (Martins et al., 2003). The results were expressed as AU.

2.5 Brix

Sugar content was determined using a refractometer with direct reading display (American optical ABBE 10450 Refractometer, Buffalo, NY, USA) and was expressed as °Brix and sugar concentration (g/L). Briefly, honey samples were diluted to 50% (w/v) with distilled water, centrifuged for 5 minutes at 5000 x g and subjected to sugar content determination. A 7% (w/v) solution of glucose was used as the standard.

2.6 Size-exclusion chromatography

Sepharose 4B (Sigma-Aldrich) was packed into a column (24 x 1.6 cm) and was equilibrated with distilled water at 1ml/minute prior to use. Briefly, 2mls of a 50% (w/v) honey solution supernatant that was diluted in 0.15M sodium chloride and spun at 13000 x g for 5 minutes was loaded onto the column. The fractions (3ml) were eluted with distilled water and monitored at 280nm.

A standard curve for molecular weight determination was generated using a protein kit (Gel Filtration HMW Calibration kit, GE Healthcare) containing ferritin (440 kDa), catalase (240 kDa), aldolase (158 kDa), and albumin (66 kDa).

2.7 Antioxidant activity

The antioxidant activity was measured using the ORAC (oxygen radical absorbance capacity) assay as described by Huang et al. (2002) and the application note (Held, 2005) from BioTek (Winooski, VT) with modifications. Immediately prior to use, the fluorescein stock solution (4x 10^{-3}mM), which was wrapped in foil and stored at 4°C, was diluted 1:100 with 75mM phosphate buffer (pH 7.4) to obtain a working fluorescein solution. A Trolox stock solution was prepared by measuring 0.25g of Trolox and

dissolving it in 50ml of 75mM phosphate buffer (pH 7.4) to obtain a 0.02M Trolox stock solution.Working Trolox solutions (20 – 0.625μM Trolox concentrations) were prepared prior to use with the same phosphate buffer.

In terms of plate usage, the exterior wells were filled with 300μl of distilled water and were not used for experimental determination to avoid errors due to light scattering at the plate edges. To the interior wells of the plate, 150μl of the freshly prepared working fluorescein solution was added using a 12-channel micropipette. In addition, 25 μl of the suitably diluted sample and Trolox were added to the sample and standard wells, and 25 μl of 75mM phosphate buffer (pH 7.4) was added to the blank containing wells. The plate was then allowed to equilibrate by incubating it for a minimum of 30 minutes in the Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc, Winooski, VT) at 37°C. Following incubation, the reaction was initiated by the addition of 25 μl of the freshly prepared AAPH solution (0.414g of AAPH dissolved in 10ml of 75mM phosphate buffer, pH 7.4) with a 12-channel micropipette to all experimental wells to result in a final volume of 200 μl.

Fluorescence was measured from the bottom of the plate at a sensitivity setting of 60 every 1.5 minutes for 60 minutes with emission and excitation wavelengths of 528 and 485nm, respectively. Prior to each reading, shaking at maximum intensity for 10 seconds took place.

KC4 Data Reduction Software (BioTek Instruments, Inc, Winooski, VT) was used to obtain the raw fluorescence values, and Microsoft Excel was used to calculate the Net area under the curve (AUC) of the samples and standard. ORAC values were calculated using the following equations.

AUC was calculated as follows:

$$AUC = 0.5 + (R2/R1) + (R3/R1) + (R4/R1) + ... + 0.5(Rn/R1)$$

where R1 is the fluorescence measurement taken at the initiation of the reaction and Rn is the last fluorescence measurement.

The net AUC was calculated as follows:

$$\text{Net AUC} = \text{AUC}_{\text{sample or standard}} - \text{AUC}_{\text{blank}}$$

Final ORAC values were calculated by using the regression equation obtained from the Trolox standard curve, and were expressed as μmol Trolox equivalents (TE) per gram of honey.

2.8 Solid-phase extraction

Honey samples were subjected to solid-phase extraction prior to total phenolic content analysis. Briefly, honey samples were prepared (25% v/v) in acidified water (pH 2.3) and spun for 2 minutes at 13000 x g in an Eppendorf microcentrigfuge. Prior to addition of the supernatant onto the Waters Oasis HLB 3cc Extraction Cartridges (SPE cartridge, Oasis HLB from Waters, Milford, MA), the cartridges were conditioned and equilibrated by adding and drawing through 1ml of pure methanol and 1ml of distilled water, respectively. After loading 1ml of the 25% (v/v) honey sample supernatant onto the cartridge, the cartridge was washed with 1ml of 5% methanol in water (v/v) to remove the sugars and polar constituents in honey. Samples were eluted with 1ml of pure methanol and were stored at 4°C.

2.9 Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu method as described by Singleton and Rossi (1965) with modifications, using gallic acid as the standard (Singleton et al., 1999).

The working Folin-Ciocalteu reagent was prepared just prior to use by diluting the concentrated Folin-Ciocalteu reagent 1:17 with distilled water. A gallic acid standard curve was run alongside the samples (500-31.25 µg/ml). Briefly, 200µL of the corresponding gallic acid solution, the methanol fraction eluted from solid phase extraction (SPE), or distilled water which was used for the blank was mixed in a 4.5ml tube with 2.4ml of the freshly prepared working Folin-Ciocalteu reagent. After exactly one minute, 420µL of sodium bicarbonate (20 % (w/v)) was added to each test tube. Each test tube was inverted three times and was incubated in the dark at room temperature for one hour.

After incubation, 910µL of distilled water was added to each test tube, the test tube was inverted three times and absorbance was measured at A_{765}nm using a spectrophotometer (Ultrospec 3100 Pro). The quantity of phenolics was estimated in relation to a gallic acid standard curve and the results were expressed as µg gallic acid equivalents (GAE) per g of honey.

2.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to electrophoresis, honey fractions obtained from size-exclusion chromatography were subjected to 80% ethanol precipitation by adding 6.5 volumes of 95% ethanol to 1 volume of sample solution. The ethanol solutions were cooled to -20°C

for at least 12 hours and were centrifuged at 13 000 x g for 20 minutes. The ethanol was removed from the pellet and the samples were dried at 85°C. The pellets were resuspended in sample buffer (pH 6.8) containing 4% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) ß-mercaptoethanol and 0.01% (w/v) bromophenol blue in 0.25M Tris-HCl and heated at 95°C for 5 minutes.

SDS-polyacrlamide gel electrophoresis was carried out according to Laemmli (1970) using 10% (w/v) polyacrylamide separating and 5% (w/v) stacking gels. These gels contained 0.1% (w/v) SDS. Honey samples were loaded onto each well (30µl), and were electrophoresed for 1.5 hours at a constant current of 100 Volts.

Protein bands were visualized by Coomassie Brilliant Blue R-250 (BioRad) and destained with 7% acetic acid/ 40% methanol. Estimation of the protein molecular mass was performed using a protein standard kit (PageRuler Prestained Protein Ladder, Fermentas).

2.11 Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS)

LC-ESI-MS analyses were carried out with a Bruker HCT Ultra LC/MS instrument operating in the negative ion mode with a capillary exit voltage of -128.5 Volts and a skimmer voltage of -40.0 Volts. Honey components in the sample were separated on an Agilent 160 ZORBAX Eclipse XDB- C18, 4.6 x 50 mm column with 2mmol/L formic acid (pH 2.7) (A) and methanol (B) as solvents (HPLC grade, Merck). LC was performed within a retention time of 20 minutes using the following elution gradient: 0-3 minutes 22% B, 10 minutes 100% B, 12 minutes 100% B, 13 minutes 22% B. The separation was done at room temperature with a flow rate of 1ml/min and all

chromatograms were recorded at 254nm. MS was acquired with an accumulation time of 558 μs with a mass ion scan ranging from 100 m/z to 750 m/z.

2.12 Statistical Analysis

A Principal component analysis was performed using SPSS version 17.0. Pearson r correlation analyses were performed using the statistical program Graph-Pad Instat version 3.05 (GraphPad Software Inc.). Unless specified, a significance level of $P < 0.05$ was employed.

3. Results

Part I:

The specific aim of Part I of the thesis was to investigate the relationship between antioxidant activity, honey colour, total phenolic content and Maillard reaction products, so as to elucidate the components involved in the antioxidant activity of honey.

3.1 Physical-chemical characteristics of honeys used in the study

A set of 22 honeys from various floral sources were characterized based on sugar concentration, honey colour, total phenolic content and browning index which estimates the content of Maillard reaction products (MRPs). The honeys chosen for characterization included honeys that varied from white to dark brown in colour (0.038 to 1.16 AU at A_{560}-A_{720}, respectively). Upon arrival, the honey samples were assigned a number. The list of honeys is presented in Table 1 (Materials and Methods section).

3.1.1 BRIX

BRIX is a common method in the beekeeping industry that is used to calculate the concentration of reducing sugars. The sugar concentration of honeys diluted to 50% (w/v) were measured using a refractometer and expressed in units of °Bx (g sugar/ 100 g solution). The °BX units were then converted to sugar concentration (g/L) (Table 2).

Table 2: BRIX measurements of honey samples using a refractometer

Honey Sample	**Sugar concentration** (50% w/v honey) (g/L)	**Brix** (100% honey)
Artificial sugar (7% w/v)	74.4	14.4
77	443.8	76.8
149	456.9	78.8
76	459.9	79.2
116	453.3	78.2
08 3	452.6	78.1
112	450.4	77.8
118	446.4	77.2
105	457.1	78.8
114	444.3	76.9
08 5	447.4	77.3
119	447.9	77.4
08 6	447.4	77.3
08 2	435.6	75.5
92	453.4	78.2
08 8	450.3	77.8
123	446.7	77.2
08 9	452.9	78.2
08 1	440.8	76.3
08 4	457.9	78.9
08 7	456.6	78.7

Samples were measured at 25.9 – 27.2°C. Brix values were adjusted to temperature using the conversion factor: °Brix + (°C-20)*0.07

As shown in Table 2, the BRIX values of 100% honey varied from 75.52 to 79.24 °Brix. The data obtained are in agreement with those published in a number of studies (Silva et al., 2009; Saxena et al., 2010). According to these values, honey contains high amounts of reducing sugars with a slight variation among the floral sources.

3.1.2 Honey colour and browning index (MRPs)

Honey colour as defined by visual assessment varied from white to dark brown. The almost white honeys originated from clover and alfa alfa, honeys of yellow to light amber in colour originated from sunflower, canola, wildflower, and borage, the medium amber coloured honeys were from manuka, leafy spurge and dandelion, and the brown to dark brown honeys were from buckwheat origin (Figure 3).

a) b) c) d) e)

Figure 3: Colour of honey. a) white/colourless, b) light yellow, c) dark yellow/light amber, d) medium amber, e) brown to dark brown.

The colour intensity of honey results from the presence and concentration of pigments, which include phenolics, flavonoids and MRPs. The colour intensity can be determined spectrophotometrically by evaluating the net absorbance at A_{560}-A_{720}nm. The results obtained for honey colour measurements are shown in Table 3.

In addition to phenolics, MRPs are also known to influence honey colour. Beekeepers are well aware of the fact that honeys of light colour often became darker upon storage. Moreover, the change in colour is also notable during honey processing and pasteurization, which is known to facilitate the Maillard reaction and non-enzymatic browning (Turkmen et al., 2006). As of the current literature, the relationship between MRPs and honey colour in raw, unheated honey has not been investigated. There is no experimental data in the literature supporting the notion and the involvement of MRPs in the browning of honey upon storage. To determine the potential presence of MRPs in

unheated honey and their influence on honey colour, the MRP content was assessed based on the browning index and was related to the colour of honey.

Table 3: Colour characterization (A_{560}-A_{720}nm) and browning index (A_{450}-A_{720}nm) of honey samples

	Honey Sample	A_{560}-A_{720}nm [a]	A_{450}-A_{720}nm [a]
Dark	77	1.16 ± 0.04	3.51 ± 0.10
	149	0.88 ± 0.02	3.6 ± 0.1
	76	0.72 ± 0.05	3.05 ± 0.24
	116	0.68 ± 0.03	2.73 ± 0.19
	177	0.66 ± 0.02	2.18 ± 0.26
Medium	08-3	0.40 ± 0.02	1.78 ± 0.09
	148	0.39 ± 0.01	1.41 ± 0.06
	112	0.24 ± 0.01	0.82 ± 0.02
	118	0.191 ± 0.009	0.64 ± 0.02
	105	0.14 ± 0.01	0.56 ± 0.02
	114	0.13 ± 0.01	0.50 ± 0.03
Light	08-5	0.11 ± 0.01	0.43 ± 0.04
	119	0.10 ± 0.01	0.32 ± 0.04
	08-6	0.10 ± 0.01	0.26 ± 0.03
	08-2	0.094 ± 0.005	0.26 ± 0.02
	92	0.09 ± 0.01	0.34 ± 0.03
	08-8	0.089 ± 0.008	0.29 ± 0.02
	123	0.087 ± 0.006	0.325 ± 0.004
	08-9	0.046 ± 0.007	0.13 ± 0.01
	08-1	0.043 ± 0.007	0.12 ± 0.01
	08-4	0.040 ± 0.005	0.12 ± 0.01
	08-7	0.038 ± 0.008	0.11 ± 0.02

a. Data shown as means (AU) ± SD of at least five independent determinations ($n \geq 5$). Values obtained for A_{560}-A_{720}nm and A_{450}-A_{720}nm correspond to 50% (w/v) honey solutions. Honeys are arranged from darkest to lightest in colour according to A_{560}-A_{720}nm values.

As a result of the heterogenous mixture of chemical compounds formed in the Maillard reaction, there is no official method to estimate MRP content. The most commonly used method is a spectrophotometric measurement of absorbance at wavelengths between A_{420} to A_{480}nm, which is the wavelength range where brown pigments show a broad peak (Manzocco et al., 2001). Therefore, to estimate the content of brown pigments in honey, the net absorbance at A_{450}-A_{720}nm was measured and expressed as AU (Table 3).

As shown in Table 3, the brown pigments absorbing in the wavelengths characteristic of MRPs were present in all tested honeys. Their content was dependent on honey colour with the highest levels being observed in the dark honeys and the lowest levels in the light honeys. In the extreme cases, up to a 30 fold difference in the level of brown pigments between the lightest (#08-7) and darkest honey (#77) was observed with values ranging from 0.11 to 3.51 AU (Table 3). This is the first evidence that Maillard reaction-like products (MRLPs) occur in unheated honey and that their content differs significantly among honeys of different colour and originating from various floral sources. The term 'Maillard reaction-like products' was used to describe the brown pigments as the chemical structure and origin of these pigments are largely unknown.

The darker coloured honeys, which are rich in pigments (flavonoids and phenolic acids), have been demonstrated to show higher phenolic content as well as higher antioxidant activities compared to light coloured honeys (Gheldof, & Engeseth, 2002; Beretta et al., 2005). In order to further investigate the relationship between MRLPs and honey colour, it was important to determine the level of phenolics in these honeys.

3.1.3 Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu method. Honey solutions diluted to 25% (v/v) were subjected to solid phase extraction (SPE) to remove reducing sugars that could interfere with Folin-Ciocalteu analysis. The Folin-Ciocalteu method measures the intensity of blue colour of a solution which results from the reduction of metal oxides by phenolics (Singleton et al., 1999). The higher the content of phenolics and the higher the reducing capacity of honey the darker the colour of the resultant reaction mixture. A gallic acid standard curve was run alongside the samples (refer to Appendix A, Figure A-1). Total phenolic content was estimated from the standard curve and was expressed as μg GAE/ g of honey (Table 4).

Table 4: Total phenolic content of honey as determined by Folin-Ciocalteu

	Honey Sample	A_{560}-A_{720}nm [a]	Total phenolic content (μg GAE/ g of honey)[b]
Dark	77	1.16 ± 0.04	513.25 ± 8.03
	149	0.88 ± 0.02	408.03 ± 12.39
	76	0.72 ± 0.05	318.74 ± 7.04
	116	0.68 ± 0.03	253.88 ± 1.72
	177	0.66 ± 0.02	163.14 ± 15.56
Medium	08-3	0.40 ± 0.02	320.41 ± 12.09
	148	0.39 ± 0.01	186.68 ± 12.53
	112	0.24 ± 0.01	156.39 ± 16.45
	118	0.191 ± 0.009	165.10 ± 0.92
	105	0.14 ± 0.01	144.77 ± 3.83
	114	0.13 ± 0.01	106.97 ± 3.60
Light	08-5	0.11 ± 0.01	144.55 ± 36.71
	119	0.10 ± 0.01	80.82 ± 3.49
	08-6	0.10 ± 0.01	68.03 ± 12.26
	08-2	0.094 ± 0.005	84.86 ± 25.40
	92	0.09 ± 0.01	81.90 ± 6.58
	08-8	0.089 ± 0.008	79.55 ± 1.09
	123	0.087 ± 0.006	81.22 ± 4.66
	08-9	0.046 ± 0.007	51.21 ± 18.64
	08-1	0.043 ± 0.007	70.10 ± 21.52
	08-4	0.040 ± 0.005	80.38 ± 36.40
	08-7	0.038 ± 0.008	49.78 ± 13.61

a. Data shown as means (AU) ± SD of at least five independent determinations (n≥5). Values obtained correspond to 50% (w/v) honey solutions.

b. Data shown as means (μg Gallic Acid Equivalents/ g of honey) ± SD of at least three independent determinations tested in duplicate (n≥6).

As shown in Table 4, the concentration of phenolics was the highest in the dark honeys and lowest in the light honeys. In the extreme cases, up to a 10 fold difference in total phenolic content was observed where the values ranged from 49.78 to 513.25 μg GAE/g, respectively (honey #08-7 and honey #77, respectively). The results observed here indicate an association between honey colour and the content of phenolic chromophores in honey. This is significant because honey colour, which reflects the presence of pigments, is a known predictive marker of honeys antioxidant activity (Gheldof & Engeseth, 2002; Blasa et al., 2006).

3.1.4 Antioxidant activity of honey

Honey colour and phenolic content have been shown to be strongly correlated with antioxidant activity (Gheldof & Engeseth, 2002; Blasa et al., 2006; Bertoncelj et al., 2007). On the other hand, in beverages such as coffee, the overall antioxidant power has been influenced by Maillard reaction products (Borrelli et al, 2002b; Delgado-Andrade et al., 2005). Thus, it was of interest to determine the influence of these parameters on the antioxidant activity of raw, unheated honey.

