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Identifying Iron-Binding Antioxidants in New Zealand Honeydew Honey



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A thesis submitted in fulfilment
of the requirements for the degree
of
Doctor of Philosophy in Pharmaceutical Analysis
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by

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"And your Lord (Allah) taught the honey bee to build its cells in hills, on trees, and in (men's) habitations; Then to eat of all the produce (of the earth), and find with skill the spacious paths of its Lord: there issues from within their bodies a drink of varying colours, wherein is healing for men: verily in this is a sign for those who give thought. (Surat an-Nahl (The Bee), 68-69)

In addition, the Prophet Muhammad said:

'Honey is a remedy for every illness and the Qur'an is a remedy for all illness of the mind, therefore I recommend to you both remedies, the Qur'an and honey.'
(Hadith Bukhari)

ABSTRACT

Iron is an essential element for human physiological function. However, excess free iron could participate in the formation of reactive oxygen species via the Fenton reaction and is thought to be the primary mechanism for the pathogenesis of diseases related to iron excess. Prevention of the oxidative damage caused by reactive oxygen species can be accomplished by using selective iron-binding compounds. Beech honeydew honey has been found to have an exceptionally high level of iron-binding antioxidant activity. Due to that, the focus of the research in this thesis has been to isolate the compound(s) responsible for the activity and identify their molecular structure.

The stability of the antioxidants on exposure to heat and high and low pH was investigated to get a better understanding of the conditions which could be used when developing suitable techniques for the isolation of the iron-binding antioxidants.

The isolation of the antioxidants was first attempted with XAD-2 resin. The results of solid phase extraction using XAD-2 resin showed that the activity is mainly found in the honey effluent, the fraction not retarded by the resin, indicating that hydrophilic components contributed to the iron-binding antioxidant activity. The phenolic compounds isolated from this resin did not exhibit iron-binding antioxidant activity.

When the antioxidants could not be isolated from the XAD-2 resin, size exclusion chromatography was used to separate the honey components based on their molecular sizes. The technique was successful in separating antioxidants from free sugars in honey. However, size exclusion chromatography did not give any significant information regarding the identity of the antioxidants. Thus, two types of chromatography (reversed phase and anion exchange) were used in sequence to aid the separation of components of the active fraction from size exclusion chromatography. This procedure however was not compatible with subsequent mass spectrometry since a high level of sodium chloride existed in the final

fraction, even after desalting using reversed phase column chromatography. Due to that, other means of chromatography were trialled in order to obtain a salt-free fraction prior to mass spectrometry. The first procedure was modified by switching the sequence of the columns used, and different mobile phases without salt were used. This second procedure was successful in isolating the antioxidants and the compounds have been characterised by mass spectrometry.

Mass spectrometry revealed that the antioxidants isolated were compounds of low molecular weight (*i.e.* 255, 283 and 227) which exhibited peaks with high intensity. Nuclear magnetic resonance (NMR) spectroscopy of the active fraction showed that the antioxidant may possess a 1,4-disubstituted aromatic ring. However, full elucidation of the structure of the compound was not possible because of the very low mass of the material that had been isolated.

Other than the information gained from mass spectrometry and NMR, the antioxidant also could be characterised by forming the negative ion at pH 9.5–10. The UV absorbance at 220 and 280 nm, but not at 360 nm is indicative that the antioxidant possess a phenolic ring but not a flavonoid in the molecular structure.

Complete structural elucidation of the iron-binding antioxidants in honeydew honey could initiate a better understanding of the mechanism of action of the antioxidants. This could increase the use of honeydew honey in pharmaceutical and nutraceutical products as an iron-binding antioxidant. There is also the possibility that the identified compound(s) could be synthesised for pharmaceutical use, or serve as lead compounds for the development of other pharmaceutical iron-binding antioxidants.

ACKNOWLEDGEMENTS

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My utmost gratitude to the Biology and Chemistry technicians; Kerry Allen, Colin Monk, Judith Patrow, Lyne Parker, Wendy Jackson and Pat Gregard who have generously helped in every single way for me to complete the research. Not forgetting Cheryl Ward, the librarian, who has guided me on my thesis formatting.

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Finally, heaps of thanks to my husband and precious daughter, Wan and Sarah who have been supportive and cooperative in making this research a success. This PhD journey has taught me that they are more important to me than anything else in the world. Both of you are the jewels in my heart.

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LIST OF ABBREVIATIONS

Abs	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BHT	Butylated hydroxytoluene
C	Celsius
D ₂ O	Deuterim oxide
DMSO- _{d6}	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EGCG	(-)-Epigallocatechin-3-gallate
ESI	Electrospray ionisation
Fe ²⁺	Ferrous ion
Fe ³⁺	Ferric ion
FPLC	Fast protein liquid chromatography
FRAP	Ferric reducing antioxidant power
HCL	Hydrochloric acid
H ₂ O ₂	Hydrogen peroxide
mAU	Milli absorbance
MRPs	Maillard Reaction Products
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
O ₂ ^{•-}	Superoxide radical
•OH	Hydroxyl radical
ORAC	Oxygen radical absorbance capacity
PG	Propyl gallate
PSDVB	Polystyrene divinylbenzene
ROS	Reactive oxygen species
SEC	Size exclusion chromatography
SPE	Solid phase extraction
TBHQ	tert-butylhydroquinine
UV/Vis	Ultraviolet/visible

CHAPTER 1

INTRODUCTION

In recent years, studies have revealed that the progression of certain degenerative diseases, including certain cancers, is related to the presence of free ferrous (Fe^{2+}) ions in the biological system. These free Fe^{2+} ions catalyse the formation of $\cdot\text{OH}$ radicals, the most reactive free radical, through the Fenton reaction which attacks lipid, proteins and DNA of the cells.

The damage to cells by $\cdot\text{OH}$ radicals can be prevented by antioxidants. Antioxidants which sequestered iron and stop it giving rise to $\cdot\text{OH}$ radicals give better protection than antioxidants which just scavenge free radicals after they have been formed. Studies on the antioxidant activity of honey from various types and origins have attracted much attention. Beech honeydew honey has been found to have a high level of iron-binding antioxidant activity, thus it has the potential to stop the formation of $\cdot\text{OH}$ radicals catalysed by free Fe^{2+} ions. This thesis describes a study conducted to find the nature of the component(s) of honeydew honey responsible for this activity.

1.1 Research background

1.1.1 Reactive oxygen species

A free radical is a molecule with an unpaired electron in an outer orbital (Halliwell & Gutteridge, 1999b). Its inherent tendency to attain molecular stability by acquiring or donating an electron from or to another molecule renders it highly reactive (Levine & Kidd, 1985) compared with a non-radical. This characteristic has made free radicals capable of initiating a chain reaction (Halliwell, 1989).

The most important reactive oxygen species in biological system are superoxide anion radicals ($\text{O}_2^{\cdot-}$) and hydroxyl radicals ($\cdot\text{OH}$), whereas hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$) are known as non-radical reactive oxygen species.

These free radical are the products of the reduction of molecular oxygen which be initiated by heat, UV radiation, oxygen or transition metals.

1.1.2 *The hazardous effect of iron*

Chemically, $\cdot\text{OH}$ can be generated by reaction between free Fe^{2+} and H_2O_2 , which is called the Fenton reaction. In general, iron is an essential metal that is needed by living organisms for biological functions such as enzyme activity (Machlin & Bendich, 1987) and the electron transport system (Pietrangelo, 2002). In humans and other animals, iron is responsible for the transport of oxygen in the form of haemoglobin, myoglobin and haemerythrin (also spelled as hemerythrin). These various biological functions however bring some drawbacks. Iron that exists in a free form has been reported as a cause of certain diseases (Weinberg, 1999).

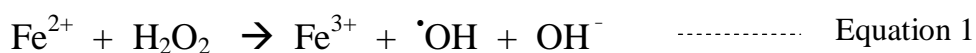
Chemically, iron belongs to the transition metal group. The transition metals are referred to as the *d*-block because the electrons partly fill the *d*-orbitals. The availability of *d*-orbitals to accept or donate electrons gives transition metals the ability to vary oxidation states by getting oxidised and reduced easily. Iron can have oxidation states from -2 to +6 (Spiro & Saltman, 1974). However, only the two principal oxidation states, the +2 (ferrous) and +3 (ferric) states are found commonly in biological environments. This is because the (+2) and (+3) oxidation states of iron are stable in aqueous solution (Zepp *et al.*, 1992).

Iron has a high affinity for a dioxygen (O_2) molecule (Liu & Hider, 2002) or its derivatives (Aisen, 2007). In biological environments, O_2 derivatives exist in various forms such as hydrogen peroxide (H_2O_2) and superoxide ($\text{O}_2^{\cdot-}$). $\text{O}_2^{\cdot-}$ is produced in mitochondria (Lenaz *et al.*, 2002) and is known as a poorly reactive oxygen radical (Fridovich, 1995). On the other hand, H_2O_2 is a waste product of metabolism, poorly reactive, and has an ability to cross the cell membranes (Halliwell *et al.*, 2000). Iron, that has the ability to be involved in reduction and oxidation (redox) reactions, could convert these O_2 derivatives to $\cdot\text{OH}$.

The hydroxyl radical, $\cdot\text{OH}$, has been implicated as the most reactive ROS. The rapidity of action and the non-specific reactivity of the $\cdot\text{OH}$ makes it particularly dangerous. The reactivity of $\cdot\text{OH}$ radicals is so great that, if formed in living

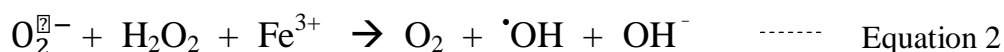
systems, they will react immediately with whatever biomolecule is in their vicinity, producing secondary radicals of variable reactivity (Halliwell & Gutteridge, 1992). Their ability to abstract a hydrogen atom from membrane lipids (Agil *et al.*, 1995) will cause cell injury or death. Therefore, $\cdot\text{OH}$ is believed to be an etiological agent for several diseases such as Alzheimer's disease (Bartzokis *et al.*, 2004), Parkinson's disease (Whitnall & Richardson, 2006), atherosclerosis (de Valk & Marx, 1999), cardiovascular disease (Cai & Harrison, 2000) and cancer (Halliwell, 2007). It may also be involved in natural ageing (Altamura & Muckenthaler, 2009).

The formation of $\cdot\text{OH}$ is only governed by the existence of free iron in the cellular environment in the presence of H_2O_2 , in the Fenton reaction (Equation 1). It is interesting to note that H_2O_2 itself does not induce lipid peroxidation. In the Fenton reaction, ferrous ion (Fe^{2+}) acts as a catalyst.



In order for the Fenton reaction to occur *in vivo*, the iron must be in a sufficiently reduced oxidation state, as Fe^{2+} ion. Biological reducing agents such as ascorbate, glutathione and nicotinamide adenine dinucleotide (NADH) can enhance the reaction by generating or re-generating reduced states of iron (*i.e.* Fe^{2+} ion), making it available for the Fenton reaction (Andrade Jr *et al.*, 2005; Svingen *et al.*, 1979). The re-generation of the reduced state of iron ensure that even at trace levels such as those *in vivo*, the supply of iron is not exhausted.

Another source of $\cdot\text{OH}$ formation is through the Haber-Weiss reaction (Equation 2):



In the Haber-Weiss reaction, the ferric ions (Fe^{3+}) (*i.e.* the oxidised form of iron formed in the Fenton reaction) are reduced back to Fe^{2+} ions by $\text{O}_2^{\cdot-}$ and then react with H_2O_2 to produce $\cdot\text{OH}$ (the Fenton reaction). This cycling of iron from one valence state to the other is what causes the repetitive formation of $\cdot\text{OH}$ (Figure 1.1).

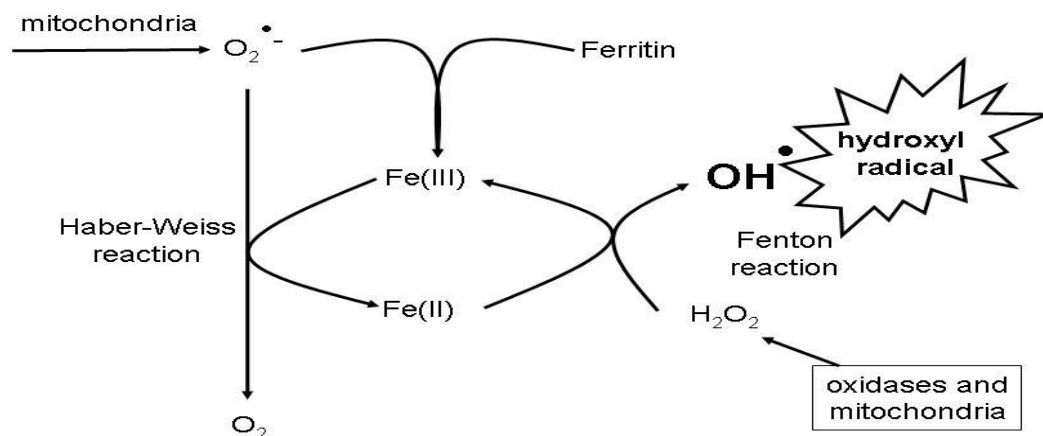


Figure 1.1: Catalysis and autocatalysis in the Haber-Weiss and Fenton reactions leading to the production of the $\cdot\text{OH}$ radical, including the liberation by $\text{O}_2^{\cdot-}$ of free iron from ferritin. The figure was adapted from Kell, (2009).

1.1.3 Lipid peroxidation mechanism

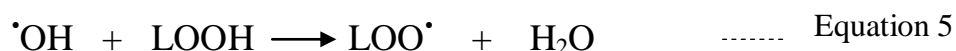
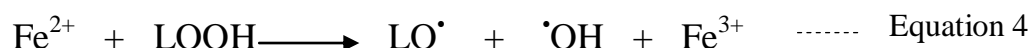
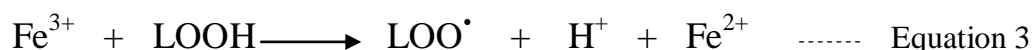
Membrane lipid is a vital part of every cell and cell organelles. It mostly consists of polyunsaturated fatty acids (PUFA). PUFA have been reported to be more susceptible than saturated fatty acids are to oxidation due to the fact that they have multiple double bonds which give higher electron densities and therefore are a prime target for free radical reaction (Schafer *et al.*, 2000).

Free radical-mediated lipid peroxidation proceeds by a chain mechanism, that is, one initiating free radical can oxidize many molecules of lipids (Niki, 2009). The common chain reactions of lipid oxidation regardless of the cause species are comprised of three phases: initiation, propagation and termination, as shown below.

Initiation of lipid peroxidation occurs when a hydrogen atom at a bis-allylic methylene group in double bonds of polyunsaturated fatty acids is abstracted by a free radical or oxidising agent to form an alkyl radical (Welch *et al.*, 2002b).

The reaction occurs at the surface of the membrane lipid (Gutteridge, 1995), in contact with Fe^{2+} in the aqueous phase. The $\cdot\text{OH}$ radical then abstracts the hydrogen atom of the lipid, and combines with it to form water, leaving the lipid radical behind, hence the propagation stage of the lipid peroxidation starts as depicted in Section 1.1.3.

The relatively stable lipid hydroperoxide which is formed in the propagation stage in the normal lipid peroxidation can react with iron (Equations 3 and 4) and $\cdot\text{OH}$ (Equation 5) (Marnett, 1999). Therefore, the stability of the lipid hydroperoxide is much less in the presence of iron, where it can be reduced by Fe^{2+} ions to alkoxy ($\text{LO}\cdot$) or $\cdot\text{OH}$ (Marnett, 1999; Winston *et al.*, 1983), or oxidised to peroxy ($\text{LOO}\cdot$) by Fe^{3+} . These free radicals formed can further react with other lipid or biomolecules (*e.g.* DNA and proteins). These lipid radicals formed will continue the process of the chain reaction (propagation) and termination stages as illustrated above.



Although the intermediate free radicals will be neutralised from the cell system in the termination stage (c) as described in Section 1.1.3, the damage caused to membrane lipid in the propagation stage results in abnormality of the shape of membrane proteins (Dean *et al.*, 1997) and the breakage of DNA strands (Chevion, 1988). This is because the oxidation of the membrane lipid will cause the free radicals that are formed in the propagation stage (*e.g.* $\cdot\text{OH}$, $\text{LOO}\cdot$, $\text{LO}\cdot$ and $\text{L}\cdot$) to enter the cell, and thus attack the intracellular components (*e.g.* DNA and proteins). For instance, if the $\cdot\text{OH}$ radical attacks DNA, the chain reactions propagate through DNA and cause chemical alteration of the bases (Cadet *et al.*,

2010) which leads to mutation, blockage of DNA replication and causes strand breakage (Halliwell, 1989). Figure 1.2 illustrates the fate of the cellular components in the presence of the $\cdot\text{OH}$ radical in the cell. A similar process occurs with proteins as well, where the $\cdot\text{OH}$ radical attacks the amino acids (the building block of proteins), hence increases the amount of oxidised protein (Floyd & Carney, 1992).

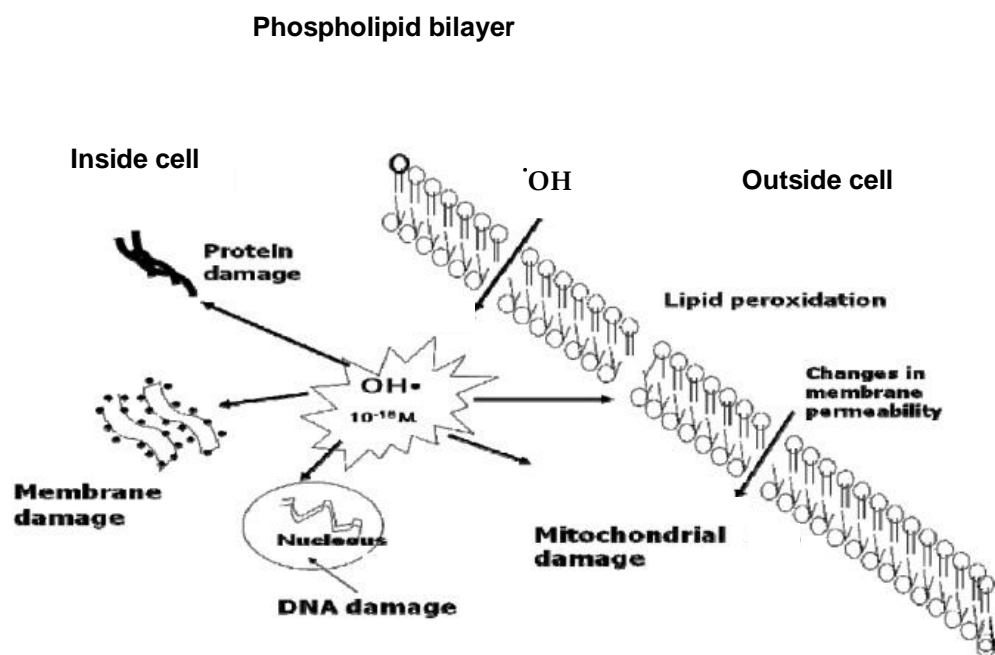


Figure 1.2: The fate of the cell components in the presence of $\cdot\text{OH}$ radical. The figure was adapted from Repetto *et al.* (2012).

Another alternative way for the DNA and protein to get damaged is through their reaction with other lipid peroxidation products, such as aldehydes (*e.g.* malonaldehyde and hydroxynonenal), isoprostanes, and many more (Burcham, 1998; Montine *et al.*, 2004; Traverso *et al.*, 2004). These compounds are known to be toxic to the cells and are active promoters of the disease process (Burcham *et al.*, 2002).

1.1.4 Iron regulation and factors responsible for the dysregulation of iron in humans

Due to pathophysiological problems when iron ions exist in free form, the human body has developed a homeostasis mechanism to keep a balance of iron. Iron must be well stored and transported. The iron-binding proteins such as transferrin (the iron transport protein) and ferritin (the iron storage protein) are important for preventing the iron from undergoing an uncontrolled redox reaction. For the endogenous prevention of formation of free radicals, the body has enzymes such as catalase to destroy H_2O_2 and superoxide dismutase to neutralise the superoxide ($\text{O}_2^{\cdot-}$) radical.

Even though the body has developed the homeostasis system to regulate the utilisation of iron, there are several conditions when the iron status can change which leads to free radical formation thus causing more damage to the cells:

- (i) the depletion of activity of catalase which converts H_2O_2 to water. Catalase can be inhibited by ($\text{O}_2^{\cdot-}$) formed through the xanthine oxidase reaction (Kono & Fridovich, 1982). Xanthine oxidase is an enzyme which catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming $\text{O}_2^{\cdot-}$ in the first stage and H_2O_2 in the second. This condition would increase the concentration of $\text{O}_2^{\cdot-}$ and H_2O_2 in the cell. As mentioned earlier, H_2O_2 could penetrate cell membranes, thus making it more readily accessible in the cells, therefore it can release iron by degrading the haem proteins (Gutteridge, 1986; Halliwell & Gutteridge, 1999b).
- (ii) the release of iron from ferritin and transferrin by $\text{O}_2^{\cdot-}$ (Biemond *et al.*, 1984; Thomas *et al.*, 1985). It has been reported that NADH (a reducing agent) also might facilitate the release of iron from ferritin (Stohs & Bagchi, 1995).
- (iii) the extrinsic factors that can contribute to the generation of free radicals in humans due to lipid peroxidation such as polluted

environment and lifestyles. A polluted environment contains several such factors such as carbon dioxide emissions, industrial pollutants, cigarette smoke (passive smoker) and UV radiation exposure (Dellinger *et al.*, 2001). Lifestyles such as stress, trauma, excessive alcohol consumption, high intake of red meat, deep-fried foods, processed foods with fortified iron and excessive exercising also could add to this effect (Powers *et al.*, 2004).

- (iv) hereditary diseases such as haemochromatosis (iron is not bound to transferrin in the serum) and thalassemia (the condition associated with the abnormality of haemoglobin which needs regular blood transfusions) have been reported to cause accumulation of free iron in the body.

With respect to the problems caused by free iron as explained above, the major consideration to solve the problem of Fe^{2+} -mediated lipid peroxidation is the importance of binding agents that can bind the free Fe^{2+} ions in a way that makes them not catalytic. To prevent the formation of $\cdot\text{OH}$ radicals through the Fenton reaction in the initiation stage of lipid peroxidation, the Fe^{2+} ions which play the major role as a catalyst must be bound up. However, it must be stressed that in the complex, the iron must be unreactive, and the complexing agent must not promote any pro-oxidant effect such as reducing ability and/or autoxidation since the hazardous effect of iron can be seen physiologically when iron is not properly chelated and reacts with naturally occurring free radicals like O_2^{\ominus} in cells (Kell, 2009).

1.1.5 The importance of antioxidants

An antioxidant may be defined as ‘any substance that when present at low concentrations, compared with those of the oxidisable substrate, significantly delays or inhibits oxidation of that substrate’ (Halliwell & Gutteridge, 1999a). Antioxidants have an important role in scavenging free radicals that are being produced within the living cells, and/or preventing the formation of these free radicals.

All antioxidants can be generally classified in term of their mechanism of action as primary (type 1 or chain breaking) antioxidants or secondary (type 2 or preventive) antioxidants. Chain-breaking antioxidants either endogenous or exogenous act by scavenging free radicals and commonly are divided into enzymatic and non-enzymatic antioxidants. Enzymes such as superoxide dismutase, catalase and glutathione peroxidase commonly work closely together (Drevet, 2006). Superoxide dismutase works by catalysing the dismutation of the superoxide anion ($O_2^{\ominus-}$) to hydrogen peroxide (H_2O_2). Catalase converts the hydrogen peroxide to water, while glutathione peroxidase reduces lipid hydroperoxides to their corresponding alcohols.

The typical non-enzymatic chain-breaking antioxidants which are derived from the diet are vitamin E, vitamin C, β -carotene and phenolic compounds. They mainly provide hydrogen atom(s) or electron(s) that can be easily donated to the chain-propagating peroxy (LOO^\bullet), alkoxy (LO^\bullet), and hydroxyl radicals (de Beer *et al.*, 2002; Leopoldini *et al.*, 2004). The radicals formed in the antioxidant by this donation are stable and do not abstract more hydrogen atoms from PUFA (Figure 1.3).

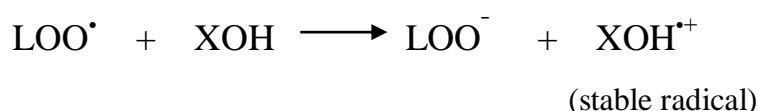


Figure 1.3: Reaction mechanism of phenolic compound with lipid peroxy radical by hydrogen transfer. XOH =antioxidant; LOO^\bullet =lipid peroxy radical; $LOOH$ =lipid hydroperoxide.

On the other hand, preventive antioxidants are generally the compounds that bind to transition metal ions, particularly iron and copper, to prevent the initiation of lipid peroxidation chain reactions (de Beer *et al.*, 2002). The common endogenous iron-binding antioxidants are proteins such ferritin, transferrin and exogenous iron-binding compounds such as some naturally occurring phenolic compounds (*e.g.* quercetin, catechin, rutin) and synthetic metal chelators such as ferrioxamine and desferrioxamine that play an important role in controlling the free metal ions in the cells' environment. Figure 1.4 shows the structure of the iron-ferrioxamine complex.

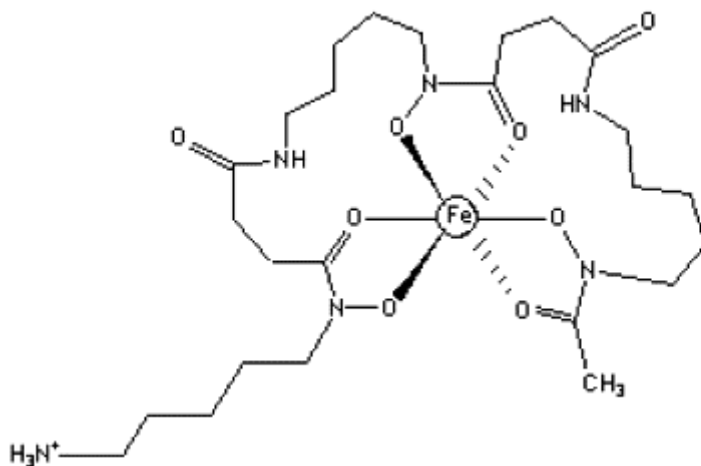


Figure 1.4: Ferrioxamine-Fe³⁺ complex structure. The figure was adapted from Cozar *et al.* (2006).

1.2 Significance of the present study

As briefly explained above, because the $\cdot\text{OH}$ radical is so dangerous once it formed, it is of importance to protect the cells against $\cdot\text{OH}$. Chain-breaking antioxidants do not offer such protection due to the fact that $\cdot\text{OH}$ radicals are short lived and might not be scavenged efficiently by chain breaking antioxidants before they cause damage (Sies, 1997). Therefore, one of the strategies is to inhibit $\cdot\text{OH}$ formation in the initiation stage of lipid peroxidation and other damaging reactions by means of sequestering transition metals ions. Sies (1997) has noted that metal chelation is a major means of controlling lipid peroxidation and DNA fragmentation.

Many studies in cell culture experiments, animal models and human clinical trials have demonstrated that the oxidative stress level or diseases caused by iron are reduced when treated with iron-binding antioxidants (Afanas'ev *et al.*, 1995; Fibach & Rachmilewitz, 2010; Kalpravidh *et al.*, 2010; Zacharski *et al.*, 2008; Zhang *et al.*, 2010). For instance, baicalin and quercetin significantly reduced the iron content in the liver and kidney of iron-overloaded mice, indicating that these flavonoids act by binding to iron, thus preventing further injury to these organs due to iron excess (Zhang *et al.*, 2011).

The potential role of iron chelation in cancer therapy has been reviewed by Buss *et al.* (2003), and only recently, Heli *et al.* (2011) reviewed the importance of iron

chelators in patients suffering from the diseases involving iron-induced free radicals.

Recently, research by Branguolo & Molan (2010) has discovered that New Zealand Beech honeydew honey has an exceptionally high level of iron-binding antioxidant activity. However, the study was not of sufficient depth to explain which compounds are involved in the high activity observed in an iron-binding assay. The study was only limited to the crude honey and neither fractionation nor purification has been attempted. Therefore, the study needed to be extended into more detail by proper analytical techniques that could isolate the iron-binding antioxidants. The present study was undertaken to explain why honeydew honey has such high iron-binding activity and to identify the compounds involved in the binding mechanism. A better understanding of the mechanism by which honeydew honey antioxidant compounds inhibit iron-induced lipid peroxidation could increase the nutraceutical use of honeydew honey and provide lead compounds for pharmaceutical development.

1.3 Research questions

- i) What are the types of compounds in honeydew honey that are involved in its iron-binding antioxidant activity?
- ii) How do the antioxidant(s) contribute to the iron-binding antioxidant activity? What functional groups are involved?
- iii) What is the molecular size of the iron-binding antioxidant(s) present in honeydew honey?

1.4 Objectives of the research

- i) To determine the stability of the iron-binding antioxidants to heat and pH in honeydew honey
- ii) To isolate the iron-binding antioxidant(s) in honeydew honey using activity-led fractionation with various chromatographic techniques.
- iii) To characterise the isolated iron-binding antioxidant(s) by spectroscopic techniques (mass spectrometry and nuclear magnetic resonance spectroscopy).

CHAPTER 2

REVIEW OF LITERATURE

2.1 Honey

Honey is described by the Codex Alimentarius as the natural sweet substance produced by honeybees from the nectar of plants or from secretions of living parts or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in honeycombs to ripen and mature (Codex Alimentarius Commission, 1996).

The main difference between nectar honey and honeydew honey is the source of the raw material collected by the bees for the production of honey. For nectar honey, the main source is from flower nectar, whereas honeydew honey is made when bees collect honeydew droplets secreted by phloem sap-sucking insects of the order Hemiptera. Insects in the order Hemiptera comprise 4,000 species such as aphids and cicadas. In New Zealand, honeydew honey is produced by a scale insect (*Ultracoelostoma assimile*) that lives in the tree bark of *Nothofagus sp.* that grow in the South Island.

2.1.1 Composition of honey

Regardless of the difference in types of honey discussed above, all honeys qualitatively contain similar components such as sugars, proteins, peptides, amino acids, organic acids, some vitamins, minerals, phenolic compounds and Maillard reaction products (MRPs) (Gheldof *et al.*, 2002). The enzymes that are constituents of honey are amylase, invertase, catalase and glucose oxidase (Bogdanov *et al.*, 2008). The variations in composition and property of honeys greatly depends on their raw materials (*i.e.* nectar or honeydew sources) (Lachman *et al.*, 2010; Sanz *et al.*, 2005; Tuberoso *et al.*), the type of honeybee (*i.e.* *Apis*, *Meliponinae* or *Trigona* species) (Truchado *et al.*, 2011; Vit *et al.*, 1998;

Vit *et al.*, 1997) and hemipteran species (Dhami *et al.*, 2011), also on geographical origins and climatic condition of the sources (Al *et al.*, 2009).

Sugars constitute a major percentage of honey total mass and the remainder consists of a small percentage of other minor compounds as illustrated in Figure 2.1.

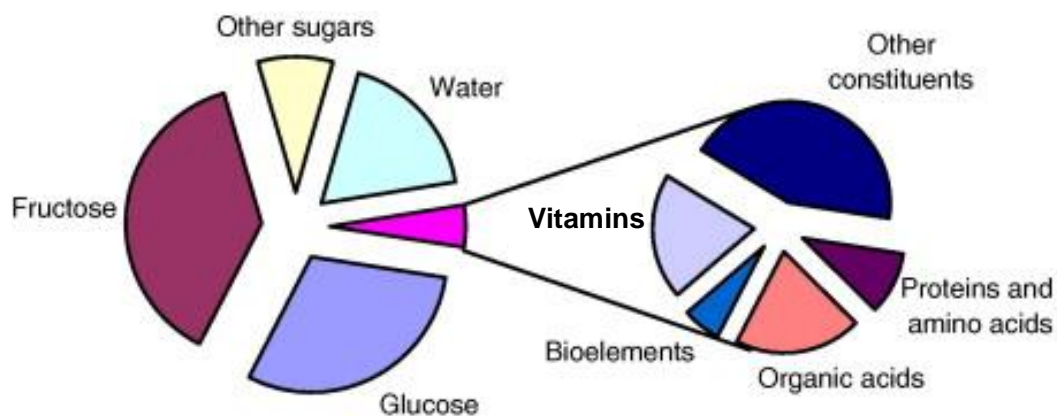


Figure 2.1: Average percentage shares of honey constituents. The figure was adapted from Kujawski & Namiesnik (2008).

The chemical composition of honey is complex and varies widely among honeys. It is well documented for the trace organic compounds such as phenolic compounds, proteins, peptides, amino acids, vitamins and volatile compounds present at different concentrations and/or absent from one type of honey to another (Ciulu *et al.*, 2011; Gil *et al.*, 1995; Tuberoso *et al.*, 2010; Yao *et al.*, 2004a). The concentration of organic compounds also varies even between samples of honey from the same botanical species (Yao *et al.*, 2004b). This characteristic has led to a number of authors using specific compound(s) present at higher level in the corresponding honeys as a potential marker to characterised botanical or geographical origin of the honeys. In addition, different properties of the honeys are expected because the composition of the active compounds in honey from different locations is likely to be different.