The antioxidant activity was established using the high-throughput Oxygen Radical Absorbance Capacity (ORAC) assay. The honey's radical scavenging activity was analyzed using the peroxyl radical generator, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), and was expressed as Trolox equivalent (TE) units. A Trolox standard curve was run alongside the samples (refer to Appendix A, Figure A-2).

As shown in Table 5, the dark honeys demonstrated the greatest antioxidant activity and the light honeys demonstrated significantly lower antioxidant levels. The ORAC values differed 10 fold among the honeys tested and ranged from a value of 1.99 µmol TE/g for a light coloured honey (#08-9) up to 19.77 µmol TE/g for a dark coloured honey (#76). The darkest coloured honeys, mainly those of buckwheat origin, were found to be the most potent against peroxyl radicals, with values ranging from 12.75 to 19.77 µmol TE/g (Table 5).

These results suggest a relationship between honey colour and antioxidant activity as determined by ORAC. Moreover, the concentration of phenolics and MRLPs seemed to correlate with honey colour. In order to demonstrate the involvement of these three parameters (honey colour, phenolic content and MRLPs) on the antioxidant activity of honey, a principle component analysis was employed.

Table 5: Summary of results for honey colour, total phenolic content and antioxidant activity

	Honey Sample	A_{560}-A_{720}nm [a]	Total phenolic content (μg GAE/ g of honey) [b]	ORAC (μmol TE/g of honey) [c]
Dark	77	1.16 ± 0.04	513.25 ± 8.03	19.13 ± 0.96
	149	0.88 ± 0.02	408.03 ± 12.39	12.75 ± 0.43
	76	0.72 ± 0.05	318.74 ± 7.04	19.77 ± 0.84
	116	0.68 ± 0.03	253.88 ± 1.72	17.41 ± 1.11
	177	0.66 ± 0.02	163.14 ± 15.56	8.26 ± 0.41
Medium	08-3	0.40 ± 0.02	320.41 ± 12.09	8.19 ± 0.32
	148	0.39 ± 0.01	186.68 ± 12.53	7.62 ± 0.55
	112	0.24 ± 0.01	156.39 ± 16.45	4.59 ± 0.25
	118	0.191 ± 0.009	165.10 ± 0.92	4.41 ± 0.27
	105	0.14 ± 0.01	144.77 ± 3.83	5.31 ± 0.23
	114	0.13 ± 0.01	106.97 ± 3.60	5.37 ± 0.33
Light	08-5	0.11 ± 0.01	144.55 ± 36.71	4.66 ± 0.23
	119	0.10 ± 0.01	80.82 ± 3.49	2.75 ± 0.15
	08-6	0.10 ± 0.01	68.03 ± 12.26	3.73 ± 0.15
	08-2	0.094 ± 0.005	84.86 ± 25.40	3.53 ± 0.13
	92	0.09 ± 0.01	81.90 ± 6.58	5.85 ± 0.34
	08-8	0.089 ± 0.008	79.55 ± 1.09	4.64 ± 0.19
	123	0.087 ± 0.006	81.22 ± 4.66	4.62 ± 0.21
	08-9	0.046 ± 0.007	51.21 ± 18.64	1.99 ± 0.09
	08-1	0.043 ± 0.007	70.10 ± 21.52	2.98 ± 0.11
	08-4	0.040 ± 0.005	80.38 ± 36.40	3.04 ± 0.09
	08-7	0.038 ± 0.008	49.78 ± 13.61	3.36 ± 0.14

a. Data shown as means (AU) ± SD of at least five independent determinations (n≥5). Values obtained correspond to 50% (w/v) honey solutions.

b. Data shown as means (μg Gallic Acid Equivalents/ g of honey) ± SD of at least three independent determinations tested in duplicate (n≥6).

c. Data shown as means (μmol Trolox Equivalents/ g of honey) ± SEM (n≥ 7). Each trial was tested in triplicate. RSD is <15% for the values obtained.

3.1.5 Relationship between the antioxidant activity of honey with honey colour, total phenolic content and Maillard reaction products

As the antioxidant activity appears to be influenced by honey colour, which in turn is indicative of the content of phenolics and MRPs, it was of interest to test statistically the relationship between these parameters by employing a principle component analysis. As shown in Table 6, the correlation matrix revealed that all four variables showed a strong correlation with one another. The first principal component was found to be extremely large, accounting for 95% of the variance.

Table 6: Component loading matrix

Variable	Loading on First Principal Component
Honey colour	0.992
MRPs	0.991
Total phenolics	0.967
ORAC	0.955

According to the correlation matrix (Table 7), the highest correlation has been observed between honey colour and MRLPs (0.980), followed by total phenolic content and honey colour (0.963), MRLPs and total phenolics (0.950), and ORAC and MRLPs (0.938) (Table 7). Regarding antioxidant activity, ORAC demonstrated the highest correlation with MRLP content and honey colour with values of 0.938 and 0.931, respectively.

Table 7: Correlation matrix

	Honey colour	MRPs	Total phenolics	ORAC
Honey colour	1.000	0.980	0.963	0.931
MRPs	0.980	1.000	0.950	0.938
Total Phenolics	0.963	0.950	1.000	0.860
ORAC	0.931	0.938	0.860	1.000

The strong correlation of these parameters with the antioxidant activity supports the idea that honey colour is a predictive marker of antioxidant activity. As ORAC demonstrated a highly significant correlation with MRLPs, this is the first evidence of a significant contribution of MRLPs to the overall antioxidant capacity of raw, unheated honey. This novel observation suggests that non-enzymatic browning may be chiefly responsible for the colour of unheated honey, and as a result, appears to be a major contributor to the antioxidant activity of honey.

A version of this chapter has been published.
Brudzynski & Miotto (2011). *Food Chemistry*. 124: 869-874.

Part II:

The observation of a strong correlation between the antioxidant activity of honey and the content of MRLPs, total phenolics and honey colour prompted further investigations on the chemical composition of MRLPs in honey and their role in the antioxidant activity.

The specific aims of Part II of the thesis were as follows:

A. Isolation of components involved in the antioxidant activity of honey
B. Demonstration of the formation of MRPs/ melanoidins in honey and their contribution to antioxidant activity
C. Chemical characterization of MRPs/melanoidins

3.2 Methodological approach towards the isolation and characterization of components involved in the antioxidant activity of honey

In order to isolate a honey's antioxidants, size-exclusion chromatography (SEC) on Sepharose 4B was employed and the fractions obtained were analyzed for antioxidant activity using the ORAC assay. Based on the results obtained so far, it was hypothesized that MRLPs will be present among the antioxidant compounds. From the literature, MRPs are aggregates and polymers formed via crosslinking between low molecular weight chromophores and HMW non-coloured biopolymers (protein), and polycondensation of repeating units of furans and pyrroles (Tressl et al., 1998; Hofmann, 1998b; Adam et al., 2005). In this context, SEC appeared to be the method of choice because (1) it allows for the separation of high molecular weight aggregates/complexes, which we assumed we are dealing with and (2) SEC allows the continuous determination of the molecular size of compounds exhibiting antioxidant activity, unlike methods such as dialysis and dia-/ultrafiltration, which rely on the cut-off limits of the filtration

membrane to estimate the molecular size. Thus, SEC and activity-guided fractionation was the method employed in this study.

The scheme that was used for the fractionation of honey components is shown in Figure 4. Briefly, 2mls of honey diluted to 50% (w/v) with 0.15M NaCl was loaded onto the column. The fractions (3ml) were eluted with distilled water and monitored at 280nm. The identified active fractions of known molecular size were further analyzed for (a) phenolic content using Folin-Ciocalteu, (b) Maillard reaction products using the browning index (net absorbance at A_{450}-A_{720}nm), (c) protein content using SDS-PAGE and (d) the presence of sugar, amino sugars and honey specific flavonoids by LC-ESI-MS (Figure 4).

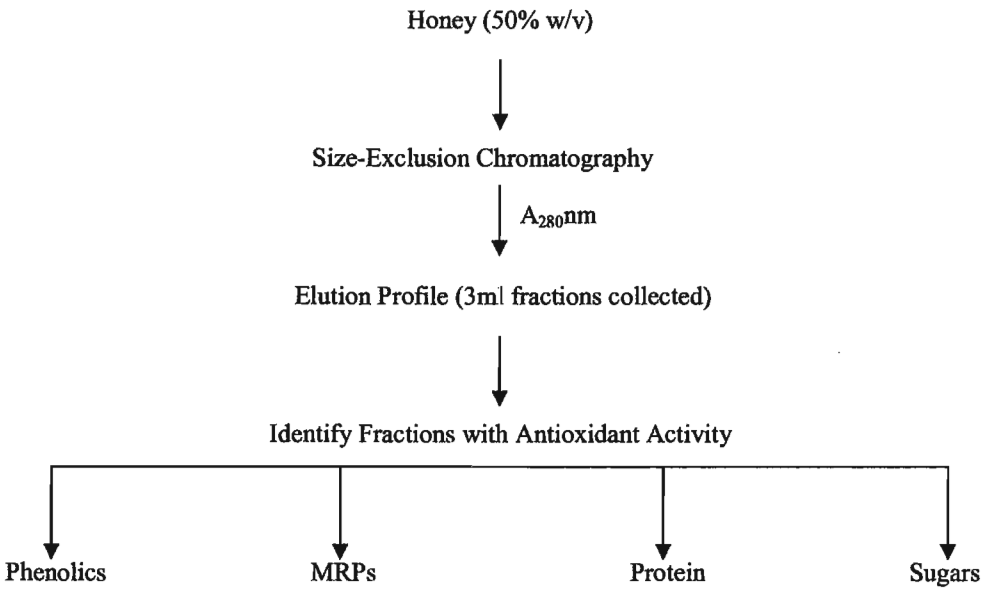


Figure 4: Scheme for the characterization of honey fractions

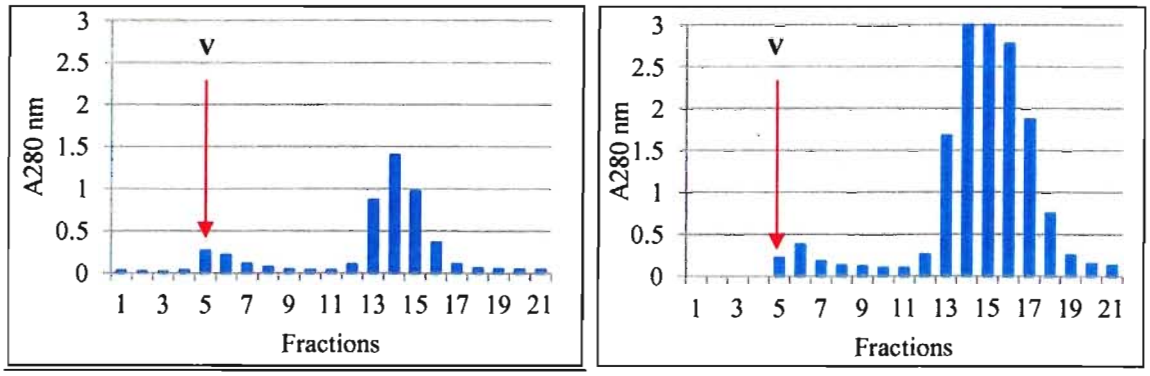
3.2.1 Characterization of the antioxidant components in unheated honey obtained from SEC as analyzed at 280nm

Size-exclusion chromatography (SEC) was conducted using a Sepharose 4B resin. The fractions that eluted from the column were monitored at 280nm, which is the

absorbance maximum that is characteristic of proteins, phenolics, flavonoids and their derivatives. SEC appeared to be the method of choice because it allows the separation of chemical components on a molecular weight basis. Using this technique, the low molecular weight compounds were retained on the column and were eluted after the molecules of high molecular weight.

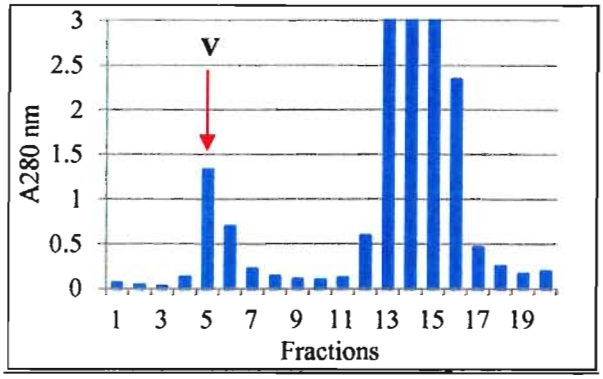
By monitoring the fractions that eluted from the SEC column at 280nm, differences in the chemical composition of the honeys were demonstrated. As shown in Figure 5, the chromatographic profiles of the three unheated honeys that differed in their colour were in general, similar. All honeys demonstrated profiles that consisted of a minor peak in the void volume (F5) and a major peak appearing in fractions F13 to F17 (Figure 5a-c). The main difference between the elution profiles obtained for the different honeys was the amount of UV absorbing components. The unheated darker honeys (#148 and #177) indicated higher levels of UV absorbing components, particularly in the major peak (F13-F17), compared to the light honey, which gave rise to a major peak that was half the intensity of that observed for the darker honeys. In addition, the dark buckwheat honey also demonstrated the highest level of UV absorbing components in the minor peak (F5), in turn indicating the presence of a significant amount of very high molecular weight components.

All fractions that eluted from the column were tested for their antioxidant activity using the 96-well format ORAC assay. The results indicated that the activity was only observed in fractions that eluted in the major peak (F13 to F15 and F13 to F16 for the light and darker honeys (#148 and #177), respectively) (Table 8). The ORAC values were shown to differ up to 7 fold between the light and dark coloured honeys (#08-5 and #177) with values ranging from 437 to 2979 umol TE/ L and 404 to 2742 umol TE/ L, for fraction F14 and F15, respectively.



a) Honey #08-5 (light)

b) Honey #148 (medium)



c) Honey #177 (dark)

Figure 5: Elution profiles demonstrating the differences in A280nm of honey fractions obtained from size-exclusion chromatography.
V: void volume

While the majority of fractions obtained for unheated honeys were colourless, the unheated dark honey (#177) was the exception. The dark honey gave rise to light brown coloured fractions in both the minor and major peaks (F5, F13 to F15). The brown pigments absorbing in the wavelength characteristic of MRPs were present in all light brown coloured fractions. Regarding antioxidant activity, the brown coloured fractions that eluted in the major peak (F13 to F15) for unheated dark buckwheat honey (#177) demonstrated the greatest antioxidant activity (Table 8). From these results, the colour and the level of brown pigments seemed to directly influence the observed antioxidant activity.

Table 8: Level of brown pigments, phenolic content and antioxidant activity of unheated honey fractions

Honey Fraction	Brown pigments (AU) (A_{450}-A_{720}nm)[a]	Total phenolic content (μg GAE/ ml)[b]	ORAC (μmol TE/ L)[c]
Honey #177			
13	0.14 ± 0.05	118.60 ± 5.04	1547 ± 220
14	0.23 ± 0.06	135.00 ± 4.77	2979 ± 202
15	0.19 ± 0.03	108.06 ± 2.79	2742 ± 308
16	0.14 ± 0.04	63.49 ± 5.60	1125 ± 67
Honey #148			
13	0.05 ± 0.01	48.14 ± 2.56	1175 ± 165
14	0.092 ± 0.009	62.64 ± 5.93	1479 ± 48
15	0.11 ± 0.01	65.27 ± 6.64	1287 ± 147
16	0.08 ± 0.03	55.43 ± 3.92	967 ± 19
Honey #08-5			
13	0.015 ± 0.005	39.53 ± 3.23	363 ± 11
14	0.017 ± 0.006	45.97 ± 3.26	437 ± 46
15	0.017 ± 0.005	39.84 3.96	404±54

a. Data shown as means (AU) ± SD of two independently collected samples from the column tested in duplicate (n=4).
b. Data shown as means (μg GAE/ ml) ± SD of four independent trials tested in duplicate (n=8).
c. Data represented as means (μmol TE/L of 100% fraction) ± SD of at least three independent trials tested in triplicate (n≥9). RSD is <15% for the values obtained.

3.2.2 Molecular size of the active fractions

To obtain information regarding the size of the active components in honey, and to suggest that they belong to MRPs/melanoidins, a standard curve for molecular weight determination was generated under the same chromatographic conditions using the following standards: ferritin (440 kDa), catalase (240 kDa), aldolase (158 kDa) and bovine serum albumin (66 kDa) (refer to Appendix A, Figure A-3). From the standard curve, approximate molecular weights of the components in the fractions of interest were determined.

From the molecular weight standard curve, the size of the antioxidant components were estimated to be of much higher molecular weight than anticipated, ranging from

approximately 66 to 232 kDa (F13 to F17) (Table 9). In addition, brown components from unheated dark buckwheat honey eluted in the same volume that blue dextran (2 000 kDa) eluted (F5). This result in turn suggested that brown MRLPs of very HMW, which exhibited high UV absorbance at 280nm, were present in unheated dark honey.

Table 9: Molecular weight of components as determined from the SEC standard curve

Honey Fraction	Approximate molecular weight of components (kDa)
13	180 – 232
14	140 – 180
15	109 – 140
16	85– 109
17	66 – 85

These results suggest that the active fractions of dark buckwheat honey (#177) contain high molecular weight MRLPs. It was demonstrated that the components in these fractions absorb at A_{280}nm, contain brown pigments (A_{450}-A_{720}nm), exhibit antioxidant activity (ORAC), and are of high molecular weight (as estimated from the standard curve). As the Maillard reaction is known to occur in heat-treated foods and is associated with the formation of MRPs/melanoidins, these honeys were subjected to heat-treatment for the purpose of generating melanoidins. The MRLPs generated in heat-treated honey were then compared to those present in unheated honey, particularly dark buckwheat honey.

3.3 Methodological approach towards the identification of melanoidins in honey and their contribution to the antioxidant activity

The Maillard reaction is known to occur in heat-treated foods and is associated with the formation of brown pigments-melanoidins. Melanoidins can be characterized by their absorbance maximum (A420 -A450nm), high molecular weight, darkening of the initial colour, increased content of UV absorbing compounds, and antioxidant activity. As

heat-treatment is the critical element to generate melanoidins, the above characteristic parameters were compared between heat-treated and unheated honeys.

3.3.1 The effect of heat-treatment on honey colour and the level of browning of honey

The effect of heat-treatment on the colour and brown pigment formation was analyzed first using whole honey. The same three honeys (light-coloured dandelion, #08-5, medium-coloured manuka, #148, and dark-coloured buckwheat, #177) that differed in their initial colour and demonstrated different characteristics based on the chosen criteria were subjected to heat-treatment at 121°C for 30 minutes.

By visual observation, heat-treatment of the 50% (w/v) honey solutions resulted in the darkening of colour of honey in comparison to the unheated control group (Figure 6). The changes in honey colour were supported by the observed increase in net absorbance (A_{560}-A_{720}nm). The spectrophotometric measurements showed about a two-fold increase in colour values compared to the control (Table 10).

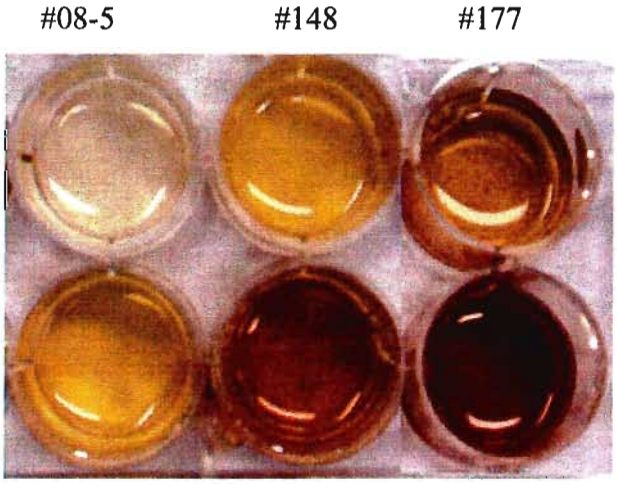


Figure 6: Colour of honey samples (50% w/v) before (top) and after heat-treatment (bottom) at 121°C for 30 minutes.

As MRP/melanoidin formation is known to occur at high temperatures, it was anticipated that heat-treatment would result in an overall increase in brown pigment formation. As shown in Table 10, heat-treatment did cause a change in brown pigment levels however the anticipated result that an increase in brown pigments would be observed for all heat-treated honey samples was not the case.

Heat-treatment resulted in about a 2.5 and 1.5 fold increase in the level of brown pigments for the light dandelion (#08-5) and medium manuka (#148) honeys, respectively, as demonstrated by the net absorbance (A_{450}-A_{720}) values (Table 10). In contrast, an unexpected 1.2 fold decrease in brown pigment content was observed for the dark buckwheat honey (#177) (Table 10). In the latter case, a substantial brown pellet was observed following heat-treatment and was thus removed prior to taking honey colour and browning measurements.

Table 10: Honey colour (A_{560}-A_{720}) and level of brown pigments (A_{450}-A_{720}) before and after heat-treatment of 50% (w/v) honey solutions

Honey Sample	A_{560}-A_{720} [a]	A_{450}-A_{720} [a]
08-5	0.16 ± 0.01	0.54 ± 0.01
08-5 Treated	0.31 ± 0.01	1.36 ± 0.06
148	0.39 ± 0.01	1.41 ± 0.06
148 Treated	0.77 ± 0.03	2.08 ± 0.64
177	0.66 ± 0.02	2.18 ± 0.26
177 Treated	1.19 ± 0.25	1.78 ± 0.70

a. Data shown as means (AU) ± SD of three independently prepared samples. Values determined for 50% (w/v) honey solutions.

The unexpected decrease in brown pigment levels may be due to the formation and removal of insoluble MRPs, presumably of high molecular weight, from the dark buckwheat honey. Despite the removal of the brown precipitate, the brown colour of honey still prevailed. It appears that heat-treatment resulted in the formation of new brown coloured products that differ in their solubility from those present in unheated honey (soluble versus insoluble).

3.3.2 The effect of heat-treatment on the antioxidant activity of honey

Heat-treatment of honey at mild temperatures (50-70°C) was demonstrated to result in an increase in brown pigment formation which coincided with an increase in antioxidant activity (Turkmen et al., 2006). In the present study, heating honeys at a much higher temperature (121°C) also resulted in the formation of new brown pigments, however, an overall increase was only observed for the light and medium coloured honeys. From this result, it appears that the level of soluble brown pigments formed is dependent on the initial colour of honey as well as the time and temperature of heating.

As honey colour and the level of brown pigments showed a strong relationship with the overall antioxidant activity of unheated honeys, it was anticipated that heat-treated honeys, which darkened following heating, would show greater antioxidant activities compared to the unheated control. As expected, the ORAC values that were obtained for the heat-treated honeys demonstrated about a 1.5 fold overall increase in value compared to the control (Table 11). Despite the removal of an insoluble brown pellet from the dark buckwheat honey (#177), an increase in antioxidant activity still prevailed.

Table 11: Antioxidant activity of honey before and after heat-treatment

Honey Sample	**ORAC**[a] (μmol TE/ L of honey)
08 5	3143 ± 457
08 5 Treated	5568 ± 760
148	11113 ± 1614
148Treated	16391 ± 2338
177	16054 ± 1593
177Treated	24266 ± 917

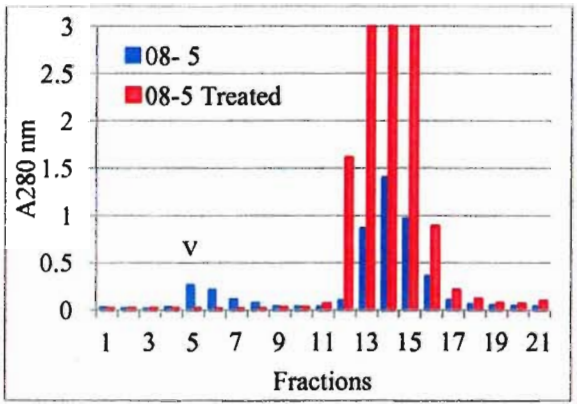
a. Data shown as means (μmol TE/ L) ± SD of at least three independent trials tested in triplicate (n≥9). RSD is <15% for the values obtained.

These results demonstrated that heat-treatment of honey resulted in the generation of new brown pigments that exhibit antioxidant activity. In order to determine whether

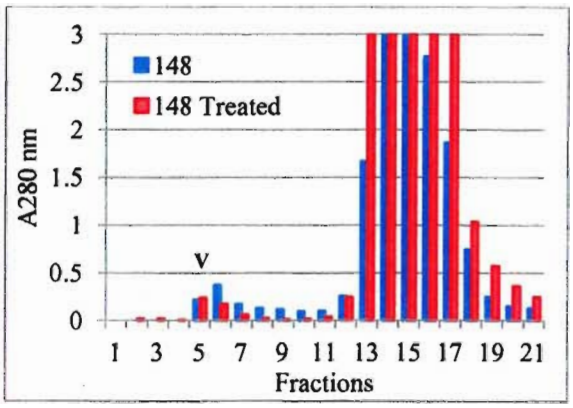
these newly formed compounds are melanoidins, the heat-treated honeys were subjected to SEC fractionation to establish the size of the heat produced antioxidant components.

3.3.3 SEC of heat-treated honey

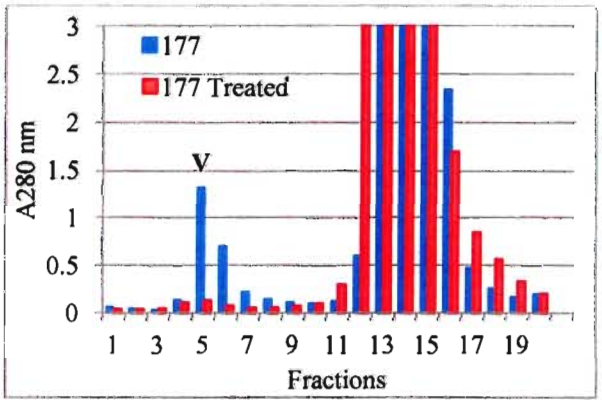
The heat-treated honey fractions were analyzed for changes in colour, level of brown pigments, changes in the UV profile and antioxidant activity. The above characteristics were compared between unheated and heat-treated honey fractions.



a) Honey #08-5 (light)



b) Honey #148 (medium)



c) Honey #177 (dark)

Figure 7: Elution profiles demonstrating the differences in A280nm of honey fractions obtained from size-exclusion chromatography before and after heat-treatment.
V: void volume

By visual observation of the fractions that eluted from the column for the heat-treated honeys, a notable difference in the chemical properties of unheated versus heat-treated honeys was observed. The presence of brown coloured fractions after heat-treatment compared to colourless (#08-5 and #148) or light brown fractions (#177) of the unheated honeys, as well as a heat induced increase in UV absorbance at 280nm for these fractions, demonstrated the first evidence of a change in honeys properties (Figure 7).

Heat-treatment of honey resulted in more intense brown coloured fractions for the dark buckwheat honey (#177), specifically in F12 to F16, and the introduction of light brown fractions in F13 to F17 and F13 to F15 for the medium (#148) and light (#08-5) honeys, respectively. In addition, heat-treatment also resulted in the loss of the light brown fraction in the void volume (F5) of the elution profile for the dark buckwheat honey. The differences in the intensity of browning before and after heat-treatment are supported by the browning index (net absorbance at A_{450}-A_{720}nm) values obtained for the fractions that eluted in the major peak (Figure 8, Table 12).

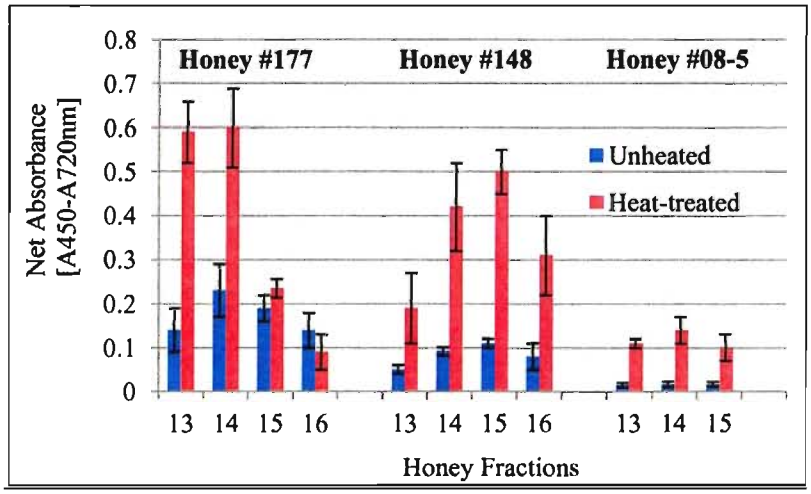


Figure 8: Level of brown pigments in unheated and heat-treated honey fractions that eluted in the major peak.
Data represents means (AU) ± SD of two independently collected samples tested in duplicate (n=4).

Table 12: Level of brown pigments in unheated and heat-treated honey fractions that eluted in the major peak

MRPs (A_{450}-A_{720}nm)		
Honey Fraction	**Unheated**	**Heat-treated**
Honey #177		
13	0.14 ± 0.05	0.59 ± 0.07
14	0.23 ± 0.06	0.60 ± 0.09
15	0.19 ± 0.03	0.235 ± 0.02
16	0.14 ± 0.04	0.09 ± 0.04
Honey #148		
13	0.05 ± 0.01	0.19 ± 0.08
14	0.092 ± 0.009	0.42 ± 0.10
15	0.11 ± 0.01	0.50 ± 0.05
16	0.08 ± 0.03	0.31 ± 0.09
Honey #08-5		
13	0.015 ± 0.005	0.11 ± 0.01
14	0.017 ± 0.006	0.14 ± 0.03
15	0.017 ± 0.005	0.10 ± 0.03

a. Data shown as means (AU) ± SD of two independently collected samples tested in duplicate (n=4).

The influence of colour on the antioxidant activity of fractions became even more complex following heat-treatment of honey. Heat-treatment resulted in the darkening of honey fractions and/or the introduction of new brown coloured fractions, particularly in the major peak. For unheated honey, the dark honey (#177) demonstrated the highest ORAC value in fractions F14 and F15. On the other hand, heat-treatment of the medium coloured honey (#148) demonstrated the highest ORAC value (4710 umol TE/ L) as well as the greatest increase in ORAC in fraction F14 (Figure 9, Table 13). Heat-treatment seemed to influence the antioxidant activity of the light and medium coloured honeys to a greater extent, as ORAC values were demonstrated to differ 7 fold in F14 between these two honeys (683 to 4710 umol TE/ L for the light and medium honey F14, respectively).

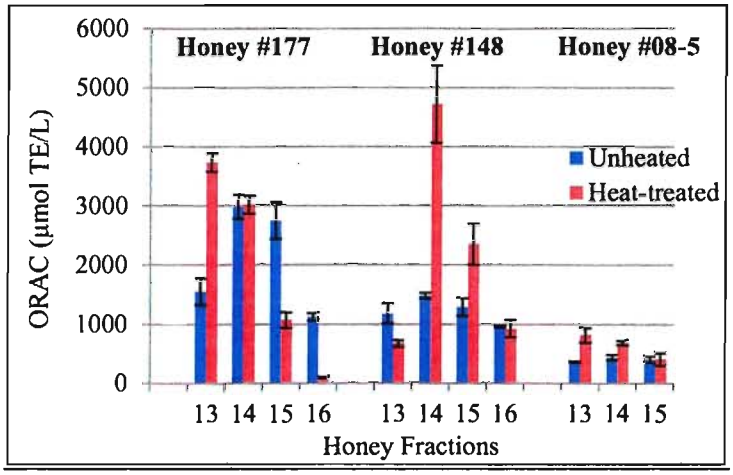


Figure 9: Antioxidant activity of unheated and heat-treated honey fractions obtained from SEC.

Data represented as means (µmol TE/L of 100% fraction) ± SD of at least three independent trials tested in triplicate (n≥9). RSD is <15% for the values obtained.

Table 13: Antioxidant activity of unheated and heat-treated honey fractions obtained from SEC

ORAC (µmol TE/ L)[a]		
Fraction	**Unheated Honey**	**Heat-treated Honey**
Honey #177		
12	ns	1258 ± 187
13	1547 ± 220	3730 ± 155
14	2979 ± 202	3017 ± 147
15	2742 ± 308	1073 ± 132
16	1125 ± 67	102 ± 8
Honey #148		
13	1175 ± 165	674 ± 56
14	1479 ± 48	4710 ± 645
15	1287 ± 147	2345 ± 339
16	967 ± 19	921 ± 139
Honey #08-5		
13	363 ± 11	814 ± 122
14	437 ± 46	683 ± 34
15	404 ± 54	409 ± 102

a. Data shown as means (µmol TE/ L of 100% fraction) ± SD of at least three independent trials tested in triplicate (n ≥ 9). RSD is < 15% for the values obtained.

The colour of the honey fraction did not appear to significantly correlate with the observed antioxidant activity of heat-treated honey. Heat-treatment of honey from different floral origins appears to give rise to different results. For example, heat-treatment of the light dandelion honey (#08-5) and the medium manuka honey (#148) resulted in light brown coloured fractions (F13 to F15 and F13 to F17) that either showed an increase or a slight decrease in antioxidant activity (Figure 9, Table 13). For the light honey, heat-treatment resulted in moderately higher ORAC values in F13 and F14, and no change in F15. Similarly, heat-treatment of the medium honey resulted in a significant increase in ORAC in F14 and F15, and a slight decrease in F13. Thus, in turn heat-treatment of the light and medium honeys appears to have accelerated the formation of brown pigments in the major peak fractions, which resulted in an increase, for the most part, in antioxidant activity.

On the other hand, dark buckwheat honey was the exception and gave rise to a different outcome. Among the major peak fractions that demonstrated antioxidant activity in unheated dark buckwheat honey (F13 to F16), only F13 demonstrated a significant increase in ORAC following heat-treatment (paired t-test, $p<0.001$). Moreover, heat-treatment of this honey caused a significant decrease in the antioxidant activity in F15 and F16 (Tukey-Kramer multiple comparison test, $p<0.001$) and virtually no change in ORAC in F14 (Figure 9, Table 13). To possibly support the generation of new brown antioxidant pigments of a different molecular size in this honey, a fraction of higher molecular weight, F12, which did not show sufficient antioxidant activity in unheated dark buckwheat honey, demonstrated a change in colour (from colourless to brown) and the introduction of a measurable level of antioxidant activity following heat-treatment.

These results in turn may suggest that heat-treatment resulted in the generation of new brown pigments of higher molecular weight that exhibited antioxidant activity, as well as a loss in brown antioxidant pigments originally present in unheated honey,

A version of this chapter has been published.
Brudzynski & Miotto (2010). *Food Chemistry*. Doi:10.1016/j.foodchem.2010.09.049

specifically in F15 and F16, that caused a significant decrease in ORAC in these fractions. From these results it appears that the dark buckwheat honey behaves in a different manner compared to the other two honeys and that the initial composition of honey is a major influencing factor.

Heat-treatment appears to have resulted in the formation of brown MRLPs of HMW that exhibit antioxidant activity. Based on the characteristic change in colour, increase in UV absorbance, increase in brown pigment formation and the overall increase in antioxidant activity following heat-treatment, it has been suggested that melanoidins were formed in heat-treated honey and possibly in unheated dark buckwheat honey. In order to gain information on the main components involved in the honey melanoidin structure, the unheated and heat-treated honeys were analyzed for proteins, carbohydrates and phenolics.

3.4 Methodological approach towards the identification of the main components involved in the melanoidin structure

Data in the literature define melanoidins as carbohydrate-based nitrogen-containing high molecular weight polymers that are formed in the final stage of the Maillard reaction (Chandra et al., 2008). In addition, polyphenols are an important part of certain types of melanoidins, such as those found in coffee (Nunes & Coimbra, 2007; Gniechwitz et al., 2008b). In order to characterize the main components involved in the melanoidin structure in honey, the Folin-Ciocalteu method was used to determine the presence and concentration of polyphenols, SDS-PAGE was employed to determine the presence and level of proteins and the presence of carbohydrates was determined using LC-ESI-MS.