Senanayake (2006) has successfully identified the composition of the New Zealand Beech honeydew honey using gas chromatography-mass spectrometry (GC-MS) as shown in Table 2.1. However, it should be borne in mind that the variation in compounds detected is also suggested to depend on the method of

analysis (Kaškonienė & Venskutonis, 2010). For example, Weston *et al.* (2000) who used high performance liquid chromatography for New Zealand Beech honeydew honey did not detect compounds detected by Senanayake (2006). Weston *et al.* (2000) could only detect a few phenolic compounds, namely benzoic acid, *p*-coumaric, *p*-hydroxybenzoic acid, cinnamic acid, pinobanksin, pinocembrin, chrysin and galangin. The differences could be due to the different batch of honeydew honey used or to the method of analysis.

Table 2.1: Organic compounds found in New Zealand Beech honeydew honey as detected by Senanayake (2006) using GC-MS.

phenyllactic acid	hexanedioic acid
methyl syringate	decanoic acid
phenylacetic acid	heptanedioic acid
salicylic acid	octanedioic acid
4-hydroxyphenylacetic acid	dodecanoic acid
indole acetic acid	nonanedioic acid
<i>trans,cis</i> -abscisic acid	decanedioic acid
4-methoxyphenyllactic acid	myristic acid
2-methoxybenzoic acid	palmitic acid
4-methoxybenzoic acid	stearic acid
2-methoxyacetophenone	dehydroabiatic acid
4-hydroxyphenylacetic acid	pimaric acid
nonanoic acid	sandaracopimaric acid
	isopimaric acids

2.1.2 Medicinal use of honey

Honey is considered to be an ancient folk treasure that serves various functions in the lives of mankind. 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 (Henriques *et al.*, 2006; Molan, 1999), has been demonstrated to be the result of its antibacterial activity (Molan, 1992). Honey shows bacteriostatic and bactericidal activities against a broad range of microorganisms, including both Gram-positive and Gram-negative bacteria (Cooper *et al.*, 2002; Molan, 1992; Taormina *et al.*, 2001; Willix *et al.*, 1992) and two antibiotic-resistant bacteria: methicillin-resistant *Staphylococcus aureus* (MRSA) (Maeda *et al.*, 2008; Natarajan *et al.*, 2001) and vancomycin-resistant *enterococci* (VRE) (Williams *et al.*, 2009).

In addition to antibacterial activity, honey has also been reported to exhibit several other biological activities such as antioxidant (Baltrusaityte *et al.*, 2007; Bertoneclj *et al.*, 2007; Blasa *et al.*, 2007; Gheldof & Engeseth, 2002a), antitumor (Fukuda *et al.*, 2011; Jaganathan & Mandal, 2009), antifungal (Brady *et al.*, 2004; Candiracci *et al.*, 2012) and anti-inflammatory activity (Kassim *et al.*, 2010; van den Berg *et al.*, 2008).

2.1.3 Antioxidant property of honey

In food technology, honey has been used to prevent enzymatic browning (Chen *et al.*, 2000; Jeon & Zhao, 2005; Oszmianski & Lee, 1990) caused by polyphenol oxidase (PPO), a copper-containing enzyme which occurs widely in fruits and vegetables. One of the mechanisms of action that has been suggested in preventing the enzymatic browning is the binding of honey antioxidant compounds to copper (Cu^{2+}) ions in the PPO molecule (Gacche *et al.*, 2009; Goetghebeur & Kermasha, 1996). Honey also is used in preventing the oxidation of lipid in meat products (Antony *et al.*, 2000a; Johnston *et al.*, 2005; McKibben & Engeseth, 2001) as well as to keep the meat from being spoilt by microorganisms (Nagai *et al.*, 2006).

For the last few years, honey has been studied extensively for its antioxidant activity. The interest in the beneficial effects of honey antioxidants stem from their ability to protect against the development of disease by scavenging free radicals in the body (Alvarez-Suarez *et al.*, 2012; Blasa *et al.*, 2007; Gheldof & Engeseth, 2002a, 2002b; Gheldof *et al.*, 2003; Schramm *et al.*, 2003). A recent study by Zhou *et al.* (2012) showed that honey protects from damage to DNA caused by hydroxyl radicals. In addition, honey has been reported to have an ability to scavenge superoxide radicals (Hegazi & Abd El-Hady, 2007; Inoue *et al.*, 2005; Nagai *et al.*, 2006), hydroxyl radicals (Nagai *et al.*, 2006) and suppress the oxidation of low density lipoprotein (Hegazi & Abd El-Hady, 2007).

Two common antioxidant functions that have been researched in honey are free radical scavenging and metal binding. The free radical scavenging activity of honey has been studied more than its metal binding property. A vast body of literature has been cited in favour of free radical scavenging activity of different

types of honey. However, very few studies have focused on the effect of honey on metal binding activity. Only recently, a study by Brangoulo & Molan (2010) has opened a new dimension in honey research by showing that honey has an Fe^{2+} -binding property. Other bee products such as propolis also have been reported to have Fe^{2+} -binding activity (Geckil *et al.*, 2005).

2.2 Potential iron-binding antioxidants in honey

According to coordination chemistry, binding to metal requires the presence of two or more atoms on the same molecule capable of metal binding (Flora & Pachauri, 2010). Oxygen, nitrogen, and sulphur atoms of molecules are most commonly the metal ligands. It has been established that the oxygen atom is a good ligand for the ferric ion (Fe^{3+}), whereas nitrogen and sulphur atoms have a high affinity for the ferrous ion (Fe^{2+}) (Welch *et al.*, 2002a; Welch *et al.*, 2002b).

It has been suggested that the compounds in honey that contribute to the antioxidant properties are phenolic compounds (Aljadi & Kamaruddin, 2004; Bertoneclj *et al.*, 2007; Lachman *et al.*, 2010; Piljac-Zegarac *et al.*, 2009; Yao *et al.*, 2011), amino acids (Baltrusaityte *et al.*, 2007; Meda *et al.*, 2005; Perez *et al.*, 2006; Saxena *et al.*, 2009), peptides (Ates *et al.*, 2001; Oszmianski & Lee, 1990) and Maillard reaction products (Brudzynski & Miotto, 2010c; Mathew *et al.*, 1998). The observed strong relationship between these compounds and the radical scavenging activity of honeys has made these compounds the potential antioxidants in honey. However, some authors suggested that the antioxidant activity in honey is the result of the combined activity between this wide range of compounds (Antony *et al.*, 2002; Ferreira *et al.*, 2009; Gheldof & Engeseth, 2002a; Mathew *et al.*, 1998; Saxena *et al.*, 2009), although the chemical identity of compounds responsible for the antioxidant activity still requires further investigation.

2.2.1 Polyphenols

Numerous phenolic compounds have been identified in honey. Table 2.2 shows briefly the common phenolic compounds found in honey.

Generally, phenolic compounds possess a benzene ring with one or more hydroxyl groups on the aromatic ring (Andjelkovic *et al.*, 2006). Plant phenolics are primarily derived from the shikimate and phenylpropanoid metabolic pathways. They can be from phenolic acids which are simple molecules, of low molecular weight, consisting of a single aromatic ring, to flavonoids and complex compounds such as tannins, for example, condensed tannin and hydrolysable tannin. They also are commonly found in a conjugated form with sugars and organic acids.

It has been established that the efficiency of phenolic compounds as antioxidants, either scavenging free radicals or binding to metals, is highly dependent on their molecular structure. Figure 2.2 shows the basic skeleton of phenolic and flavonoid compounds.

Table 2.2: Phenolic compounds commonly found in honey.

Phenolic acids	Flavonoids
Protocatechuic acid ⁹	Quercetin ^{3,4,5,7}
Methyl syringate ²	Myricetin ^{1,7}
4-hydroxybenzoic acid ⁹	Chrysin ^{3,4,5,6,7}
Abscisic acid ⁴	Apigenin ⁶
Coumaric acid ^{3,4,5,6,7,9}	Luteolin, ⁷
Cinnamic acid ⁴	Galangin ^{3,4,5}
Ellagic acid ¹	Pinobanksin ⁴
Chlorogenic acid ^{3,5}	Pinocembrin ^{4,7}
Caffeic acid ^{3,5,7,9}	Kaempferol ^{3,4,5,6,7}
Syringic acid ^{3,4,5}	Rutin ⁷
Vanillic acid ^{4,7}	Pinostrobin ⁸
Ferulic acid ^{3,5,7}	Hesperitin ^{3,5,7}
Gallic acid ^{3,5,7,9}	Naringenin ^{3,5,7}
Synapic acid ^{3,5,7}	Tricetin ¹⁰
Gentisic acid ⁵	Isorhamnetin ¹⁰

¹Vit *et al.* (1997), ²Inoue *et al.* (2005), ³Socha *et al.* (2009), ⁴Gheldof *et al.* (2002), ⁵Socha *et al.* (2009), ⁶Baltrusaityte *et al.* (2007), ⁷Tenore *et al.* (2012), ⁸Fahey & Stephenson (2002), ⁹Zhou *et al.* (2012), ¹⁰Yao *et al.* (2003).

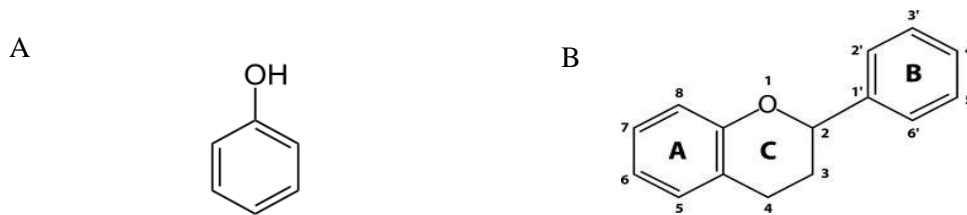
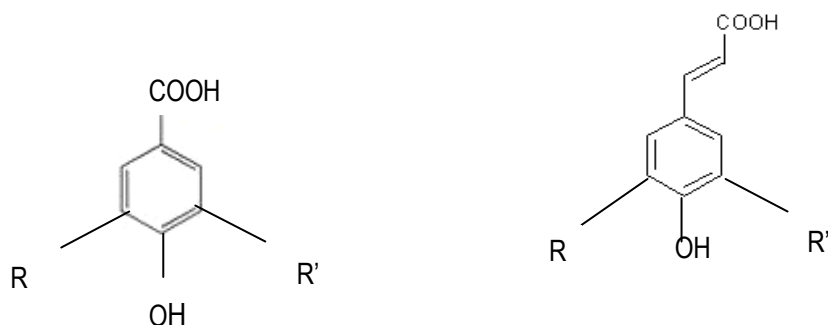


Figure 2.2 : Basic skeleton of (A) phenol and (B) flavonoid

Phenolic acids include two major subgroups, hydroxybenzoic acids and hydroxycinnamic acids. Table 2.3 shows the differences between these two compounds and their derivatives.

Table 2.3: Structural differences between hydroxybenzoic acid and hydroxycinnamic acid and their derivatives (Pereira et al., 2009).



R = R' = H; *p*-hydroxybenzoic acid

R = OH, R' = H; protocatechuic acid

R = OCH₃, R' = H; vanillic acid

R = R' = OH; gallic acid

R = R' = OCH₃; syringic acid

R = R' = H; *p*-coumaric acid

R = OH, R' = H; caffeic acid

R = OCH₃, R' = H; ferulic acid

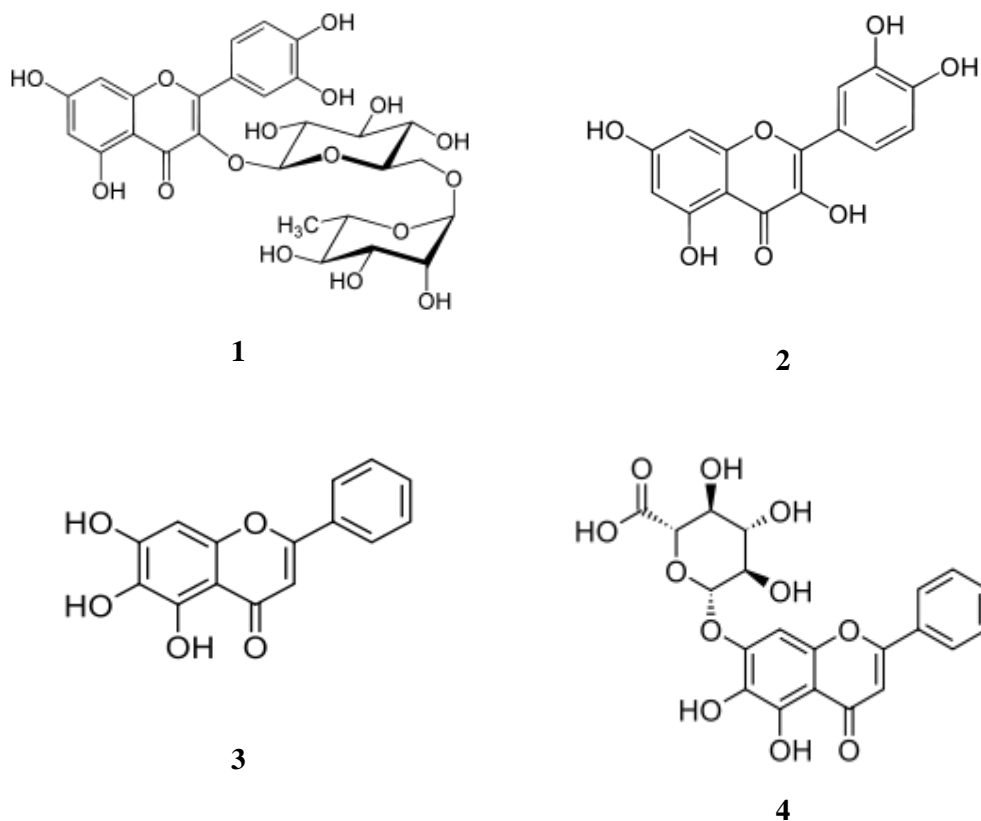
R = R' = OCH₃; sinapic acid

The typical nomenclature for flavonoids presents six major subclasses based on the substitution patterns of ring C (Table 2.4). The position of ring B as well as the oxidation state of the furan ring is important in this classification. Within each class, individual flavonoids are identified and characterised by the conjugation patterns of the hydroxyl groups on the A and C rings as well as the hydroxylation and conjugation patterns of the B ring.

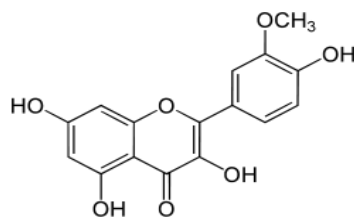
Table 2.4: Major subclasses of flavonoids, adapted from Corcoran et al. (2012).

Flavonoid subclass	Structure	C-ring unsaturation	C-ring functional groups	example
Flavan-3-ols (Flavanols)		None	3-hydroxy 3-O-gallate	(+)-Catechin (+)-Gallocatechin
Flavanones		None	4-oxo	Naringenin Hesperitin
Flavones		2-3 double bond	4-oxo	Apigenin Luteolin
Isoflavones		2-3 double bond	4-oxo	Daidzen Genistein
Flavonols		2-3 double bond	3-hydroxy 4-oxo	Myricetin Quercetin Kaempferol
Anthocyanins		1-2, 3-4 double bonds	3-hydroxy	Cyanidin Delphinidin Petunidin

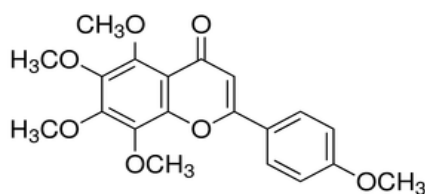
One interesting feature that these phenolic compounds can have is the ability to be conjugated with sugars (*i.e.* glucose, rhamnose and galactose). It is common to find flavonoids in a glycosylated form. For example, rutin (**1**) is the glycoside form of quercetin (**2**). The ability to exist in various forms (glycoside or aglycone) contributes to different kind of activities. For instance, baicalein (**3**) is more efficient in binding Fe^{2+} ions as compared with its glycoside form, baicalin (**4**) (Perez *et al.*, 2009).



In some conditions, the flavonoids can also be methylated (*e.g.* isorhamnetin (**5**)) or methoxylated (*e.g.* tangeretin (**6**)) (Häberlein & Tschiersch, 1998). These conditions obviously will give different effects on activity. The attachment of hydroxyl groups and/or sugars obviously will alter the chemistry of the phenolic compounds to make them become more hydrophilic. On the other hand, methoxylated flavonoids have been reported to exhibit a hydrophobic property (Li *et al.*, 2006).



5



6

To date, there are 8,000 phenolic compounds that have been structurally identified (Bravo, 1998; Harborne & Williams, 2000). This is not surprising as phenolic compounds are so diverse and are found in almost all plants. Among the groups, flavonoids comprise the largest group of phenolic compounds and have been the most extensively researched due to their potential as health-promoting compounds. The research on flavonoids began when the longitudinal epidemiology study conducted by Hertog *et al.* (1993) on Eastern Netherland's elderly men reported that the regular consumption of flavonoids may reduce the risk of death from coronary heart disease. Even though the results of epidemiological studies were not consistent with each other, it appears in biochemical studies that flavonoids possess a wide array of biological activities such as antioxidant, anti-inflammatory, antibacterial, antitumour, oestrogenic and other activities (Bors *et al.*, 1997; Buer *et al.*, 2010; Cotelle, 2001; Harborne & Williams, 2000; Havsteen, 2002; Jovanovic *et al.*, 1994).

The antioxidant activity of flavonoids has been proved to work either by scavenging the radicals or binding with metal ions (particularly iron and copper) that are involved in the production of reactive oxygen species (ROS) (Rice-Evans

et al., 1996, 1997). However, it has also been shown that flavonoids can have dual mechanisms of radical scavenging and binding to metals (Guo *et al.*, 2007; Perez *et al.*, 2009; Zhang *et al.*, 2006). The molecular sites for each biological activity are different and only the probable site for metal-binding is the focus in this review.

To establish the structure-relationship activity of Fe²⁺-flavonoids complexes, the first attempt was made by van Acker *et al.* (1996). They emphasized that 3-OH in the C ring and a catechol group (3', 4'-dihydroxy) in the B ring are more important chelation sites than 5-OH.

However, as numerous studies have been conducted on flavonoids, there are some variable results in the literature concerning the preferred site for iron binding (Andjelkovic *et al.*, 2006; Kawabata *et al.*, 1996; Khokhar & Owusu Apenten, 2003; Leopoldini *et al.*, 2006b, 2011; van Acker *et al.*, 1998). For example, Andjelkovic *et al.* (2006) showed that flavonoids that have an hydroxyl group in position 3 of ring B did not bind to iron, which may be due to the low negative charge density on the binding site. They also observed the importance of the catechol and galloyl groups: flavonoids without these groups did not show any complex formation with iron. In contrast, Leopoldini *et al.* (2011) reported that the catechol group was a poor iron binding site of quercetin. Instead, they suggested that 3-OH and 4-keto (oxo) and 5-OH and 4-keto positions showed greater iron-binding ability.

Despite the apparent inconsistency between authors regarding the iron-binding sites for flavonoids, there are three established metal binding sites reported in the literature for flavonoids: i) 3-OH and 4-oxo, ii) 3', 4'-dihydroxyl (catechol group), and iii) 5-OH and 4-oxo as shown in Figure 2.3.

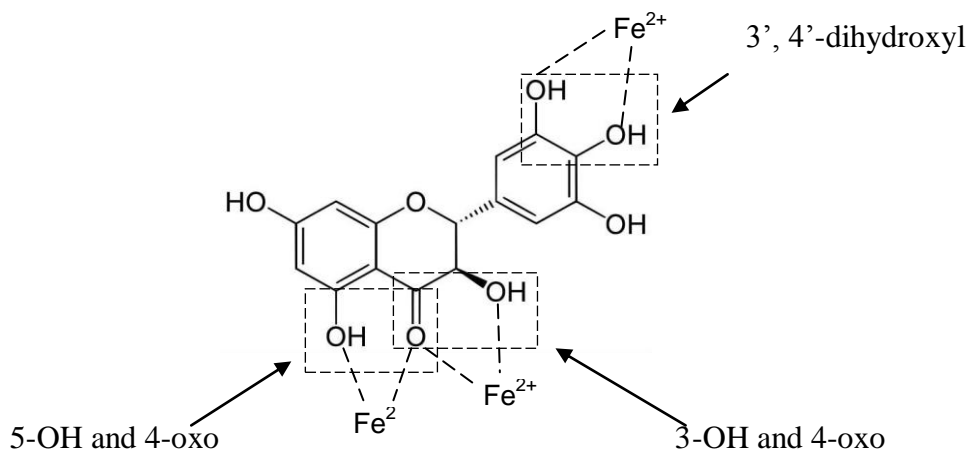
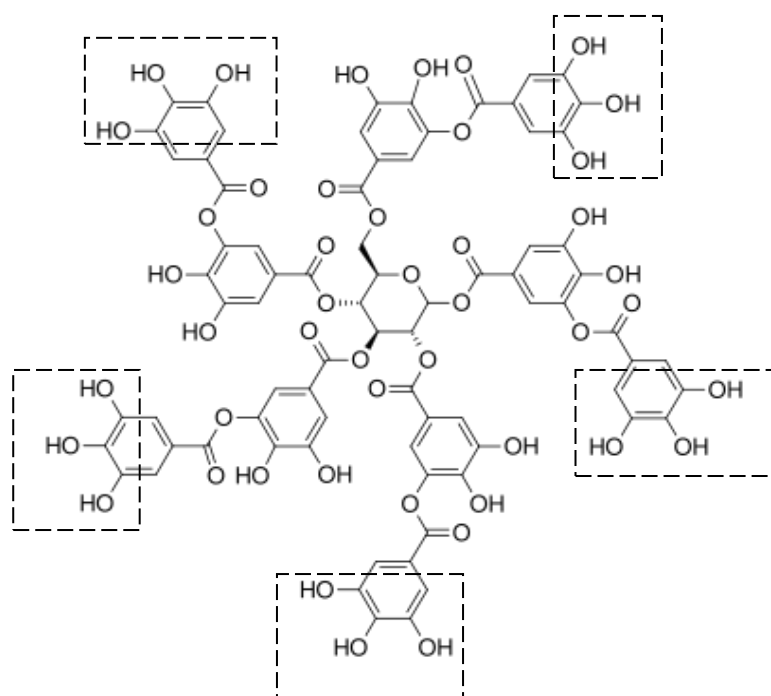


Figure 2.3: Structural requirements of flavonoids in iron-binding antioxidant activity.

Another additional chelating site was the 6, 7 hydroxyl groups of ring A as Perez *et al.* (2009) found that at pH 7.2 baicalein bound to Fe^{2+} ions more strongly than ferrozine did.

As for phenolic acids, Malesev & Kuntic (2007) showed that the galloyl group is the basic site for metal binding which is in agreement with Andjelkovic *et al.* (2006). This had been reported earlier by South & Miller (1998) who also found that the binding activity of tannic acid (**7**) to Fe^{2+} ions was at the adjacent hydroxyls on the galloyl groups (the dotted boxes in structure **7**). Andjelkovic *et al.* (2006) studied the complexation ability between phenolic acids and iron by calculating binding constants of the complexes, and reported that the phenolic acids that do not have catechol or galloyl groups (*e.g.* vanillic acid, syringic acid and ferulic acid) did not show any complex formation with iron.



7

2.2.2 Amino acids, proteins, peptides and proteinaceous compounds

Plant exudates contain compounds such as proteins, peptides, amino acids, citrate, phytochelatins and siderophores which have an ability to bind metals. In addition, it has been generally known that plants produce certain metal-binding compounds in order to cope with the excess or deficient level of metals in plant tissue (Budesinsky *et al.*, 1980; Sharma & Dietz, 2006).

2.2.2.1 Proteins

Apparently, honey is known to contain a small amount of proteinaceous components which originate from bees and plants (Iglesias *et al.*, 2004; Iglesias *et al.*, 2006; White Jr & Kushner, 1967). According to White Jr & Kushner (1967) who determined the molecular weight of the protein in floral honey, protein originating from the bee is in the range of 40–240 kDa, whereas proteins from plants are larger molecules (98 and above 400 kDa). Until now, literature on the identification of the metal-binding proteins in honey has been sparse. Even though plant exudates should contain metallothioneins (metal-binding proteins in

plants) which function as a detoxifier by binding to metals in response to metal accumulation in plants (Grill *et al.*, 1985), it has been reported that metallothioneins have a limited affinity, for only several types of metals (*i.e.* zinc, cadmium and copper) (Clemens, 2001).

2.2.2.2 Peptides

Peptides are considered as small proteins with usually between 2-50 amino acid residues. They are formed after heat or hydrolysis treatment of proteins. Peptides also can occur naturally, synthesised as such or resulting from proteolysis of larger proteins. They occur in plants.

It is interesting to note that Oszmianski & Lee (1990) have isolated a small molecular weight peptide (600 Da) from honey that was proposed to be the component responsible for the inhibition of polyphenol oxidase (PPO) activity in white grapes and fresh apple cuts. The finding of Oszmianski & Lee (1990) was in agreement with that of Ates *et al.* (2001) who also found that honey peptides with molecular weight of 600 Da inhibited mushroom PPO.

The class of peptide components that has a metal-binding ability is the phytochelatins. They are small peptides that commonly exist in plants for detoxification and heavy metals homeostasis (Cobbett & Goldsbrough, 2002; Grill *et al.*, 1985). Their general structure is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2-11$) (see Figure 2.4) and they are synthesized from reduced glutathione (GSH) in a transpeptidation reaction (Grill *et al.*, 1985). Walker & Welch (1987) observed that low molecular weight compounds in lettuce leaves formed complexes with zinc and other trace metals, and came to the conclusion that the compounds could be phytochelatins since they have a partial similarity to the phytochelatins that have been identified by Grill *et al.* (1985).

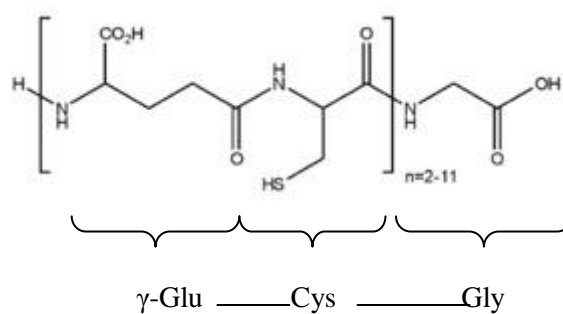


Figure 2.4: Primary structure of phytochelatins. The figure was adapted from Department of Plant Physiology Universität Bayreuth (2009).

2.2.2.3 Amino acids

Amino acids such as proline, have been reported to exist in honey (Bouseta *et al.*, 1996; Hermosin *et al.*, 2003). To date, the published literature on honey amino acids with antioxidant activity has been very scarce. Meda *et al.* (2005) found a higher correlation between radical scavenging activity and proline ($r=0.75$) than between the scavenging activity and total phenolic content. Perez *et al.* (2006) also found correlation between the radical scavenging activity of honey and the content of proline ($r=0.571$), total free amino acids ($r=0.825$) and proteins ($r=0.300$), suggesting that amino acids could possibly play a role in scavenging activity. However, no metal-binding test with proline or free amino acids was carried out by Meda *et al.* (2005) and Perez *et al.* (2006). Even though the above findings were only focused on radical-scavenging activity, many studies have reported that free amino acids could possess metal-binding activity as well. Figure 2.5 shows the free amino acids commonly found in honey.

A few studies have reported that other free amino acids could possess metal-binding activity. For example, some sulphur amino acids such as *S*-methyl-L-cysteine, DL-ethionine, L-cysteine and DL-penicillamine have a binding activity with transition metals (Lenz & Martell, 1964). Wu *et al.* (2003) demonstrated that amine and carboxylic acid groups in the α -position relate to the metal-binding property of α -amino acids, but other functional group such as the imidazole group in histidine (His) can enhance their binding activity. Pazos *et al.* (2006) also noted

that tryptophan (Trp) and histidine have dual mechanisms, chelating and scavenging activity, whereas glycine and methionine have a binding property.

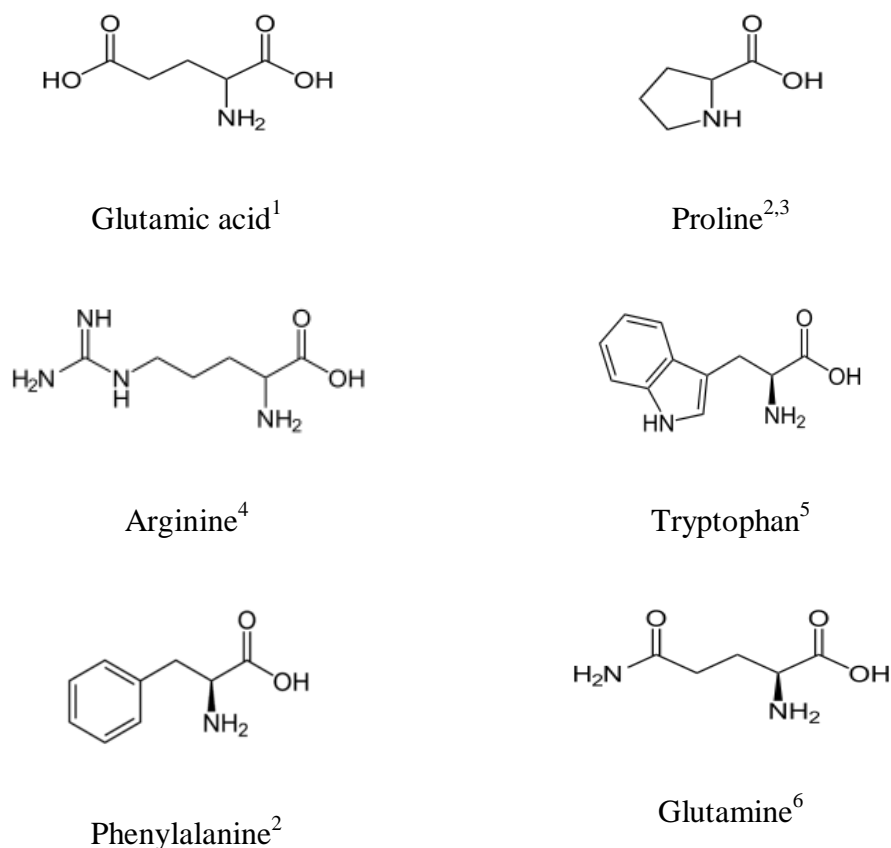


Figure 2.5: Free amino acids commonly found in honey.

¹Iglesias *et al.* (2004), ²Bouseta *et al.* (1996), ³Hermosin *et al.* (2003), ⁴Bernal *et al.* (2005), ⁵Pazos *et al.* (2006), ⁶Kelly *et al.* (2010).

It is well known that the biological activity of proteins and peptides is dependent on the type of amino acid residues they contain (Elias *et al.*, 2008; Shahidi & Zhong, 2008; Sovago *et al.*, 2012). Kim *et al.* (2007) have isolated the iron-binding peptides from whey protein after enzymic hydrolysis. They determined that the components with highest iron-binding activity contained higher levels of lysine (Lys), alanine (Ala) and phenylalanine (Phe). Vattem *et al.* (2001) used enzyme-digested chicken muscle to investigate the role of cysteine (Cys) sulfhydryl groups in iron binding. They concluded that the sulfhydryl group is the leading component in iron binding activity, whereas other amino acid residues

may play a minor role. However, the finding of Vatterm *et al.* (2001) was contradicted by Wu *et al.* (2003) who found that cysteine could also be a pro-oxidant. This is due to the sulfhydryl group being able to reduce Fe^{3+} to Fe^{2+} .

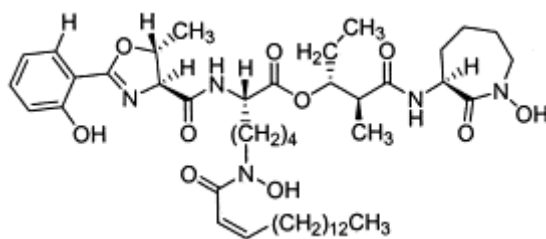
2.2.2.4 Siderophores

Microorganisms (*i.e.* bacteria and fungi) and plants produce siderophores, low molecular weight compounds (500-1500 Da) (Hider & Kong, 2009), when cells are limited for iron. These aid in the sequestering and transport of iron (Drechsel & Jung, 1998; Guerinot *et al.*, 1990).

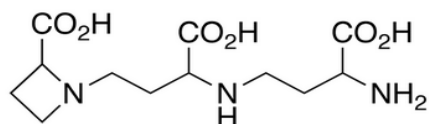
The differences between bacterial, fungal and plants siderophores are in the functional groups. For example, bacterial siderophores, such as mycobactin (**8**), consist of catecholate, hydroxamate and carboxylate groups. On the other hand, siderophores produced by plants (*e.g.* nicotianamine (**9**), mugineic acid and avenic acid) do not contain catecholate or hydroxamate groups (Drechsel & Jung, 1998). Fungal siderophores generally consist of a hydroxamate group (*e.g.* ferrichrome (**10**) (*Wikimedia Commons: Ferrichrome*, 2011)), but some contain a catecholate group (*e.g.* pistillarin), and others (*e.g.* rhizoferrin (**11**)) contain a citrate group.