3.4.1 The effect of heat-treatment on the overall phenolic content of honey

There are a number of studies demonstrating that phenolic acids are the main factors responsible for the antioxidant activity of honey (Gheldof & Engeseth, 2002; Beretta et al., 2005; Vela et al., 2007). As heating of food systems is known to change the chemical properties of the components in the system, such as the solubility of phenolics, it was of interest to determine the influence of heat-treatment on the overall level of phenolics in honey.

The concentration of phenolics in the heat-treated honeys varied between honeys of different floral origin with the highest level being observed in the medium and dark honeys (#148 and #177) (Table 14). Heat-treatment resulted in about a two-fold increase in phenolic content for all honeys compared to the control. Despite the formation and removal of an insoluble brown pellet following heat-treatment of the dark buckwheat honey (#177), an increase in phenolic content, comparable to that observed for the medium honey, was still demonstrated.

Table 14: Total phenolic content of unheated and heat-treated honey

Honey Sample	**Phenolic Content**[a] (μg GAE/ml of honey)
08 5	131.40 ± 2.30
08 5 Treated	239.23 ± 7.52
148	272.25 ± 18.27
148Treated	483.88 ± 24.88
177	317.21 ± 30.26
177Treated	644.03 ± 21.42

a. Data shown as means (μg GAE/ ml) ± SD of three independent trials tested in duplicate (n=6).

Since honey contains a certain amount and type of components, the observed overall higher amount of phenolics after heat-treatment could be explained, at least in

part, by the formation of MRLPs with a phenolic type structure. It is possible that phenolic condensation plays a role in the browning of honey and the formation of melanoidins during heat-treatment. The observed overall increase in antioxidant activity following heat-treatment also supports the notion that formation of melanoidins occurred in heat-treated honey.

3.4.2 Phenolic content in active fractions of unheated and heat-treated honey

As phenolics have been demonstrated to be involved in the melanoidin structure in coffee, and since heat-treatment demonstrated an overall increase in the level of phenolics, it was of interest to determine if the active honey fractions contain phenolic compounds. The influence of phenolics on the antioxidant activity was compared between the active fractions of unheated and heat-treated honey.

After subjecting honey to SEC, which removed the small components that may interfere with Folin-Ciocalteu analysis, the active fractions were subjected to total phenolic testing. The total phenolic content was expressed as μg GAE/ml and the results obtained are shown in Table 15.

The concentration of phenolics in the major peak for all unheated honeys varied greatly depending on the floral source, where the dark buckwheat honey demonstrated the greatest level of phenolics in F14 (135 μg GAE/ml) (Figure 10, Table 15). For unheated honey, lower amounts of phenolics were present in fractions belonging to the light honey (#08-5) as compared to the darker coloured honeys (#148 and #177), where up to a 3 fold difference in the phenolic content was demonstrated between the lightest and darkest honey in these fractions. The difference in phenolic content observed in the unheated honey fractions appeared to be directly influenced by colour, as the brown coloured fractions of the dark honey (#177) demonstrated the greatest level of phenolics

Table 15: Total phenolic content of unheated and heat-treated honey fractions

	Phenolic content[a]	
Honey Fraction	**Unheated**	**Heat-treated**
	Honey #177	
12	36.74 ± 1.76	106.12 ± 2.51
13	118.60 ± 5.04	251.71 ± 5.51
14	135.00 ± 4.77	200.70 ± 12.50
15	108.06 ± 2.79	88.53 ± 4.93
16	63.49 ± 5.60	44.65 ± 5.59
	Honey #148	
13	48.14 ± 2.56	59.30 ± 1.16
14	62.64 ± 5.93	108.84 ± 2.68
15	65.27 ± 6.64	106.90 ± 7.21
16	55.43 ± 3.92	81.71 2.94
	Honey #08-5	
13	39.53 ± 3.23	93.02 ± 13.19
14	45.97 ± 3.26	80.85 ± 1.4
15	39.84 ± 3.96	58.45 2.01

a. Data shown as means (µg GAE/ ml of fraction) ± SD of three independent trials.

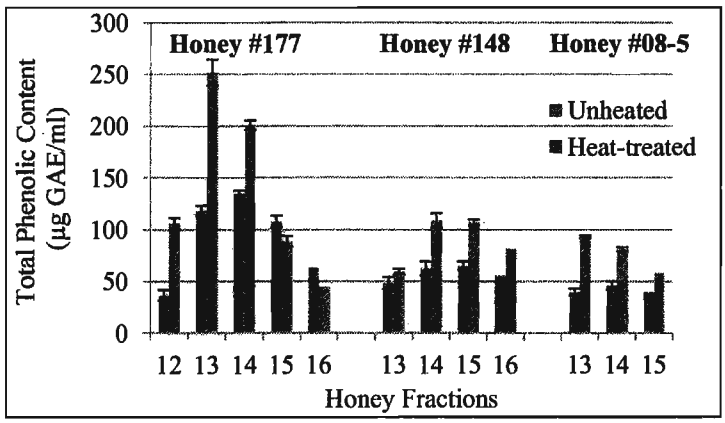


Figure 10: Total phenolic content of unheated and heat-treated honey fractions
Data represents means (µg GAE/ml of fraction) ± SD of three independent trials.

After heat-treatment, the phenolic levels increased in the major peak fractions of the light dandelion and medium manuka honeys, and in fractions of higher molecular weight (F12-F14) for dark buckwheat honey (Figure 10, Table 15). In the latter case, a slight decrease in phenolic content was observed in several fractions, particularly in F15 and F16 of heat-treated dark buckwheat honey. Despite the reduction in phenolic levels in F15 and F16 of dark buckwheat honey, an overall increase in phenolic content in the major peak fractions (F13-F15, light; F13-F16; medium and F12-F16, dark honey) for heat-treated honey was observed (paired t-test, $p<0.01$). From these results, phenolics appear to influence the colour of both unheated and heat-treated honey fractions, as well as the observed antioxidant activity. In order to demonstrate the relationship between these parameters, a Pearson r correlation was employed.

3.4.3 Relationship between the phenolic content, MRLP levels and antioxidant activity of active fractions of unheated and heat-treated honey

To test statistically the relationship between phenolic content, the level of browning and antioxidant activity of the active fractions of unheated and heat-treated honey, the Pearson r correlation was used (Table 16). Overall, a strong correlation between phenolics and antioxidant activity was observed in unheated and heat-treated honeys ($R^2= 0.58$, $p<0.001$). In spite of this, a much stronger correlation was observed between phenolic content and antioxidant activity in unheated fractions than between phenolic content and antioxidant activity in heat-treated fractions ($R^2=0.791$, $p<0.001$ and $R^2=0.528$, $p<0.006$, respectively) (Table 16). It appears that heat-treatment of dark buckwheat honey resulted in the loss of some phenolic antioxidants, as the Pearson r correlation indicated a weaker relationship between these two parameters following heat-treatment.

Similarly, a comparable trend was observed between the level of browning and antioxidant activity of unheated and heat-treated honey fractions. As shown in Table 16, a

stronger correlation was observed between the level of browning and antioxidant activity in unheated fractions than between the level of browning and the antioxidant activity in heat-treated fractions (R^2=0.875, p<0.001 and R^2 =0.707, p<0.005, respectively). Regarding antioxidant activity, ORAC demonstrated the highest correlation with the content of brown pigments in both unheated and heat-treated honeys (R^2= 0.875, p< 0.001 and R^2 = 0.707, p<0.005, respectively). From this result, it appears that MRLPs contribute to the antioxidant activity of both unheated, particularly dark buckwheat honey, and heat-treated honeys.

Table 16: Summary of the correlation analyses between ORAC, total phenolic content and MRLP content of unheated and heat-treated honey fractions

Parameter	**R^2**	**Significance**
Unheated Honey		
ORAC: Phenolic	0.791	0.001
ORAC: MRP	0.875	0.001
Phenolic : MRP	0.829	0.001
Heat-treated Honey		
ORAC:Phenolic	0.528	0.006
ORAC: MRP	0.707	0.005
Phenolic : MRP	0.719	0.001
Unheated and Heat-treated		
ORAC:Phenolic	0.578	0.001
ORAC: MRP	0.592	0.001
Phenolic : MRP	0.724	0.001

Lastly, the Pearson r correlation also suggests a strong relationship between the phenolic levels and the content of brown pigments in both unheated and heat-treated honey fractions (R^2= 0.829, p<0.001 and R^2= 0.719, p<0.001, respectively), as well as the strongest correlation overall (R^2= 0.724, p<0.001 unheated and heat-treated). Given that the antioxidant activity of honey appears to be strongly influenced by both the presence of phenolics and MRLPs, this may in turn suggest that these two structures are chemically related.

In order to support the formation of melanoidins in unheated dark buckwheat honey and in all thermally treated honeys, the HMW brown coloured fractions, which were demonstrated to contain phenolics and exhibit antioxidant activity, were tested for protein content. Given the high molecular weight of these fractions obtained from SEC, it is possible that they contain protein, as the size of protein in honey is in the range of 13 to 94 kDa (Iglesias et al., 2006).

3.4.4 Protein in active fractions of unheated and heat-treated honey

Enzymes, protein and amino acids are known to be present in honey. In fact, the identification of honey proteins is currently being looked into as an authenticity marker for a physiologically active food (Mohammed, & Babiker, 2009). As the current results suggest that MRLPs are present in the coloured active fractions of unheated (dark honey) and heat-treated honeys, and given the high molecular weight of components in these fractions, to support the presence of melanoidins, it is important to prove that proteins are present in these fractions as MRP formation requires them.

Considering that the size of proteins in honey are in the range of 13 to 94 kDa (Iglesias et al., 2006) and therefore can be easily visualized using gel electrophoresis, one-dimensional 10 % SDS-PAGE was employed in this study. Prior to subjecting the fractions that eluted in the major peak to SDS-PAGE, the protein in the selected fractions were precipitated by 80% ethanol precipitation. For estimation of protein molecular weight, a molecular weight calibration curve was used (refer to Appendix A, Figure A-4).

The protein profiles that were obtained from SDS-PAGE are shown in Figures 11-13. Differences in the number of protein bands were revealed between the unheated honeys of different floral origin. Two prominent bands corresponding to apparent molecular weights of 56-60 kDa and 82 kDa were detected for all three honeys, while

only the dark buckwheat honey fractions demonstrated bands at 26 kDa and 32 kDa in all major peak fractions and a faint band at 180 kDa in F14 (Figure 11a). The abundance of these proteins in the honey fractions not only differed between honeys from different origins but also between fractions of the same honey, as indicated by the intensity of the Coomasie Blue staining. Therefore, based on the intensity of the electrophoretic bands, F14 for all honeys showed the highest amount of protein, while fractions beyond that (F15-F16) showed lower amounts (Figures 11-13).

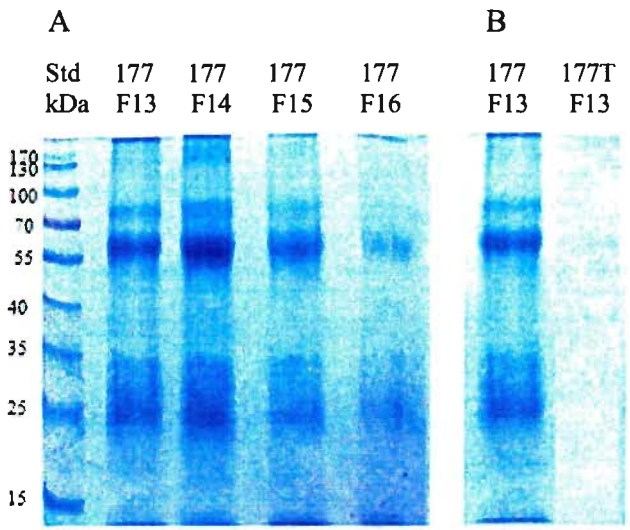


Figure 11: SDS-PAGE protein profile of the dark buckwheat fractions that eluted from SEC*. Molecular weight standards (in kDa) are shown at the left of the gel.
A. Protein profile of unheated dark buckwheat fractions. B. Comparison between fraction F13 of unheated (177) and heat-treated (177 T) dark buckwheat honey.
* Fractions were subjected to 80% ethanol precipitation and were concentrated 8-fold. 10% (w/v) polyacrlamide separating and 5% (w/v) stacking gels were used.

For all unheated honeys, a diffuse band at 56-60 kDa was observed on the SDS-PAGE gel for all major peak fractions (Figures 11-13). However, the width of the diffuse band, in terms of protein mobility within the gel, was observed to a lesser extent for light clover (#08-5) honey fractions (Figure 13). On the other hand, for the dark buckwheat honey (#177) fractions, the 26 kDa and 32 kDa protein bands also appeared as diffuse bands (Figures 11a). A similar observation pertaining to honey proteins on SDS-PAGE was demonstrated by other authors (Marshall & Williams, 1987; Baroni et al., 2002). As distinct protein bands were not observed by SDS-PAGE, this may suggest that several

proteins of a similar molecular weight, most likely differing by slight modification, are represented in the diffused bands.

SDS-PAGE also revealed the influence of heat-treatment on honey proteins. As shown in Figures 11-13, heat-treatment of honey caused a disappearance of protein bands, as well as a loss in protein content as indicated by a decrease in the band intensity following Coomasie Blue staining. For all heat-treated honeys, a faint band at 56-60 kDa appeared following heat-treatment, which was shown to be the most abundant protein in unheated honey fractions. Moreover, beyond F13 for all heat-treated honeys, a notable decrease in the number of bands as well as the amount of proteins was observed for all active honey fractions, particularly for dark buckwheat and light dandelion honey fractions, and to a lesser extent for medium manuka honey (Figure 11-13). Thus, heat-treatment of honey caused both a reduction in the number of protein bands and a reduction in protein content in these fractions.

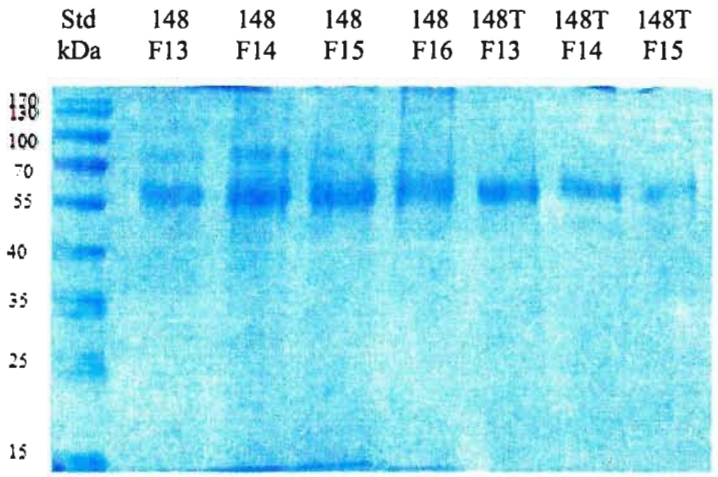


Figure 12: SDS-PAGE protein profile of the unheated (148) and heat-treated (148 T) medium manuka fractions that eluted from SEC*. Molecular weight standards (in kDa) are shown at the left of the gel.
* Fractions were subjected to 80% ethanol precipitation and were concentrated 8-fold.
10% (w/v) polyacrlamide separating and 5% (w/v) stacking gels were used.

Dark buckwheat honey demonstrated the most drastic reduction in protein as evidenced by SDS-PAGE. As shown in Figure 11b, heat-treatment reduced the number of

protein bands from four to one in F13 and resulted in the complete disappearance of protein bands in fractions F14-F16. Thus, honey proteins, specifically those in dark buckwheat honey, appear to be vulnerable to heat-treatment, as heat-treatment resulted in the reduction in protein content as well as the disappearance of protein bands.

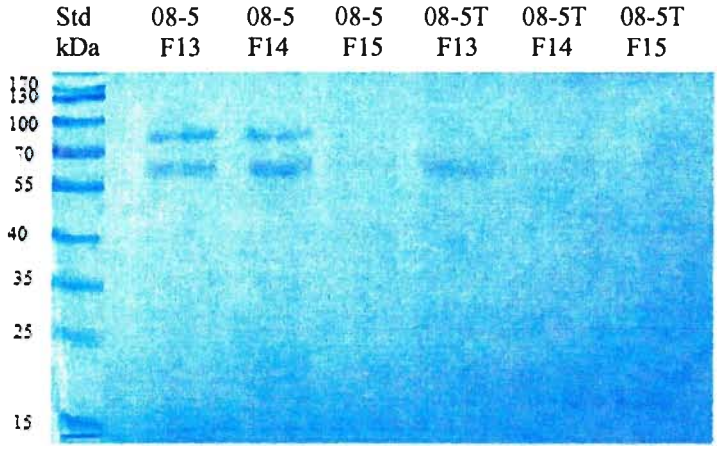


Figure 13: SDS-PAGE protein profile of the unheated (08-5) and heat-treated (08-5 T) light clover fractions that eluted from SEC*. Molecular weight standards (in kDa) are shown at the left of the gel.

* Fractions were subjected to 80% ethanol precipitation and were concentrated 8-fold. 10% (w/v) polyacrlamide separating and 5% (w/v) stacking gels were used.

The disappearance of protein bands from the SDS-PAGE gel following heat-treatment, particularly for dark buckwheat honey fractions, was an interesting observation that was made. Given that our hypothesis is correct, that heat-treatment caused an increase in polymerization to give rise to higher molecular weight complexes/polymers, such as melanoidins, the loss of protein in these fractions after heat-treatment may support this phenomenon.

As phenolic acids were also detected in these HMW coloured fractions, it is possible that these compounds may interact with proteins to form phenolic-protein complexes, which may be able to explain the diffused bands observed on the gel for unheated honey fractions. Due to this observation, it was of interest to investigate whether phenolic acids are able to form complexes with proteins in unheated honey, and

whether their involvement is responsible for the formation of high molecular weight complexes after heat-treatment.

In an attempt to resolve the diffused bands observed by SDS-PAGE for unheated honey and to explain the disappearance of bands observed for heat-treated honey fractions, a mixture of three alcohols (24% ethanol: 21% isopropanol: 24% methanol) was employed as the solvent extraction method to remove phenolic compounds from the fractions of dark buckwheat honey before (F13) and after heat-treatment (F12-F14). Following solvent extraction, electrophoresis on a 10% (w/v) polyacrlamide separating and 5% (w/v) stacking gel was run for one hour at a constant current of 110 Volts.

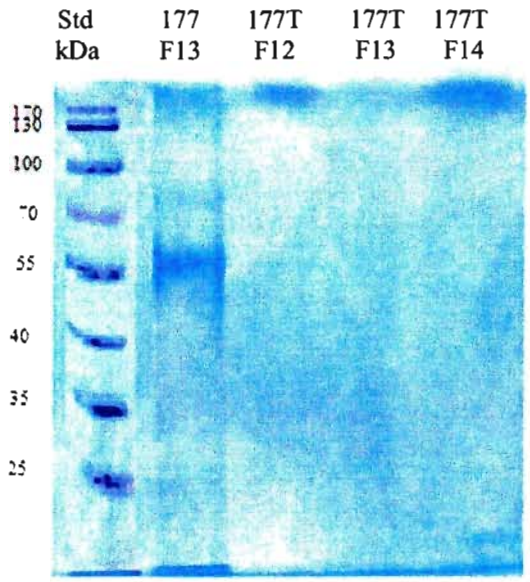


Figure 14: Protein profile of the unheated (177) and heat-treated (177 T) fractions of buckwheat honey following phenolic extraction using a mixture of three alcohols (24% ethanol: 21% isopropanol: 24%methanol). Molecular weight standards (in kDa) are shown at the left of the gel.
*Fractions were concentrated 8-fold. 10% (w/v) polyacrlamide separating and 5% (w/v) stacking gels were used.

The SDS-PAGE results that were obtained following the solvent extraction using a mixture of three alcohols (ethanol, isopropanol and methanol) are shown in Figure 14. For unheated dark buckwheat honey, the results demonstrated an intense coloured protein

band at 56-60 kDa, and three distinct, but faint bands at 26 kDa, 32 kDa and 82 kDa in fraction F13 (Figure 14). In contrast, heat-treatment of dark buckwheat honey showed the complete absence of protein bands in fractions F12-F14 on the separating gel, however, the presence of a clearly visible band between the stacking and resolving gel was observed.

From these results, it appears that the removal of some phenolics using a three alcohol extraction lessened the extent of band diffusion in unheated honey F13. On the other hand, despite the removal of some phenolics, this method does not appear to be able to separate the proteins from the HMW protein polymer stuck between the stacking and resolving gel. It appears that protein-phenolic complexation occurred in unheated honeys resulting in the formation of soluble complexes which appeared as diffused bands on the SDS-PAGE gel. In addition, following heat-treatment, the formation of a protein polymer that stuck between the stacking and resolving gel suggests a potentially less soluble complex of HMW.

3.4.5 Sugars, amino sugars and honey specific flavonoids by LC/MS in active fraction 15 of dark buckwheat honey (#177)

To suggest that melanoidins are one of the main factors that contribute to the antioxidant activity of honey, it is important to demonstrate that all of the components that are required for MRP formation are present in the active honey fractions. Fraction 15 of the unheated dark honey was selected for LC-ESI-MS as it showed high antioxidant activity, high amounts of phenolic content and the presence of protein.

The total ion current (TIC) chromatogram for active fraction 15 was obtained using an elution gradient involving formic acid and methanol, and was measured at

254nm, as many phenolics and flavonoids show their UV absorption maxima at this wavelength. The chromatogram that was obtained is shown in Figure 15.

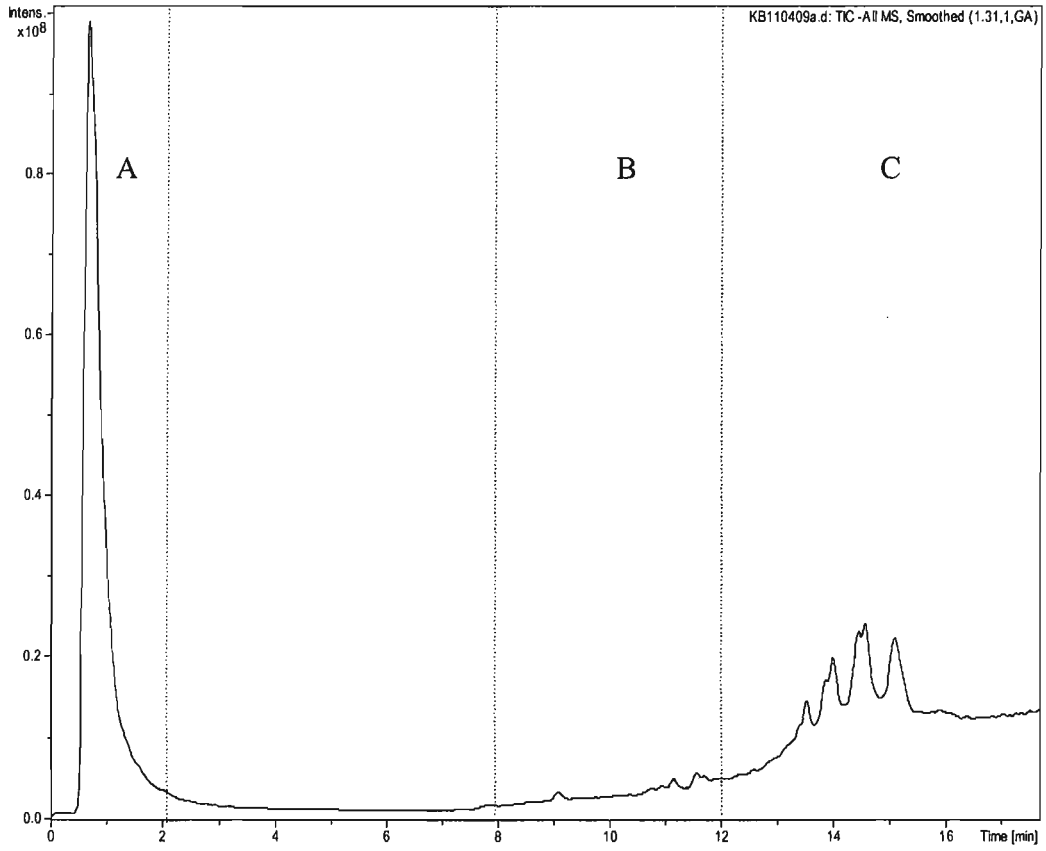


Figure 15: Total ion current chromatogram (UV, 254nm) of active fraction 15 obtained from dark buckwheat honey #177.

The TIC chromatogram shown in Figure 15 indicates the presence of several groups of components a) the hydrophilic compounds that eluted under the two minute interval as represented by an intense peak, b) the hydrophilic/hydrophobic compounds that eluted between 8-12 minutes, and c) the hydrophobic compounds which eluted from 12-20 minutes and are represented by a cluster of peaks. The presence of both highly hydrophilic and hydrophobic compounds in this active fraction indicates a diverse set of compounds that may take part in the formation of high molecular weight complexes.

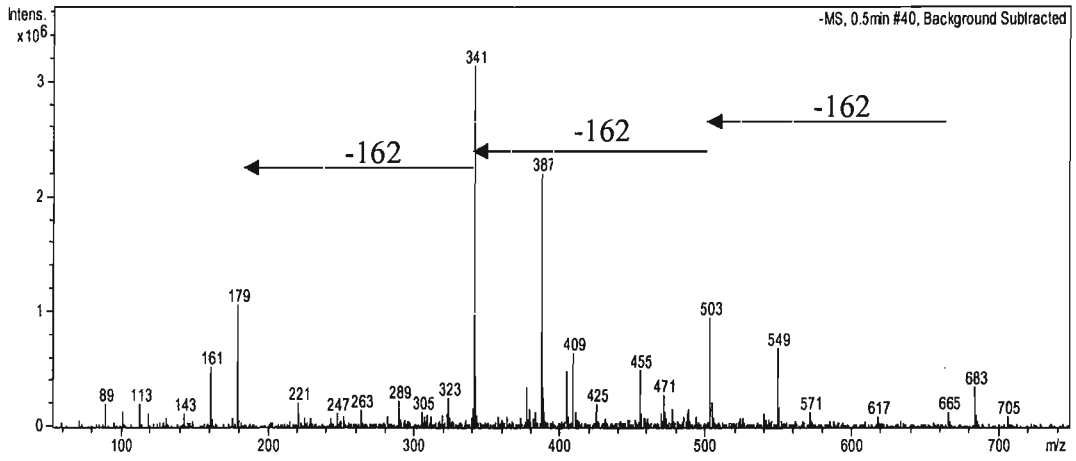


Figure 16: ESI-MS in negative ion mode of the hydrophilic peak (R_t= 0.5min) of active fraction 15 of #177.

Figure 16 shows a mass spectrum obtained by electrospray ionization in negative mode of the hydrophilic peak that eluted at 0.5 minutes as indicated in the chromatogram (Figure 15). The ESI-MS obtained for the hydrophilic peak showed ion peaks that indicated three consecutive losses of 162 amu from the ion at m/z 665 [M-162-162-162]. The loss of 162 amu is typically observed for anhydrous hexose moieties. As three consecutive losses of 162 amu were observed, this demonstrates the degree of polymerization (DP) of hexoses starting from DP2 (m/z 341) up to DP4 (m/z 665) (Figure 16). Further DP were not observed due to the MS scan range of 100 to 750 m/z used in this study. Therefore, these results suggest the presence of oligomers of hexose moieties in this active honey fraction, which is significant because oligomers may act as substrates, either directly or through their degradation, in the formation of Maillard reaction products.

In addition to the indication that oligomers are present in this active fraction, a number of fragment ions, specifically m/z 89, 113, 143 and 161 that are characteristic of the fragmentation pattern of hexoses, have been observed in this mass spectrum (Figure 16). This is an important observation because another important contributor to the formation of MRPs is the degradation of sugars, specifically glucose. It is known that the

degradation products of glucose can result in the formation of highly reactive dicarbonyls, such as methylglyoxal and glyoxal that are able to further react with both each other and other components to create MRPs. These reactive dicarbonyls, methylglyoxal and glyoxal have been previously identified in Manuka honey as the principle antibacterial components (Mavric et al., 2008; Adams et al., 2009). As the degradation products of hexose moieties form highly reactive dicarbonyls, the indication of hexose moieties in this active fraction provides further support of the appropriate conditions for the Maillard reaction to take place.

Moreover, numerous fragment ions that are characteristic of trisaccharides have also been observed in this mass spectrum (Figure 16). It is known that honey contains mostly monosaccharides, specifically glucose and fructose, however, a number of oligosaccharides including maltotriose and melezitose have previously been identified in honey (Arias et al., 2003). As a result, the presence of the fragmentation pattern typical of trisaccharides as demonstrated by Verardo et al., (2009), adds additional support to the required conditions for Maillard reaction. It is possible that the degradation products of the trisaccharides may provide additional means for the Maillard reaction to take place.

In addition to sugars, honey contains a very diverse set of components including numerous flavonoids and phenolic acids and their glycosides. Figure 17 shows a mass spectrum obtained by electrospray ionization in negative mode of a hydrophilic/ hydrophobic peak that eluted at 10.7 minutes as indicated in the TIC chromatogram (Figure 15). The ESI-MS obtained for the peak at 10.7 minutes showed a fragmentation pattern involving the ions at m/z 387 and m/z 255 (M-132). The loss of 132 amu indicates the loss of a pentose moiety. Based on the retention time of a commonly found flavonoid in honey, pinocembrin, it appears that pinocembrin glycosides are present in this fraction. As components other than sugars are also present in this active fraction, this adds to the variety of components that may take part in the formation of polymers.

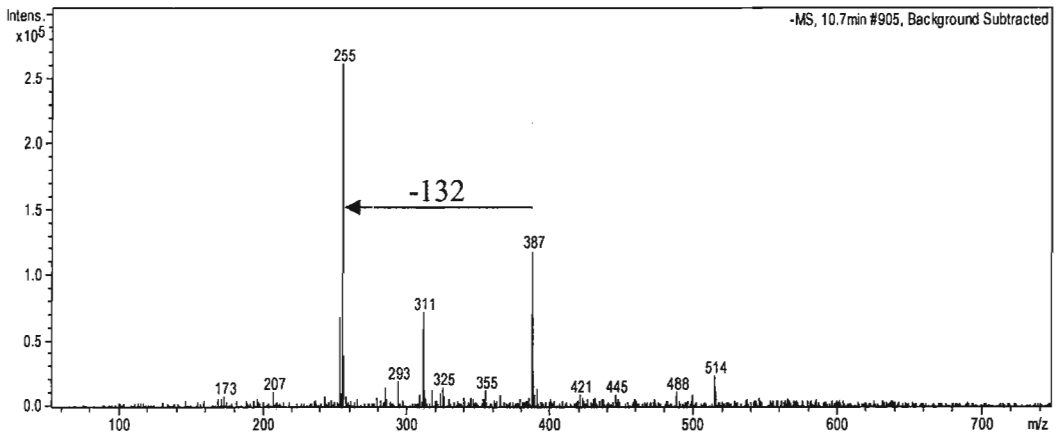


Figure 17: ESI-MS in negative ion mode of a hydrophilic/hydrophobic peak obtained for (R_t = 10.7min) active fraction 15 of #177.

LC-ESI-MS analysis provided information about a number of components that potentially have the ability to take part in the Maillard reaction. The results demonstrated the presence of oligosaccharides as well as their degradation products, and the presence of flavonoid glycosides. Ultimately, these components may contribute to the formation of HMW polymers that exhibit antioxidant activity.

Discussion:

There is a growing public interest on the effects of natural antioxidant components in food products. Honey has the potential to serve as a dietary antioxidant because it has demonstrated antioxidant activities comparable to that of many fruits and vegetables, in terms of its ORAC value (Gheldof & Engeseth, 2002). Numerous antioxidants have been identified in honey, mainly the polyphenols (flavonoids and phenolic acids), but also other minor constituents, such as vitamins C and E, enzymes (peroxidase, glucose oxidase, catalase), carotenoids and products of the Maillard reaction (Gheldof et al., 2002; Aljadi & Kamaruddin, 2004). Despite the identification of these antioxidant components, the individual components could not account for the total antioxidant activity of honey. It has been suggested that several antioxidants in honey may act synergistically to account for the observed activity (Gheldof et al., 2002).

The purpose of the present study was to elucidate the main chemical components involved in the antioxidant activity of honey and to establish their relationship with each other. In order to gain an understanding of the factors that influence the antioxidant activity of honey, a large study involving 22 honey samples from various floral sources were chosen and characterized based on sugar concentration, honey colour, phenolic content, browning index and antioxidant activity.

4.1 Identification of compounds with antioxidant activity and analysis of their relationship with each other

A set of 22 honeys were first characterized based on their total sugar concentration and colour. In the beekeeping industry, these two parameters are generally used to distinguish between honeys of floral versus honeydew origin, and to categorize honeys into groups based on colour for marketing purposes. The honey samples were subjected to testing for sugar concentration by using the Brix method, which is based on a refractometric measurement. By determining the Brix values, it was demonstrated that the honeys selected for this study contained between 75.52 and 79.24°Brix. These values were in agreement with those published in several recent reports that showed values ranging between 76.2 and 82.2°Brix (Silva et al., 2009; Saxena et al., 2010).

According to the National Honey Board and the Canadian Honey Council, honeys are typically classified into five colour categories: white/colourless, light yellow, dark yellow/light amber, medium amber, and brown to dark brown based on their visually perceived colour measured using the Pfund scale. However, as the Pfund scale gives imprecise and approximate colour values, in the present study, the colour of honey was established spectrophotometrically by determining the net absorbance (A_{560}-A_{720}nm) according to the method originally described by Huidobro and Simal (Pérez et al., 2007).

The honey samples that were used in this study showed a broad variety of colours established by measuring the net absorbance (A_{560}-A_{720}nm) and ranging from a net absorbance value of 0.038 AU for the lightest coloured honey (#08-7) up to 1.16 AU for the darkest coloured honey (#77). The relationship between the spectrophotometric measurements and the visually perceived honey colour is illustrated in Table 3. Based on these measurements, the honeys were divided into three groups: light coloured honeys with net absorbance values ranging from 0.038 to 0.12 AU, medium coloured honeys with net absorbance values ranging from 0.12 to 0.5 AU, and dark coloured honeys with net absorbance values ranging from 0.5 to 1.16 AU.

The colour of honey emerged as an important factor that influences the functional properties of honey, specifically the antioxidant activity (Gheldof & Engeseth, 2002; Blasa et al., 2006). Previous studies have demonstrated that honeys from different floral sources display similar phenolic profiles, however, differ in their richness of individual phenolic compounds (Gheldof, et al., 2002; Baltrusaityte et al., 2007). Darker coloured honeys, in general, contained higher concentrations of phenolics and exhibited higher antioxidant activities (Frankel et al., 1998; Gheldof & Engeseth, 2002).

To analyze the complex relationship between floral origin, content of individual phenolic acids and antioxidant activity, it was necessary to determine the total level of phenolics in the set involving 22 honeys. This was done by using the Folin-Ciocalteu method. As the Folin-Ciocalteu method is commonly used to estimate the level of phenolics, this method was employed in the present study so that the results could be easily related to the data obtained by other authors. The total phenolic content ranged from 49.78 to 513.25 μg GAE/g between the lightest (#08-7) and darkest honeys (#77). The darker coloured honeys, mainly those of buckwheat origin, consistently contained the highest level of phenolic content. Similar levels of phenolics were demonstrated in honeys differing in colour and floral source by several authors with values ranging from

46 to 796 mg GAE/kg between the lightest and darkest honeys, with buckwheat honeys belonging to the group containing the higher level of phenolics (from 456 to 796 mg GAE/kg) (Gheldof & Engeseth, 2002; Beretta et al., 2005).

In addition to phenolics, Maillard reaction products also contribute to the colour and antioxidant activity of many food products such as coffee (Gniechwitz et al., 2008b). Maillard reaction products, specifically melanoidins, result from non-enzymatic browning and contribute to the darkening of colour in food products. The appearance of these brown pigments has been demonstrated to coincide with an increase in the antioxidant activity, thus indicating a relationship between these two parameters (Manzocco et al., 2001). The role of Maillard reaction products on the antioxidant activity of honey has not yet been investigated. Honey appeared to be an ideal natural system to study the formation of Maillard reaction products, specifically melanoidins, due to the high concentration of reducing sugars, glucose and fructose, and the presence of lower amounts of free amino acids and proteins, conditions that are generally agreed upon to promote the rate of Maillard browning (O'Brien and Morrissey, 1989). Given that the required substrates for Maillard reaction to occur are present, it appeared that MRPs may in turn contribute to both the colour and antioxidant activity of honey.