Generally, most siderophores have a higher affinity for Fe^{3+} than Fe^{2+} . However, the phytosiderophore nicotianamine preferentially binds Fe^{2+} rather than Fe^{3+} (Hider & Kong, 2009; von Wiren *et al.*, 1999). This is due to its role as a Fe^{2+} transporter (Curie *et al.*, 2009) which translocates Fe^{2+} in phloem of all plants (Stephan & Scholz, 1993). Nicotianamine is present in the sieve tube of plants (Schmidke *et al.*, 1999).

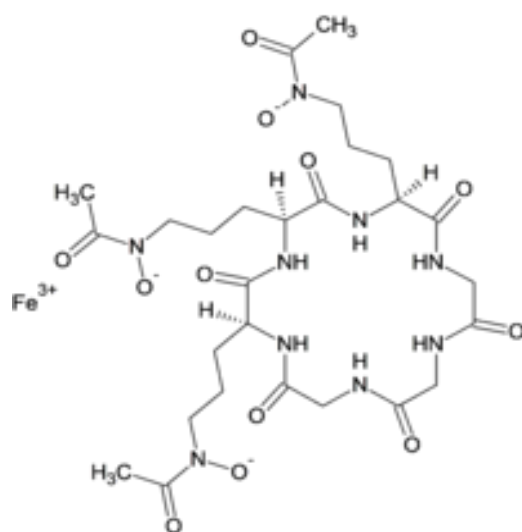
Unlike other phytosiderophores such as mugineic acid which is specifically secreted by graminaceous plant roots (Ma & Nomoto, 1996) to acquire iron, nicotianamine does not. Nicotianamine is biosynthesised from three molecules of S-adenosylmethionine by nicotianamine synthase. It exists almost ubiquitously in plants. Nicotianamine forms a stable complex with Fe^{2+} and does not undergo redox cycling, which is the important characteristic of preventing the Fenton reaction from occurring (von Wiren *et al.*, 1999).



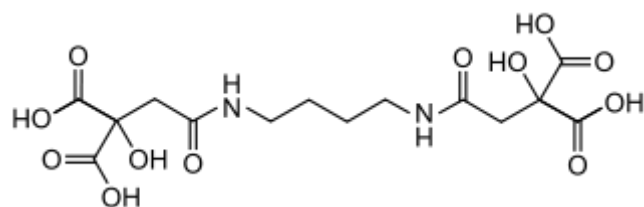
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Since the content of honeydew originates solely from the phloem sap and is extracted out by aphids using the sieve tube, the possibility of nicotianamide existing in honeydew and from this being transferred to honeydew honey is high.

There have been several reports that honey contains microorganisms including bacteria and fungi. Recently, Saksinchai *et al.* (2012a) have isolated a sugar-tolerant yeast species (*Zygosaccharomyces siamensis*) from Thailand raw honeys. The authors suggested that the yeast species originated from honey bees and/or pollen and nectar. The same research team also have isolated two new species of the genus *Candida* in the *Zygoascus* clade (*i.e.* *Candida lundiana* *sp. nov.* and *Candida suthepensis* *sp. nov.*) from raw honey in Thailand (Saksinchai *et al.*, 2012b).

It has been reported that a black sooty mould (a type of fungus) is a common microorganism found in New Zealand Beech honeydew and honeydew honey (Moar, 1985; Morales *et al.*, 1988). With regard to this, Moar (1985) suggested that sooty mould is characteristic of the Beech honeydew honey. Hence, there is a reason to believe that these microorganisms possibly produce siderophores with high affinity for iron (Van Ho *et al.*, 2002) for their survival (Olaitan *et al.*, 2007).

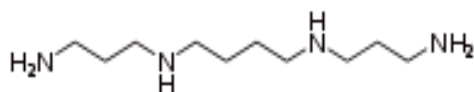
2.2.2.5 Polyamines

Polyamines such as spermine (**12**), spermidine (**13**) and putrescine (**14**) are non-protein nitrogenous compounds with low molecular weight that are ubiquitous in cells. They have been proposed to have a role in plant growth (Gaboriau *et al.*, 2004) and developmental processes (Kalac & Krížek, 2003). The concentration of putrescine has been reported to increase under cadmium stress in wheat leaves (Groppa *et al.*, 2007). A study by Hsu & Kao, (2007) suggested that spermidine and spermine are able to protect from cadmium-induced oxidative damage in rice leaves through the avoidance of generation of hydrogen peroxide and the reduction of cadmium uptake. Polyamines have been proposed to have a role in heavy metal defence in plants (Groppa *et al.*, 2001).

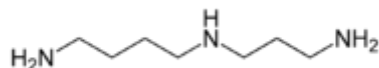
The polyamines always exist as cations at physiological pH (Belle *et al.*, 2004). This characteristic gives these compounds a high affinity for negatively charged

membrane phospholipid, thus stabilising the membrane (Sharma & Dietz, 2006). However, it has been reported that polyamines do not form a complex with a zwitterion phospholipid (*e.g.* phosphatidylcholine) (Tadolini, 1988).

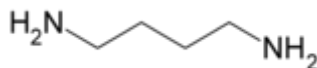
The role of polyamines should not be underestimated as Belle *et al.* (2004) demonstrated that the formation of thiobarbituric acid-reactive substances (TBARS), a byproduct of lipid peroxidation, induced by Fe²⁺-or Fe²⁺-EDTA, was inhibited by a relatively high concentrations of spermine and spermidine.



12



13



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2.2.3 Maillard reaction products (MRPs): melanoidins

Another class of compounds which has been thought to exert antioxidant activity in honey is Maillard reaction products (MRPs), specifically melanoidins (Brudzynski & Miotto, 2010a). Melanoidins are polymeric brown compounds generated in the late stages of the Maillard reaction from reducing sugars and proteins or amino acids during food processing and preservation. Earlier on, it was thought that melanoidins were only formed by the typical reaction between amino acids and sugar (Cämmerer *et al.*, 2002), however, more recent studies have

shown that phenolic compounds are also involved in the structure of melanoidins (Brudzynski & Miotto, 2010a; Takenaka *et al.*, 2005).

Melanoidins with a radical scavenging property with a molecular weight between 85–232 kDa have been isolated from Canadian buckwheat honey (Brudzynski & Miotto, 2010b). They have been shown to exhibit several biological activities such as antioxidant (Brudzynski & Miotto, 2010a, 2010b; Morales, 2005; Morales *et al.*, 2005), antibacterial (Rufian-Henares & de la Cueva, 2009) and antihypertensive activity (Rufian-Henares & Morales, 2007).

The metal-binding property of melanoidins has been studied more in model systems (Maillard *et al.*, 2007; Migo *et al.*, 1997; Ramonaityte *et al.*, 2009; Takenaka *et al.*, 2005) rather than real food system. Morales *et al.* (2005) reported that melanoidins could have an iron-binding property. Which type of sugar there is in the backbone of melanoidins influences the binding activity of melanoidins to iron. Takenaka *et al.* (2005) also have shown that melanoidins in coffee could bind to zinc. Maillard *et al.* (2007) have found that melanoidins from a glucose-cystein model system stopped the catalytic reaction of copper ions (Cu^{2+}). Since melanoidins are so diverse and complex in nature, the components responsible for the iron-binding activity remains unclear.

2.2.4 Organic acids

Gheldof *et al.* (2002) have suggested that organic acids such as gluconic, citric and malic acids might contribute to the antioxidant activity in honey. Apparently, organic acids, such as citric and malic acids exist in low quantities in honey (del Nozal *et al.*, 1998; Nozal *et al.*, 2003a; Nozal *et al.*, 2003b; Suarez-Luque *et al.*, 2002). Citric and malic acids have been reported to bind iron and prevent peaches and nectrines from skin discoloration due to free iron in fruits cells (Cheng & Crisosto, 1997).

2.3 General features of iron ligands

In general, iron ligands can be characterized as bidentate (*i.e.* two coordination sites), tridentate (*i.e.* three coordination sites) and hexadentate (*i.e.* six coordination sites), the hexadentate coordination being the most stable form of iron complexes (South & Miller, 1998). Devanur *et al.* (2008) reported that an $\cdot\text{OH}$ radical formed from the complex of the weakly chelated Fe^{3+} ion with deferiprone (*i.e.* a bidentate iron chelator), where the deferiprone does not completely chelate all the six coordination sites of the Fe^{3+} ion, hence the unbound site(s) of Fe^{3+} ion are able to be reduced to Fe^{2+} ion by the reducing agent. This was in agreement with Graf *et al.* (1984), namely that the availability of a coordination site in the iron complexes promote the formation of the $\cdot\text{OH}$ radical in the Fenton reaction.

However, it also has been reported that the valence of iron can influence the property of metal complexes too (de Souza & de Giovani, 2004; South & Miller, 1998). For example, the study by van Acker *et al.* (1996) has showed that the complex between Fe^{2+} and ethylenediaminetetraacetic acid (EDTA) was stable, however this was contradicted by the study by Graf *et al.* (1984) who found that the $\cdot\text{OH}$ radical was formed from the Fe^{3+} -EDTA complex. It has been established theoretically that there is a preference for either Fe^{2+} or Fe^{3+} for making complexes to different type of ligands (Spiro & Saltman, 1974). Basically, Fe^{2+} ion prefers nitrogen-containing ligands such as 2,2'-bipyridyl and 1,10-phenanthroline, whereas Fe^{3+} ion has high affinity to hydroxamate oxygen atoms (Liu & Hider, 2002).

2.4 Compounds found in honey known for antioxidant properties

A few compounds in honey have been identified as possessing a specific antioxidant mechanism. For instance, methyl syringate was identified as having a scavenging activity for superoxide radicals in Manuka honey (Inoue *et al.*, 2005). Catalase, which generally occurs in all honeys, is widely known as a peroxide scavenger. Salicylic acid which has been found in honeys (Senanayake, 2006; Venema *et al.*, 1996) also has been reported to regulate redox balance and protect from oxidative damage in rice plants (Yang *et al.*, 2004). Gheldof *et al.* (2002)

demonstrated that approximately 16% of the peroxy scavenging activity in Buckwheat honey was contributed by the protein fraction.

2.5 Analytical determination of antioxidants

2.5.1 Bioactivity-guided fractionation

The majority of the biologically active natural compounds have been isolated using bioactivity-guided fractionation. In bioactivity-guided fractionation, the sample extract or a mixture of unknown molecules is fractionated using any separation techniques and the biological activities of the fractions are subjected to specific biological assay to determine the active fraction in each step of the separation process. This procedure is also useful to detect any changes in the activity of bioactive compounds due to the purification which either may lead to the loss or enhancing of its bioactivity (Nwodo *et al.*, 2011; Nwodo *et al.*, 2010).

2.5.2 Methods for testing antioxidant activity

In the area of antioxidant research, a large number of bioassay systems are available (Carocho & Ferreira, 2013; Moon & Shibamoto, 2009). Generally *in vitro* assays can be divided into two groups: (i) measuring the ability of compounds to donate an electron or hydrogen atom to a known radical such as DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid), or to ferric ions (Fe^{3+}); or (ii) testing the ability of compounds to inhibit the action of catalysts such as iron or copper ions that promote oxidation. According to Antolovich *et al.* (2002), antioxidant activity cannot be measured directly but rather by the effects of the antioxidant in controlling the extent of oxidation which was followed by a comparison of the observed effects of a known standard. The assays should be simple, rapid, reliable, reproducible, sensitive, meaningful and, most importantly, predictive.

Various antioxidant assays have been used to test antioxidant activity in honeys. The assays involve different mechanisms and some are specific to a specific radical. Table 2.5 provides information on the antioxidant assays widely used for honey.

Table 2.5: Antioxidant assays commonly used to test antioxidant activity in honey.

Assay	Mechanism	Advantage	Disadvantage	References
<i>Radical scavenging activity</i>				
DPPH ¹	The measurement is based on the scavenging ability of antioxidants to reduce DPPH [•] radical to hydrazine. The reducing mechanism involves H atom donation from antioxidants. Purple DPPH turns to yellow in the presence of antioxidants and can be measured at 517 nm.	<ul style="list-style-type: none"> •Test is simple and rapid. •Useful as a preliminary screening assay. •The results are highly reproducible and comparable to other radical scavenging assay (e.g. ABTS). •DPPH radical is stable and does not need to be generated prior to assay. 	<ul style="list-style-type: none"> •Not a competitive reaction. •Not useful in measuring the antioxidant activity of the matrix which is not soluble in alcoholic reaction medium (e.g. plasma)(Sánchez-Moreno, 2002). •Interaction of antioxidants with DPPH is mediated by steric hindrance. •DPPH radical does not mimic the radical in biological system since DPPH is stable. 	<p>Bertoncelj <i>et al.</i> (2007) Aljadi & Kamaruddin (2004) Estenvinho <i>et al.</i> (2008) Socha <i>et al.</i> (2009) Perez <i>et al.</i> (2006) Vela <i>et al.</i> (2007) Al <i>et al.</i> (2009) Saxena <i>et al.</i> (2009) Meda <i>et al.</i> (2005) Tuberoso <i>et al.</i> (2011) Ferreira <i>et al.</i> (2009) Zalibera <i>et al.</i> (2008) Guerrini <i>et al.</i> (2009) Pichichero <i>et al.</i> (2009)</p>

Table 2.5 continued

ABTS ²	Mechanism is based on the inhibition by antioxidants of the absorbance of radical cation ABTS ^{•+} (Sánchez-Moreno, 2002). ABTS radical decolorises in the presence of antioxidants.	<ul style="list-style-type: none"> •Capable of testing the antioxidant activity of hydrophilic and lipophilic compounds. •the reaction is pH independent 	•ABTS does not resemble the radical found in the biological system.	Zalibera <i>et al.</i> (2008) Socha <i>et al.</i> (2009)
ORAC ³	Measures the ability of the antioxidants to protect against oxidation induced by the peroxy radical.	The only method that uses an area under curve (AUC) technique for quantitation.	•The oxidative reaction does not mimic the biological system	Gheldof <i>et al.</i> (2002) Gheldof & Engeseth (2002a) Gheldof <i>et al.</i> (2003)
FRAP ⁴	Measures the reducing ability of the antioxidants to reduce ferric-2,4,6-tris(2-pyridyl)-1,3,5-triazine (Fe ³⁺ -TPTZ) to the ferrous complex.	<ul style="list-style-type: none"> •simple, economic and reducible method. •can be applied to both plasma and plant extracts. •does not dependent on enzymatic and non-enzymatic reaction to generate free radicals prior to the evaluation. 	<ul style="list-style-type: none"> •The method has its limitations, especially in measurements under non-physiological pH value (pH 3.6). •not able to detect slowly reactive polyphenolic compounds and thiols. 	Bertoncelj <i>et al.</i> (2007) Aljadi & Kamaruddin (2004) Kucuk <i>et al.</i> (2007) Blasa <i>et al.</i> (2007) Estenvinho <i>et al.</i> (2008) Saxena <i>et al.</i> (2009) Tuberoso <i>et al.</i> (2009) Ferreira <i>et al.</i> (2009) Pichichero <i>et al.</i> (2009)

Table 2.5 continued

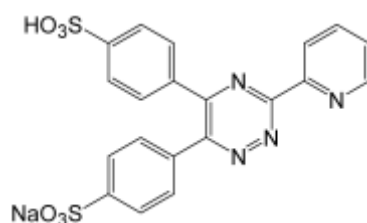
Superoxide (O ₂ ^{•-}) scavenging activity	The mechanism is based on the competition kinetics of O ₂ ^{•-} reduction of cytochrome c (probe) and O ₂ ^{•-} scavenger (antioxidant).	• O ₂ ^{•-} is a common radical in biological system, so this assay could represent the antioxidant reaction biologically.	•the method is not suitable for quantifying non-enzymatic antioxidant	Inoue <i>et al.</i> (2005) Kucuk <i>et al.</i> (2007) Nagai <i>et al.</i> (2001)
<i>Iron-binding assay</i>				
Iron-chelating assay	Mechanism is based on the Fe ²⁺ chelating activity of antioxidants. Ferrozine is commonly used as a positive control.	•a competitive reaction between antioxidants and Ferrozine for binding to Fe ²⁺ when mixed.	•not representing chelating of iron in a physiological medium. •will not show antioxidant activity if binding of Fe ²⁺ does not stop catalysis of the Fenton reaction.	Ita (2011) Zhou <i>et al.</i> (2012)
Iron-binding assay based on lipid peroxidation	Mechanism is based on the antioxidants preventing lipid peroxidation from occurring by binding to Fe ²⁺ which catalyses this.	•lipid emulsion mimics the membrane lipid in physiology system.	•pH used in the assay is lower (pH 5.0) than physiological pH (nearly neutral pH).	Brangoulo & Molan (2010)

¹ 2,2-diphenyl-1-picrylhydrazyl, ² 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ³Oxygen radical absorbance capacity,

⁴Ferric reducing antioxidant power

2.5.2.1 Fe^{2+} -binding antioxidant assay

There are several *in-vitro* assays to measure Fe^{2+} -binding antioxidant activity described in the literature. In many cases, ferrozine (sodium 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulphonate) (**15**), a ferroin-type reagent is used as a comparative standard (Ita, 2011; Karamac, 2007; Valvi *et al.*) for Fe^{2+} -binding activity as ferrozine can form soluble complexes with Fe^{2+} quantitatively (Schilt & Hoyle, 1967). Ferrozine reacts only with Fe^{2+} ion and the reaction yields a stable complex that is magenta in colour, and absorbs strongly at 550 nm (White & Flashka, 1973). Another ferroin reagent that has been used in a small number of cases for Fe^{2+} -binding antioxidant assay is 4,7-diphenyl-1,10-phenanthroline (Morales *et al.*, 2005).



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A few different approaches have been taken to measure the Fe^{2+} -binding activity in the assay. The most widely used method is based on the estimation of the decrease in the maximal absorbance of the complex between antioxidant and Fe^{2+} ion and ferrozine (Gulcin, 2006). The Fe^{2+} -binding compounds compete for the Fe^{2+} ions, leading to the disruption of the Fe^{2+} -ferrozine complex formation, thus decreasing the magenta color of the complexes. Measurement of the decrease in the colour after 10 min of reaction therefore allows the estimation of the Fe^{2+} -binding ability of the compounds.

However, it should be borne in mind that not all substances binding iron will give inhibition of the Fenton reaction. Simply measuring binding of iron does not assure that there is an antioxidant action. This is because some chelators leave the iron still capable of catalysing the Fenton reaction (Devanur *et al.*, 2008). For example, Fe^{3+} -adenosine triphosphate (ATP) complex is a well known redox

cycling agent for the Fenton reaction (Cheng & Breen, 2000). Almeida *et al.* (2005) have demonstrated that the Fe^{2+} -citrate complex formed $\cdot\text{OH}$ in which it attacked the DNA of the isolated rat liver mitochondria.

An Fe^{2+} -binding antioxidant assay can also be conducted with the use of a lipid emulsion. Considering the need for a reliable and biologically relevant antioxidant assay, a new assay simulating the oxidative damage on cell membrane in the presence of free ion has been developed by Branguolo & Molan (2010). The assay is based on the Fenton reaction-mediated lipid peroxidation which involves Fe^{2+} ion that acts as a catalyst to accelerate the lipid peroxidation process in the emulsion. It can measure the iron-binding antioxidant activity of substances using a Fe^{2+} ion-catalysed β -carotene-linoleic acid model system. Linoleic acid was used to mimic the membrane lipid of living cells. The degree of inhibition of this reaction shows the amount of removal of Fe^{2+} from catalytic activity.

The Fe^{2+} -binding antioxidant assay used in the present research was based on the method of Branguolo & Molan (2010). β -Carotene is added into an emulsion of linoleic acid. Antioxidants that bind to Fe^{2+} ions will allow the colour of β -carotene to be retained after 10 minutes incubation. This indicates that Fe^{2+} ion have been bound and prevented from catalysing the lipid peroxidation reaction. The colour of the β -carotene emulsion will fade in the absence of Fe^{2+} -binding antioxidants because it is destroyed in the iron-catalysed lipid peroxidation chain reaction. The absorbance at 450 nm is measured to estimate the amounts of remaining β -carotene colour after 10 minutes incubation. Ferrozine is used as a comparative standard for Fe^{2+} -binding activity. The assay was described in detail in Section 3.2.1. Unlike assays that just measure Fe^{2+} binding, which may not inactivate the catalytic activity of the Fe^{2+} , this assay measures inactivation of Fe^{2+} catalytic activity.

2.6 Analytical separation of antioxidants

The design of the analytical procedure for the identification of antioxidant compounds in biological materials will depend very much on the target analyte of interest. Basically, it involves three steps: extraction from the sample or sample clean-up, analytical separation to isolate the antioxidants and elucidation of the

structure of the antioxidants. Common chromatographic techniques in use for the isolation of antioxidants are thin layer chromatography (TLC), liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE) with ultraviolet-visible wavelength (UV-Vis) spectrophotometry being the most commonly used mode of detection of separated components.

The analytical determination of phenolic compounds in honey has been approached successfully based on both chromatographic and spectrophotometric methods (Avila, 2006). HPLC has been the method of choice because it can be used for both separation and quantitation of phenolics. Furthermore, this method provides high accuracy and reproducibility for routine work (D'Arcy, 2005), is versatile and is of relatively low cost. The introduction of reversed-phase columns has considerably enhanced HPLC separation of different classes of organic compounds (Molnar-Perl, 2000; Molnar & Horvath, 1977), in conjunction with aqueous acetonitrile or methanol as modifiers (Escarpa & Gonzalez, 2001). With regard to the detection system employed in HPLC, it should be emphasized that UV-Vis spectrophotometry and mass spectrometry (MS) are the methods commonly used for the detection of antioxidants (Andlauer *et al.*, 1999; Careri & Mangia, 2003; Shui & Leong, 2005), although the electrochemical detection system has been used to a lesser extent (Shao *et al.*, 2010).

Many know that LC-MS is said to provide a powerful tool for the rapid identification of novel, biologically active metabolites from natural sources (Ryan *et al.*, 1999). Thus, high performance liquid chromatography using reversed phase columns with mass spectrometry (HPLC-MS) has been commonly used for structural characterisation of many phytochemicals (Andlauer *et al.*, 1999). Nuclear magnetic resonance (NMR) spectroscopy is a non-destructive technique which could be applied in determination of the molecular structure of isolated antioxidant compounds (Jahnke, 2007). However, it requires a relatively high purity and high concentration of antioxidant in the sample (Antolovich *et al.*, 2002), limiting its usage when the concentration of antioxidants exists in trace amounts in samples.

CHAPTER 3

MATERIALS AND GENERAL METHODS

This chapter outlines the materials and general methods used throughout this study. The specific methods for specific determinations are given in the relevant chapters.

3.1 Materials

3.1.1 Honey sample

Honeydew honey was obtained from New Zealand beekeepers. The honey was received in June 2009 and was given an identification number (HD20). It was stored at -21°C in a dark, dry place throughout the study.

3.1.2 Chemicals

The following chemicals and solvents (HPLC grade) were used throughout the research and commercially obtained; methanol (Sharlau), acetonitrile (Merck), ethanolamine (BDH). Acetone, hydrochloric acid, sodium hydroxide and Tris (hydroxymethyl methylamine) were purchased from Ajax Finechem. In all cases, deionised water was used for the chromatography work.

3.1.3 Instruments

The chromatography work was carried out on AKTA FPLC system (GE Healthcare). All columns used were from GE Healthcare.

3.2 Methods

3.2.1 Assay for the measurement of iron-binding antioxidant activity

The iron-binding activity of honeydew honey was assayed based on the assay developed by Brangoulo & Molan (2010) with some modifications. Ferrozine (1.5

mmol/l) was used as the standard. Into 1 ml of β -carotene solution (0.1 g in 3 ml chloroform) was mixed with 0.02 g of linoleic acid and 0.2 g of Tween 40 (Sigma Aldrich) in the round bottomed flask. Chloroform (BDH Chemicals) was removed by using the rotary evaporator at 40°C for 15 minutes. Following evaporation, 25 ml of 0.5 mol/l sodium acetate buffer pH 5 was added to the mixture, and gently vortexed to form an emulsion. The blank emulsion was prepared using the same method but without β -carotene in the chloroform.

Ferrous chloride (Merck) solution (0.5 mmol/l) was prepared by dissolving 12.6 mg of FeCl_2 in 100 ml of 0.5 mol/l sodium acetate buffer pH 5. This gave the stock concentration of 1 mmol/l. Then, 25 ml of the stock FeCl_2 solution was transferred into a 50 ml volumetric flask and 0.5 mol/l sodium acetate buffer pH 5 was added to make it up to 50 ml to get the 0.5 mmol/l of the FeCl_2 solution (in Section 8.3.3.2 lower concentrations of FeCl_2 were used to increase the sensitivity of the antioxidant activity of the isolated compounds).

Ferrozine (Sigma Aldrich) standard stock solution was prepared by dissolving 37 mg of Ferrozine in 10 ml of 0.5 mol/l sodium acetate buffer pH 5. To get the concentration of 1.5 mmol/l Ferrozine, 2 ml from the stock Ferrozine solution was transferred into a 10 ml volumetric flask and 0.5 mol/l sodium acetate buffer pH 5 was added to make it up to 10 ml.

The assay was done in a 96 well microtitre plate. Briefly, 10 μl of 1.5 mmol/l FeCl_2 solution was pipetted into each well including the well for blank and standard. Then, 50 μl of 0.5 mol/l sodium acetate buffer pH 5 was added to the wells in the first row (A1, A2, A3, and A4) as a control. To the wells in the next row (B1, B2, B3, and B4), 50 μl of 1.5 mmol/l Ferrozine solution was added as the standard. Following that, 50 μl of honey solution or fractions was added to the wells in the following row (C1, C2, C3 and C4). Blank emulsion (150 μl) was added to all wells in the first column (*i.e.* A1 – H1, A5 – H5, and A9 – H9) to serve as blanks. To all other wells 150 μl of emulsion containing β -carotene was added. Consequently, the plate was shaken at 120 rpm to avoid any bubbles forming in the wells if the high rotation was used. The absorbance at 450 nm was used and the values were measured at 0 min and 10 min with an automated BMG plate reader.

The setting up of the 96-well flat-bottomed microtitre plate was shown in Figure 3.1.

		Columns											
		1	2	3	4	5	6	7	8	9	10	11	12
Rows	A	B	CONTROL			B	S7			B	S15		
	B	L	FERROZINE			L	S8			L	S16		
	C	A	S 1			A	S9			A	S17		
	D	N	S2			N	S10			N	S18		
	E	K	S3			K	S11			K	S19		
	F	“	S4			“	S12			“	S20		
	G	“	S5			“	S13			“	S21		
	H	“	S6			“	S14			“	S22		

S=sample

Figure 3.1: The illustration of the microtitre plate for the iron-binding antioxidant activity assay.

3.2.1.1 Calculations

The damage (%) or bleaching of β -carotene was calculated as:

$$\text{Damage \%} = 100 \times [1 - (A_t/A_0)]$$

A_t and A_0 were respectively the absorbance at determined time $t=10$ minutes and $t=0$ minute.

The protective effects of samples were evaluated as:

$$\text{Protective effect \%} = 100 \times [(D_{\text{control}} - D_{\text{sample}}) / D_{\text{control}}]$$

D_{control} and D_{sample} were respectively the damage obtained in the control and sample.

A typical result sheet from an iron-binding antioxidant assay is shown in Appendix 1.

3.2.2 Acetone extraction

Ten grams of honey was mixed with 10 ml of water. One ml of this honey solution was then mixed with 1 ml of acetone or 70% (v/v) acetone. Then, more

acetone (approximately 10 ml) was added. At this stage, the mixture was like a sticky paste. The mixture was centrifuged at 10 000 rpm for 10 min. Supernatant was put on a rotary evaporator at 35°C until all acetone was dried off. The dried extract was dissolved in 1 ml of water and tested for iron binding assay.

3.2.3 Glucose oxidase test

A glucose oxidase (Thermotrace) test was used to determine if glucose was present in the fractions, so that the elution volume of the sugar in honey could be determined. All fractions were tested for free glucose. The glucose reagent (500 µl) was mixed with 25 µl of each fraction, in small test tubes, and incubated at 37°C for 15 minutes. If glucose was present, the mixture turned a pink colour after 15 minutes. For a blank, water was used instead of the fractions.

3.2.4 Silver nitrate test

Silver nitrate (BDH Chemicals) test was used to determine if sugars other than glucose were present in the fractions. Fractions 30-50 were selected for testing due to the assumption that other sugars could elute at different elution volume than glucose. The fractions were spotted on a filter paper, then a spot of 1% silver nitrate solution followed by a spot of 2% ethanolic sodium hydroxide solution were dropped on the fraction spots. The fraction spots which turned to intense brownish colour represented sugars in the fractions. For a blank, 30% acetonitrile was used instead of the fractions.

3.3 Statistical analysis

In the measurements of the antioxidant activities, at least two independent assays in were made, and results were calculated as means of these measurements. Microsoft Excel software statistical calculations were carried out in order to calculate mean, standard deviation and standard error of mean.

CHAPTER 4

EFFECT OF TEMPERATURE AND pH ON IRON-BINDING ANTIOXIDANTS IN HONEYDEW HONEY

4.1 Introduction

It is well known that honey contains various organic compounds which are potentially iron-binding antioxidants (as described in Chapter 2). These compounds could be expected to differ in their stability with respect to temperature and pH.

Temperature higher than room temperature (*i.e.* above 18–22°C) is normally applied to honey during extraction, drying processes, pasteurization or other experimental procedures. Recently, temperature above 25°C has come into use as a tool to optimize the chromatographic conditions such as to reduce the analyte retention, efficiency and selectivity of the columns (Castells *et al.*, 2004; Greibrokk & Andersen, 2003; Heinisch *et al.*, 2006; Wolcott *et al.*, 2000). Mallipattu *et al.* (2010) used a temperature of 40°C in ion exchange chromatography for the separation of phenolic acids in food samples. It was found by del Nozal *et al.* (1998) that the retention of organic acids in honeys was decreased accordingly with increasing column temperature in ion exchange and reversed phase columns.

Chromatography techniques such as ion exchange and reversed phase chromatography (*i.e.* ion pairing mode) use high and low pH (in term of buffer usage) in their separation mechanism. The widely used XAD-2 column chromatography technique for extracting phenolic compounds in honey uses acidified water at pH 2 purposely to suppress the ionization of phenolic compounds in honey so that the phenolic compounds will be non-ionic and thus be adsorbed on the resin (Ferreira *et al.*, 2009).

However, high temperature and extreme pH might lead to degradation or instability to the organic compounds. Some of phenolic compounds and proteins are susceptible to degradation under high temperature and alkaline pH (Elias *et al.*, 2008; Makkar & Becker, 1996; Zhu *et al.*, 1997). High temperature and pH affect the stability of antioxidants due to oxidation of the compounds (Reblova, 2012), transform the phenolic compounds into chalcones (Jurd & Geissman, 1963) and cause epimerization to occur (Sang *et al.*, 2005), or unfold the tertiary structure of proteins (Elias *et al.*, 2008; Fagain, 1995). These conditions could influence the antioxidant activity of the compounds.

For these reasons, it was considered worthwhile to conduct a simple experiment to find the stability of honeydew honey at temperatures and pH that mimic the proposed analytical conditions (*i.e.* extraction/isolation and purification work) before the real procedures were carried out. This was of importance because the chromatography work that was planned to be used varied in extraction conditions, for example the use of temperature higher than room temperature for drying the extracts, or washing-elution conditions with a specific pH of the mobile phase.

The objective of this study was to investigate the effect on the iron-binding activity of honeydew honey solution of varying temperatures and pH values that are commonly applied in experimental work for antioxidants. The influence of temperature and pH on the stability of iron-binding antioxidants can be distinguished experimentally by exposing the honey solutions at an indicated temperature and pH for a period at least longer than the chromatography and processing time. The information gained from this study would help in choosing suitable experimental conditions to prevent the loss or degradation of the antioxidants during the isolation and purification procedures.

4.2 Methods

4.2.1 Sample preparation

Five grams of the honeydew honey (HD20) was dissolved in 50 ml of purified water to give a solution of honey that was 0.1 g/ml. The honey solution was stirred with a magnetic stirrer to get a homogeneous solution.

4.2.2 The temperature effect

Samples of honey solution (10 ml) prepared as described in Section 4.2.1 were individually placed in 25 ml glass bottles and incubated in the water bath (Julabo PC, Life Technologies) at 40°C, 70°C and 90°C. Samples of 2 ml of each of these were removed after 5, 20, 40, 60, and 120 min and quickly chilled in a container filled with ice cubes and then subjected to iron-binding assay immediately. The temperatures were chosen considering the standard temperatures mainly used in the chromatography and processing procedures. Control samples were prepared in the same way without temperature treatment (left at room temperature at 14°C) and tested for antioxidant activity at the same time as the samples that had been incubated. All tests and assays were run in duplicate.

4.2.3 The pH effect

Honey solutions prepared as described in Section 4.2.1 were made acidic (pH 1 and 2) or alkaline (pH 8, 9, 10, 11, 12 and 12.6) by addition of 1.0 mol/l hydrochloric acid or 1.0 mol/l sodium hydroxide solution. The iron-binding activity in both acidic and alkaline pH was determined after standing at room temperature (14°C) for 5, 45 and 70 min. Honey solution without pH adjustment was used as a control. Its pH was 5.2 ± 0.06 .