Determination of the Maillard reaction product levels is typically based on the degree of browning by measuring the net absorbance at (A_{450}-A_{720}nm) (Martins et al., 2003). Due to the fact that the chemical structure and origin of these brown pigments are largely unknown, in this study the term 'Maillard reaction-like products' (MRLP) was used to describe compounds that absorbed at A_{450}- A_{720} nm. The level of brown pigments varied greatly among the 22 honeys ranging from 0.11 AU for the lightest honey (#08-7) up to 3.51 AU for the darkest honey (#77). This result provided the first evidence that MRLPs are present in unheated honey and thus responsible, at least in part, for honey colour.

From these results, it appeared that the level of brown pigments correlated with the colour of honey and the level of phenolics, where the darker coloured honeys showed higher levels of brown pigments as well as higher levels of phenolics. Therefore, as the next step in this study, the relationship between the antioxidant activity and MRLPs, in the context of honey colour and phenolic content, was investigated.

The 22 honey samples were screened for their antioxidant activity using the ORAC assay. The ORAC assay was employed in this study because it is a highly sensitive fluorescence based method that can analyze a large number of samples in a short period of time, and because it requires small testing volumes, it has the ability to minimize cross-reactions between samples and reagents. Moreover, the ORAC assay demonstrated superiority to other methods because it takes a free radical reaction to completion and can utilize different free radical generators or oxidants in the reaction mixture to test a different set of parameters (Cao et al., 1997).

All honeys tested in the present study were demonstrated to possess antioxidant activity. As indicated by the ORAC values, the dark honeys demonstrated the greatest antioxidant activities and the light honeys demonstrated significantly lower antioxidant levels (from 1.99 μmol TE/g up to 19.77 μmol TE/g for the lightest and darkest coloured honeys, respectively). The ORAC values were consistently higher for honeys of buckwheat origin (12.75, 17.41, 19.13 and 19.77 μmol TE/g). These results are in agreement with those of other authors who obtained ORAC values ranging from 2.00 to 21.07 μmol TE/g between the lightest and darkest coloured honeys, and from 7.47 to 16.95 μmol TE/g for honeys of buckwheat origin (Gheldof & Engeseth, 2002; Beretta et al., 2005).

In order to determine the influence of each parameter (honey colour, phenolics, MRLPs) on the antioxidant activity of honey, a principle component analysis was employed. The principle component analysis revealed that all four variables showed a strong correlation with one another. The correlation values between honey colour and MRLPs, total phenolic content and honey colour, MRLPs and total phenolics, and ORAC and MRLPs were 0.980, 0.963, 0.950 and 0.938, respectively. These results represent the first evidence of a significant contribution of MRLPs to the colour and overall antioxidant activity of unheated honey.

The overall extremely significant correlation between the four parameters (honey colour, phenolic content, MRLPs and ORAC) supported our view that compounds which represent specific groups/parameters may be chemically related and/or represent a chemical entity that involves the incorporation of components into a higher molecular weight complex. Such an example would be the integration of polyphenols into the melanoidin/MRP complex. However, at this point in our study, no such suggestion could be made as the molecular size and chemical properties of the so-called MRLPs was not known.

4.1.2 Melanoidins in heat-treated honey

It is generally assumed that the Maillard reaction requires heat in order to occur. The effects of thermal treatment on the formation of melanoidins in a number of foods have been extensively studied; such an example is coffee (Bekedam et al., 2006; Gniechwitz et al., 2008b). As the current results suggest the presence of MRLPs in unheated honey, it was important to show that the Maillard reaction can be accelerated during heat-treatment to provide evidence for the origin of melanoidin formation in unheated honey. To the best of our knowledge, no information on the presence of melanoidins in heat-treated honey exists. Recently, Turkmen et al. (2006) showed that heating honey at 50-70°C resulted in the formation of brown MRP pigments which

correlated with an increase in antioxidant activity. Despite this observation, no information regarding the structure of the brown antioxidant pigments in heated honeys were described.

The initial observation made following heat-treatment of honey at 121°C for 30 minutes was the change in colour. It was demonstrated that heat-treatment resulted in visually darker coloured honeys. The darkening of honey was anticipated to be due, at least in part to, nonenzymatic browning, as was previously demonstrated by Turkmen et al., (2006). To provide evidence that the darkening of honey was related to the Maillard reaction, the level of brown pigments was determined by measuring the net absorbance at A_{450}-A_{720}nm. The results obtained for the heat-treated light and medium honeys revealed an overall increase in the level of brown pigments following heat-treatment (from 0.54 to 1.36 AU and 1.41 to 2.08 AU for the light and medium honeys, respectively). Unlike the results obtained for the light and medium honeys, heat-treatment of the dark honey resulted in an unexpected overall decrease in the level of browning (from 2.18 to 1.78 AU) which coincided with the formation of an insoluble brown precipitate. It is possible that thermal treatment in the case of the dark honey, which was enriched in MRLPs, may have promoted the formation of high molecular weight polymers which ultimately became insoluble and precipitated out of solution, thus reducing the overall content of brown pigments.

As phenolics can undergo chemical changes upon thermal treatment, and since they contribute to both the colour and antioxidant activity of honey, it was of interest to determine the influence of heat-treatment on the level of phenolics. It was demonstrated that heat-treatment resulted in an overall increase in phenolic content for all honeys (from 131 to 239 μg GAE/ml, 272 to 484 μg GAE/ml, and 317 to 644 μg GAE/ml, for the light, medium and dark honeys, respectively). Despite the overall decrease in the level of brown pigments for the dark honey, heat-treatment resulted in an increase in the level of

phenolics. As honey contains a certain amount and type of components, the observed overall higher level of phenolics after treat-treatment suggests that heat-treatment altered the chemical properties of honey.

To further investigate whether heat-treatment accelerated the formation of melanoidins in honey, the antioxidant activity of the honeys was determined. As heat-treatment is known to promote the Maillard reaction, and since melanoidins typically exhibit antioxidant activity, it was anticipated that the heat-treated honeys would demonstrate higher antioxidant activities than the control. From the ORAC values, it was revealed that heat-treatment resulted in an overall increase in antioxidant activity for all honeys. Again, despite the decrease in brown pigment levels for the heat-treated dark honey, an increase in antioxidant activity was still observed. The results obtained for the light and medium honeys in this study are in agreement with those observed by Turkmen et al., (2006) who demonstrated a correlation between the antioxidant activity and the increase in brown pigment formation following heat-treatment. For the dark honey, it is possible that in addition to the formation of new brown coloured products that exhibited antioxidant activity, heat-treatment may have also resulted in the formation of insoluble complexes that precipitated out of the honey solution and caused a decrease in the overall level of brown pigments.

These results demonstrated that heat-treatment of honey resulted in an overall increase in the level of phenolics and antioxidant activity for all heat-treated honeys tested, as well as an overall increase in the degree of browning for the light and medium honeys. The exception was the dark honey which showed an overall decrease in the degree of browning and the formation of insoluble complexes. Thus far, the characteristics that were demonstrated following the heat-treatment of honey satisfy the description of melanoidins.

To further investigate the properties of honey melanoidins, and to determine whether the MRLPs in unheated dark honey belong to the melanoidin class of compounds, it was necessary to estimate the approximate molecular size of the components responsible for the antioxidant activity in heat-treated honey and relate their chemical properties to the antioxidant components in unheated honey.

4.1.3 Isolation and characterization of honey melanoidins

Melanoidins are defined as carbohydrate-based, high molecular weight brown macromolecules that are formed in the advanced stages of the Maillard reaction (Borrelli et al., 2002a). Melanoidins are usually characterized based on the following criteria: molecular size, degree of browning and antioxidant activity. Based on this description, the identification of high molecular weight brown coloured polymers would provide the first piece of evidence suggesting the presence of melanoidins in unheated honey. However, in order to suggest that the MRLPs that are formed in unheated honey are melanoidins and thus contribute to the antioxidant activity, it would be required to demonstrate that the MRLPs in unheated honey are similar in their chemical nature to the melanoidins formed in heat-treated honey.

To isolate the melanoidins in honey, size-exclusion chromatography using a Sepharose 4B column was employed in combination with activity-guided fractionation. Three honeys that differed in their initial colour as well as floral source were selected for this experiment: light honey (dandelion), medium honey (manuka), and dark honey (buckwheat). The honeys were heat-treated and the components from both unheated and heat-treated honeys were fractionated separately by using SEC, and all fractions obtained from SEC were analyzed for antioxidant activity using the ORAC assay. The properties of the components in the identified active fractions obtained for both unheated and heat-treated honeys were compared by analyzing the molecular size of the active components

and the level of browning. In addition, as an overall increase in phenolic content was observed for whole honey following heat-treatment, the total phenolic content was also determined for the active fractions.

4.1.4 Antioxidant activity and molecular weight of the active components in unheated and heat-treated honey

The use of SEC in combination with activity-guided fractionation allowed the initial characterization of honeys antioxidant components. By subjecting the honeys to SEC, several fractions possessing antioxidant activity were identified. For all honeys, the antioxidant activity was observed in fractions that eluted between F13 and F15. The active fractions of the dark honey exhibited the highest ORAC values (1125 to 2979 μmol TE/L), while the lighter coloured honeys demonstrated much lower ORAC values (363 to 437 μmol TE/L and 967 to 1479 μmol TE/L for the light and medium honeys, respectively).

Heat-treatment resulted in a significant increase in the antioxidant activity in several of these fractions. It was demonstrated that heat-treatment resulted in moderately higher ORAC values in F13 (363 to 814 umol TE/ L) and F14 (437 to 683 umol TE/ L) for the light coloured honey, and significantly higher ORAC values in F14 (1479 to 4710 umol TE/ L) and F15 (1287 to 2345 umol TE/ L) for the medium coloured honey. The change in ORAC values of the active fractions belonging to the unheated and heat-treated medium and light honeys was demonstrated to be highly significant (ANOVA, $F [7, 20] = 81.14$, $p<0.0001$ and $F [5, 13] = 19.72$, $p<0.0001$, for the medium and light coloured honeys, respectively).

In contrast, heat-treated dark honey was the exception. For the dark honey, fractions F12 and F13 demonstrated a significant increase in ORAC value following heat-treatment (from no observed activity to 1258 μmol TE/ L and 1547 to 3730 umol TE/ L for fractions F12 and F13, respectively) (Tukey-Kramer multiple comparison test, $p<0.001$). On the other hand, F15 and F16 demonstrated a significant decrease in antioxidant activity following heat-treatment (from 2742 to 1073 umol TE/ L and 1125 to 102 umol TE/ L for fractions F15 and F16, respectively). Thus, heat-treatment altered the chemical properties of the antioxidant components.

Determination of the molecular weight distribution revealed that the antioxidant components were of much higher molecular weight than anticipated, ranging from approximately 66 to 232 kDa. Even more surprising was the observation that the active components following heat-treatment of honey were of similar molecular weight (66 to 298 kDa). As a result, SEC provided the first indication that the heat produced antioxidants are of a similar molecular weight to the antioxidant compounds in unheated honey, thus suggesting that the MRLPs in unheated dark honey may belong to the melanoidin group of compounds.

4.1.5 Degree of browning and colour change in heat-treated honeys

By comparison of the heat-treated fractions to the unheated control, the first evidence of a change in honeys properties to account for the observed change in antioxidant activity of the fractions was demonstrated. It was revealed that heat-treatment resulted in a change in the colour of the active fractions towards darker hues as well as an increase in the level of brown pigments (A_{450}-A_{720}nm), presumably due to non-enzymatic browning.

Fractionation of the heat-treated light and medium coloured honeys revealed brown coloured fractions in the major peak that eluted between F13 and F16 which were colorless in unheated honey. The darkening of colour of the active fractions appeared to correlate with the increase in the level of brown pigments as measured at $A_{450\text{-}720}$ nm. As a result, this observation implied that heat-treatment of these honeys led to the accelerated generation of brown HMW melanoidins.

On the other hand, heat-treatment of the dark honey, which already showed brown coloured fractions, resulted in more intense brown coloured fractions in the major peak as well as a loss of the very high molecular weight brown pigments that eluted in the void volume of the column. Despite the darkening of colour of the active fractions for the dark honey, the level of brown pigments ($A_{450\text{-}A720}$ nm) did not appear to follow the trend. An increase in the level of brown pigments was only observed in the brown coloured fractions of higher molecular weight (F13-F14) and virtually no change in the level of brown pigments in the coloured fractions of lower molecular weight (F15-F16). In the latter, a change in the level of browning was not observed despite the darkening of colour of these fractions.

As a result of this observation, it is possible to conclude that brown pigments are not only chromophores that contribute to the colour of honey. Heat-treatment stimulated the generation of new chromophores of HMW which was associated with an increase in absorbance in the UV range at 280 nm. Moreover, these new chromophores, specifically in the light and medium heat-treated honeys, were demonstrated to exhibit antioxidant properties. Thus, it appeared that heat-treatment accelerated the formation of HMW melanoidin antioxidants in the light and medium honeys.

Heat-treatment of the dark honey resulted in the loss of the very HMW brown polymers that eluted in the void volume for the unheated dark honey. It is possible that

thermal treatment resulted in the degradation of these very HMW coloured polymers to yield new HMW brown polymers, which may explain the increase in the level of brown pigments in F13 and F14. Alternatively, heat-treatment may have also accelerated the polymerization of the very HMW polymers or the lower molecular components, such as those eluting in F15 and F16, to result in the formation of very HMW insoluble components and HMW antioxidant components, respectively. The latter may be able to provide reasoning for the simultaneous loss of brown pigments in F15 and F16 and the marked increase in the level of brown pigments in F13 and F14 after heat-treatment. From this observation, it appeared that the initial composition of honey played a major role in determining the fate of melanoidins in heat-treated honey. It was suggested that the dark honey already contained melanoidins, and thus heat-treatment resulted in the simultaneous formation and degradation of melanoidin compounds.

4.1.6 Browning and antioxidant activity

As melanoidins are known to exhibit antioxidant activity, it was of interest to evaluate the relationship between these two parameters. Heat-treatment of the light and medium honeys resulted in an increase in the degree of browning (A_{450}-A_{720}nm) in all fractions that eluted between F13 to F16 as well as an increase or no change in the antioxidant level. On the other hand, the dark honey showed a significant increase in both the degree of browning and ORAC in fractions F12 and F13 of high molecular weight, and a significant decrease in the degree of browning and antioxidant activity in fractions of lower molecular weight (F15 and F16). For the dark honey, which contained marked amounts of brown pigment in unheated honey, heat-treatment appeared to have led to the formation of very high molecular weight insoluble polymers that were excluded (by centrifugation) prior to SEC chromatography. Ultimately, lower amounts of brown pigments were observed after heat-treatment for this honey, specifically in F15 and F16. Similarly, the ORAC values were the highest in F12 and F13, but significantly lower in F15 and F16.

The Pearson r correlation revealed a strong relationship between the level of browning and antioxidant activity in both the unheated and heat-treated fractions of HMW (R^2=0.875, $p<0.001$ and R^2 =0.707, $p<0.005$, respectively). These results support the notion that heat-treatment resulted in the generation of melanoidins of higher molecular weight that exhibited antioxidant activity. Therefore, depending on the initial content of brown pigments in unheated honeys, heat-treatment either generated more brown pigment antioxidants or led to their loss.

4.1.7 Phenolic content in unheated and heat-treated honey fractions

Data in the literature suggests that polyphenols are an integral part of the melanoidin complex in foods such as coffee (Bekedam et al., 2008; Gniechwitz et al., 2008b). In the present study, a strong correlation between MRLPs, phenolic content and antioxidant of whole unheated honeys was established. As such a strong correlation was demonstrated between these parameters, it was of interest to investigate whether phenolics are involved in the melanoidin complex in honey.

The antioxidant components in unheated and heat-treated honeys were separated using SEC and were analyzed for the presence of phenolics. As SEC separates components on a molecular weight basis, the presence of phenolics in the active fractions of HMW might suggest their incorporation, either by covalent or non-covalent interactions, into the melanoidin structure.

For the active fractions of unheated honey, the concentration of phenolics varied greatly depending on the floral source, where the dark buckwheat honey demonstrated the greatest level of phenolics in F14 (135 μg GAE/ml). While the active major peak fractions of unheated dark honey contained the highest level of phenolics, the light and

medium honeys contained 29% and 54% of the total phenolics present in the major peak for the dark honey. Indeed, the darker honeys contained much higher levels of phenolics.

Heat-treatment caused a significant increase in the phenolic content in the active fractions of the light and medium honeys, and in fractions of higher molecular weight (F12-F14) for the dark honey. On the other hand, a slight decrease in phenolic content was demonstrated in fractions F15 and F16 of heat-treated dark honey. From these results, the level of phenolics appeared to correlate with the antioxidant activity.

Losses of the natural antioxidants from the dark honey during heat-treatment could be compensated for, at least in part, by the formation of MRPs that exhibit antioxidant activity (Manzocco et al., 2001; Turkmen et al., 2006). In this case, the phenolic antioxidants in some fractions may have taken part in the formation of melanoidins, as a significant increase in the level of browning, phenolic content and ORAC was observed in F13 of heat-treated dark honey. The presence of phenolics in the HMW melanoidin fractions isolated from roasted coffee has been demonstrated by several authors to be due to the incorporation of phenolics, particularly chlorogenic acid, into the melanoidin structure (Bekedam et al., 2008; Gniechwitz et al., 2008b).

Moreover, the thermal processing conditions, such as the time and temperature, greatly influence the degradation of compounds, such as the reduction/ degradation of ascorbic acid in tomato puree (Anese et al., 1999) or chlorogenic acid in dark roasted coffee beans (del Castillo et al., 2002). Although these studies demonstrated the loss of some naturally occurring antioxidants, they suggested that the antioxidant activity can be maintained by the simultaneous formation of Maillard reaction products. Even though the results are not conclusive, it might be speculated that heat-treatment of honey led to the increased incorporation of phenolics into the melanoidin structure.

Despite the reduction in phenolics for the dark honey in F15 and F16, the Pearson r correlation revealed a strong correlation between phenolic content and antioxidant activity in both unheated and heat-treated fractions (R^2=0.791, $p<0.001$ and R^2=0.528, $p<0.006$, respectively). However, the stronger relationship between these parameters was demonstrated for unheated honey fractions. As the Pearson r correlation indicated a loss of a strong relationship between phenolic content and antioxidant activity after heat-treatment of honey, this suggests that the dark honey lost some phenolic antioxidants due to heat-treatment.

The Folin-Ciocalteu method that was used in the present study detects all water-soluble phenolic groups in the sample, including structures that consist of a phenolic type structure, such as MRPs (Sahin et al., 2009). Therefore, the increase in phenolic content which correlated with the darkening of honey and antioxidant activity could be explained, at least partially, by the formation of melanoidins with a phenolic type structure. The incorporation of phenolic acids, such as chlorogenic acid, into the melanoidin structure has been previously suggested for roasted coffee (Bekedam et al., 2008; Gniechwitz et al., 2008b) or lipid peroxidation products in tomato melanoidins (Adams et al., 2005).

Based on the results obtained from SEC, it could be concluded that the phenolic components are associated with the melanoidin structure and eluted in the same fractions that possess antioxidant activity. Since honey phenolics exhibit antioxidant properties on their own, this observation supports their ability to contribute to the observed antioxidant activity.

4.1.8 Evidence demonstrating the presence of melanoidins in honey

Based on the characteristic change in colour, UV absorbance, brown pigment formation and antioxidant activity following heat-treatment, it was suggested that the heat produced antioxidants, as well as the antioxidant brown pigments in unheated dark

honey, were in fact melanoidins. This study has provided the first evidence suggesting that melanoidins are present in unheated and heat-treated honey and contribute to the antioxidant activity. These results were demonstrated to be in agreement with those demonstrated by other authors who indicated a positive relationship between melanoidin formation, the degree of browning and antioxidant activity (Borrelli et al., 2002b; Manzocco et al., 2001).

4.1.9 Characterization of the structural components in honey melanoidins

Currently, three theories regarding the melanoidin structure were suggested (1) polymers made up of repeating units of furans or pyrroles (Tressl et al., 1998), (2) carbohydrate-based melanoidin skeletons (Cämmerer & Kroh, 1995; Cämmerer et al., 2002) and (3) protein-based melanoidin skeletons (Hofmann, 1998a).

In order to gain information regarding the main components involved in the honey melanoidin structure, the SEC fractions from the unheated and heat-treated honeys were analyzed for the presence of proteins, carbohydrates and phenolics.

4.1.10 Proteins in the melanoidin fractions

To further characterize the components involved in the honey melanoidin structure, the protein profile of unheated and heat-treated honey fractions were analyzed by SDS-PAGE. Given the high molecular weight of the active fractions, it was anticipated that these fractions did contain protein. From the SDS-PAGE gels, it was demonstrated that all active fractions contained protein, however, differences in the number of protein bands was revealed between the honeys of different floral origin.

An important observation was that the protein bands appeared as fuzzy, diffused bands on the SDS-PAGE gel. This observation has been demonstrated previously in honey by other authors (Marshall & Williams, 1987; Baroni et al., 2002). As a result of the observation that the protein pellet remained brown in colour following 80% ethanol precipitation, specifically for the dark honey, and because phenolics were detected in these fractions by the Folin-Ciocalteu method, it was assumed that phenolic compounds were, at least in part, responsible for the band diffusion observed by SDS-PAGE. It is known that some polyphenols are able to form water-soluble complexes with proteins which appear as fuzzy, diffused bands on both native and SDS-PAGE gels (Kusuda et al., 2006).

In comparison to unheated honey fractions, where SDS-PAGE detected proteins in all major peak fractions, heat-treatment of honey demonstrated a loss in the protein content for all active honey fractions. The most drastic reduction in protein content was revealed for the dark honey, which showed that heat-treatment caused the reduction in the number of protein bands from four to one in fraction F13, and the complete disappearance of bands in fractions F14 – F16. Similar observations were reported for roasted coffee, where protein content decreased as a result of roasting and was suggested to be due to protein breakdown or protein polymerization (Borrelli et al., 2002b; Montavan et al., 2003).

The disappearance of protein bands from the SDS-PAGE gel after heat-treatment of honey, specifically dark honey, is an interesting phenomenon. It is possible that the loss in protein content after heat-treatment supports the notion that polymerization to form high molecular weight polymers/complexes occurred. In addition, as the protein bands on SDS-PAGE appeared as diffuse bands in both unheated and heat-treated active honey fractions, this provides additional support for the involvement of phenolics in the melanoidin structure. With this in mind, and in an attempt to resolve the diffused bands

on the SDS-PAGE gels, the fractions from unheated and heat-treated dark honey were subjected to a solvent extraction involving a mixture of three alcohols to remove phenolics from the protein pellet.

SDS-PAGE following the solvent extraction revealed two separate scenarios. On one hand, the mixture of three alcohols was able to remove some of the contaminating phenolic compounds from the unheated dark honey fraction (F13), as evidenced by the lessened extent of band diffusion. On the other hand, the solvent extraction method resulted in the complete absence of bands on the resolving gel for the heat-treated dark honey fractions (F12-F14), however, it revealed the presence of a well defined high molecular weight polymer stuck at the top of the resolving gel. From this result, it appears that the mixture of three alcohols was able to remove some phenolics from the unheated honey fractions however, protein-phenolic complexation still occurred as evidenced by the diffused band. For the heat-treated honey fractions, it appears that this method was unable to sufficiently separate the protein from the high molecular weight polymer which suggested the presence of a less soluble complex of high molecular weight.

Therefore, the present results suggest that protein-phenolic complexation occurred in unheated honey which resulted in the formation of soluble complexes. Moreover, heat-treatment appeared to have resulted in the increased binding of phenolics to proteins as a HMW polymer was observed by SDS-PAGE and was unable to be disrupted by the solvent extraction method employed.

4.1.11 Carbohydrates in the melanoidin fractions

As carbohydrates are the most important substrate involved in the formation of melanoidins, it was of great interest to detect the presence of carbohydrates in the

unheated active fractions, specifically for the dark honey, to support the origin of melanoidins in honey. Carbohydrates can be incorporated into the backbone structure of melanoidins as an intact sugar or as a sugar degradation product (Tressl et al. 1998; Cämmerer & Kroh, 1995; Cämmerer et al., 2002). To detect the presence of carbohydrates in the active fractions of unheated dark honey, LC-ESI-MS was employed.

The LC total ion chromatogram belonging to the unheated dark honey fraction (F15) indicated the presence of three groups of components: hydrophilic, hydrophilic/hydrophobic and hydrophobic. Upon further analysis of the hydrophilic and hydrophilic/hydrophobic group of components, LC-ESI-MS revealed that this fraction contained oligosaccharides and their degradation products, as well as flavonoid glycosides.

The presence of fragment ions that are characteristic of the fragmentation pattern of hexoses and trisaccharides in the mass spectrum was of great significance. The fragmentation pattern of monosaccharides, disaccharides, trisaccharides and oligosaccharides have been extensively studied by Verardo et al., (2009) using LC/ESI tandem mass spectrometry in negative ion mode where they demonstrated a list of fragmentation ions for hexoses (m/z 179, 161, 143, 113, 89), sucrose (m/z 341, 353 179), maltose (m/z 341, 161), meleziotose (m/z 323, 179) or maltotriose (m/z 341, 161) that were identical to those observed in the current study. The presence of fragment ions characteristic of hexoses and trisaccharides provides support for the origin of melanoidin formation in honey.

Conclusion:

The novel finding in this study was the evidence that high molecular weight melanoidins contribute to the antioxidant activity of both unheated and heat-treated

honey. The use of SEC and activity-guided fractionation allowed the antioxidant components of unheated and heat-treated honey to be characterized as being high molecular weight brown coloured compounds that strongly absorb at both 280nm and 450nm. The simultaneous presence of phenolics, proteins and sugars in the HMW active fractions of unheated honey suggests that these components represent a chemical entity that exerts its antioxidant activity synergistically. Due to SEC separating the components based on molecular size, these results suggest for the first time the involvement of phenolics, either by covalent or non-covalent interaction with the melanoidin structure, in the antioxidant activity of honey.

Heat-treatment resulted in darker coloured fractions that contained phenolic levels which correlated with the observed change in the antioxidant activity. Polyphenols have been shown to be involved in protein modification and were observed as diffused bands on the SDS-PAGE gels. Heat-treatment simultaneously resulted in the loss in protein due to complexation by phenolics and the formation of a HMW polymer which could not enter the separating SDS gels. The finding that polyphenols and proteins together with brown pigments eluted in the same high molecular fractions from SEC provides further support to the notion that proteins participated in the formation of phenolic-protein-carbohydrate polymers. From these results, it appears that heat-treatment promoted the Maillard reaction and the formation of phenolic-protein complexation to generate melanoidins that involve phenolic chromophores.

The presence of oligosaccharides of various degrees of polymerization, hexose fragmentation products, as well as the presence of protein in the active unheated fractions from SEC supports the initial requirements for melanoidin formation. As higher levels of phenolics were present in the fractions that demonstrated higher antioxidant activities after heat-treatment, heat-treatment appears to have accelerated the incorporation of phenolics into the melanoidin skeleton. The fate of melanoidins appears to be dependent

on the initial concentration of melanoidins in honey. Heat-treatment resulted in the formation of melanoidins which correlated with an increase in antioxidant when very low or no melanoidins were present (light and medium honeys) while heat-treatment of the dark honey which originally contained melanoidins resulted in the simultaneous degradation and formation of melanoidins.

In summary, these results have indicated for the first time that the initial concentration of melanoidins is an important factor that determines the fate of melanoidins. The first evidence that phenolic acids may be primarily involved in the formation of honey melanoidins has been suggested. As phenolics, proteins and carbohydrates were simultaneously detected in the brown coloured fractions of HMW, this supports the notion that phenolics may exert their antioxidant action by interacting with melanoidins, rather than acting independently. Although the linkage of the HMW polymer is not known, it is possible that through either covalent or non-covalent linkages, these aggregates or complex structures exhibit their antioxidant activity.

References Cited:

Adams, A., Borrelli, R.C., Fogliano, V., & De Kimpe, N. (2005). Thermal degradation studies of food melanoidins. *J. Agric. Food Chem.,* 53, 4136-4142.

Adams, C.J., Manley-Harris, M., & Molan, P.C. (2009). The origin of methylglyoxal in New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohyd. Res.,* 344, 1050-1053.

Alonso, A. M., Guillén, D. A., Barroso, C. G., Puertas, B., & García, A. (2002). Determination of antioxidant activity of wine byproducts and its correlation with polyphenolic content. *J. Agric. Food Chem.*, 50, 5832–5836.

Aljadi, A.M., & Kamaruddin, M.Y. (2004). Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. *Food Chem.,* 85, 513-518.

Al Somal, N., Coley, K.E., Molan, P.C., & Hancock, B.M. (1994). Susceptibility of Helicobacter pylori to the antibacterial activity of manuka honey. *J. R. Soc. Med.,* 87, 9-12.

Anese, M., Manzocco, L., Nicoli, M.C., Lerici, C.R. (1999). Antioxidant properties of tomato juice as affected by heating. *J. Sci. Food Agric.,* 79, 750-754.

Anklam, E. (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chem.,* 63, 549-562.

Arias, V.C., Castells, R.C., Malacalza, N., Lupano, C.E., & Castells, C.B. (2003). Determination of oligosaccharide patterns in honey by solid-phase extraction and High-Performance Liquid Chromatography. *Chromatographia*, 58, 797-801.

Baltrušaitytė, V., Venskutonis, P.R., & Čeksterytė, V. (2007). Radical scavenging activity of different floral origin honey and beebread phenolic extracts. *Food Chem.,* 101, 502-514.

Baroni, M. V., Chiabrando, G. A., Costa, C., & Wunderlin, D. A. (2002). Assessment of the floral origin of honey by SDS–PAGE immunoblot techniques. *J. Agric. Food Chem.*, 50, 1362–1367.

Bekedam, E.K., Loots, M.J., Schols, H.A., van Boekel, M.A.J.S., & Smit, G. (2008). Roasting effects on formation mechanisms of coffee brew melanoidins. *J. Agric. Food Chem.,* 56, 7138-7145.

Bekedam, E.K., Schols, H.A., van Boekel, M.A.J.S., & Smit, G. (2006). High molecular weight melanoidins from coffee brew. *J. Agric. Food Chem.,* 54, 7658-7666.

Beretta, G., Granata, P., Ferrero, M., Orioli, M., & Facino, R.M. (2005). Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. *Anal. Chim. Acta.,* 533, 185-191.

Bertoncelj, J., Doberšek, U., Jamnik, M., & Golob, T. (2007). Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. *Food Chem.,* 105, 822-828.

Blasa, M., Candiracci, M., Accorsi, A., Piacentini, M.P., Albertini, M.C., & Piatti, E. (2006). Raw *Millefiori* honey is packed full of antioxidants. *Food Chem.,* 97, 217-222.

Borrelli, R.C & Fogliano, V. (2005). Bread crust melanoidins as potential prebiotic ingredients. *Mol. Nutr. Food Res.49*, 673-678.

Borrelli, R.C., Fogliano, V., Monti, S.M., & Ames, J.M. (2002a). Characterization of melanoidins from a glucose-glycine model system. *Eur. Food Res. Technol.,* 215, 210-215.

Borrelli, R.C., Mennella, C., Barba, F., Russo, M., Russo, G.L., Krome, K., Erbersdobler, H.F., Faist, V., & Fogliano, V. (2003). Characterization of coloured compounds obtained by enzymatic extraction of bakery products. *Food Chem. Toxicol.,* 41, 1367-1374.

Borrelli, R.C., Visconti, A., Mennella, C., Anese, M., & Fogliano, V. (2002b). Chemical characterization and antioxidant properties of coffee melanoidins. *J. Agric. Food Chem.,* 50, 6527-6533.

Brands, C.M.J., Wedzicha, B.L., & van Boekel, M.A.J.S. (2002). Quantification of melanoidin concentration in sugar-casein systems. *J. Agric. Food Chem.,* 50, 1178-1183.

Brudzynski, K. (2006). Effect of hydrogen peroxide on antibacterial activities of Canadian honeys. *Can. J. Microbiol.,* 52, 1228-1237.

Bulut, L., & Kilic, M. (2009). Kinetics of hydroxymethylfurfural accumulation and color change in honey during storage in relation to moisture content. *J. Food Process. Pres.,* 33, 22-32.

Cämmerer, B., Jalyschkov, V., & Kroh, L.W. (2002). Carbohydrate structures as part of the melanoidin skeleton. *International Congress Series.,* 1245, 269-273.

Cämmerer, B., & Kroh, L.W. (1995). Investigation of the influence of reaction conditions on the elementary composition of melanoidins. *Food Chem.,* 53, 55-59.

Cao, G., Sofic, E., & Prior, R.L., (1997). Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Bio. Med.,* 22, 749-760.

Castro-Vásquez, L., Díaz-Maroto, M.C., González-Viñas, M.A., De La Fuente, E., & Pérez-Coello, M.S. (2008). Influence of storage conditions on chemical composition and sensory properties of citrus honey. *J. Agric. Food Chem.,* 56, 1999-2006.

Chandra, R., Bharagava, R.N., & Rai, V. (2008). Melanoidins as major colourant in sugarcane molasses based distillery effluent and its degradation. *Bioresource Technol.,* 99, 4648-4660.

Chen, X-M., & Kitts, D.D. (2008). Antioxidant activity and chemical properties of crude and fractionated Maillard reaction products derived from four sugar-amino acid Maillard reaction model systems. *Ann. N.Y. Acad. Sci.,* 1126, 220-224.

Chen, L., Mehta, A., Berenbaum, M., Zangerl, A.R., & Engeseth, N.J. (2000). Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetable homogenates. *J. Agric. Food Chem.,* 48, 4997-5000.

Cooper, RA., Molan, P. C., & Harding, K.G. (2002). The sensitivity to honey of Gram-positive cocci of clinical significance isolated from wounds. *J. Appl. Microbiol.,* 93, 857-863.

D`Arcy, B.R. (2005). Antioxidants in Austrailian floral honeys-Identification of health-enhancing nutrient components. Rural Industries Research and Development Corporation Publication No. 05/040.

Del Castillo, M.D., Ames, J.M., & Gordon, M.H. (2002). Effect of roasting on the antioxidant activity of coffee brews. *J. Agric. Food Chem.,* 50, 3698-3703.

Delgado-Andrade, C., Rufián-Henares, J.A., & Morales, F.J. (2005). Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. *J. Agric. Food Chem.,* 53, 7832-7836.

Efem, S. (1988). Clinical observations on the wound healing properties of honey. *Br. J. Surg.,* 75, 679-681.

Fallico, B., Zappalà, M., Arena, E., & Verzera, A., (2004). Effects of conditioning on HMF content in unifloral honeys. *Food Chem.,* 85, 305-313.

Frankel, S., Robinson, G.E., & Berenbaum, M.R. (1998). Antioxidant content and correlated characteristics of 14 monofloral honeys. *J. Apic. Res.,* 37, 27-31.

French, V.M., Cooper, R.A., & Molan, P.C. (2005). The antibacterial activity of honey against coagulase-negative staphylococci. *J. Antimicrob. Chemother.*, 56, 228-231.

Gheldof, N., & Engeseth, N.J., (2002). Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of *in vitro* lipoprotein oxidation in human serum samples. *J. Agric. Food Chem.,* 50, 3050-3055.

Gheldof, N., Wang, X-H, & Engeseth, N.J. (2002). Identification and quantification of antioxidant components of honeys from various floral sources. *J Agric. Food Chem.,* 50, 5870-5877.

Gniechwitz, D., Reichardt, N., Meiss, E., Ralph, J., Steinhart, H., Blaut, M., & Bunzel, M. (2008a). Characterization and fermentability of an ethanol soluble high molecular weight coffee fraction. J Agric Food Chem 56:5960–5969

Gniechwitz, D., Reichardt, N., Ralph, J., Blaut, M., Steinhart, H., & Bunzel, M. (2008b). Isolation and characterisation of a coffee melanoidin fraction. *J. Sci. Food Agric.*, 88, 2153-2160.