After 70 min, each of the acidic and alkaline honey solutions was titrated back to its original pH (approximately pH 5.2) with 0.1 mol/l sodium hydroxide or 0.1 mol/l hydrochloric acid to see whether the activity is reversible. The iron-binding activity was measured immediately after each time period (5, 45, 70 min), and after titration back to the original pH of the honey.

To make sure that the activity came totally from antioxidants in treated honey, and not from the changes of assay pH, blank samples were prepared at the same studied pH. Deionised water (50 ml) was made acidic by adding of 1.0 mol/l hydrochloric acid until the pH meter showed the desired pH (pH 1 and 2). A similar approach also was used to make blank samples at the desired alkaline pH by adding 1.0 mol/l sodium hydroxide into deionised water (50 ml). The blanks were not titrated back to pH 5.2 or pH 7.0. The iron-binding activity for blank

solutions was measured. All tests and assays were run in duplicate. The blank solution tests were run in triplicate.

4.3 Results and discussion

4.3.1 Effect of temperature on the iron-binding antioxidant activity of honeydew honey solution

To measure the stability of the iron-binding antioxidants with elevated temperature, honey solutions were incubated at 40°C, 70°C and 90°C for 5, 20, 40, 60, and 120 min, and compared with a non-heated honey solution (control). The iron-binding activity in honeydew honey after exposure to different temperatures is shown in Figure 4.1. It can be seen that temperature affects iron-binding activity of honeydew honey. Half of the activity was lost in 2 hours at 70°C and most of the activity was lost in a very short period of time at 90°C. It could be noted that honey solutions heated at 40°C retained high activity constantly throughout 120 min, but the activity was a bit lower than in the control. This was in agreement with Decker *et al.* (2005) that some antioxidant compounds such as phenolic compounds are stable at temperature below 60°C.

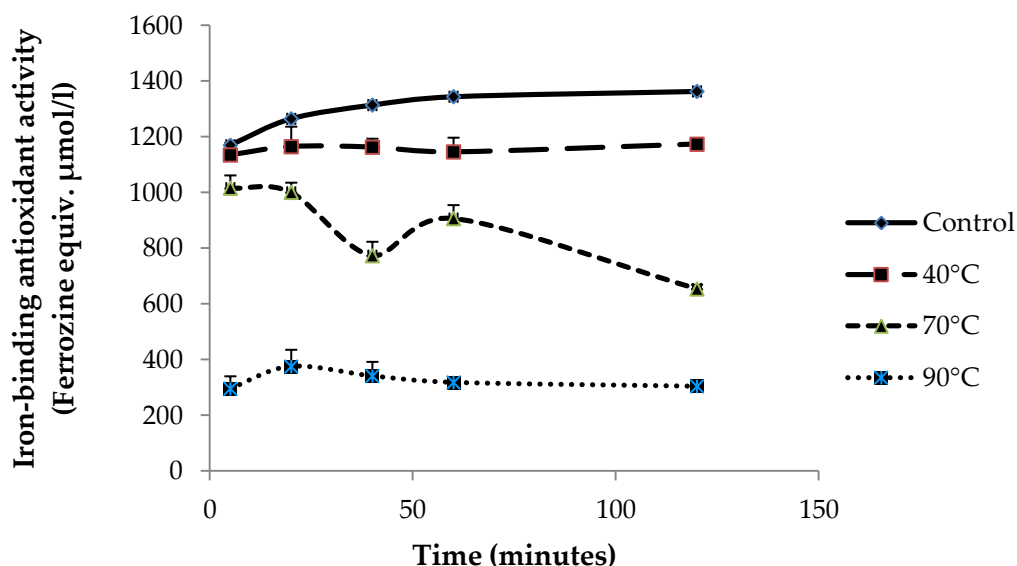


Figure 4.1: The effect of exposure to elevated temperature for various periods of time on iron-binding antioxidant activity of honeydew honey solutions. Honey solution without heat treatment was used as a positive control. Results are means \pm standard error of mean of two determinations.

On the other hand, the decrease in activity was more noticeable in honey solution heated at 90°C than those at 40°C and 70°C, where 63% of the activity was lost at 90°C in 5 min. Loss of activity at 70°C and 90°C could be due to the partial degradation or completely loss of certain iron-binding antioxidants at these temperatures. The vulnerability of antioxidants at high temperature has been documented. Reblova *et al.* (2012) found that antioxidant activity of some phenolic acids decreased with increasing temperature.

Rohn *et al.* (2007) showed that glycosides of quercetin in onions were prone to degradation under high temperature (180°C), which might due to the changes of the glycone structure. Different types of flavonoid glycosides have been detected in some floral honeys produced by either *Apis mellifera* or *Melipona sp.* honey bees (Truchado *et al.*, 2008; Truchado *et al.*, 2009; Truchado *et al.*, 2010; Truchado *et al.*, 2011). Even though the authors never tested the isolated glycosides for any metal-binding activity, recent finding by Sivasothy *et al.* (2012) revealed that a dimeric flavonol glycoside (Spectaflavoside A) isolated from the rhizomes of *Zingiber spectabile* is a potent iron-binding agent. This finding at least supports the ability of flavonoid glycosides to bind iron.

Hamama & Nawar (1991) studied the effect of heating on synthetic phenolic antioxidants, namely butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ), and found that the TBHQ underwent extensive degradation when heated at 185°C for 60 min, and gave rise to numerous decomposition products. The authors also found that the concentration of the volatile antioxidant compounds studied declined after 50 min of heating at the same temperature. This finding should be of concern since honey contains volatile compounds (Soria *et al.*, 2005) that have potential in contributing to the antioxidant activity (Goff & Klee, 2006).

Several proteins and peptides also are known to be heat sensitive (Abu-Jdayil *et al.*, 2002; Korhonen *et al.*, 1998). Lyophilized crude protein extracts of white beans subjected to a temperature of 121°C for 20 min reduced the ferrous-binding capacity almost 2.3-fold (Arcan & Yemenicioglu, 2007). The authors suggested that the conformational changes of the protein due to high temperature had taken

place and reduced the accessibility of metal-chelating groups to ferrous ions in white bean proteins.

It also should be noted that at temperatures of 70°C and 90°C the formation of Maillard reaction products (MRPs) was likely to have occurred in the solutions by the fact that the honey solutions turned to brown in colour. MRPs are reported to form when honey is treated at higher temperature (Antony *et al.*, 2000b; Turkmen *et al.*, 2006). In fact, MRPs (*i.e.* melanoidins) are known to have an iron-binding activity (Kim & Lee, 2009; Maillard *et al.*, 2007; Rufian-Henares & de la Cueva, 2009). Therefore, it is suggested that the formation of different types of MRPs at 70°C and 90°C in the present study may have occurred, which did not have significant iron-binding antioxidant activity due to the fact that the activity did not increase in the honey solutions treated at 70°C and 90°C.

However, it should be clearly borne in mind that MRPs are complex compounds that form under complex reactions. The reactions depend on the types of sugars and amino acid residues present (Maillard *et al.*, 2007; Morales *et al.*, 2005), reaction temperature (Benzing-Purdie *et al.*, 1985) and time (Kim & Lee, 2009), thus giving different level of activity (Turkmen *et al.*, 2006; Yanagimoto *et al.*, 2002). The stability of MRPs formed or the substrates for the formation of MRPs (*i.e.* sugar and amino acids) under different high temperatures also varied (Sang Kim & Soon Lee, 2009). Lan *et al.* (2010) showed that at high temperatures (80-130°C), peptides and amino acids tend to degrade, therefore the formation of MRPs will be less in the reaction mixture. Nevertheless, Nicoli *et al.* (1997) suggested that even though the activity of natural antioxidants will be significantly reduced after exposing to heat, the overall antioxidant properties will be maintained by the development of MRPs.

In the present study, it was evident that the activity observed at 40°C was from antioxidants, and unlikely to be from MRPs. It is because at this temperature and length of heat exposure (120 min), the formation of MRPs will be very slow. This was supported by Benzing-Purdie *et al.* (1985) who also mentioned that a low yield of melanoidins was formed at 22°C in 9 months. The lower in activity at 40°C compared with the control over the same length of time could be due to the oxidation of the antioxidants (Reblova, 2012; Réblová, 2006). Sang *et al.* (2005)

showed that (–)-epigallocatechin-3-gallate (EGCG) oxidized at 37°C under neutral pH and transformed to EGCG dimers and other products.

In general, the results of the study showed that the activity was decreased more extensively with the increasing of the temperatures (90°C > 70°C > 40°C), which indicates that the iron-binding antioxidants in honeydew honey are thermo-labile. It was concluded care needed to be taken in the future work to maintain the temperature less than 40°C to avoid any degradation of the antioxidants.

4.3.2 Effect of pH on the iron-binding antioxidant activity of honeydew honey solution

4.3.2.1 Colour changes in honey solutions

The honey solution before making into acidic or alkaline was initially light yellow in colour. When 1.0 mol/l sodium hydroxide was added into the honey solution, the solution rapidly developed a brownish colour. The intensity of the colour increased with an increase in pH. The honey solution at pH 12.6 was amber-like in colour. In contrast, honey solutions treated with acidic pH (pH 1 and pH 2) did not change their colour.

The colour changes of the honey solutions under alkaline condition could be attributed to the present of chromophore which was altered under alkaline conditions (Chethan & Malleshi, 2007). It has been reported that phenolic compounds such as anthocyanins have an ability to change their colours depending on the pH of the medium (Borkowski *et al.*, 2005; Hurtado *et al.*, 2009; Lapidot *et al.*, 1998). Anthocyanins (Socha *et al.*, 2009) and pigments (Iurlina *et al.*, 2009) exist in honey. (–)-Epicatechin, (+)-catechin and their dimers, epicatechin-(4β-8)-epicatechin and epicatechin-(4β-6)-epicatechin changed their colours when dissolved in simulated intestinal juice (alkaline pH), to become light brown and rapidly turned to dark brown (Zhu *et al.*, 2002). The iron-bound siderophores of the alkaliphilic bacteria (*Candalkalibacillus thermarum*) also were reported to produce an orange pigment colour at pH 9 (McMillan *et al.*, 2010).

4.3.2.2 Iron-binding activity in acidic and alkaline honey solutions

The results of the study of the effect of pH on honeydew honeys are summarized in Figure 4.2. They show that the iron-binding antioxidant activity remained active at all pH values. The highest activity was given by honey solution treated at pH 12.6.

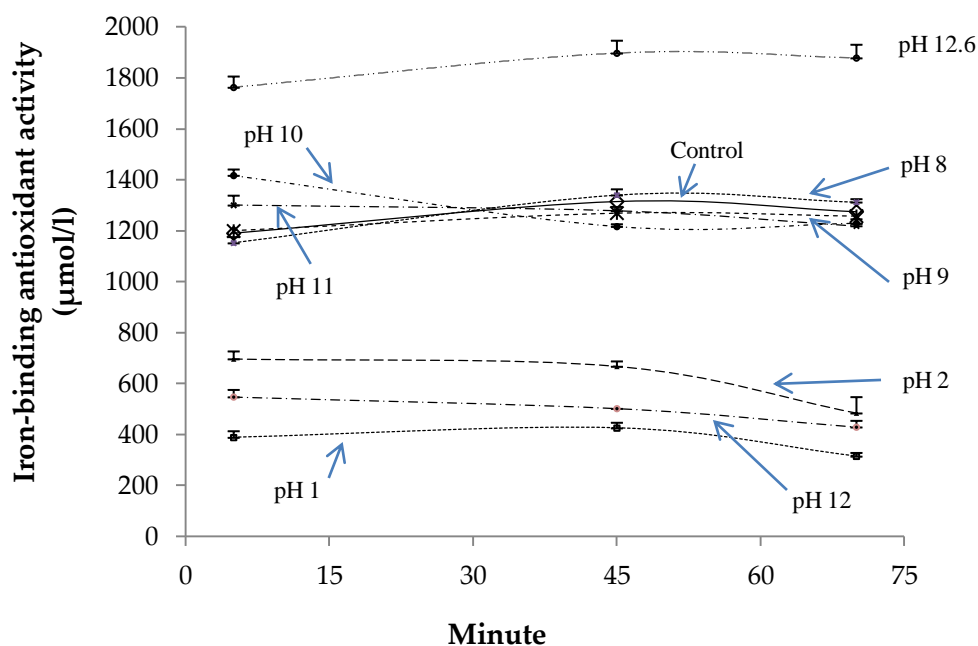


Figure 4.2: The effect of various levels of pH for various periods of time on iron-binding antioxidant activity of honeydew honey solutions. The assays were done at the pH values shown. Honey solution without pH adjustment was used as a positive control. Results are means \pm standard error of mean of two determinations.

It should be noted in the present study that the activity at pH 12.6 was higher than that in the control. This result should be interpreted with caution since the value given at that particular pH may be due to an error in the assay. This was because the pH of the assay was not adjusted back to pH 5 and the assay relied on the buffer to do this, thus there may have been too much alkalinity at pH 12.6 for it to be neutralised.

The pH at which the antioxidant assay is run can no doubt alter the reactivity of the Fe^{2+} ions, therefore it would change the overall mechanism of the antioxidant assays (Decker *et al.*, 2005; Ozcelik *et al.*, 2003; Wong & Kitts, 2001). In this study, the pH plays an important role in the iron-binding assay as the reaction

mixture must be maintained at pH 5.0. This is because Fe^{2+} ions favour low pH for their solubilisation and stability since at high pH (*i.e.* pH higher than neutral pH) the formation of iron hydroxides is likely to occur (Gayer & Woontner, 1956). The oxidation rate of Fe^{2+} ions also occur slowly under acidic pH (Stumm & Lee, 1961). Care should be taken at any stages in the assay in order to maintain the assay pH. Therefore, the buffer at pH 5.0 was preferentially used rather than distilled water in all reagents prepared.

Due to the possibility that the assay pH may be altered when assaying honey solution at pH 12.6, thus giving such a high value for activity, the assay was repeated with the blank solutions. The blank solutions at all studied pH values were tested for activity to make sure that the results given by honey solutions were trustworthy. The blank samples were left for 5 min before assaying for the activity, so that the results were comparable with honey treated at the same pH and time. Deionised water was used as a control and tested in a similar way as with the blanks.

The activity given by blank solutions at similar pH is shown in Figure 4.3. It can be clearly seen that the activity given by blank solutions at pH 12 and 12.6 were higher than with the honey solutions treated at the same pH. The blank solution at pH 1 also gave some iron-binding activity, but at a lower value. Conversely, blank solutions at pH 2–11 and water showed a pro-oxidant effect.

The pH of the mixture of each of the blank solutions and buffer for assay reagents (0.5 mol/l acetate buffer, pH 5) also was determined to get the estimated final pH in the assay. This was done by conducting the model system experiment that mimics the reaction condition in the assay. The proportional volume of the blank sample and buffer (pH 5) similar to the actual volume in the assay for sample and reagents were mixed and left for 10 min (similar to the assay completion time). The pH of the mixture at 10 min was assumed to represent the actual reaction pH in the assay.

It shows that pH 1, pH 12 and 12.6 of the blank solutions have altered the final pH of the assay (Table 4.1). This results at least in concordance with the activity given by blank solutions of these pH values (Figure 4.3). Therefore, it could be

suggested that the changes of the assay pH have altered the overall mechanism in the assay.

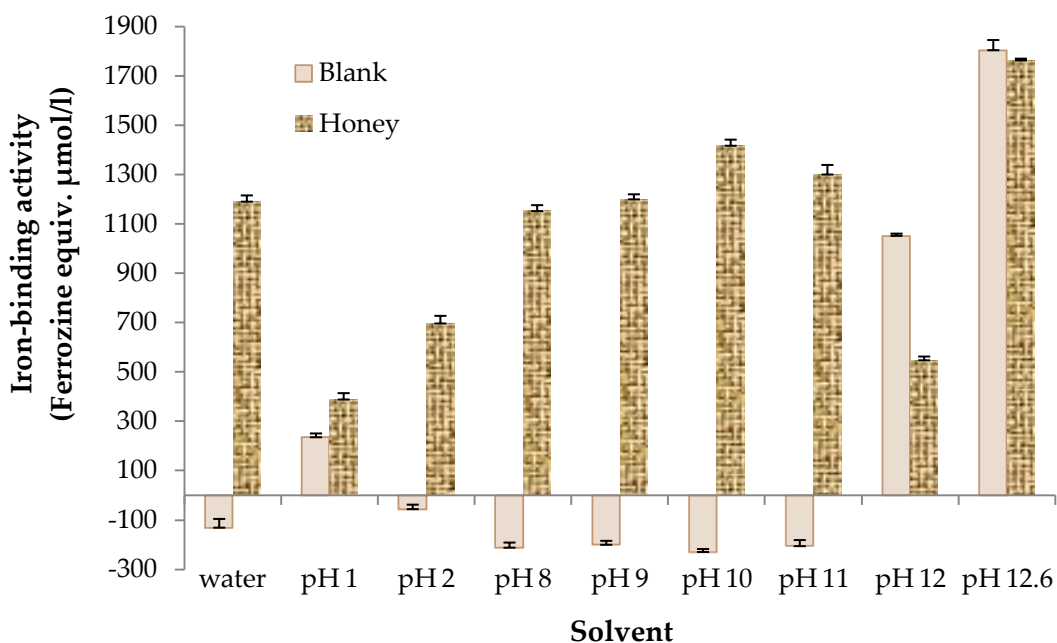


Figure 4.3: The effect of the pH of blank solutions on the iron-binding activity. The results for the water blank at various pH values were compared with those honey solutions treated at the same pH. Water and honey solution without pH adjustment were used as a positive control for blanks and honey-treated pH. Results are expressed as mean \pm standard error of mean of two determinations.

Table 4.1: Final pH of the blank mixture in the model system of the iron-binding assay. Results are expressed as mean \pm standard error of mean of three determinations.

pH	
Solution	Final pH in the assay
1.0	4.9 ± 0.14
2.0	5.04 ± 0.1
8.0	5.00 ± 0.01
9.0	5.01 ± 0.08
10.0	5.02 ± 0.05
11.0	5.03 ± 0.07
12.0	5.15 ± 0.05
12.6	12.14 ± 0.04

The high iron-binding activity given by blank solution at pH 12.6 could possibly be due to the formation of ferrous and/or ferric hydroxides in the assay occurring when sodium hydroxide was added (Cooper, 1937; Gayer & Woontner, 1956; Leussing & Kolthoff, 1953). Therefore, the activity given by the blank at pH 12.6 could have actually been from the formation of Fe^{2+} and/or Fe^{3+} hydroxides complexes in the assay. Nevertheless, the result given by honey solution at pH 12.6 was not comparable to others due to the alteration of the assay pH. The blank solution at pH 1 that gave some activity value may be due to the small changes of pH in the assay from the optimal pH for reaction (Welch *et al.*, 2002a).

The negative value given by blank solutions at pH 2–11 and deionised water indicates that pro-oxidant effect occurred in the assay (Figure 4.3). Theoretically, blank solutions at pH 8–11 should promote the formation of iron hydroxides in the assay as discussed above, but there was no activity observed in the blanks at these pH. They should have formed hydroxides of iron, however, the equilibrium process of iron ions is known to occur more slowly in acidic pH (Stumm & Lee, 1961), hence the formation of iron hydroxides is slow. This is concordance with the final pH in the assay for these blank solutions was not altered and maintained at optimum pH of the assay (Table 4.1).

Therefore, the pro-oxidant effect at these pH could be explained by the oxidation of Fe^{2+} ions (Welch *et al.*, 2002a; Yang & Chasteen, 1999). Welch *et al.*, (2002a) studied the effect of pH on the oxidation of Fe^{2+} ions, and found that the oxidation of Fe^{2+} ions increases as the pH of the solution was increased. However, the author's studies only limited at pH 6.5–7.5, therefore the effect of alkaline pH on the Fe^{2+} ions oxidation was unknown. Apparently, by looking at the level of pro-oxidant effect at pH 8–11, which increased as the pH become more alkaline, therefore it could be suggested partially that oxidation occurred and in agreement with Welch *et al.*, (2002a). The negative value given by water and pH 2 could be due to the oxygen ligands that bind to iron, which in theory promotes the oxidation of Fe^{2+} ions.

For the purpose of this study, only honey solutions treated at pH 2–11 were comparable to each other as far as the pH of the assay is concerned, hence it could be suggested that the results shown by those honey solutions at these pH values

(Figure 4.2) were genuine and could be due to the antioxidants. It shows that the activity was stable throughout pH range of 8–11 within the test period (70 min), indicating that the antioxidants were stable at alkaline pH. The activity at alkaline pH also was slightly higher than activity given by the control (honey solution without the pH altered). Honey solution at pH 2 also gave activity, but at lower level.

The stability of the antioxidant activity of phytochemicals in alkaline medium has earlier been reported. Azizah *et al.*, (1999) noticed that the radical scavenging activity from cocoa products was stable at pH 9 and 11. Arabshahi-D *et al.*, (2007) also showed that radical scavenging activity from mint leaves and carrot were higher at pH 9 than pH 4, indicating that the antioxidants were stable at pH 9. Kumamoto *et al.*, (2001) reported that catechins were stable at pH ranging from 6 to 12, which gave higher antioxidant activity.

4.3.2.3 Reversibility and irreversibility of iron-binding activity

The reversibility of iron-binding activity in honey treated with acidic (pH 2) and alkaline (pH 8–11) pH after 70 min of treatment was determined. The length of time (70 min) is more than enough time for the equilibration of ionization to be achieved. When the pH of acidic or alkaline honey solutions was titrated back to the natural pH of honey (pH 5.2) with 0.1 mol/l sodium hydroxide or 0.1 mol/l hydrochloric acid, the activity was found to be reversible (Figure 4.4). However, the activity did not reach the same value as given by control. This could be due to the structural change of some iron-binding antioxidants related to pH, which disabled them to accept the proton (H^+) from the acid (Jurd & Geissman, 1963).

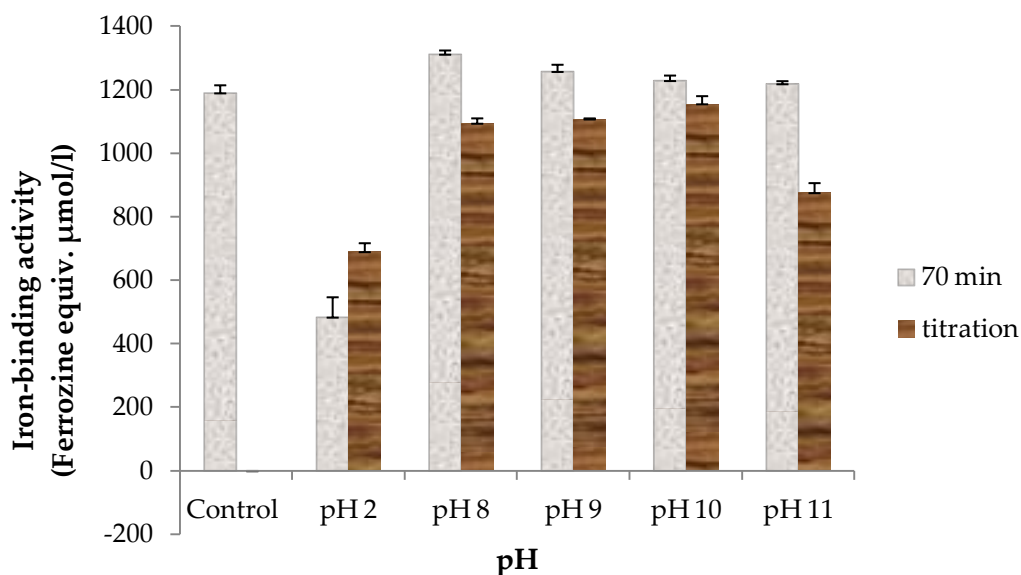


Figure 4.4: The reversibility of the iron-binding activity of honeydeew honey solutions after adjusting the pH of the honey to various values then restoring the pH to the original pH of honey (pH 5.2). Honey solution without pH adjustment was used as a positive control. Results are means \pm standard error of mean of two determinations.

The activity was higher when honey solution at pH 2 was titrated to pH 5.2 than it was at pH 2. It was assumed that at pH 2, the ionization of carboxylic acid and phenol groups was suppressed. At pH 5.2, carboxylic acid group starts to ionize, therefore giving rise in the iron-binding activity.

Honey solutions at pH 8–11 returned their colour back to the colour of honey control. Iron-binding activity in honeydeew honey can be recovered from acidic or alkaline pH to the pH of honey most probably due to the reversible reaction that involves deprotonation or protonation of the molecules (Glazer & Smith, 1961; Tanford & Roberts, 1952). Ferulic acid at pH 11 regenerates its original spectra when neutralized to pH 7 or 9 (Friedman & Jurgens, 2000).

4.4 Conclusion

The effect of temperature and pH on the iron-binding activity of honeydeew honey was investigated as the preliminary information of the stability nature of iron-binding antioxidants. The results showed that the activity was decreased with increasing temperature, indicating that the iron-binding antioxidants were thermo-labile. It is interesting to note that the iron-binding antioxidants were stable at

alkaline pH (pH 8–11). The data proves that the iron-binding antioxidants were not destroyed totally by altering the pH from alkaline to acidic pH, as the activity, even though not 100%, could be recovered once the pH was returned to the pH of the honey.

The results of the present study of the effect of pH also emphasize the importance of controlling the pH of the assay. The change of pH in the assay medium due to the addition of sample with high or low pH which is not similar to the assay pH, will lead to the mistaken conclusion that the honey solution treated at such pH, and not the pH changes in the assay, is responsible for the antioxidant activity. Therefore, to avoid any meaningless conclusion of the results when involving pH of the sample solution, the results must be compared with the blank solution at the same pH that is used for the extraction.

This finding is useful to understand the behaviour of the iron-binding antioxidants aimed to be isolated throughout this research project with regard to temperature and pH, so that the analysis work could be planned based on this finding. It was concluded that chromatography analysis under low and high pH is feasible.

CHAPTER 5

EXTRACTION OF IRON-BINDING ANTIOXIDANTS FROM HONEYDEW HONEY USING AMBERLITE[®] XAD-2 RESIN

5.1 Introduction

Many researchers have claimed that flavonoids or other phenolic compounds might act as antioxidants in honey. The dual properties of phenolic compounds as both radical scavengers and metal chelators (Rice-Evans *et al.*, 1997) has raised interest in isolating the compounds from many plants. One of the traditional techniques to isolate phenolic compounds from a sample matrix is by using liquid-liquid extraction or solvent extraction. This technique is easy to perform, however problems arise when the extraction of the phenolic compounds using this technique is interfered with by sugars that exist in high concentration relative to phenolics in the crude samples that could be co-extractive in certain cases.

Alternatively, another technique that has been widely used is solid phase extraction (SPE). This technique is said to eliminate several disadvantages of liquid-liquid extraction that have been previously encountered. Solid phase extraction is an adsorptive technique. Its mechanism of retention is due to reversible hydrophobic, polar and ionic interactions between analyte and the adsorbent (Fontanals *et al.*, 2007). Common adsorbents such as silica coated with a C18, hydrocarbon macroporous polymers or carbon are widely employed depending on the suitability between analyte and the adsorbent on the efficiency of the extraction process.

Amberlite[®] XAD-2 is a non-ionic, macroporous polymeric adsorbent which possesses a large surface area and is widely used in the SPE technique. Unlike with other adsorbents such as C18-coated silica that can be loaded in a small cartridge with a small amount of resin (0.1–0.5 g), a glass column is preferred with XAD-2 to perform extraction due to the high mass of the XAD-2 resin that needs to be used. The mechanism of adsorption of XAD-2 is through the

hydrophobic interaction with the resin (Kennedy, 1973). In addition, pi-pi (π - π) bonding between aromatic ring(s) in the compounds with the aromatic ring in the polystyrene structure plays a role in the retention as well (Fontanals *et al.*, 2007).

It has been found that high recoveries of phenolic compounds, particularly flavonoids from plant matrices, are possible when these are extracted with Amberlite XAD-2 resin (Gray, 1978). Ferreres *et al.* (1991) carried out several extractions with XAD-2 to isolate flavonoids from honeys and reported that the technique successfully recovered flavonoids to a degree of more than 90% in most cases. Since then, the use of XAD-2 has become the most widely used technique for extracting flavonoids in honey (Ferreres *et al.*, 1996; Ferreres *et al.*, 1993; Ferreres *et al.*, 1994; Ferreres *et al.*, 1991; Martos *et al.*, 1997; Martos *et al.*, 2000). XAD-2 resin has been shown to be an effective adsorbent for removal of the flavonoids from plant extracts and foods rich in sugars regardless of whether flavonoids are in their aglycone or glycosides form (Tomás-Barberán *et al.*, 1992). The XAD-2 technique also has been applied with success in food matrices with a high sugar content such as fruit jam (Silva *et al.*, 2000; Tomas-Lorente *et al.*, 1992) and fruit purees (Andrade *et al.*, 1998). The efficiency of this technique has been presented by many researchers in isolating phenolic compounds, particularly flavonoids, from the plant matrix.

The aim of this section of work was to isolate the component(s) of honeydew honey that could be responsible for the iron-binding antioxidant activity, using XAD-2 resin in the expectation that they would be phenolic molecules.

5.2 Materials and methods

5.2.1 Preparation of the resin

Amberlite[®] XAD-2 (porosity 9 nm, particle size 0.3 – 1.2 mm; 100gm) was purchased from Supelco (Bellefonte, USA). The resin was soaked in 50% aqueous methanol overnight before use. The resin slurry was then loaded into a glass column (2.5 cm internal diameter x 45 cm length) to give a bed height of 13 cm (approximately 70 ml of bed volume). The excess solvent was run out of the column until the resin was settled, then the resin was covered with solvent to

about 1 cm above the top of the resin. This was done to allow space for when the resin swelled throughout the procedure. After standing for 1 hour, the resin was washed with many bed volumes of distilled water to remove the 50% aqueous methanol.

5.2.2 Fractionation of honey on Amberlite® XAD-2 resin

There are no published standard procedures in term of resin:honey ratio, solution volume for washing steps and elution volume for extracting phenolic compounds using XAD-2 resin. For this work, the honey sample (20 g) was mixed with acidified water pH 2 (adjusted with HCl) (100 ml) until it was totally fluid. The homogenate was filtered through Whatman No.1 paper to remove any solid particles, and honey solution (70 ml) was passed through the column filled with Amberlite XAD-2 that has been prepared earlier. The flow rate used was 1 ml/min. The first 70 ml of solution that eluted off from the column was discarded. This was the distilled water that was the void volume. By this time, the honey solution was fully loaded onto the column. Then, the column was washed with 70 ml of acidified water pH 2 to remove the sugars and non-adsorbed organic compounds. During this stage, effluent was collected (approximately 70 ml). Then, distilled water (70 ml) was used to further remove any polar compounds. All washings fractions (with acidified water pH 2 and distilled water) were collected (approximately 70 ml of each washing step). The adsorbed phenolic compounds were eventually eluted with methanol (200 ml) and dried under vacuum at 35°C. The fraction eluted with methanol was re-dissolved in deionised water (70 ml) to get the same volume relative to the honey solution used (70 ml). All fractions were tested for iron-binding antioxidant activity in the same volume relative to the volume of honey solution loaded onto the column. The activity was expressed as Ferrozine equivalent ($\mu\text{mol/l}$). The extraction flow is summarized in Figure 5.1.

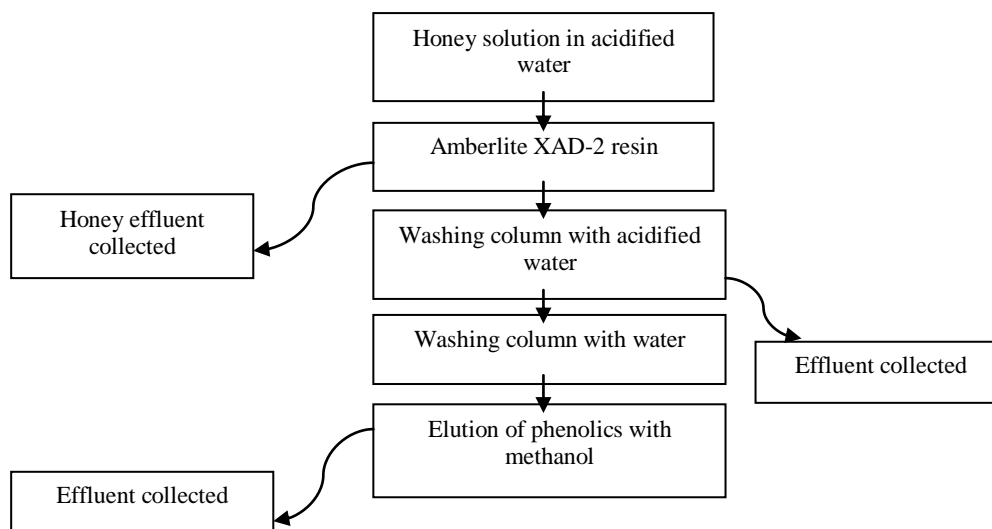


Figure 5.1: Flow chart for extraction of phenolic compounds from honey using XAD-2.