Gonzales, A.P., Burin, L., & del Pilar Buera, M. (1999). Color changes during storage of honeys in relation to their composition and initial color. *Food Res. Int.*, 32, 185-191.

Gupta, J.K., Kaushik, R., & Joshi, V.K. (1992). Influence of different treatments, storage temperature end period on some physico-chemical characteristics and sensory qualities of Indian honey. *J. Food Sci. Technol. India,* 29, 84-87.

Halliwell, B., & Gutteridge, J.M. (1999). "Free Radicals in Biology and Medicine" Oxford University Press, Oxford.

Heim, K.E., Tagliaferro, A.R., & Bobilya, D.J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem.*, 13, 572-584.

Held, P. (2005). Performing oxygen radical absorbance capacity (ORAC) assays with Synergy HT multi-detection microplate reader. Application note from Biotek. http://www.biotek.com/resources/tech_res_detail.php(id=161).

Hodge, J.E. (1953). Chemistry of browning reactions in model systems. *J. Agric. Food Chem.*, 1, 928-943.

Hofmann, T. (1998a). Studies on the relationship between molecular weight and the color potency of fractions obtained by thermal treatment of glucose/amino acid and glucose/protein solutions by using ultracentrifugation and color dilution techniques. *J. Agric. Food Chem.*, 46, 3891-3895.

Hofmann, T. (1998b). Studies on melanoidin-type colorants generated from the Maillard reaction of protein-bound lysine and furan-2-carboxaldehyde-chemical characterisation of a red coloured domaine. *Z. Lebensm Unters Forsch A.,* 206, 251-258.

Homma, S., Terasawa, N., Kubo, T., Yoneyama-Ishii, N., Aida, K., & Fujimaki, M. (1997). Changes in chemical properties of melanoidins by oxidation and reduction. *Biosci. Biotechnol. Biochem.,* 61, 533-535.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J., and Prior, R. (2002). High-throughput assay of Oxygen Radical Absorbance Capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.* 50, 4437-4444.

Iglesias, M.T., Martín-Alvarez, P.J., Polo, M.C., de Lorenzo, C., & Pueyo, E. (2006). Protein analysis of honeys by fast protein liquid chromatography: Application to differentiate floral and honeydew honeys. *J. Agric. Food Chem,* 54, 8322-8327.

Jeffrey, A.E., & Echazarreta, C.M. (1996). Medical uses of honey. *Rev. Biomed.,* 7, 43-49.

Jing, H., & Kitts, D. D. (2004). Antioxidant activity of sugar–lysine Maillard reaction products in cell free and cell culture systems. *Arch. Biochem. Biophys.*, 429, 154–163.

Kroh, L.W., Fiedler, T., & Wagner, J. (2008). α-dicarbonyl compounds- key intermediates for the formation of carbohydrate-based melanoidins. *Ann. N. Y. Acad. Sci.,* 1126, 210-215.

Kuntcheva, M.J., & Obretenov, T. D. (1996). Isolation and characterization of melanoidins from beer. *Z Lebensm. Unters. F. A.*, *202*, 238-243.

Kusuda, M., Hatano, T., & Yoshida, T. (2006). Water-soluble complexes formed by natural polyphenols and bovine serum albumin: Evidence from gel electrophoresis. *Biosci. Biotechnol. Biochem.*, 70, 152-160.

Labrinea, E.P., & Georgiou, C.A. (2005). Rapid, fully automated flow injection antioxidant capacity assay. *J. Agric. Food Chem.*, 53, 4341-4346.

Lachman, J., Kolihová, D., Miholová, D., Košata, J., Titěra, D., & Kult, K. (2007). Analysis of minority honey components: Possible uses for the evaluation of honey quality. *Food Chem.*, 101, 973-979.

Lachman, J., Orsák, M., Hejtmánková, A., & Kovářová, E. (2010). Evaluation of antioxidant activity and total phenolics of selected Czech honeys. *LWT - Food Sci. Technol.*, 43, 52-58.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature*, 227, 680-685.

Lavelli, V. (2002). Comparison of the antioxidant activities of extra virgin olive oils. *J. Agric Food Chem.*, 50, 7704-7708.

Lindenmeier, M., Faist, V., & Hofmann, T. (2002). Structural and functional characterization of pronyl-lysine, a novel protein modification in bread crust melanoidins showing *in vitro* antioxidative and phase I/II enzyme modulatin activity. *J. Agric. Food Chem.*, 50, 6997-7006.

Lusby, P.E., Coombes, A., & Wilkinson, J.M. (2005). Bactericidal activity of different honeys against pathogenic bacteria. *Arch. Med. Res.*, 36, 464-467.

Lynn, E.G., Englis, D.T., & Milum, V.G. (1936). Effect of processing and storage on composition and color of honey. *J. Food Sci.,* 1, 255-261.

Manzocco, L., Calligaris, S., Mastrocola, D., Nicoli, M.C., & Lerici, C.R. (2001). Review of non-enzymatic browning and antioxidant capacity in processed foods. *Trends Food Sci. Tech.,* 11, 340-346.

Marceau, E., & Yaylayan, V.A. (2009). Profiling of α-dicarbonyl content of commercial honeys from different botanical origins: Identification of 3,4-dideoxyglucoson-3-ene (3,4-DGE) and related compounds. *J. Agric. Food Chem.,* 57, 10837-10844.

Marshall, T., & Williams, K.M. (1987). Electrophoresis of honey: Characterization of trace proteins from a complex biological matrix by silver staining. *Anal. Biochem.,* 167, 301-303.

Martins, S.I.F.S., Jongen, W.M.F., & van Boekel, M.A.J.S. (2000). A review of Maillard reaction in food and implications to kinetic modelling. *Trends Food Sci. Technol.,* 11, 364-373.

Martins, S.I.F.S, Martinus A.J.S. van Boekel . (2003). Melanoidins extinction coefficient in the glucose/glycine Maillard reaction. *Food Chemistry,* 83, 135-142.

Mavric, E., Wittmann, S., Barth, G., & Henle, T. (2008). Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. *Mol. Nutr. Food Res.,* 52, 483-489.

Mohammed, S.E.A.R., & Babiker, E.E. (2009). Protein structure, physicochemical properties and mineral composition of *Apis Mellifera* honey samples of different floral origin. *Aust. J. Basic Appl. Sci.,* 3, 2477-2483.

Molan, P. (1992). The antibacterial activity of honey. 2. Variation in the potency of the antibacterial activity. *Bee World,* 73, 59-76.

Molan, P. (1999). The role of honey in the management of wounds. *J. Wound Care,* 8, 415-418.

Montavan, P., Mauron, A-F., & Duruz, E. (2003). Changes in green coffee protein profiles during roasting. *J. Agric. Food Chem.,* 51, 2335-2343.

Monti, S.M., Ritieni, A., Graziani, G., Randazzo, G., Mannina, L., Segre, A.L., Fogliano, V., (1999). LC/MS analysis and antioxidative efficiency of Maillard reaction products from a lactose-lysine model system. J. *Agric. Food Chem.*, 47, 1506–1513.

Morales, F.J. (2002). Application of capillary zone electrophoresis to the study of food and food-model melanoidins. *Food Chem.,* 76, 363-369.

Morales, F.J., & Babbel, M-B. (2002). Melanoidins exert a weak antiradical activity in watery fluids. *J. Agric. Food Chem.,* 50, 4657-4661.

Mundo, M.A., Padilla-Zakour, O.I., & Worobo, R.W. (2004). Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. *Int. J. Food Microbiol.*, 97, 1-8.

Natarajan, S., Williamson, D., Grey, J., Harding, K.G., & Cooper, R.A. (2001). Healing of an MRSA-colonized, hydroxyurea-induced leg ulcer with honey. *J. Dermatol. Treat.,* 12, 33-36.

Nunes, F.M., & Coimbra, M.A. (2007). Melanoidins from coffee infusions. Fractionation, chemical characterization, and effect of the degree of roast. *J. Agric. Food Chem.,*

55, 3967-3977.

Nunes, F.M., & Coimbra, M.A. (2010). Role of hydroxycinnamates in coffee melanoidin formation. *Phytochem. Rev.,* 9, 171-185.

O'Brien, J. & Morrissey, P.A. (1989). Nutritional and toxicological aspects of the Maillard browning reaction in foods. *Crit. Rev. Food Sci. Nutr.,* 28, 211-248.

Oszmianski, J., & Lee, C.Y. (1990). Inhibition of polyphenol oxidase activity and browning by honey. *J. Agric. Food Chem.,* 38, 1892-1895.

Pérez, R.A., Iglesias, M.T., Pueyo, E., González, M., & de Lorenzo, C. (2007). Amino acid composition and antioxidant capacity of Spanish honeys. *J. Agric. Food Chem.,* 55, 360-365.

Rice-Evans, C.A., Miller, N.J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Bio. Med.,* 20, 933-956.

Rufián-Henares, J.A., & de la Cueva, S.P. (2009). Antimicrobial activity of coffee melanoidins-A study of their metal-chelating properties. *J. Agric. Food Chem.,* 57, 432-438.

Rufián-Henares, J.A., & Morales, F.J. (2007a). Angiotensin-I converting enzyme inhibitory activity of coffee melanoidins. *J. Agric. Food Chem.,* 55, 1480-1485.

Rufián-Henares, J.A., & Morales, F.J. (2007b). Antimicrobial activity of melanoidins. *J. Food Quality,* 30, 160-168.

Sahin, H., Topuz, A., Pischetsrieder, M., & Ozdemir, F. (2009). Effect of roasting process on phenolic, antioxidant and browning properties of carob powder. *Eur. Food Res. Technol.*, 230, 155-161.

Salah, N., Miller, N.J., Paganga, G., Tijburg, L., Bolwell, G.P., & Rice-Evans, C. (1995). Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys.*, 322, 339–346.

Sancho, M.T., Muniategui, S., Huidobro, J.F., & Lozano, J.S. (1992). Aging of honey. *J. Agric. Food Chem.*, 40, 134-138.

Saxena, S., Gautam, S., & Sharma, A. (2010). Physical, biochemical and antioxidant properties of some Indian honeys. *Food Chem.*, 118, 391-397.

Silici, S., Sagdic, O., & Ekici, L. (2010). Total phenolic content, antiradical, antioxidant and antimicrobial activities of *Rhododendron* honeys. *Food Chem.*, 121, 238-243.

Silva, L.R., Videira, R., Monteiro, A.P., Valentão, P., & Andrade, P.B. (2009). Honey from Luso region (Portugal): Physicochemical characteristics and mineral contents. *Microchem. J.*, 93, 73-77.

Singh, R., Barden, A., Mori, T., & Beilin, L. (2001). Advanced glycation end-products: a review. *Diabetologia*, 44, 129-146.

Singleton, V.L., Orthofer, R., and Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteau reagent. *Methods Enzymol.*, 299, 152-178.

Smaniotto, A., Bertazzo, A., Comai, S., & Traldi, P. (2009). The role of peptides and proteins in melanoidin formation. *J. Mass. Spectrom.*, 44, 410-418.

Subrahmanyam, M. (1996). Honey dressing versus boiled potato peel in the treatment of burns: A prospective randomised study. *Burns,* 22, 491-493.

Subrahmanyam, M. (1998). A prospective randomised clinical and histological study of superficial burn wound healing with honey and silver sulfadiazine. *Burns,* 24, 157-161.

Subrahmanyam, M. (1999). Early tangential excision and skin grafting of moderate burns is superior to honey dressing: A prospective randomized trial. *Burns*, 25, 729-731.

Swellam, T., Miyanaga, N., Onozawa, M., Hattori, K., Kawai, K., Shimazui, T., & Akaza, H. (2003). Antineoplastic activity of honey in an experimental bladder cancer implantation model: *In vivo* and *in vitro* studies. *Int, J. Urol.,* 10, 213-219.

Taormina, P.J., Niemira, B.A., & Beuchat, L.R. (2001). Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power. *Int. J. Food Microbiol.*, 69, 217-225.

Tonks, A.J., Cooper, R.A., Jones, K.P., Blair, S., Parton, J., & Tonks, A. (2003). Honey stimulates inflammatory cytokine production from monocytes. *Cytokine,* 21, 242-247.

Tosi, E., Ciappini, M., Ré, E., & Lucero, H. (2002). Honey thermal treatment effects on hydroxymethylfurfural content. *Food Chem.,* 77, 71-74.

Tressl, R., Wondrak, G.T., Garbe, L-A, Krüger, R-P, & Rewicki, D. (1998). Pentoses and hexoses as sources of new melanoidin-like Maillard polymers. *J. Agric. Food Chem.,* 46, 1765-1776.

Turkmen, N., Sari, F., Poyrazoglu, E.S., & Velioglu, Y.S. (2006). Effects of prolonged heating on antioxidant activity and colour of honey. *Food Chem.,* 95, 653-657.

U.S. Food and Drug Administration, Center for Drug Evaluation and Research. 510(k) Summary for Derma Sciences Medihoney Dressings with Active Manuka Honey, April 23, 2008. Retrieved October 19, 2010, from http://www.accessdata.fda.gov /cdrh_docs/pdf8/K080315.pdf

Van Boekel M.A.J.S. (2006). Formation of flavour compounds in the Maillard reaction. *Biotechnol. Adv.,* 24, 230-233.

Vela, L., de Lorenzo, C., & Pérez, R.A. (2007). Antioxidant capacity of Spanish honeys and its correlation with polyphenol content and other physicochemical properties. *J. Sci. Food Agric.,* 87, 1069-1075.

Verardo, G., Duse, I., & Callea, A. (2009). Analysis of underivatized oligosaccharides by liquid chromatography/electrospray ionization tandem mass spectrometry with post-column addition of formic acid. *Rapid Commun. Mass Spectrom.,* 23, 1607-1618.

Vinson, J.A., Mandarano, M., Hirst, M., Trevithick, J.R., & Bose, P. (2003). Phenol antioxidant quantity and quality in foods: Beers and the effect of two types of beer on an animal model of atherosclerosis. *J. Agric. Food Chem.,* 51, 5528-5533.

Weigel, K.U., Opitz, T., & Henle, T. (2004). Studies on the occurrence and formation of 1,2-dicarbonyls in honey. *Eur. Food Res. Technol.,* 218, 147-151.

Xu, Q., Tao, W., & Ao, Z. (2007). Antioxidant activity of vinegar melanoidins. *Food Chem.,* 102, 841-849.

Appendix A: Standard curves

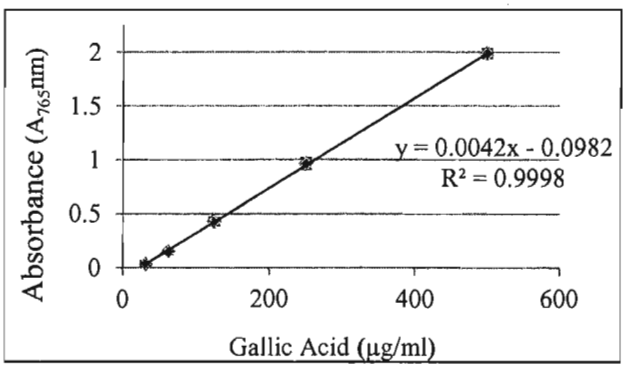


Figure A-1: Gallic acid standard curve.
Values represent the means ± SD of six independent determinations.

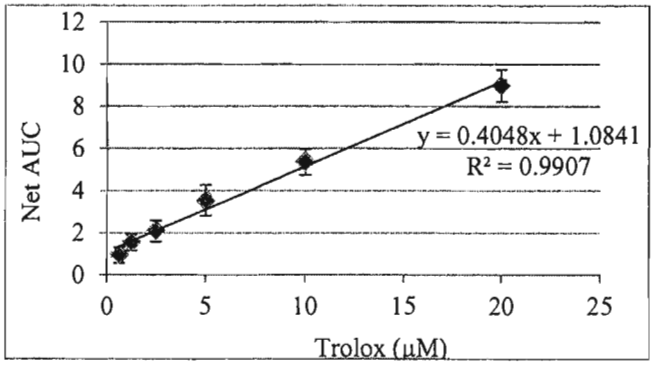


Figure A-2: Trolox standard curve.
Values represent the means ± SD of 7 independent determinations.

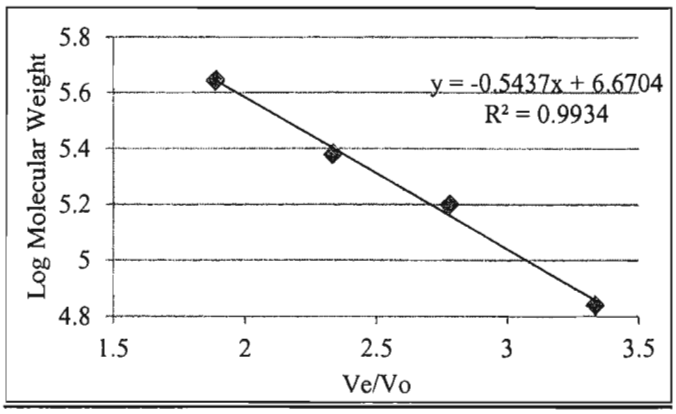


Figure A-3: Standard curve for the determination of molecular size.
Molecular weight markers eluted in the same fraction for all trials (n=3).

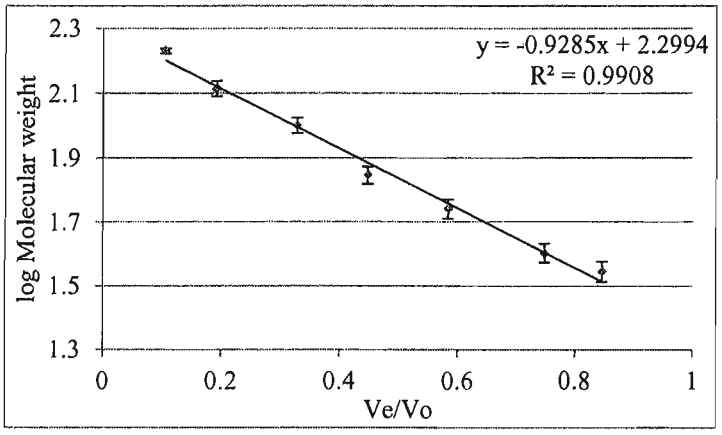


Figure A-4: Protein molecular weight standard curve. Protein standards were run on 10% acrylamide gel.

Data represents the mean ± SD of three independent determinations.