5.2.3 The effect of sample size on the extraction of iron-binding antioxidant activity

To see whether this technique could extract more phenolic compounds when honey mass was increased, the procedure was repeated as previously described in Section 5.2.2, but with 50 g of honey.

5.2.4 Extraction of iron-binding antioxidant activity under alkaline condition

Honeydew honey (20 g) was homogenised with 50 mmol/l Tris-HCl buffer, pH 9, (100 ml) until totally fluid. The homogenate was filtered through Whatman No.1 paper to remove any solid particles, and the alkaline honey solution (70 ml) was passed through a column filled with Amberlite XAD-2 that has been prepared as described earlier. The flow rate used was 1 ml/min. The first 70 ml of solution that eluted off from the column was discarded. This was the 50% aqueous methanol. At this time, the honey solution was fully loaded onto the column. Then, the column was washed with 70 ml of 50 mmol/l Tris-HCl buffer, pH 9 (70 ml). During this stage, honey effluent was collected (approximately 70 ml). The column was then eluted and the fractions were tested as described in Section 5.2.2.

5.3 Results and discussion

5.3.1 Fractionation of honey on XAD-2 resin

It was found that iron-binding compound(s), presumably flavonoids, had not been extracted from honeydew honey solution by means of solid phase extraction using Amberlite® XAD-2 resin as adsorbent. Figure 5.2 shows the iron-binding antioxidant activity in each fraction. As can be seen from these results, the methanol fraction did not show any iron-binding antioxidant activity. This prompted investigation of whether the sample size used could affect the extraction efficiency since the phenolic compounds in honey are present in a very low amount. Tomas-Barberan *et al.* (1992) also suggested increasing the amount of flavonoids standard in order to recover more flavonoids from XAD-2. Therefore, 50 g of honey was used and the extraction was repeated as described in Section 5.2.2. Figure 5.3 shows the iron-binding antioxidant activity for each fraction when the amount of honey loaded was increased to 50 g.

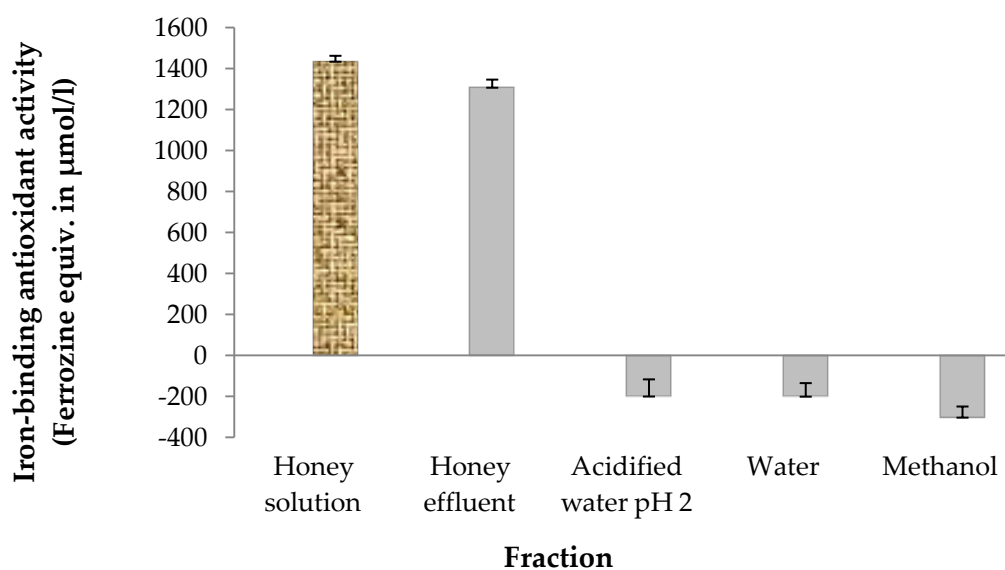


Figure 5.2: The iron-binding antioxidant activities of the fractions from the XAD-2 column chromatography extraction. For comparison, the activity of the honey solution loaded on the column is shown. Results are means \pm standard error of mean of two determinations.

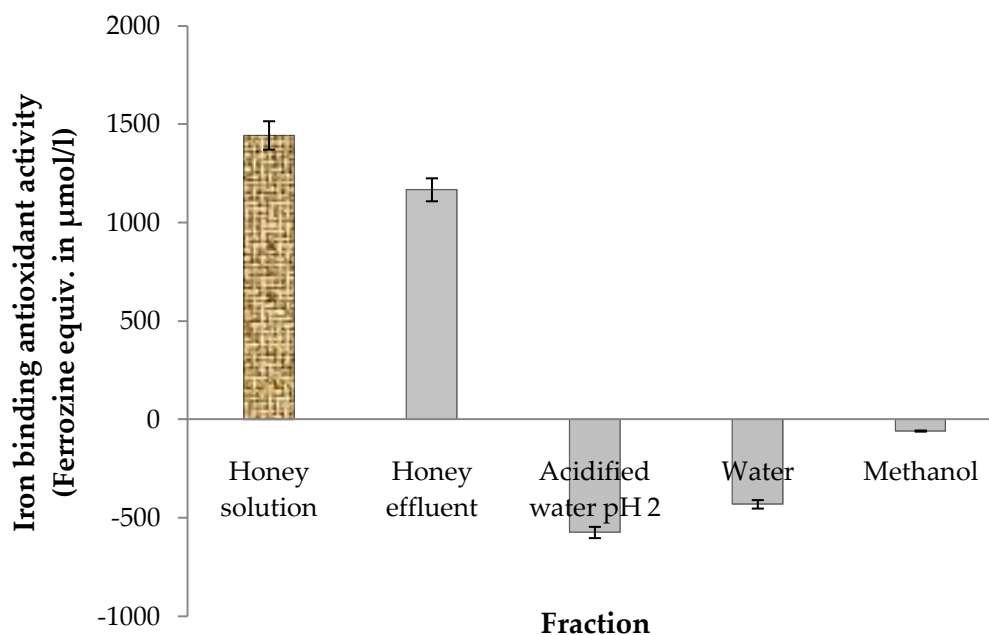


Figure 5.3: The iron-binding antioxidant activities of the fractions from the XAD-2 column chromatography extraction. For comparison, the activity of the honey solution loaded on the column is shown when honey mass was increased (50 g). Results are means \pm standard error of mean of two determinations.

Both honey loading sizes (20 g and 50 g) showed a similar trend of iron-binding antioxidant activity being only in the non-retained fraction. These results are in agreement with those of Deadman (2009) who found that the sample size did not affect the recovery of honey phenolics from XAD-2 resin. Interestingly the effluent collected whilst honey was being loaded contained a large percentage of the iron-binding antioxidant activity (approximately 95% in 20 g honey and 81% in 50 g honey). This suggested that the inability of the resin to adsorb the iron-binding antioxidant(s). Even though the iron-binding antioxidant(s) could have been lost from the column during the washing and rinsing steps, the results show that no activity was observed in both washings fractions. These results clearly indicate that iron-binding antioxidant(s) in honeydew honey simply did not adsorb on XAD-2 resin and that any phenolic compounds that eluted off from the column with methanol were not the compounds which are responsible for the iron-binding antioxidant activity. The negative values obtained from these in the assay show that the compounds which adsorbed to the XAD-2 resin have a pro-oxidant action.

Ice & Wender (1952) have pointed out that failures in flavonoids extraction with XAD-2 resin were due to (i) chemical interactions of the flavonoids with the resin,

(ii) lack of adsorption, and (iii) inability to find proper adsorbents and solvents for separation of closely related compounds. The later point seems not to be a factor in the failures of the present study as the choice of using XAD-2 resin was supported by extensive successful recovery of phenolic compounds from honey and other plants matrices from the literature. In addition, the successful extraction of phenolic compounds in the present study was indicated by the fact that a yellow fraction was eluted with methanol. However, it should be stressed that this extracted phenolics fraction was not the one that gave the iron-binding antioxidant activity.

The purpose of diluting honey with acidified water at pH 2 was to suppress the ionisation of the functional groups in the phenolics structure. The undissociated form of phenolic compounds will make the compounds more hydrophobic and thus better retained on the resin. This is of importance since Pietrzyk *et al.* (1978) have shown that ionisation could affect the retention of weak acids on the XAD-2 resin. However, in a complex matrix like honey which contains various different compounds with different chemical properties, those compounds cannot be extracted by using a single condition. Thus, the use of acidic water to solubilise honey has probably made some of the antioxidant compounds other than phenolics undergo ionisation. For example, in compounds that have an amino group, such as peptides or proteins, the amino functional group may become protonated (gets a positive charge) at pH 2. Therefore, the ionisation has made these compounds more polar and thus not retained on the column (Pietrzyk *et al.*, 1978). Even though most honey researchers suggest that phenolic compounds play an important role in antioxidant activity of honey, there have also been suggestions that amino acids could contribute to this activity (Meda *et al.*, 2005; Perez *et al.*, 2006).

Another explanation for this behaviour on extraction with XAD-2 at pH 2 is that possibly the iron-binding antioxidant(s) exists in a glycoside form in this particular honey. It is known that phenolic compounds commonly occur in a glycoside form in plants, which makes them more hydrophilic than aglycone phenolics (Blasa *et al.*, 2007; Jagota & Cheatham, 1992). The glycoside form would be less well retained on XAD-2 resin because of a lack of hydrophobicity. The work of Truchado *et al.* (2008) has demonstrated that XAD-2 resin was not

the appropriate technique in extracting the polar glycosides of flavonoids in acacia honey. The authors suspected that the hydrophobicity of the resin could be the cause of the extraction inefficiency. As an alternative, the author used C-18-coated silica which is known to be less hydrophobic than XAD-2 resin because it exhibits more hydrophilic characteristic through the silanol groups.

Results from the literature are apparently contradictory regarding the efficiency of XAD-2 to retain glycosides. The earlier work of Tomas-Barberan *et al.* (1992) showed that the chemical property of XAD-2 resin did not affect the recovery of aglycones and glycosides flavonoids from aqueous extracts in a significant manner, even though the percentage recovery of the aglycone quercetin (95%) was always higher than its glycoside form, rutin (70%). The results of Chassagne *et al.* (1996) were in agreement with those of Tomas-Barberan *et al.* (1992). Chassagne *et al.* (1996) concluded that XAD-2 resin was an efficient technique in extracting cyanoglycosides (prunasin and amygdalin) with high recovery yield of 99% and 89%, respectively. Sekiwa *et al.* (2000) also succeeded in extracting two glycoside compounds from ginger with XAD-2 resin. In contrast, Williams *et al.* (1995) found that XAD-2 resin retained only less polar glycosides from wines and fruit juices. This could suggest that the iron-binding antioxidant(s) in honeydew honey are glycosides with very polar characteristics if they are phenolics.

To find out whether the compounds with amine groups may contribute to the iron-binding antioxidant activity, the experiment was repeated using 50 mM Tris-HCl, pH 9 buffer instead of acidified water. This was possible since XAD-2 resin is stable at a wide range of pH values. The amount of honey used was 20 g and the procedure was repeated in duplicate as described in Section 5.2.4. The results are shown in Figure 5.4.

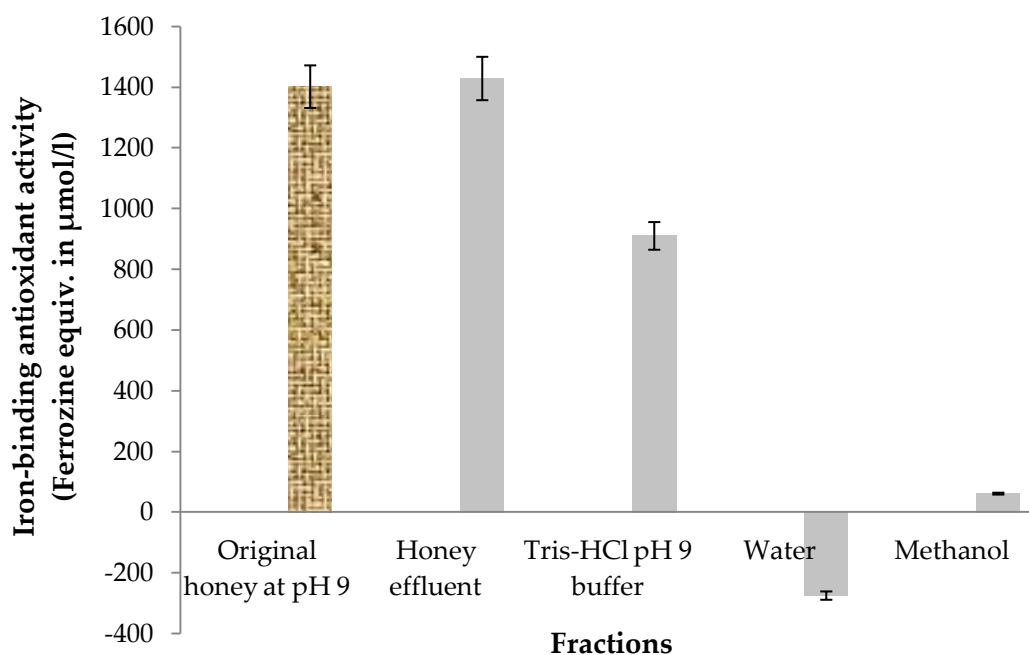


Figure 5.4: The iron binding activities of the fractions from XAD-2 column chromatography extraction at pH 9. For comparison the activity of the honey solution loaded on the column is shown. Results are means \pm standard error of mean of two determinations.

The results show that iron-binding antioxidant activity was still high in the honey effluent, however, there was a low activity observed in the methanol fraction (approximately 4% of activity). The methanol fraction could possibly contain amine compounds since the greatest retention of the amine on the XAD-2 resin occurs in basic solution where the unionised amine is present (Chu & Pietrzyk, 1974).

The buffer fraction during the washing step also showed some activity. This fraction was expected to contain sugars and organic acids which were eluted off from the column during washing with buffer. These compounds are known to ionise well at pH 9. Organic acids such as citric acid and malic acid are known to have binding affinity to iron (Cheng & Crisosto, 1997) due to the carboxylic acid group in their structure. In addition, carboxylic acid has been reported preferentially to bind iron in ionised form (Carrell *et al.*, 1988). It is interesting to note that nevertheless Tris-HCl buffer does not bind to iron (Andjelkovic *et al.*, 2006).

The slight difference in activity observed between honey solution (at pH 9) and its effluent could possibly be due to the fact that an antagonistic effect occurred between components (Grael *et al.*, 2005; Hidalgo *et al.*, 2010; Meyer *et al.*, 1998; Peyrat-Maillard *et al.*, 2003) in the honey solution which reduced the activity. This was due by the fact that 1429 $\mu\text{mol/l}$ of activity was eluted when only 1402 $\mu\text{mol/l}$ was loaded. When honey was run through the XAD-2 resin, some molecules were adsorbed, some were not adsorbed, and some weakly bound molecules would be retarded. These weakly bound molecules would elute in the buffer fraction, thus separating these antagonist molecules from the entire honey solution. This could explain at least in part why the activity in honey effluent was slightly increased after the adsorption process.

5.4 Conclusion

The present results suggest that Amberlite[®] XAD-2 resin which has been regarded as an efficient technique to extract phenolic compounds from honey was not successful in extracting the iron-binding antioxidants from honeydew honey. The phenolic compounds extracted did not show any iron-binding antioxidant activity. This could be due to conjugation of the iron-binding antioxidants to sugar moieties which make them more hydrophilic, thus they did not adsorb on to XAD-2 resin.

The poor recovery of iron-binding activity in the methanol extract when using Tris-HCl, pH 9 buffer may be due to the weak adsorption of iron-binding antioxidants on the resin. However, still most of the activity was in the honey effluent, thus was not retarded or retained by the XAD-2.

CHAPTER 6

ISOLATION OF IRON-BINDING ANTIOXIDANT(S) USING SIZE EXCLUSION CHROMATOGRAPHY

6.1 Introduction

Separation of the iron-binding antioxidant(s) from honey is quite challenging because honey contains various types of compounds. The attempts to separate the antioxidant(s) from the other compounds by the use of XAD-2 resin, described in Chapter 5, were not successful. One of the reasons may be that the abundance of sugars in honey prevented the adsorption of iron-binding antioxidant(s). Therefore, another separation technique had to be attempted in order to isolate the iron-binding antioxidant(s) without any interference from the sugar in honey.

Since honey contains components with a range of sizes, size exclusion chromatography (SEC) was tried as a technique for isolation. It is a kind of separation technique which is controlled by the size and shape of molecules and the pore size and shape of the column matrix (Eriksson, 2002). Small molecules are able to enter the pores of the column matrix and, therefore, take longer to elute, whereas larger molecules are excluded from the pores and elute earlier as shown in Figure 6.1.

SEC has been widely used as a technique to estimate the molecular weight of proteins and peptides (Irvine, 1997; Serpe & Nothnagel, 1995; Whitaker, 1963), biopolymers such as tannins (Gabetta *et al.*, 2000; Shoji *et al.*, 1999) and polysaccharides (Aguirre *et al.*, 2009; Othman *et al.*, 2010). SEC has the advantage that it can separate sample components directly in the aqueous solution without any complicated sample pre-treatment. This is of importance to avoid any denaturation of components that may occur if they were subjected to heat, solvents or extremes of pH.

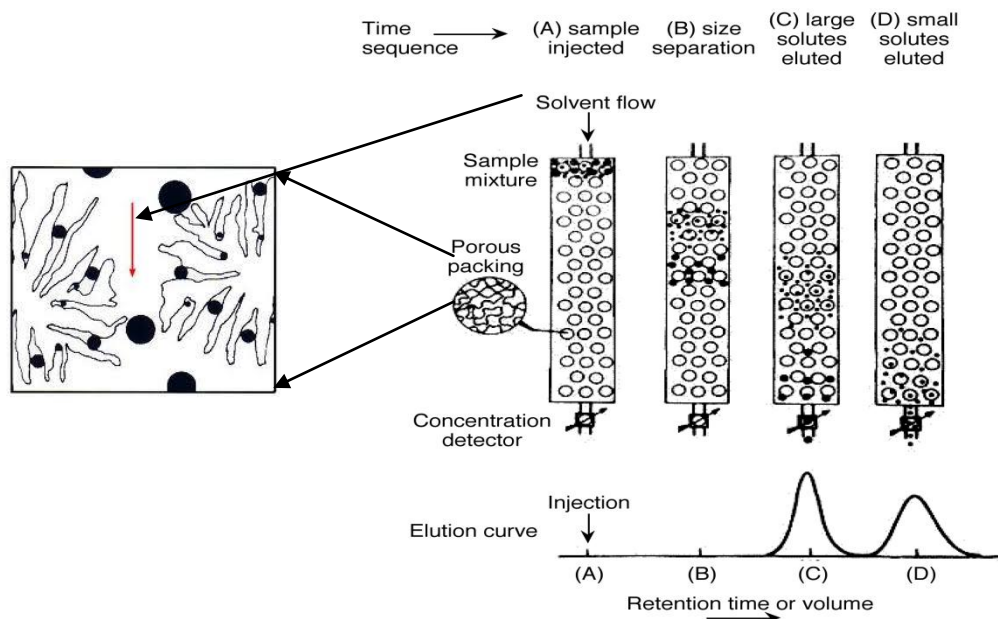


Figure 6.1: Size exclusion chromatography column. Adapted from Striegel *et al.* (2009).

There are very few reports concerning the application of SEC to honey analysis and only a few applied to isolation of components in honey. Astwood *et al.* (1998) and Morales *et al.* (2006) used SEC for determination of oligosaccharides in honey, while Iglesias *et al.* (2006) used SEC to determine the molecular weight of honey proteins. Recently Brudzynski & Miotto (2010b) used SEC and successfully separated honey melanoidins that exhibited a radical-scavenging activity.

The aim of the present work was (i) to separate the honey components according to their molecular sizes using a Superdex[®] Peptide SEC column, (ii) to identify in which fraction(s) the iron-binding antioxidant activity occurred, and (iii) to determine whether the free sugars could be separated from the iron-binding antioxidant(s).

6.2 Materials and methods

6.2.1 Preparation of honey sample

A sample of the honeydew honey, (10 g) was dissolved in water to give a final concentration of 10% (w/v) aqueous honey solution. This concentration was chosen so as to avoid introducing high viscosity of honey solution into the column. Before loading onto the column, the honey solution was filtered using a syringe membrane filter with a pore size of 0.45 μm .

6.2.2 Size exclusion chromatography

A Superdex[®] Peptide column was used with a fractionation range between 100-7000 Dalton. The void volume (V_o) of the column was estimated to be 8.1 ml according to Smyth & FitzGerald (1997). The total permeation volume of the column (V_t) was 24.0 ml. Two mobile phases (water and 30% (v/v) aqueous acetonitrile) were tried for the elution of honey components. A flow rate of 0.5 ml/min was used, with a loading of 200 μ l of honey solution. The chromatography was run at 25°C. The fractions that eluted from the column were monitored at 220 nm, 280 nm and 360 nm which is the absorbance maximum that is characteristic of peptides, proteins, phenolic acids and flavonoids that exist in honey. Fractions of 0.5 ml were collected and these were pooled based on corresponding peaks in the chromatogram. Then, each pooled fraction was brought up to the same volume as the largest pool, using the mobile phase (water or 30% acetonitrile). The pools were then tested for iron-binding antioxidant activity. Also tested was a solution prepared by making up 200 μ l of the 10% (w/v) honey solution (as loaded on the column) to the same volume as the largest pool, using mobile phase. The chromatography and the assay were carried out a further three times.

6.3 Results

6.3.1 Effect of mobile phases on the isolation of honeydew honey components

SEC is considered a mild chromatography technique because the elution can be performed in an isocratic mode, the type of mobile phases being chosen from water or other solvents or buffers depending on the nature and stability of the molecules. However, for this research, since the target was to separate iron-binding antioxidant with unknown chemical characteristics from honeydew honey, the selection of the mobile phase was limited to mild solvents to avoid any denaturation of the iron-binding antioxidants by elution conditions. To determine the effect of mobile phases on the chromatographic separation, SEC was performed with water and 30% acetonitrile.

Figure 6.2 shows the elution patterns on Superdex[®] Peptide obtained for 10% (w/v) aqueous honeydew honey solution eluted with (A) water and (B) 30% acetonitrile.

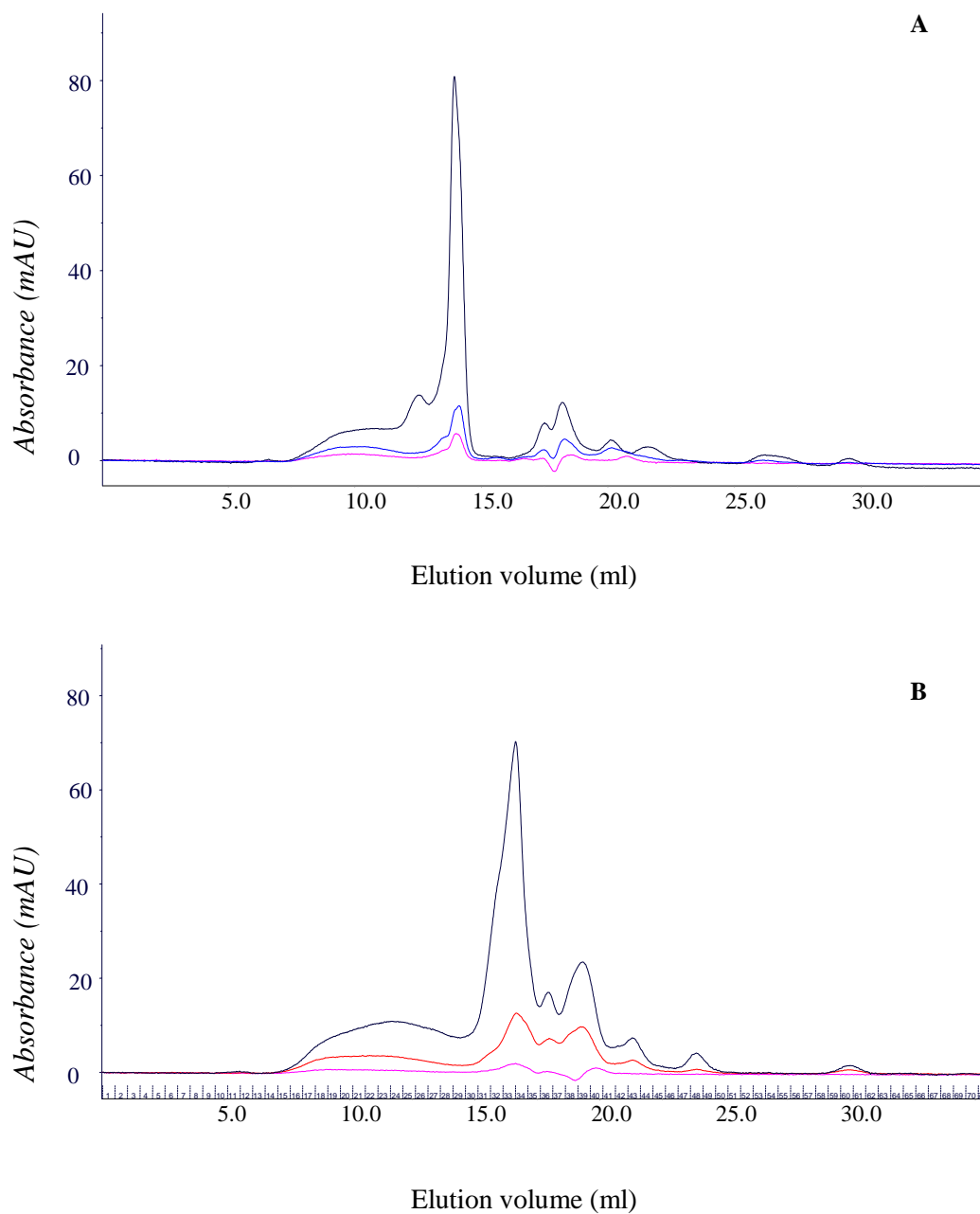


Figure 6.2: Effect of mobile phase on the elution profile of 10% aqueous honeydew honey on Superdex® Peptide. Profiles for honeydew honey eluted in: (A), water; (B), 30% acetonitrile. Upper, middle and lower lines are absorbance at 220, 280 and 360 nm, respectively.

Both eluents gave a similar profile on separating the honeydew honey components, with few unresolved peaks. The chromatograms illustrate the considerable diversity in the distribution of sizes among honey components. With 30% acetonitrile as eluent, the major peak was eluted later (16.5 ml elution volume instead of 14 ml), indicating that there had possibly been some disaggregation of

components of honey caused by this solvent. Elution with 30% acetonitrile also gave a disappearance of the peak eluting at 12.5 ml, and an increase in the size of the peaks eluting in the region of 17-20 ml. Elution with 30% acetonitrile resulted in one late-eluting peak (elution volume: 30 ml), whereas there were two late-eluting peaks (elution volume: 25.5 ml and 28.5 ml) with water as the eluent. These peaks eluted after the bed volume of the column (24.0 ml), thus were presumed to have been retarded by column (24.0 ml), thus were presumed to have been retarded by adsorption to the stationary phase.

6.3.2 Iron-binding antioxidant activity of fractions from size exclusion chromatography

Seventy fractions of 0.5 ml each were collected from the chromatography on Superdex[®] Peptide. The chromatogram was divided into eight fraction regions for assay of iron-binding antioxidant activity as shown in Figure 6.3. The tubes in each region were pooled and the pools were designated as fractions SP1, SP2, SP3, SP4, SP5, SP6, SP7 and SP8.

All eight fractions were made equal in volume before analysis for iron-binding activity. This was done by adding mobile phase to bring each fraction up to the same volume as that of the fraction with largest volume (4.5 ml). The highest iron binding activity was found to be in fraction SP2 followed by fraction SP1, SP3, and SP8 respectively (Figure 6.4). When the activity of these fractions was compared with the activity of the aqueous honey solution loaded on the column, those values were seen to be in total higher than the activity of the honey loaded on the column (335.077 ± 39.735 compared with -252.483 ± 24.557).

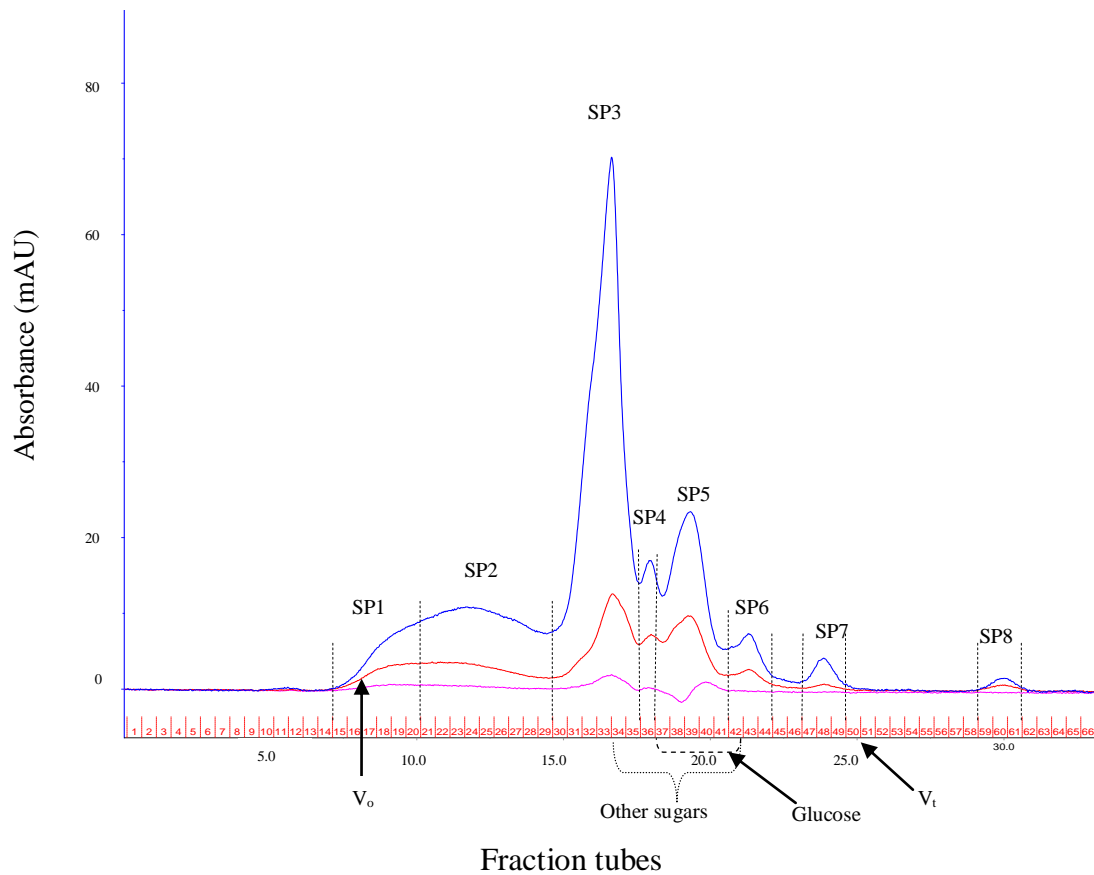


Figure 6.3: Fractions for 10% aqueous honeydew honey solution chromatographed using a Superdex® Peptide column eluted with 30% acetonitrile. Upper, middle and lower chromatograms are UV absorbance at 220, 280 and 360 nm, respectively.

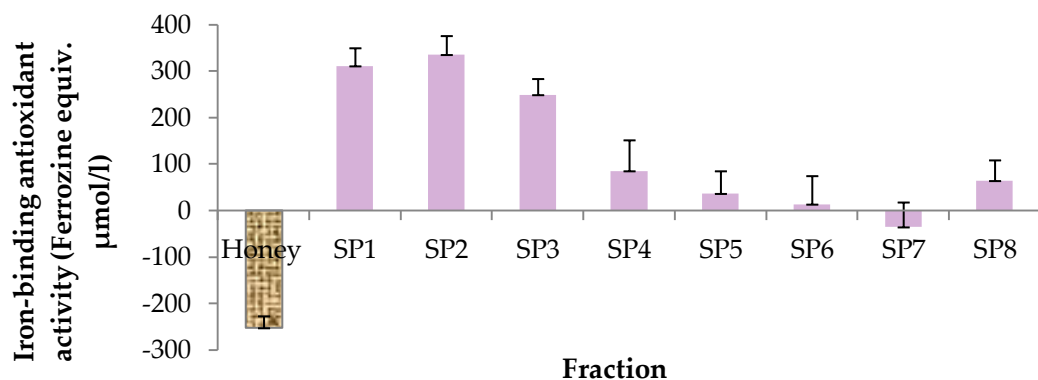


Figure 6.4: The mean ($n=4$) iron-binding antioxidant activity of the fractions collected from Superdex® Peptide, compared with that of the honey loaded on the column (all were made up to the same total volume before assaying the activity). Error bars represent the standard error of the mean.

6.3.3 Location of sugars in the eluted fractions

All individual fractions eluted with 30% acetonitrile were tested for free glucose with glucose oxidase reagent and other sugars with silver nitrate tests (see Sections 3.2.3 and 3.2.4). Glucose was visually detected in fractions 37-42, and other sugars in fractions 34-42, as shown in Figure 6.3. This indicates that the main iron-binding antioxidants had been successfully separated from the sugars in honey.

6.4 Discussion

6.4.1 Aggregation behaviour of fractions from size exclusion chromatography

It was assumed that honey components would present as individual molecules in aqueous solution so that the components could be easily separated by size exclusion chromatography according to their sizes, and then exhibit a single peak for each component. However, Figure 6.2 shows that, regardless of the mobile phases used, SP1 elutes close to the void volume (V_0) of the column. This early-eluting peak is indicative of high molecular weight compounds (Porath & Flodin, 1959). However, this peak is broad as observed for SP1 and SP2, and it was reported that such an elution pattern possibly indicates molecular aggregation (Hatano *et al.*, 2003) and/or interactions between molecules and the column matrix (Arakawa *et al.*, 2010).

Aggregation, will make the size of the resulting molecules become bigger and elute at/or near the void volume as the molecules can only enter a small portion of the internal pores of the gel, or are completely excluded (Philo, 2009). The data shows that SP1 started to elute in the void volume and continued to elute in the fractionation range of the column with the pattern of a broad peak until an elution volume of 14.5 ml. After elution of 14.5 ml, an observed increase in SP3 with almost a single peak could have possibly been due to dissociation of large aggregate that made up SP1 and SP2 from each other. It is suggested that reversible aggregation was occurring, since size exclusion chromatography involves a high dilution of the sample that over the period of time of the elution process will tend to dissociate the reversible aggregates (Philo, 2006). This possibility was explored by re-chromatographing the early and late parts of peak

SP2, *i.e.* SP2A (tubes 21–24 in Figure 6.5A) and SP2B (tubes 25–29 in Figure 6.5B). The results, shown in Figure 6.5, demonstrate that the components of peak SP2 aggregate to form the material of higher molecular weight that elute earlier (tubes 16–21) than the position of peak SP2 (tubes 21–29). Further evidence to support the view that aggregation was occurring is presented in Chapter 7.

The possible explanation to this behaviour lies in the diversity of the honey components. It is possible that each type of molecule in honey could have a tendency to form aggregation or stacking between the same type of molecules or different type of molecules through non-covalent binding, mainly hydrogen bonding, hydrophobic interaction or electrostatic interaction (Nagy *et al.*, 2012). For example, it is well known that phenolics can form a complex with proteins/peptides (Hagerman *et al.*, 1998a).

Proteins in nectar honey were reported to have a relative molar mass of 49 kDa (Iglesias *et al.*, 2006). While this size of protein could be too large for the Superdex® Peptide fractionation range (*i.e.* 100–7 000 Da) and therefore readily excluded from the column in the first place, it was found that honey also contains small peptides with a molecular mass of 600 Da which were reported to be responsible for the inhibition of the polyphenol oxidase (PPO) in apple slices (Ates *et al.*, 2001; Oszmianski & Lee, 1990). Even though the exact molecular mass of protein or peptides was not known for Beech honeydew honey, they are likely to have originated from phloem sap of the Beech trees and the bees.

On the other hand, phenolics exist in excess compared with proteins/peptides in honey (Gheldof *et al.*, 2002), with their molecular mass ranging from 500–2 000 Da (Hagerman *et al.*, 1998b). It was reported that interaction still can take place even at very low concentration of peptides (Hagerman *et al.*, 1998a; Hagerman *et al.*, 1998b; Naczka *et al.*, 2011; Rohn *et al.*, 2004; Sarni-Manchado & Cheynier, 2002; Yi *et al.*, 2006). Spencer *et al.* (1988) hypothesized that this situation was due to the fact that phenolics are polydentate ligands with a multiplicity of potential binding sites provided by the numerous phenolic groups and aryl rings of the molecules. Therefore, peptides may be covered with phenolics that interact through non-covalent forces (Baxter *et al.*, 1997), as shown in Figure 6.6A.

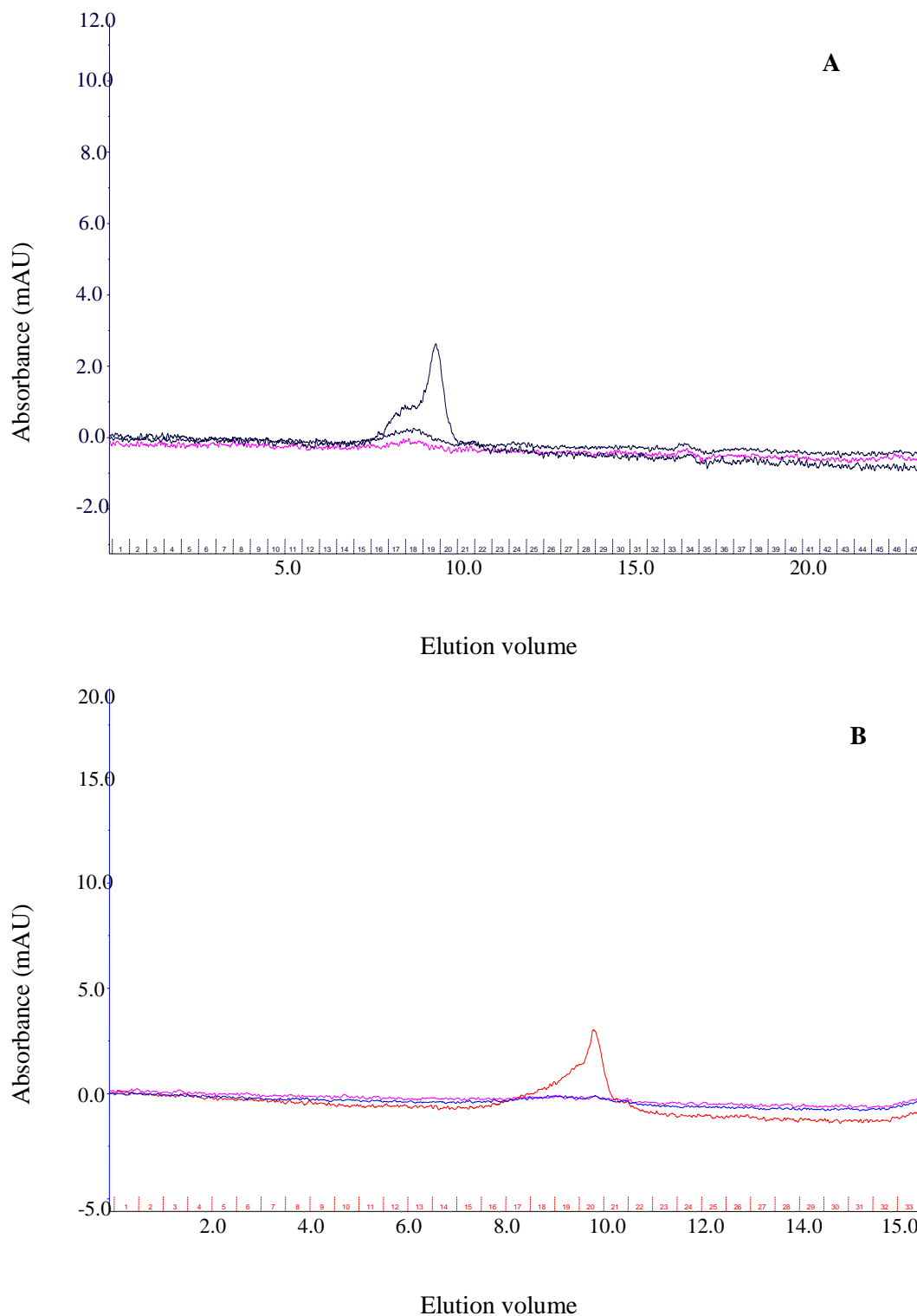
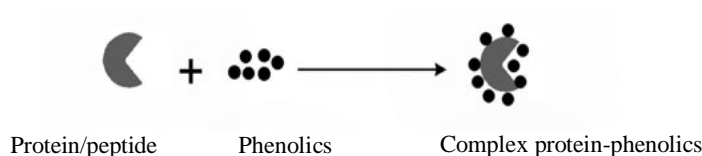


Figure 6.5: Elution profile of the re-chromatography of the early and late parts of peak SP2, i.e. SP2A (tubes 21–24) and SP2B (tubes 25–29) from Figure 6.3. Upper, middle and lower chromatograms are UV absorbance at 220, 280 and 360 nm, respectively.

Early studies of the binding of phenolics to proteins/peptides suggested the complexes will form precipitates (insoluble complexes). This condition is dependent on pH (pH 4.0 was found the optimal pH to form precipitation between bovine serum albumin and tannin) and stoichiometry (McManus *et al.*, 1985; Naczki *et al.*, 1996). However, some complexes are soluble with no precipitation involved (Philo, 2006). No precipitation was observed to occur in the fractions in the current work, suggesting that the complex was soluble in 30% acetonitrile. This might be due to the non-covalent binding between phenolics and peptides which is weak and reversible (Charlton *et al.*, 2002; Nagy *et al.*, 2012).

The presence of self-aggregated molecules (intramolecular interaction) in the sample solution could also contribute to the early-eluting peak (Figure 6.6B). It has been shown that peptides (Murphy, 2002) and phenolics (*e.g.* theaflavin) (Charlton *et al.*, 2002; Charlton *et al.*, 2000) can self-aggregate through the non-covalent binding. These situations without doubt can produce large molecular structure.

A



B

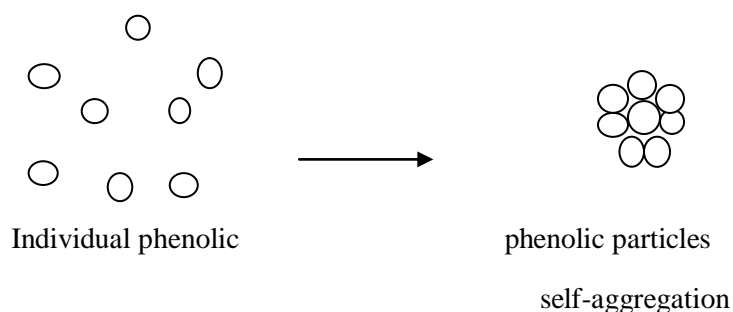


Figure 6.6: Scheme for aggregation scenario that explains the results observed in Figure 6.3. (A) Complex peptide-phenolics, adapted from Douat-Casassus *et al.* (2009) and (B) phenolics self-aggregation.

The majority of reports in the literature on the interaction of phenolics and peptides are on the interaction between tannins and proteins. Bovine serum albumin (BSA) is the commonly used protein for the model of interaction. To date, tannins have not been reported to exist in honey. In order to know whether tannins were present in honeydew honey and thus could have been involved in the aggregation observed, the honeydew honey was extracted by using absolute acetone and 70% (v/v) aqueous acetone, as tannins are very soluble in this solvent (Downey & Hanlin, 2010; Heinonen *et al.*, 1998; Naczki *et al.*, 1994; Seigler *et al.*, 1986). The iron-binding activity for the extract prepared with both solvents was negative. The absence of iron-binding antioxidant activity in the acetone extract was evidence that tannins did not exist in the extract. However, it is hard to firmly conclude anything from this assay data due to a couple of possibilities: (i) tannins could have been extracted did not have an iron-binding antioxidant property, or (ii) the concentration of tannins in the extract was too low to give the antioxidant effect in the assay. Apart from tannins, it was also likely that the aggregation was the result from low molecular weight phenolics (Bartolomé *et al.*, 2000; Hatano *et al.*, 1999; Mishra *et al.*, 2005; Rohn *et al.*, 2004).

Monitoring of size exclusion chromatography by UV absorbance showed that all fractions had a greater intensity of absorbance at 220 nm than at 280 and 360 nm. This indicates that these fractions might contain peptides, as the peptide bond absorbs strongly at 220 nm. Absorption at 280 nm indicates the presence of tyrosine or tryptophan residues in proteins, but could be due to other aromatic compounds such as phenolics. Very little absorbance occurred at 360 nm, a wavelength at which flavonoids absorb. However, conclusions as to the nature of the antioxidant compounds could not be made at this stage since there could be multiple different compounds that absorb UV light at 220 and 280 nm. For instance, carbonyl groups (mainly monocarboxylic acids) and oxygen also absorb strongly at 220 nm (Doonan, 2002).

6.4.2 Interaction of molecules with matrix of the column (non-ideal size exclusion chromatography)

In size exclusion chromatography, the separation of the molecules is assumed to be completed within one total permeation volume of the column (V_t). However, this ideal situation might not be happening when there are interactions between molecules and the column matrix. Interactions such as hydrophobic and ionic interactions commonly occur between molecules and the column matrix (Lee & Whitaker, 2004; Yumioka *et al.*, 2010). Hellberg *et al.* (1996) reported that the Superdex[®] medium which consists of crosslinked agarose could promote electrostatic and hydrophobic interactions with the molecules due to the carboxylic groups on the matrix. In the current study, fraction SP8 which eluted after the total permeation volume is clear evidence that interaction with the column matrix is occurring (Figure 6.3).

Some authors use an aqueous mobile phase such as a buffer, adding substances such as salt or arginine to the mobile phase, or adjust the pH of the mobile phase, to suppress such interactions. The addition of sodium chloride into the mobile phase is a common practice to suppress electrostatic interaction. It has been reported that sodium chloride up to 100 millimol/l can suppress electrostatic interaction between synthetic human parathyroid hormone hPTH (1-34) and a TSK-GEL G2000SWXL column (Kamberi *et al.*, 2004). Preston (1984) found that different concentrations of sodium chloride affected the elution of different fractions of gluten proteins from a Sephacryl S-300 column. Both authors, Preston (1984) and Kamberi *et al.* (2004), agreed that above the optimal limit, sodium chloride will induce hydrophobic interaction. This means that the exact dependence on sodium chloride to suppress electrostatic interaction varies from one compound to another. However, it also has been demonstrated by Cole *et al.* (1983) that a low concentration of sodium chloride (15 mM) when added in the mobile phase (1.0 millimol/l acetic acid; pH 4) could increase the extent of protein aggregation. Some mobile phase components such as phosphate and arginine which are commonly used in size exclusion chromatography to suppress the aggregation are said to be superior to sodium chloride (Arakawa *et al.*, 2010; Arakawa *et al.*, 2007; Yumioka *et al.*, 2010). However, these could interact preferentially with ferrous ions in the antioxidant assay. Phosphate and arginine

are known to have a capability to form complexes with metal ions (Andjelkovic *et al.*, 2006; Clarke & Martell, 1970; Remko *et al.*, 2008). The removal of these components from the sample extracts would be necessary prior to assay to avoid overestimation of the iron-binding antioxidant activity of fractions.

Acetonitrile, which is often used to suppress adsorption of molecules by reducing hydrophobic interactions, decreased the number of late-eluting peaks. This can be seen when comparing water (Figure 6.2A) and acetonitrile (Figure 6.2B) as eluents, where acetonitrile had decreased the elution volumes for the separation. However, acetonitrile appears unable to minimize the hydrophobic interaction that occurred for SP8. It also failed to dissociate the aggregation in SP1 and SP2, if that was occurring, to give at least a single peak in that region. However, the elution volume of SP3 increased with 30% acetonitrile, suggesting that this was disaggregated to some degree. Kamberi *et al.* (2004) reported that 10% acetonitrile was optimal for suppression of hydrophobic interactions and 30% acetonitrile was seen to induce electrostatic interactions between hPTH (1-34) and TSK-GEL G2000SWXL column. The loss of the small peak at 12.5 ml in Figure 6.2A that was eluted with water may be due to the electrostatic effect in 30% acetonitrile. The exact mechanism of interactions that take place for honey components with Superdex® Peptide matrix cannot be precisely explained from the presented results alone. The interactions with the column matrix are commonly pronounced among aromatic substances, alcohols and proteinaceous compounds including amino acids (Hellberg *et al.*, 1996; Irvine, 1997; Mant *et al.*, 1987; Stulik *et al.*, 2003). However, the results clearly confirm that the honey components have some interactions with matrix of the column. These are most likely to be primarily due to hydrophobic and electrostatic interactions in 30% acetonitrile.

6.4.3 Estimation of molecular size

The estimation of molecular size of the honey components will not be accurate if secondary interactions (hydrophobic and electrostatic) exist. For instance, it is clearly evident that the elution volume was different with different mobile phases. The shifting of the elution profile to a larger elution volume in 30% acetonitrile

obviously gave a deviation from the region of estimated molecular weight for the same peaks when eluted with water. Stronger binding to the matrix causes a greater elution volume and hence can give underestimation of the molecular weight (Arakawa *et al.*, 2010). Another example is that the late-eluting peak (SP8). This is the result of an interaction with the column matrix eluting later than total permeation volume of the column. Therefore it cannot be concluded to have a molecular weight less than 100 Da (a very small molecule). To support these claims, Smyth & FitzGerald (1997) showed through their study with a column of Superdex[®] Peptide that a different concentration of acetonitrile gave a different elution volume for the same molecules (proteins and certain amino acids), hence that differences prevented them from determining the accurate molecular size of those molecules. Lee & Whitaker (2004) also showed that different types of SEC columns (*i.e.* Sephadex G-100 and Superose 12) gave a different estimated molecular size of the red kidney bean α -amylase inhibitor protein (RKB α AI). The authors revealed that there was an ionic interaction between RKB α AI and Superose 12 matrix in low ionic strength buffers and hydrophobic interaction at higher ionic strength buffers. Furthermore, the authors reminded researchers to be cautious when using Superose 12 for molecular weight determinations.

SEC is sensitive to the elution volume where a significant error in the calculated molecular weights can be made with a small error in measuring elution volume (Holding, 1984). Hence, at this point it is not advisable to estimate the molecular size range just by looking at this SEC data alone. Misinterpretation of the data could result if the mechanism of the separation in SEC as well as the interaction with the column has not been understood.

6.5 Conclusion

Size exclusion chromatography is now a well established tool in estimating the molecular weight of organic compounds. It also can be used for observing any aggregation in the sample. It clearly indicated that the honey components may have some degree of aggregation during the separation process in the column. The use of this separation technique in the present study proved to be advantageous with respect to enabling good separation of iron-binding antioxidant(s) from

sugars. This is of importance since honey has a particularly high sugar content. However, due to the secondary interactions and aggregation behaviour of honey components, estimation of the molecular weight of iron-binding antioxidants cannot be accurately evaluated. Therefore at this time only an empirical interpretation of the data is possible.

CHAPTER 7

PURIFICATION OF IRON-BINDING ANTIOXIDANT(S) USING CONSECUTIVE REVERSED PHASE AND ANION EXCHANGE CHROMATOGRAPHY

7.1 Introduction

The information that was gained from the work described in Chapter 6 was that the iron-binding antioxidants from honeydew honey were soluble in 30% acetonitrile. It was hypothesized that there were multiple components present in the most active fraction (fraction SP2) isolated from size exclusion chromatography, possibly because of aggregation behaviour, which prevented the identification of the actual iron-binding antioxidants.

The work in this chapter was the continuation to further isolate and purify the active fraction from Superdex[®] Peptide (fraction SP2) using the application of different kinds of chromatography which would be particularly useful for further separation of the antioxidants. Reversed phase and anion exchange chromatography were chosen for this purpose. It was thought that this would help in identifying other characteristics of the antioxidants such as the hydrophobic behaviour and the ability of the antioxidants to form anions.

7.2 Methods

In all cases, the mobile phases were filtered through a 0.22 µm filter cellulose membrane filter and degassed. The sample to be chromatographed was centrifuged at 10 000 x g for 15 minutes before injection onto the column. The columns were cleaned after every 5th run to maintain the reproducibility of the peaks. The scheme for the purification protocol is depicted in Figure 7.1.

7.2.1 Reversed phase chromatography

The active fraction (fraction SP2 in Figure 6.4) from chromatography on the Superdex[®] Peptide column was further purified on a reversed phase column. A volume of 500 μ l of fraction SP2 was loaded onto a RESOURCE polystyrene divinylbenzene (PSDVB) column. The volume of the column was 3 ml. The injection volume and flow rate were kept constant at 500 μ l and 1 ml/min respectively unless otherwise stated. In all cases, linear gradient elution was carried out. The gradient used was 0-100% B in 9 min (three column volumes) with a hold at 100% B (isocratic hold) for three column volumes to elute any compounds strongly retained in the column. Between each sample run, the column was re-equilibrated with a linear gradient of 100-0% B for three column volumes with a hold at 100% A for two column volumes. The elution peaks were monitored by measuring the UV absorbance at 220, 280 and 360 nm. All individual fractions (250 μ l each) under each observed peak were pooled. The pools were each made up to the volume of the largest pool (2.25 ml) with deionised water and tested for iron-binding antioxidant activity.

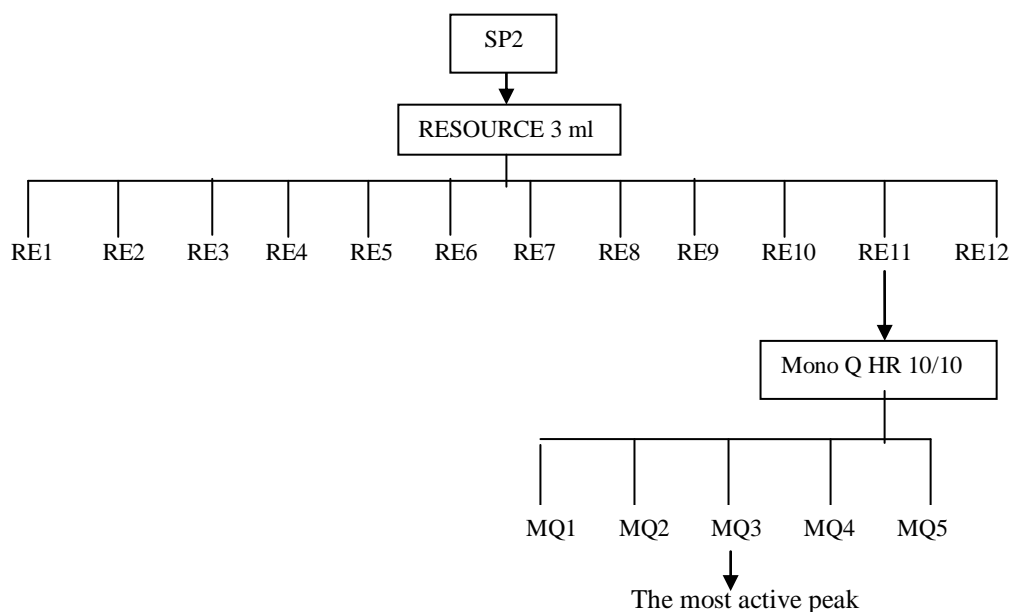


Figure 7.1: Flow diagram of scheme of purification of fraction SP2

7.2.1.1 Optimization of the separation conditions

In order to optimize the resolution, different experimental parameters influencing retention or selectivity (*i.e.* organic modifier and mobile phase composition) were used by varying one parameter at a time, keeping the other parameters constant.

7.2.2 Anion exchange chromatography (MonoQ HR 10/10)

The fraction showing highest iron-binding activity after reversed phase chromatography was further purified on an anion exchange column. The column used was Mono Q HR 10/10 with a column volume of 8 ml. Mobile phases were A (20 mmol/l ethanolamine-HCl, pH 10) and B (20 mmol/l ethanolamine-HCl, pH 10 + 1 mol/l sodium chloride). Before sample application, the column was equilibrated with mobile phase A for two column volumes (16 ml). A 250 μ l sample of the most active fraction from reversed phase (RE 11) was mixed with the same volume of mobile phase A in order to ionize the compounds to negatively charged ions before loading onto the column. At this pH value, the negatively charged ions should bind to the anionic exchanger. The injection volume and flow rate were kept constant at 500 μ l and 1 ml/min unless otherwise stated. A linear gradient elution was performed for three column volumes (24 ml), and the peaks were monitored at 220, 280 and 360 nm. All individual fractions (250 μ l each) under observed peaks were pooled, made up with deionised water to equal the volume of the largest pool and tested for iron-binding antioxidant activity.

7.3 Results and discussion

7.3.1 Optimization of the reversed phase procedure

To establish sufficient separation of iron-binding antioxidants from the Superdex[®] Peptide fraction SP2 on the reversed phase column, two parameters that were thought to influence the selectivity of the column were optimized; the type of organic modifiers and the effect of additive (*i.e.* sodium chloride) in the mobile phases.

7.3.1.1 Effect of organic modifiers on elution profile

As a normal practice in method development, the first step was to investigate the elution profile of the selected Superdex[®] Peptide fraction SP2 with different types of organic modifiers. Methanol and acetonitrile are the most commonly used organic modifiers for elution in reversed phase chromatography. The initial attempt was made with the aqueous mobile phase of 2% methanol (mobile phase A) and 100% methanol (mobile phase B). It is a common practice to include a low amount of organic modifier (not more than 5%) in a mobile phase A, to prepare the site for retention of the molecules (Amersham Biosciences, 1999).

Figure 7.2A shows the elution profile for methanol. The separation of SP2 fraction was not achieved in a reasonable elution time (18 min) when methanol was used as the organic modifier. To improve the elution profile, mobile phase containing acetonitrile was trialled, with 2% acetonitrile (mobile phase A) and 80% acetonitrile (mobile phase B). The amount of acetonitrile in mobile phase B (80%) was chosen to give the comparable data with methanol in term of the same eluotropic strength of the solvents (Bolliet & Poole, 1997; Snyder *et al.*, 1997).

Figure 7.2B shows that having 80% acetonitrile in the mobile phase B gave some degree of separation of SP2 components between 2 to 15 ml elution volume. Complete resolution of the last peak (the highest peak) was achieved. A few peaks came off after the gradient and it was assumed that these molecules were retained strongly on the stationary phase.

Because of the high cost of using acetonitrile, the eluting condition was optimized by decreasing the amount of acetonitrile in mobile phase B. Therefore, 30% and 60% acetonitrile were tried to see if there were any differences in elution profile as compared with 80% acetonitrile. It was found that no peak was observed at the same elution volume as with 80% acetonitrile when the concentration of acetonitrile was decreased. From these results, 80% acetonitrile in mobile phase B was chosen as an organic modifier throughout the study due to its ability to elute off the molecules.

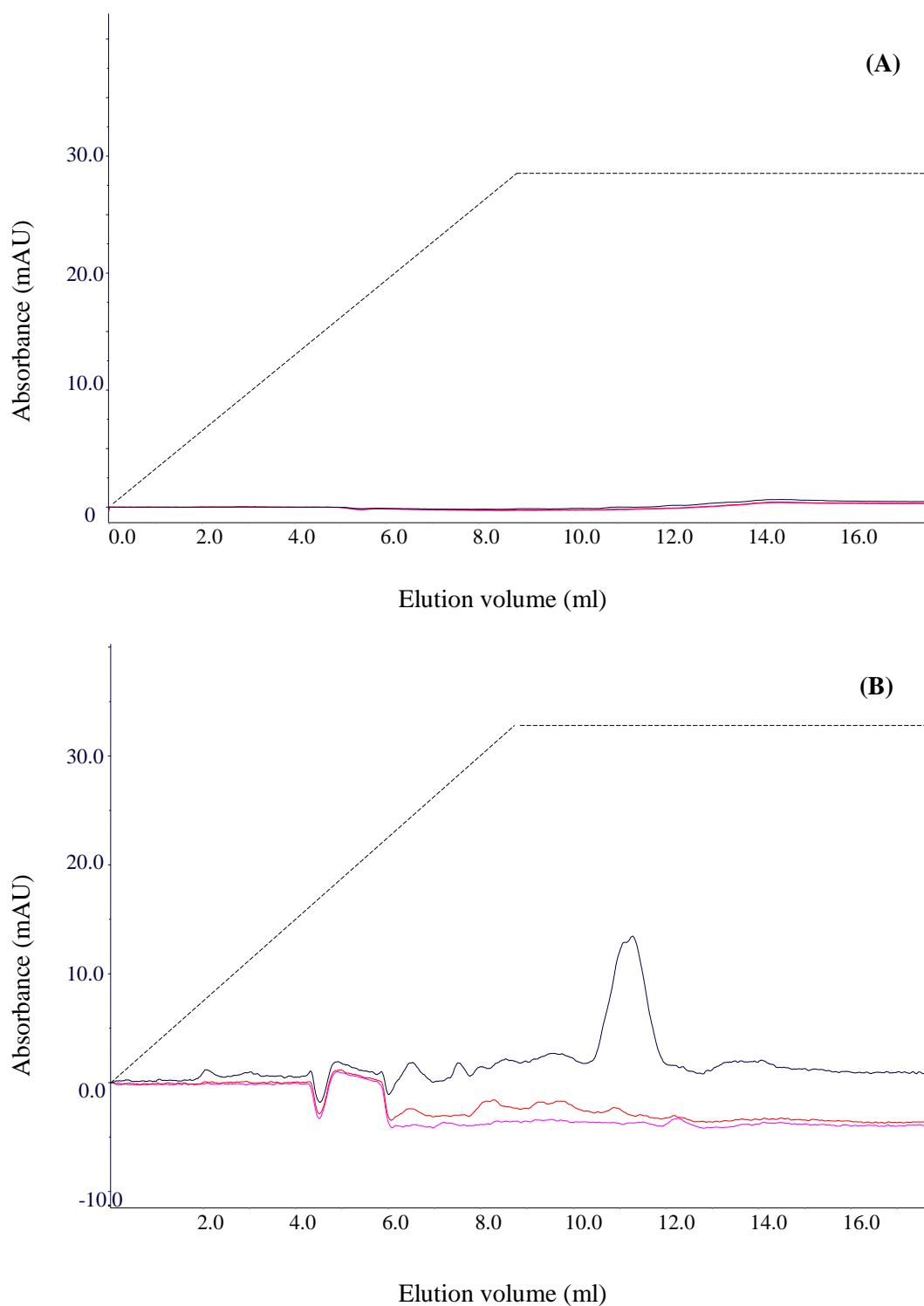


Figure 7.2: Comparison of 100% methanol (A) and 80% acetonitrile (B) in mobile phase B for the elution of fraction SP2 loaded on a RESOURCE 3 ml reversed phase column. The dotted line indicates the linear gradient from 0-100% B in 9 min and hold up at 100% B for three column volume (9 ml). Flow rate was 1 ml/min. Upper, middle and lower lines are absorbance at 220, 280 and 360 nm, respectively.

That elution was achieved in a reasonable time with 80% acetonitrile in mobile phase B could be explained by the displacement of sample molecules with acetonitrile molecules on the stationary phase. It has been established that the hydrophobic interactions between molecules and column stationary phase matrix contribute to the relative retention and column selectivity in reversed phase chromatography. However, molecules also can be retained on the matrix by additional interaction such as through pi-pi (π - π) and dipole-dipole interactions (Croes *et al.*, 2005; Neue *et al.*, 2006), especially in a special column stationary phase such as those with phenyl or cyano matrix groups.

The selection of the RESOURCE column (*i.e.* polymeric polystyrene divinylbenzene) used in this study was due to the column having a different selectivity mechanism and its potential to improve peak shape with some classes of compounds (Amersham, 1999; Yang *et al.*, 2005). Its stationary phase consists of underivatized benzene rings (Figure 7.3), and it has been reported to be more retentive than a silica C-18 stationary phase (Dorsey & Dill, 1989; Mitra, 2003). This is because the underivatized matrix is filled with π electrons that have a tendency to interact with aromatic rings of the sample molecules (Benson & Woo, 1984).

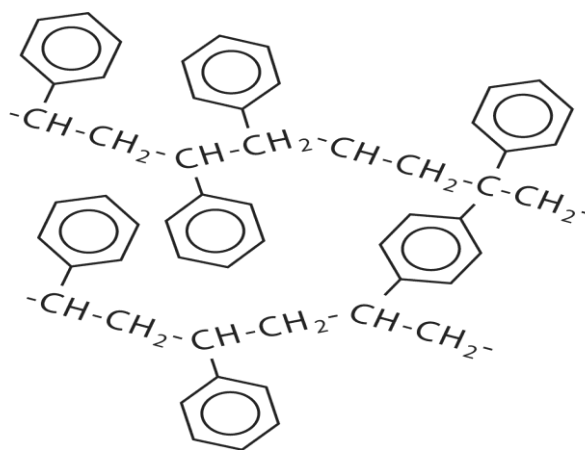


Figure 7.3: Polystyrene divinyl benzene (PSDVB) polymer. The stationary phase matrix of RESOURCE 3 ml column. The figure was adapted from Biotage (2012)

Since acetonitrile has a high density of π electrons due to its four π electrons in its cyano group (Thevenon-Emeric *et al.*, 1991; Yang *et al.*, 2005), its π electrons will replace the sample molecules on the benzene ring site on the stationary phase. Through this specific interaction the retention of the sample molecules is reduced.

Conversely, methanol does not have the π electron system that could behave similarly to acetonitrile. Furthermore, methanol has been reported to increase the retention of molecules which consist of aromatic rings and carboxylic groups on a phenyl column (Long & Henderson Jr, 2007; Yang *et al.*, 2005), thus methanol takes a longer time to elute the compounds from the column. This is probably the reason why no elution was observed with methanol compared with acetonitrile at the same elution volume.

Yang *et al.* (2005) have shown that the elution from a phenyl column for molecules which consist of aromatic rings with a carboxylic group was fully achieved when the authors used an increasing amount of acetonitrile. This led to the authors' proposal that the π - π interaction between the phenyl stationary phase and the sample molecules was suppressed when a high concentration of acetonitrile was present.

Thevenon-Emeric *et al.* (1991) have reported that π - π interaction occurs when there are at least two π electron systems existing in the chromatographic process. These systems could be between stationary phase and molecules, stationary phase and mobile phase, or stationary phase and molecules and mobile phase. When π - π interaction occurs in the system it will be more pronounced than other retention mechanisms (*e.g.* hydrophobic interaction).

Both suggestions (Thevenon-Emeric *et al.*, 1991; Yang *et al.*, 2005) seemed in agreement with the present study, where no elution was achieved with both 30% and 60% acetonitrile, indicating that a high percentage of acetonitrile controls the eluting mechanism of the molecules on the RESOURCE column. However, the elution of the molecules after the gradient with 80% acetonitrile most probably could be due to the stronger π - π interaction on the stationary phase where acetonitrile did not completely suppress the interaction between sample molecules and the stationary phase.

A sufficiently good separation of antioxidants must firstly be achieved in order to collect them in different fractions. Even though the current elution conditions could isolate the compounds, they were not sufficient to achieve a good separation for fractionation. Furthermore, the main peak which eluted after the gradient consisted of minor overlapping peaks, which indicates more than one compound existed in the fractions which were not fully separated. There could be an electrostatic interaction occurring between the molecules. To test this hypothesis, the addition of sodium chloride in low concentration in the mobile phase was tried to see if it would minimize this interaction.

7.3.1.2 *Effect of sodium chloride on the elution profile*

As discussed in Chapter 6, it is known that the addition of salt could reduce the electrostatic interaction between molecules. Therefore, sodium chloride was added in the mobile phases (A and B) to see if any improvement in peak shape and selectivity could be achieved. The concentration of sodium chloride used was 50 mmol/l, as this concentration was assumed to be sufficient to reduce any electrostatic interaction (Kamberi *et al.*, 2004).

Figure 7.4 shows the elution profile of fraction SP2 when sodium chloride was added in the mobile phases. It can be clearly seen that the shape of the peaks eluted in the gradient region were improved. The complete resolution of the main peak (the highest peak) was still not achieved, however.

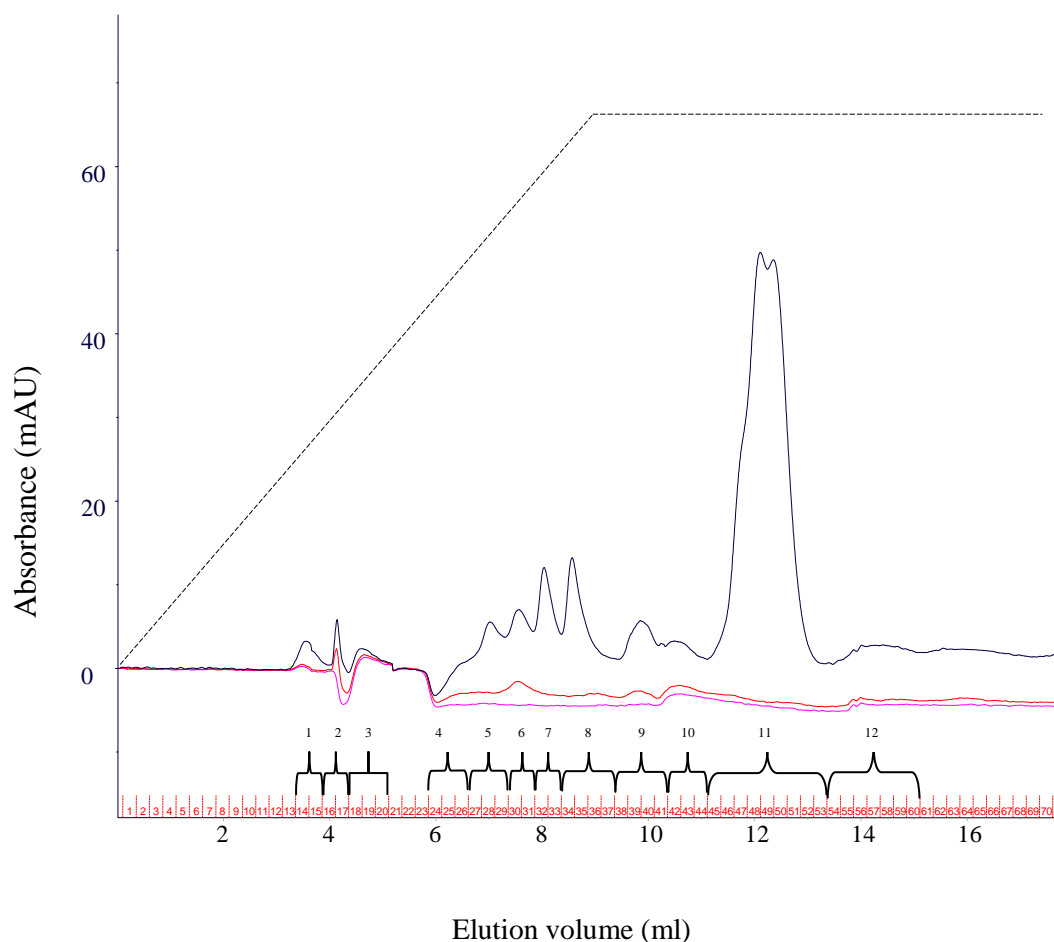


Figure 7.4: Elution profile of fraction SP2 chromatographed on a RESOURCE 3 ml reversed phase column. The eluting conditions were mobile phase A: 2% acetonitrile + 50 mmol/l sodium chloride. Mobile phase B: 80% acetonitrile + 50 mmol/l sodium chloride. The dotted line indicates the linear gradient at 0-100% B in 9 min and hold up at 100% B for three column volume (9 ml). Flow rate was 1 ml/min. Upper, middle and lower lines are absorbance at 220, 280 and 360 nm, respectively.

When the concentration of acetonitrile in mobile phase B was increased to 90%, the 90% acetonitrile and 50 mmol/l sodium chloride were slightly immiscible and formed two layers when settled. Acetonitrile is known to undergo phase separation with water when there is a high concentration of sodium chloride in the mixture (Lee *et al.*, 1991; Nagaosa & Segawa, 1993). A homogenous mobile phase solution is desirable to avoid any unwanted effect if the heterogenous mobile phase passes through the column. Therefore the optimization of the method with a higher concentration of acetonitrile than 80% in mobile phase B was not attempted.

The possible explanation of the improvement of the peak shape in the gradient region is suppression of the electrostatic interactions between sample molecules by sodium chloride. With sodium chloride, the shape of the peaks was improved. This situation also could indicate that interaction between molecules is occurring but that this has been minimized by sodium chloride. It was possible that the interaction is an electrostatic type interaction which could have originated from the components in the aqueous mixture of the crude honey at the first isolation step using Superdex[®] Peptide column (see Chapter 6). At the pH of honeydew honey (pH 5.2), carboxylic acid groups will ionize to some degree (becoming negatively charge species), whilst any amine groups will be protonated (becoming positively charge species).

The different charges will promote the electrostatic interaction between two or more molecules. It was possible that some of the isolated components in fraction SP2 were present in the form of charged molecules that interact with opposite charges through electrostatic interaction.

The addition of sodium chloride in mobile phases is not a common practice in reversed phase chromatography. One reason is because often the liquid chromatography-reversed phase system is connected to a mass spectrometer, therefore, the addition of salts in mobile phases is considered unfavourable due to the suppression of the mass spectrometry signal (Dubois *et al.*, 1996). Furthermore, the high concentration of sodium chloride at this stage was not desired as the active reversed phase fraction was to be loaded onto anion exchange chromatography, where a high salt concentration in the fractions will prevent the binding of molecules on the anion exchanger.

7.3.1.3 Iron-binding antioxidant activity of the fractions from reversed phase chromatography

Since the addition of sodium chloride in the mobile phases had given sufficient separation of the compounds, the fractionation of compounds isolated by the reversed phase could be feasible. There were twelve peaks detected on the chromatogram. Individual tubes (250 µl) under each peak were pooled and twelve pooled fractions (RE1-RE12) were obtained from the reversed phase

chromatography. All pools of fractions were made up to the same volume as the largest pool (2.25 ml) with deionised water, and tested for iron-binding antioxidant activity.

The iron-binding activity for the ten pooled fractions is shown in Figure 7.5. Fraction RE11 was the only one exhibiting iron-binding antioxidant activity, equivalent to 531.8 ± 49.5 $\mu\text{mol/l}$ Ferrozine. All the other fractions showed a negative value for the results of the assay. This negative value given by fractions RE1-RE10 and RE12 was an indication of a pro-oxidant effect (Gazzani *et al.*, 1998). It would be the result of Fe^{3+} ions formed in the assay being reduced by components in the fractions to Fe^{2+} ions to catalyse the formation of more $\cdot\text{OH}$ radicals.

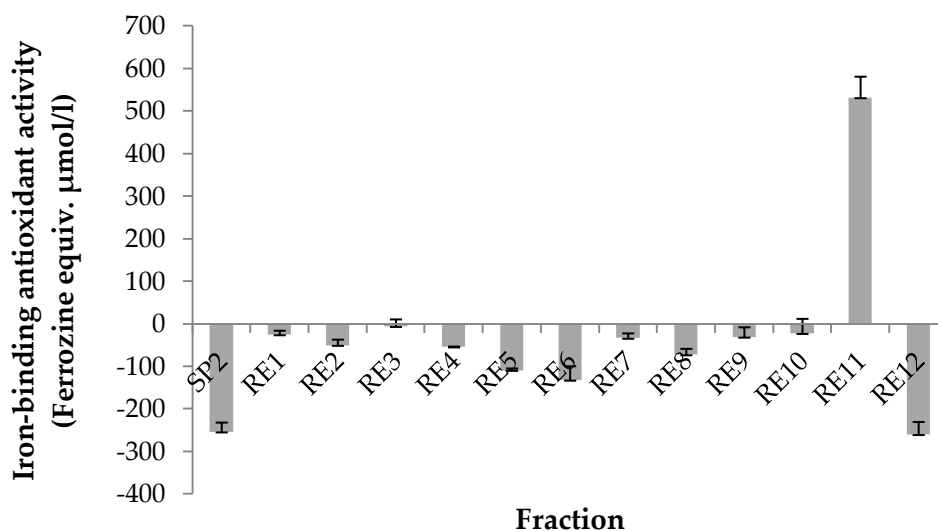


Figure 7.5: Iron-binding antioxidant activity of fractions isolated from chromatography on the reversed phase column (Figure 7.4). All reversed phase fractions including a sample of the fraction SP2 loaded on the column were tested on the same volume. Results are means \pm standard error of mean of two replicates.

Calculations were done based on the degree of dilution to show quantitatively all the activity was recovered. Results are shown in Table 7.1.

Table 7.1: Summary of the quantitative estimation of the iron-binding antioxidant activity of the fractions isolated from size exclusion chromatography on a reversed phase column. Activity is shown as Ferrozine equivalent ($\mu\text{mol/l}$) \pm standard error of measurement ($n=3$).

Activity of material from column (from Figure 6.4)	Expected activity of pooled fraction if in 0.5 ml volume loaded	Volume of pooled fraction from reversed phase chromatography (ml) (Figure 7.4)	Dilution of original loaded from pooled fraction	Measured activity in pooled fraction (in equal volume)	Loss/gain in activity in pooled fraction
Fraction SP2 = 328.6	1/9 of 328.6 = 36.5	RE1 = 1.0	0.5 ml/1.0 ml = 2 fold	-25.53 ± 9.72	pro-oxidant
		RE2 = 0.25	0.5 ml/0.25 ml = 0.5 fold	-50.38 ± 13.57	pro-oxidant
		RE3 = 0.75	0.5 ml/0.75 ml = 1.5 fold	-5.91 ± 16.69	pro-oxidant
		RE4 = 0.75	0.5 ml/0.75 ml = 1.5 fold	-54.09 ± 1.05	pro-oxidant
		RE5 = 0.5	0.5 ml/0.5 ml = no dilution	-109.14 ± 4.62	pro-oxidant
		RE6 = 0.5	0.5 ml/0.5 ml = no dilution	-132.15 ± 32.85	pro-oxidant
		RE7 = 0.5	0.5 ml/0.5 ml = no dilution	-33.72 ± 11.59	pro-oxidant
		RE8 = 1.0	0.5 ml/1.0 ml = 2 fold	-72.56 ± 14.14	pro-oxidant
		RE9 = 1.0	0.5 ml/1.0 ml = 2 fold	-31.07 ± 23.32	pro-oxidant
		RE10 = 0.75	0.5 ml/0.75 ml = 1.5 fold	-22.34 ± 34.44	pro-oxidant
		RE11 = 2.25	0.5 ml/2.25 ml = 4.5 fold	531.79 ± 49.53	gain
		RE12 = 2.25	0.5 ml/2.25 ml = 4.5 fold	-260.05 ± 29.74	pro-oxidant

A pro-oxidant is a species that causes or promotes oxidation (Decker, 1997). In the context of iron-induced oxidation, they are species that have an ability to generate more Fe^{2+} ions in the assay, either by redox cycling (*i.e.* increasing the rate of autoxidation catalysed by Fe^{2+} ions) (Chvatalova *et al.*, 2008), reducing Fe^{3+} ions to Fe^{2+} ions which are what form $\cdot\text{OH}$ radicals (Kristinova *et al.*, 2009; Moran *et al.*, 1997; Niki & Noguchi, 2000), or forming a complex with iron to become a reducing agent (Decker, 1997; Kulkarni *et al.*, 2007; Moran *et al.*, 1997).

It also is suggested that reversed phase chromatography has separated the antioxidants from the aggregates. It was suggested that the antioxidants formed aggregates with other molecules in honey during separation using size exclusion chromatography (see Chapter 6). Aggregation can occur through hydrophobic and/or hydrogen bonding between molecules. For example, interaction between phenolics and proteins has been proposed to involve such bonding (Hagerman & Butler, 1980). Acetonitrile would break these bonds, and thus disaggregate the molecules.

It should be kept in mind that honey contains various organic compounds which are potentially iron-binding antioxidant(s) (as discussed in Chapter 2). It has been reported that under certain circumstances (*e.g.* molecular structure, low concentration of antioxidants or in the presence of transition metal ions), some antioxidants (either radical scavengers or metal chelators) can also show a pro-oxidant effect *in vitro* (Amarowicz & Shahidi, 1997; Aruoma *et al.*, 1993; Cao *et al.*, 1997; Chvatalova *et al.*, 2008; Fukumoto & Mazza, 2000; Tian & Hua, 2005; Yen *et al.*, 2002). It has been clearly shown in the present study that the diluted SP2 fraction also showing a pro-oxidant effect.

The structure-activity relationship of the antioxidants has been suggested to play a significant role for their antioxidant-pro-oxidant activity. Chvatalova *et al.* (2008) reported that phenolic compounds having catechol or pyrogallol groups accelerate pro-oxidant effect catalysed by Fe^{2+} ions by way of the oxygen ligands. Moran *et al.* (1997) also demonstrated that phenolic compounds with these groups were able to chelate and reduce Fe^{3+} ions. However, these findings were conflicting with the prevailing one that catechol and galloyl groups turned out to be the groups mainly responsible for the metal-binding activity of phenolic

compounds (Andjelkovic *et al.*, 2006; Khokhar & Owusu Aparenten, 2003; Leopoldini *et al.*, 2006a; van Acker *et al.*, 1998).

The result of the present study however suggested that the pro-oxidant activity observed in the fractions was obviously related to the low concentration of the iron-binding antioxidants due to the dilution effect in the fractions which affected their binding ability with Fe^{2+} ions. This was supported by the fact that the active fraction from Superdex[®] Peptide (fraction SP2) showed a pro-oxidant activity when diluted to match the concentration of the pooled fraction (see Figure 7.5), but had shown an antioxidant activity when assayed without this dilution (see Figure 6.4). Perez *et al.* (2009) showed that this condition (low concentration of antioxidants) could affect the binding constant (the competing equilibrium between ligands and metal ions). It should be noted that the pooled fractions collected from the reversed phase chromatography had not been concentrated. The volume of all of the pools was made equal by the addition of water prior to assay, giving dilution as shown in Table 7.2. Hence, for a fraction with small pooled volume, for example RE2 (250 μl), the volume of water added relative to the fraction volume was high. Therefore, the compounds present in RE2 would have been diluted nine-fold.

To check if the dilution factor was actually responsible for the observed pro-oxidant effect, a serial dilution of crude honeydew honey was made at 0.312-10% concentration, then the iron-binding antioxidant activity of each diluted honey solution was assayed. The experiment was repeated three times. The results are shown in Figure 7.6.

The iron-binding antioxidant activity became a pro-oxidant activity when the honey concentration was decreased to about 2.5% honey solution (4 fold dilution) and lower. The pro-oxidant effect was more prominent at concentrations of 0.312-1.25%. This data thus gives clear evidence that the effect of dilution of components of honey is the major reason for the pro-oxidant effect observed in the present study.

Table 7.2: The dilution resulting from adjusting the pools of fractions in Figure 7.4 all to the same volume.

Fraction	Volume of pooled fractions (ml)	Volume of water added (ml)	Final volume (ml)	Dilution factor (fold)
RE1	1.0	1.25	2.25	2.25
RE2	0.25	2.0	2.25	9
RE3	0.75	1.5	2.25	3
RE4	0.75	1.5	2.25	3
RE5	0.5	1.75	2.25	4.5
RE6	0.5	1.75	2.25	4.5
RE7	0.5	1.75	2.25	4.5
RE8	1.0	1.25	2.25	2.25
RE9	1.0	1.25	2.25	2.25
RE10	0.75	1.5	2.25	3
RE11	2.25	-	2.25	-
RE12	2.25	-	2.25	-
Fraction SP2	0.5*	1.75	2.25	4.5

* Volume loaded on column.

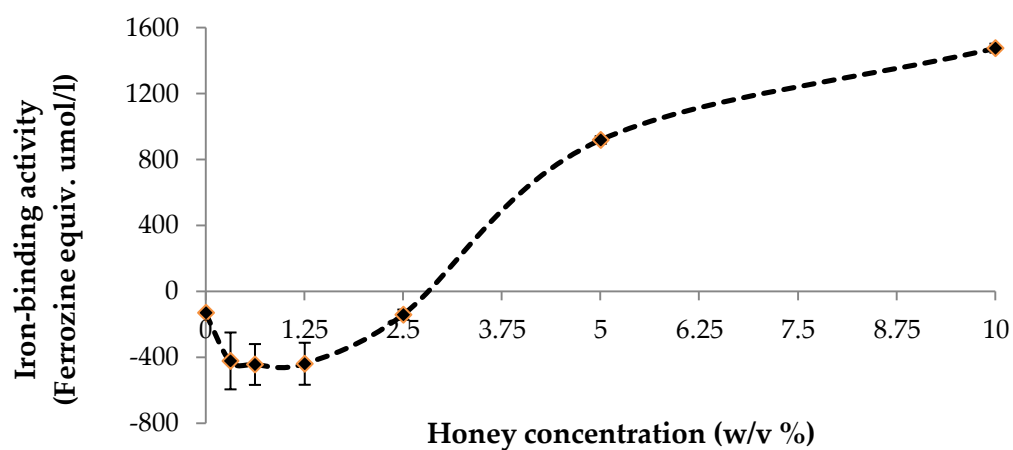


Figure 7.6: Effect of dilution on the iron-binding antioxidant activity of honeydew honey. Results are means \pm standard error of mean of three replicates.

The pro-oxidant effect in both sets of results (Figure 7.5 and 7.6) can be explained by the biphasic (antioxidant-pro-oxidant) behaviour (Kitts *et al.*, 2000; Lee & Lee, 2006) of some antioxidants, particularly phenolic compounds (Psotova *et al.*, 2003). It means that the same compound can behave as an antioxidant or a pro-oxidant at different concentration (Amic *et al.*, 2007). Many authors have discussed that this effect depends on the antioxidants to metal ions molar ratio (Hu & Kitts, 2002; Rodtjer *et al.*, 2006; Tian & Hua, 2005), where a low ratio of antioxidants to metal ions leads to a pro-oxidant effect (Fukumoto & Mazza, 2000; Kristinova *et al.*, 2009; Strlic *et al.*, 2002). This was in agreement with the present study where dilution of the fractions reduced the concentration of the antioxidants, thus lowering the ratio of antioxidant to Fe^{2+} ions in the assay. Other antioxidants that have been reported to have this biphasic effect are aloin and aloe-emodin (which were the active compounds of *Aloe* extract in a group of anthraquinones) (Tian & Hua, 2005), ascorbic acid (Yen *et al.*, 2002), caseinophosphopeptides from milk (Pihlanto, 2006), peptides from harp seal protein hydrolysates (Shahidi & Amarowicz, 1996), free amino acids (Pazos *et al.*, 2006), polyamines (*i.e.* spermine and spermidine) (Gaboriau *et al.*, 2004) and synthetic iron chelators (*e.g.* ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) (Engelmann *et al.*, 2003). In addition, it is suggested that an experiment by adding prooxidant fractions back to antioxidant fraction (RE11) to see the overall effect of having them together should be carried out.

It is interesting to note that the RE11 peak consisted of shoulder peaks (Figure 7.4). The shoulder peaks represent the overlapping of the different peaks, *i.e.* there is more than one compound existing in the RE11 fraction which were not fully separated by the reversed phase chromatography. The molecules might be very similar in hydrophobicity or π bonds. Thus, the conclusion could be made at this stage that reversed phase chromatography did not sufficiently separate the iron-binding antioxidants. Although XAD-2 resin did not extract antioxidants, the increase in activity in honey effluent indicated the resin did extract some prooxidants. Therefore, it could be a way in achieving a partial separation before starting other chromatography. Since the aim of this research was to fully purify the compounds in order to characterize them, other means of chromatography,

based on a different mechanism, needed to be used to try to separate the compounds.

7.3.1.4 Elution profile of SP1 and SP3 fractions on reversed phase chromatography

The other two Superdex[®] Peptide active fractions (SP1 and SP3) were loaded onto the reversed phase chromatography column to see if there were any differences in elution profile relative to SP2 fraction. The fractions were eluted with the same elution conditions as used for SP2 as described in Figure 7.4. The results (shown in Figure 7.7) were two very similar chromatograms obtained for the two fractions (SP1 and SP3).

Surprisingly, the reversed phase chromatograms for these two fractions were also very similar to that from the SP2 fraction. The only difference was that the SP1 fraction showed a few small peaks at 280 and 360 nm, which could distinguish the type of compounds present in SP1 from SP2 and SP3. The absorbance at 280 and 360 nm indicated that these compounds that were in the SP1 fraction eluted at 6-10 ml that were different from those in the SP2 fraction may be phenolic acids and flavonoids (Pothavorn et al., 2010).

This finding suggests that a similar group of compounds, but maybe in different proportions, existed in the three active fractions from chromatography on Superdex[®] Peptide. This could partly explain why the iron-binding antioxidant activity in these three fractions did not differ much from each other (refer to Figure 6.4 in Chapter 6), which is also consistent with the hypothesis raised in Chapter 6 that aggregation was occurring during the size exclusion chromatography isolation process.

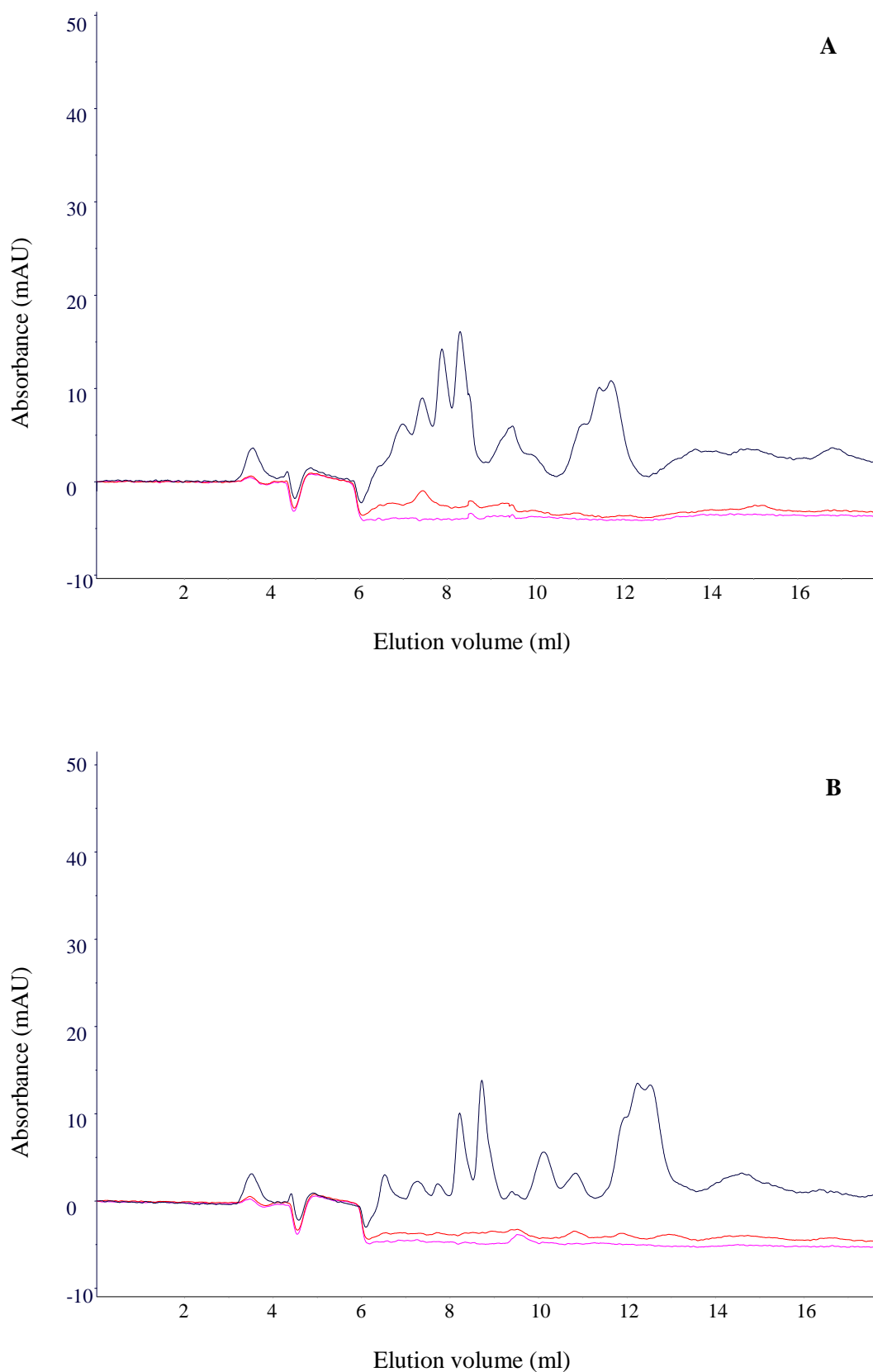


Figure 7.7: Comparison of the elution profile from chromatography on reversed phase chromatography of the other active Superdex[®] Peptide pooled fractions; SP1 (A) and SP3 (B). Upper, middle and lower lines are absorbance at 220, 280 and 360 nm, respectively. Elution conditions were similar to those in Figure 7.4.

7.3.2 *Elution profile and iron-binding antioxidant activity of fractions obtained by anion exchange chromatography*

Anion exchange chromatography is a separation technique based on the negative charge of molecules. The resin in the column commonly consists of either quarternary ammonium groups (strong bases) or amine groups (weak bases). Compounds loaded on the column which form negatively charged ions will bind onto the column through electrostatic interaction, while neutral or positively charged compounds will pass through without binding. The bound molecules can be eluted from the column with a salt gradient or a pH gradient.

Anion exchange chromatography has been reported to have been used to isolate inorganic compounds (del Nozal *et al.*, 1998) and organic acids (del Nozal *et al.*, 1998) from honey. More recently, Megherbi *et al.* (2009) used anion exchange chromatography to detect the adulteration of honey with corn syrup. Anion exchange chromatography also has been used to isolate phenolic compounds from other plant sources (Lam & Shaw, 1970; Martos *et al.*, 2005).

The most active fraction obtained from reversed phase chromatography (RE11) obtained in Section 7.3.1.3 (see Figure 7.5) was further fractionated on an anion exchange chromatography column. The column used was Mono Q HR 10/10, a strong-base anion exchanger. In this study, a salt (*i.e.* sodium chloride) gradient was used. During the elution mechanism, the chloride ions (Cl^-) from sodium chloride are exchanged for the negatively charged antioxidant molecules at the resin site.

The elution profile for the separation of the compounds in fraction RE11 is shown in Figure 7.8. There were five clear peaks of absorbance at 220 nm (MQ1, MQ2, MQ3, MQ4 and MQ5) obtained between 5–23 ml of elution volume. Peak MQ1 was concluded to contain neutral, basic, or very weakly bound negatively charged compounds since they eluted early (before one column volume). Fractions MQ2 to MQ5 contained negatively charged compounds in which MQ4 and MQ5 consisted of highly negatively charged compounds due to their late elution after the gradient. There were three minor peaks seen from monitoring at 280 nm that eluted between 10–17.5 ml. No significant peak was observed from monitoring at 360 nm, other than for neutral (unbound compounds) in MQ1.

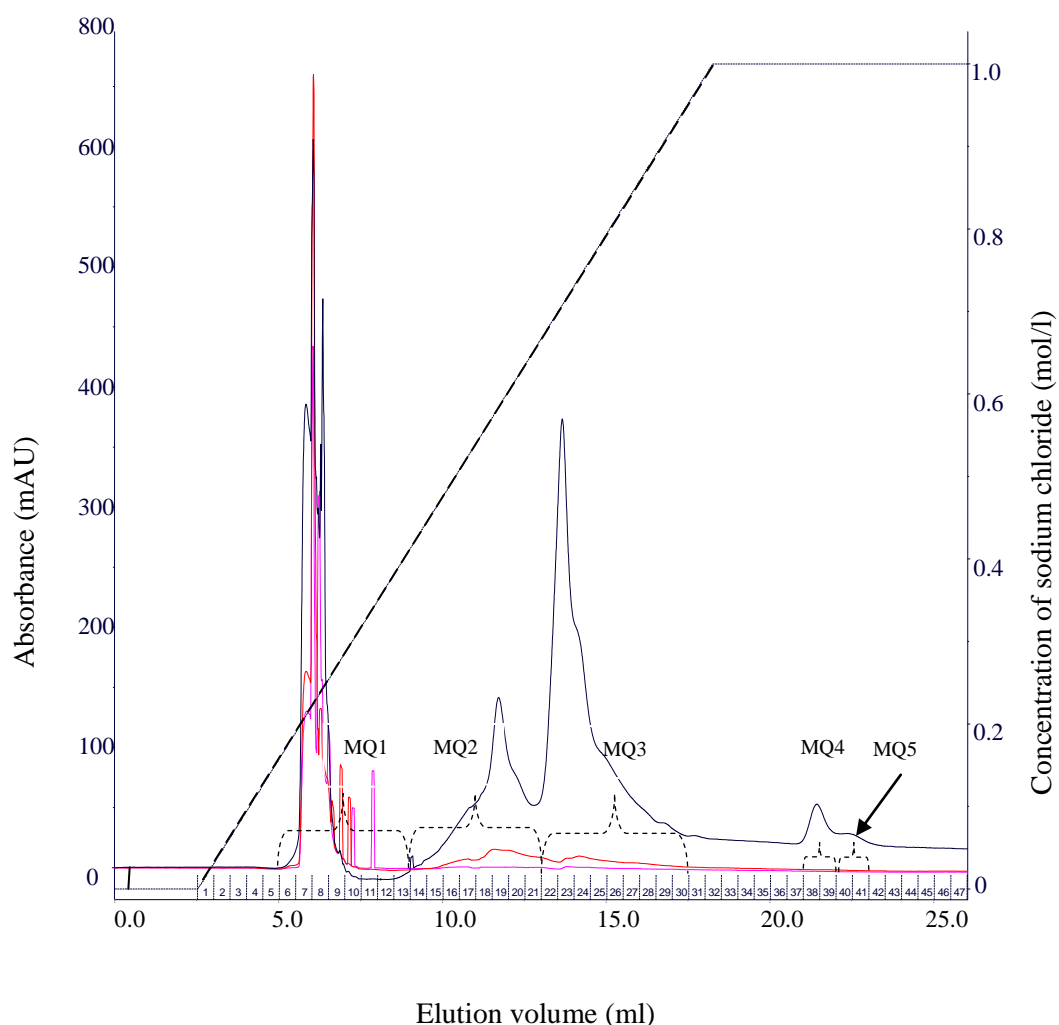


Figure 7.8: Elution profile of fraction RE11 chromatographed on a Mono Q HR 10/10 anion exchange column. The eluting conditions were mobile phases A: 20 mmol/l ethanolamine-HCl, pH 10 and B: 20 mmol/l ethanolamine-HCl, pH 10 + 1 mol/l sodium chloride. The dotted line indicates the concentration of sodium chloride in the eluent. The fraction regions are bracketed to show the fractions that were pooled to obtain the separated peaks. Upper, middle and lower elution profiles are absorbance at 220, 280 and 360 nm, respectively.

Individual fractions of 500 μ l volume were collected. These were pooled under respective peaks as shown in Figure 7.8. All pools of fractions were made up to the volume of the largest pool (2.25 ml) by addition of deionised water, and then were assayed for iron-binding antioxidant activity. Figure 7.9 shows the iron-binding antioxidant activity of these Mono Q HR 10/10 fractions. The activity was found mainly in the two major peaks, the pooled fractions MQ2 and MQ3.

MQ3, which was eluted at about 0.5 mol/l of sodium chloride, exhibited higher activity than MQ2. A pro-oxidant effect was observed for pool MQ1, while pools MQ4 and MQ5 gave very low activity. The activity given by fractions MQ2 to MQ5 revealed that negatively charged compounds contribute to the iron-binding antioxidant activity.

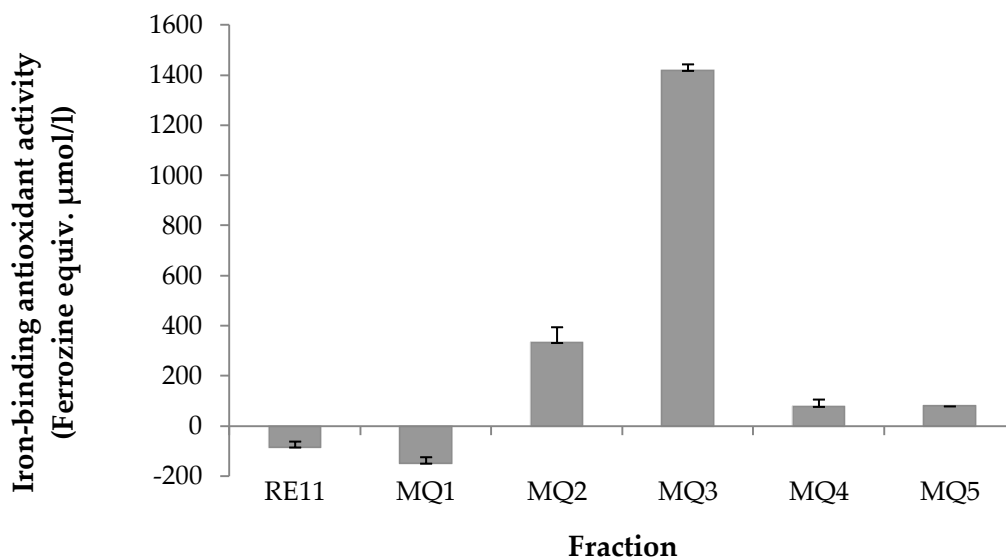


Figure 7.9: Iron-binding antioxidant activity of the fractions isolated from chromatography on the Mono Q HR 10/10 column (Figure 7.8). All fractions including a sample of the fraction RE11 loaded on the column were tested on the same volume.

Calculations were done based on the degree of dilution to show quantitatively all the activity was recovered. Results are shown in Table 7.3.

Table 7.3: Summary of the quantitative estimation of the iron-binding antioxidant activity of the fractions isolated from size exclusion chromatography on a Mono Q HR 10/10 column. Activity is shown as Ferrozine equivalent ($\mu\text{mol/l}$) \pm standard error of measurement ($n=3$).

Activity of material from column (from Figure 7.4)	Expected activity of pooled fraction if in 0.25 ml volume loaded	Volume of pooled fraction from reversed phase chromatography (ml) (Figure 7.8)	Dilution of original loaded from pooled fraction	Measured activity in pooled fraction (in equal volume)	Loss/gain in activity in pooled fraction
Fraction RE11 = 531.8	1/9 of 531.8 = 59.1	MQ1 = 2.0	0.25 ml/2.0 ml = 8 fold	-149.10 \pm 24.81	pro-oxidant
		MQ2 = 2.0	0.25 ml/2.0 ml = 8 fold	332.50 \pm 61.98	gain
		MQ3 = 2.25	0.25 ml/2.25 ml = 9 fold	1418.65 \pm 25.16	gain
		MQ4 = 0.5	0.25 ml/0.5 ml = 2 fold	77.52 \pm 28.27	gain
		MQ5 = 0.5	0.25 ml/0.5 ml = 2 fold	79.37 \pm 23.36	gain

It is likely that MQ3 consists of a mixture of compounds having carboxylic acid and/or phenol groups, since these groups will be negatively charged at pH 10, thus could be retained on the anion exchanger (Bruzzoniti *et al.*, 2001; Fritz & Takeda, 1968; Martos *et al.*, 2005; Masson, 2000; Takahashi *et al.*, 2003).

Unlike fractions in reverse phase chromatography in which the pro-oxidant effect occurred in the diluted fractions, fraction MQ1 is suggested to contain pro-oxidants, as a diluted fraction (to match the volume of the pooled fractions) and a non-diluted fraction of MQ1 both gave a pro-oxidant effect (data not shown). Table 7.4 shows the dilution made to all of the pools of fractions prior to assay.

Table 7.4: The dilution resulting from adjusting the pool of fractions in Figure 7.8 to the same volume.

Fraction	Volume of pooled fraction (ml)	Volume of water added (ml)	Final volume (ml)	Dilution factor (fold)
MQ1	2.0	0.25	2.25	1.1
MQ2	2.0	0.25	2.25	1.1
MQ3	2.25	0	2.25	0
MQ4	0.5	1.75	2.25	4.5
MQ5	0.5	1.75	2.25	4.5
Fraction RE11	0.25*	2.0	2.25	9

* Volume loaded on Mono Q HR 10/10 column

Since peak MQ3 shows shoulder peaks, this could represent slight differences in the amount of ionisation of the anionic groups in the molecules, indicating that different compounds exist in this fraction. Therefore, pool MQ3 was re-chromatographed on the same Mono Q HR 10/10 column with the same eluting conditions described in the method section (Section 7.2.2) in order to further resolve the antioxidant compounds. It was assumed that a purified compound will present as a single peak in the chromatogram (Luders *et al.*, 2005; Quinn *et al.*, 1988; Rajapakse *et al.*, 2005).

It is a common practice in chromatography to re-chromatograph poorly resolved fractions on the same type of column in order to completely resolve the compounds. Kemeny *et al.* (1984) used this technique to purify hyaluronidase, an enzyme from honeybee venom. A few authors were able to purify a single radical scavenging peptide from marine foods by re-chromatographing the fractions from a preparative reversed phase column onto an analytical reversed phase column (Mendis *et al.*, 2005; Park *et al.*, 2001; Rajapakse *et al.*, 2005; Ranathunga *et al.*, 2006). Earlier, Gauldie *et al.* (1976) used the re-chromatography approach on a cation exchange column several times until getting the purified compounds of the active peptides from bee venom.

7.3.3 *Re-chromatography profile of MQ3 fraction.*

The resulting chromatogram from re-chromatography of the fraction MQ3 from Figure 7.8 on the Mono Q HR 10/10 column is shown in Figure 7.10. Surprisingly, an almost identical elution profile was obtained as with the first chromatography (Figure 7.8). The only difference was the absorbance of fraction MQ1 was lower than in the first chromatography, and the peak designated MQ2 in the first chromatography was now partially resolved into two peaks. The peak shape of what was MQ3 to MQ5 in the first chromatography remained unchanged. The results indicated that compounds which eluted in fractions MQ1 to MQ5 were also present in fraction MQ3, and were not fully separated with one run of anion exchange. This situation supported the hypothesis that aggregation of molecules was occurring in fraction MQ3.

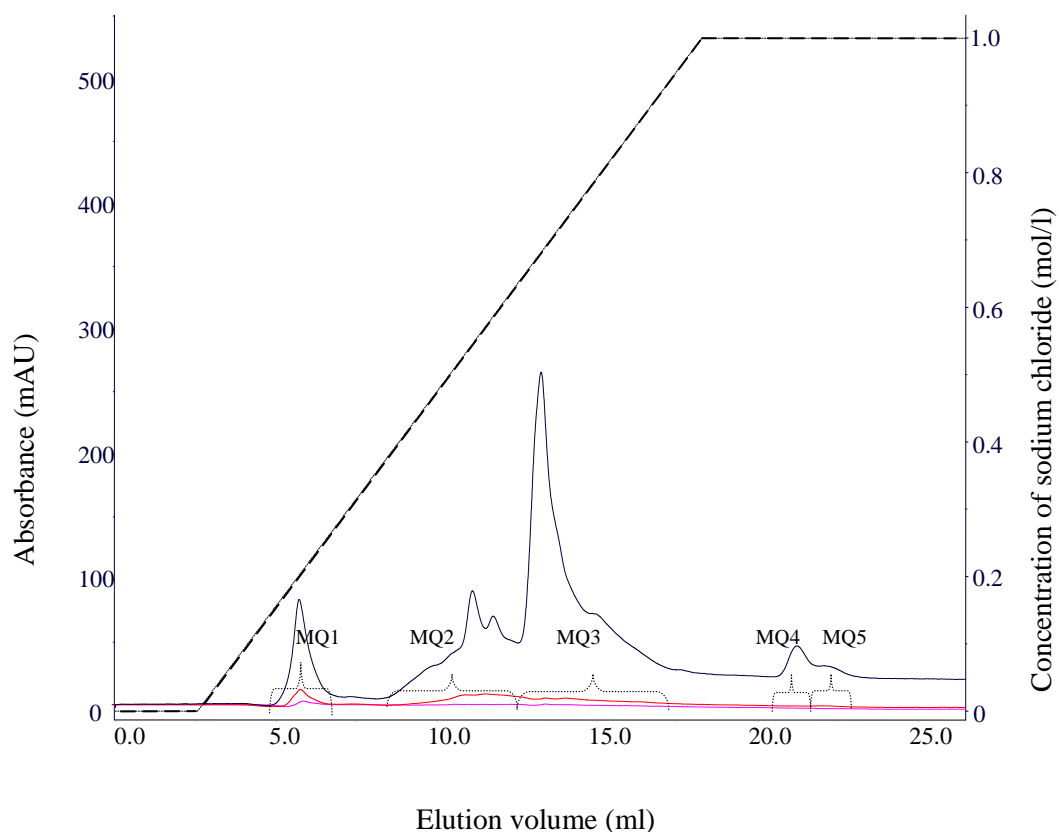


Figure 7.10: Elution profile of the re-chromatography of pooled fraction MQ3 (from Figure 7.8) on Mono Q HR 10/10. The eluting conditions were the same as in Figure 7.8. Upper, middle and lower chromatograms are UV absorbance at 220, 280 and 360 nm, respectively. The positions of peaks MQ1-MQ5 has been marked as in Figure 7.8.

To test the hypothesis that aggregation was occurring in fraction MQ3, a series of further chromatography experiments was carried out on the Mono Q HR 10/10 column. An aliquot of fraction RE11 (see Figure 7.5) was injected onto the column and the individual tubes that represented peak MQ3 were collected and pooled (see Figure 7.11A). An aliquot of the MQ3 pooled fraction was then re-chromatographed on the same column and the individual tubes representing peak MQ3 were collected and pooled (see Figure 7.11B). Finally, the aliquot of the MQ3 pooled fraction from Figure 7.11B was re-chromatographed on the same column (see Figure 7.11C). The sample loading and eluting conditions were the same as described for the chromatography shown in Figure 7.8.

The results seen in the re-chromatography profiles in Figure 7.11 (A-C) confirm the conclusion reached from those in Figure 7.10 that aggregation of molecules is

occurring in fraction MQ3 and that the aggregation was not fully separated by subsequent re-chromatography. The active fraction MQ3 contains compounds that are also eluted in fractions MQ1, MQ2, MQ4 and MQ5. However, it should be noted that the intensity of the absorbance of peaks MQ1 and MQ2 was minimised in subsequent re-chromatography (Figure 7.11B–C), indicating that less compounds of fractions MQ1 and MQ2 remained in fraction MQ3 each time it was re-chromatographed. There could have been compounds in the original MQ1 and MQ2 that were not there when MQ3 was re-chromatographed. Some components from the aggregates in MQ3 are eluted in the position of MQ1 and MQ2 but may be different compounds.

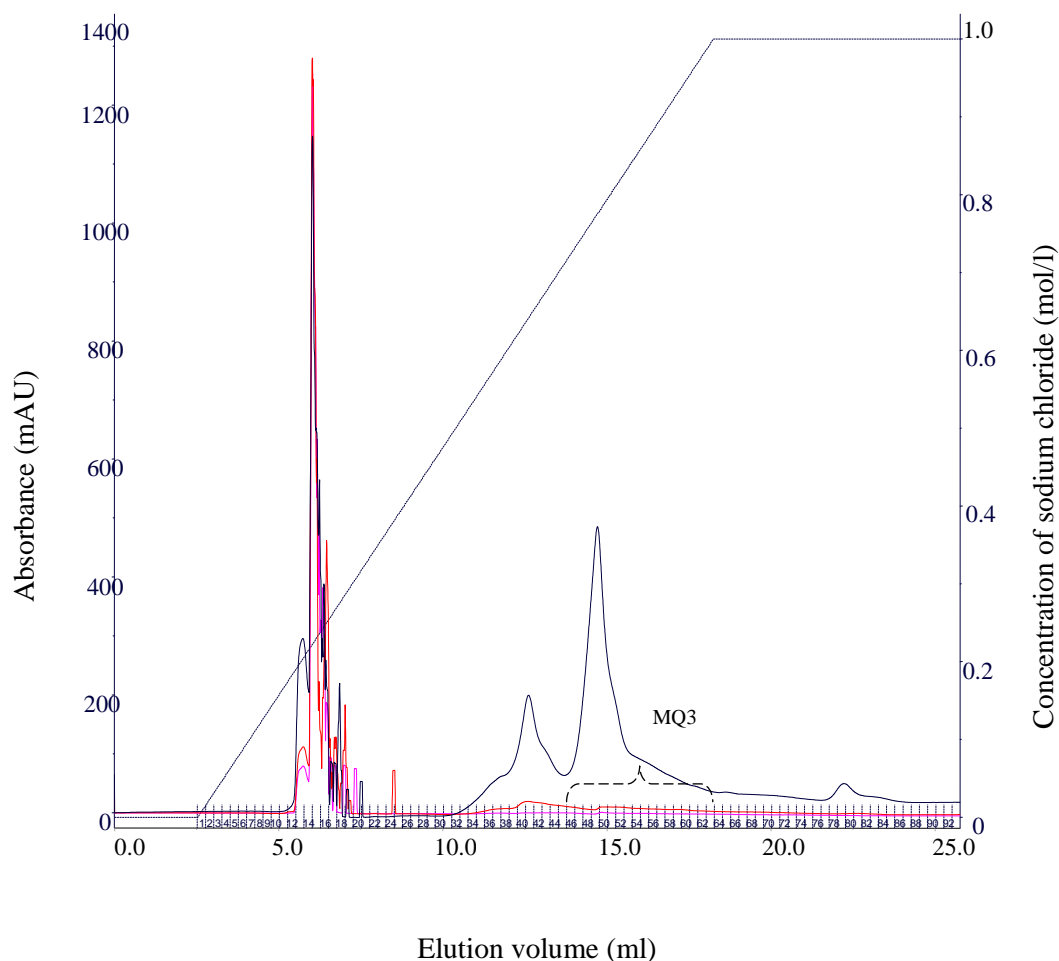


Figure 7.11A: Chromatography on the Mono Q HR 10/10 column of an aliquot of fraction RE11 (Figure 7.5). The sample loading and eluting conditions were the same as described in the methods section. Upper, middle and lower chromatograms are UV absorbance at 220, 280 and 360 nm, respectively.

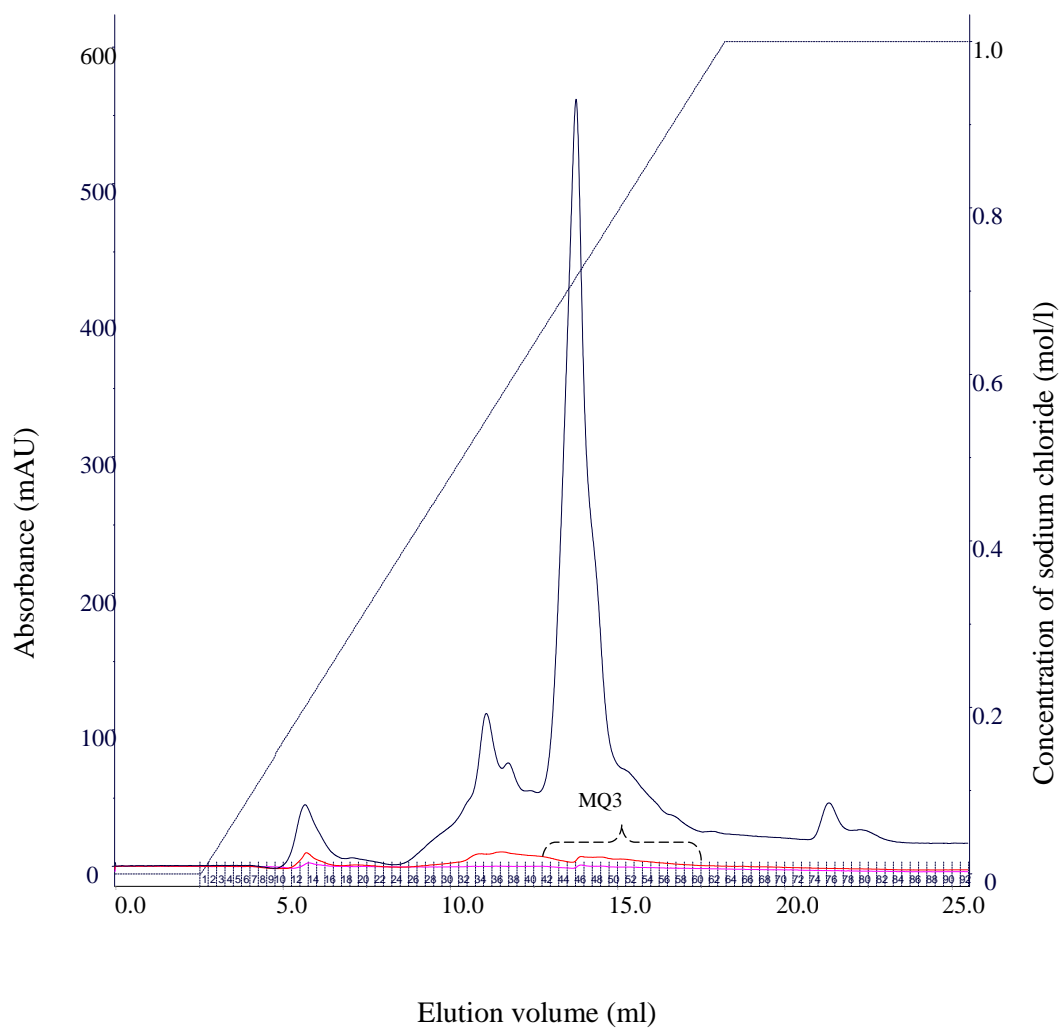


Figure 7.11B: Subsequent re-chromatography on the Mono Q HR 10/10 column of fraction MQ3 from Figure 7.11A. The sample loading and eluting conditions were the same as described in the methods section. Upper, middle and lower chromatograms are UV absorbance at 220, 280 and 360 nm, respectively.

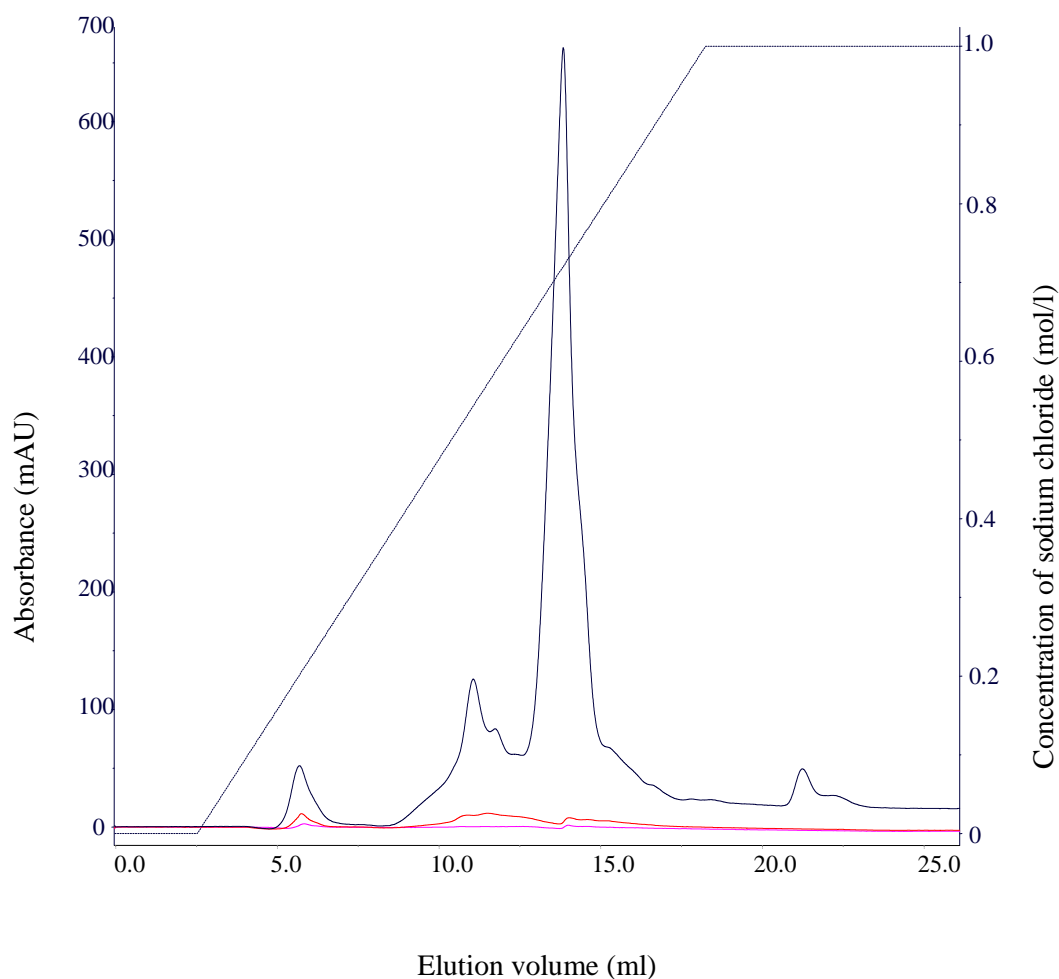


Figure 7.11C: Subsequent re-chromatography on the Mono Q HR 10/10 column of fraction MQ3 from Figure 7.11B. The sample loading and eluting condition was the same as described in method section. Upper, middle and lower chromatograms are UV absorbance at 220, 280 and 360 nm, respectively.

To check whether the dis-aggregation could be increased, a large amount (200 ml) of mobile phase A (20 mol/l ethanolamine-HCl, pH 10) was used to remove the MQ1 materials from the aggregates before starting the gradient as a type of pre-treatment for getting the purified compounds. The anion exchanger resin acts by retaining the antioxidants in the column. The chromatographic conditions remained the same as described in Section 7.2.2.

7.3.4 Elution profile of fraction RE11 after washing treatment

Figure 7.12 (A and B) shows the elution profile of RE11 fraction when subjected to prior washing with a large amount of mobile phase A. Peak MQ1 shows high UV absorbance as compared with Figure 7.8 where there was no washing treatment, indicating that more MQ1 components have been washed off. The small peaks which were previously seen in minimal amounts at 280 and 360 nm were improved in their peak shape, indicating that the aggregation was preventing the chromatograms from coming out with a good peak shape.

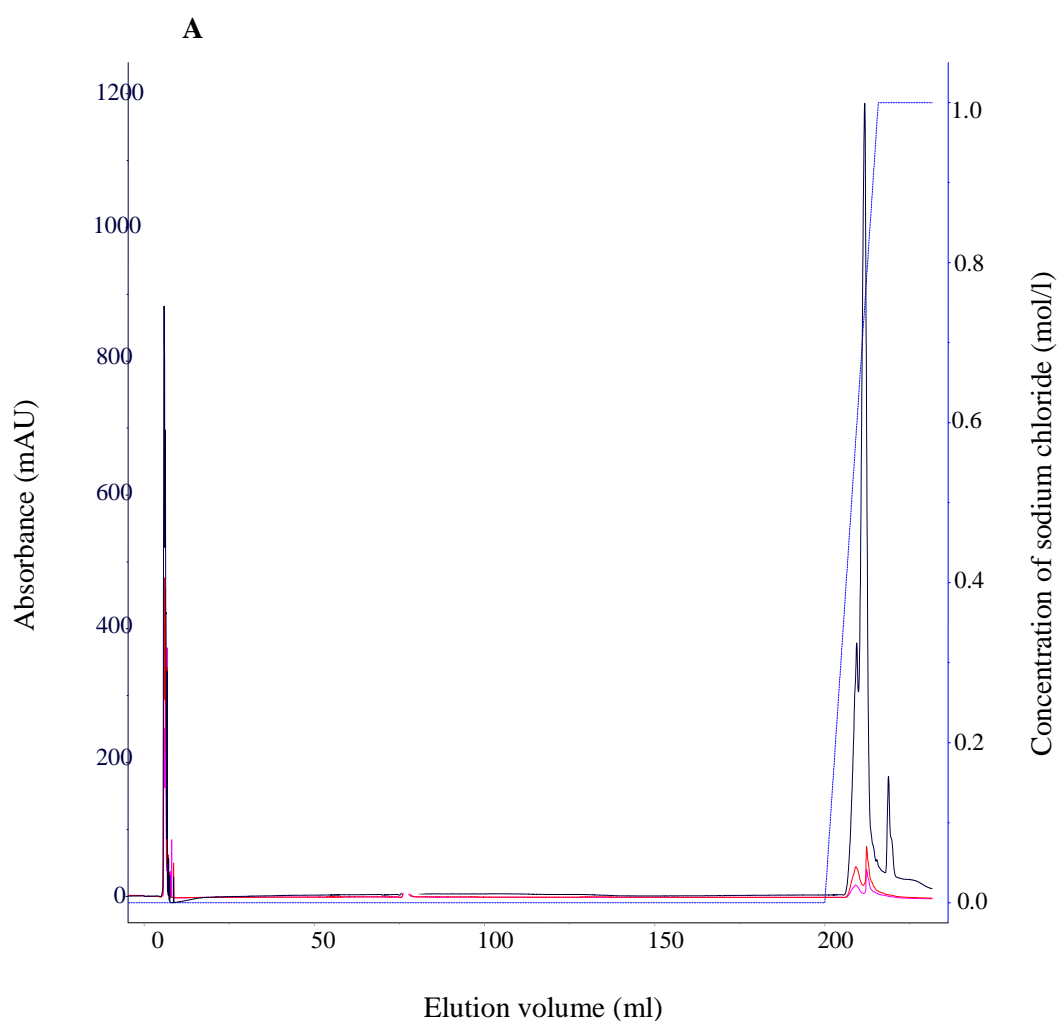


Figure 7.12A: Chromatography on the Mono Q HR 10/10 column of an aliquot of fraction RE11 (Figure 7.5) after 200 ml of washing with buffer A (20 mol/l ethanolamine-HCl, pH 10).

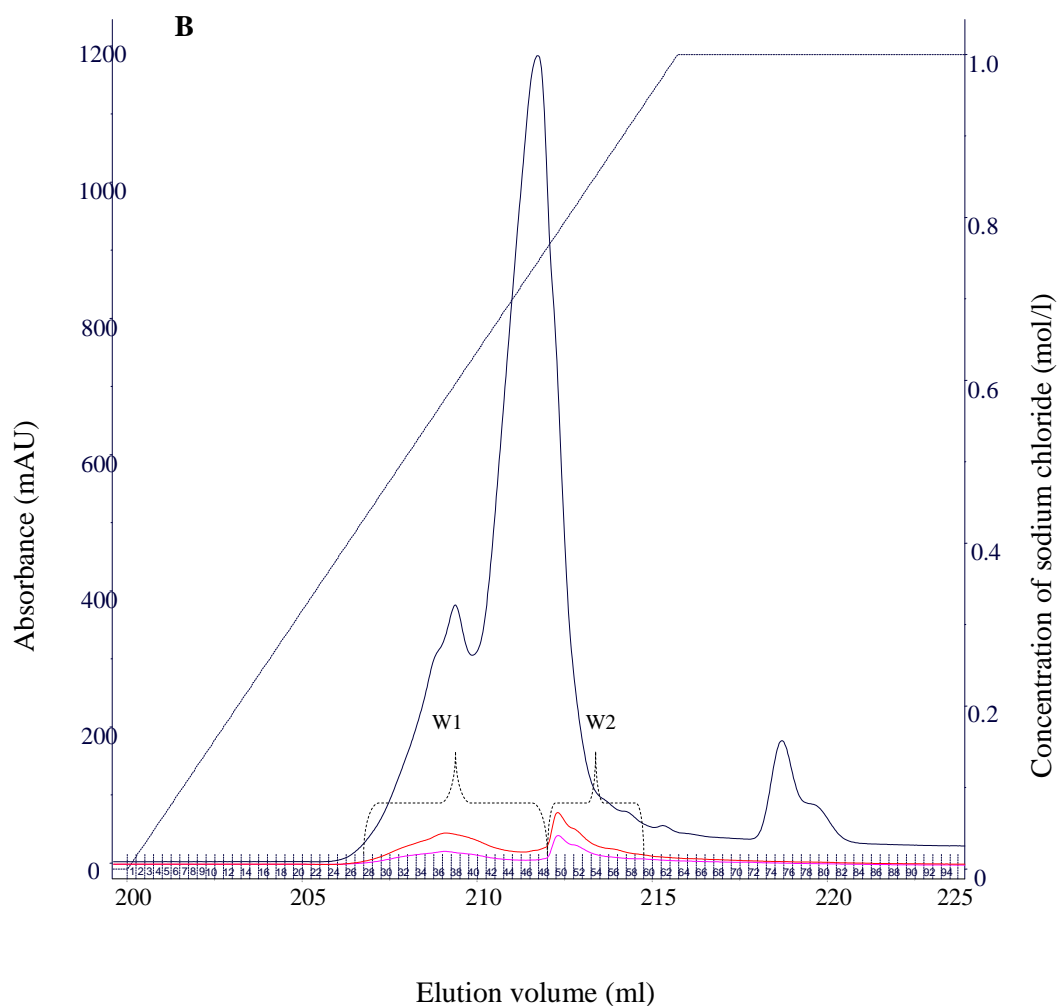


Figure 7.12B: A zoom-in of a region of the chromatogram shown in Figure 7.12A. The fraction regions in dotted brackets represent fractionation of the respective peaks (W1 and W2). Upper, middle and lower chromatograms are absorbance at 220, 280 and 360 nm, respectively.

Since the peak shapes at wavelengths 280 and 360 nm were improved, it was attempted to see if there were any differences of the activity among the individual fractions in these regions. The selected tubes (250 μ l) representing two peaks (W1 and W2) at 280 and 360 nm (Figure 7.12B-dotted brackets) were tested individually for activity instead of pooling the fractions. In this way, the activity of the fractions at least can be compared with each other and any differences in activity between individual fractions absorbing at specific wavelengths could be observed easily.

It was found that all of the tubes assayed showed iron-binding antioxidant activity without an obvious pattern of elution relating to the absorbance at different wavelengths (see Figure 7.13). The activity was spread out almost evenly in all individual tubes over the whole three wavelengths. Tube 48, which had the highest absorbance at 220 nm but low absorbance at 280 and 360 nm, did not show the highest activity related to its higher absorbance at 220 nm. The result suggested that the fractions contained a lot of different components which could be almost similar to each other in their molecular structure and/or in the amount of their ionisable groups and were not separating cleanly from each other.

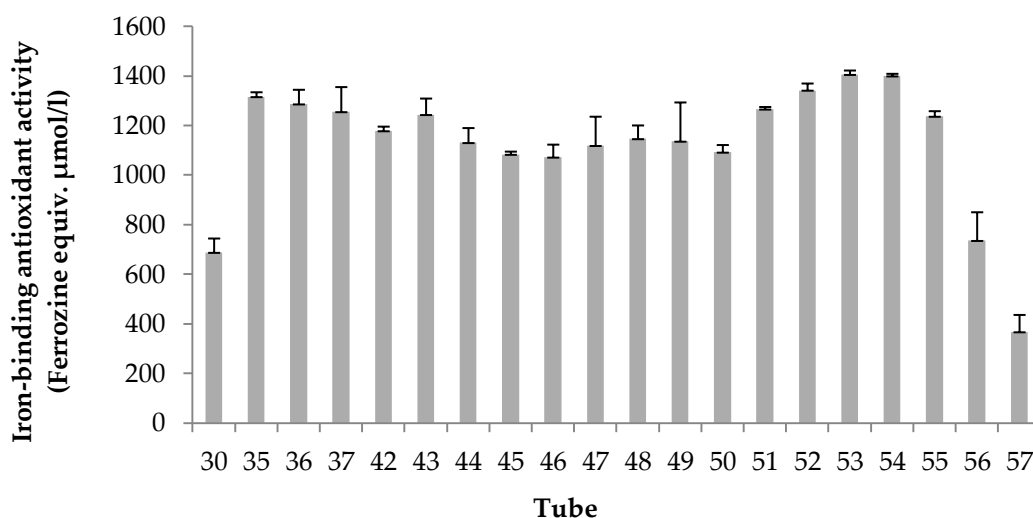


Figure 7.13: Iron-binding antioxidant activity of the individual fractions shown in Figure 7.10B. All fractions had the same volume (250 μl each). Values are given as the mean \pm standard error of mean of two replicates.

To prove that anion exchange fraction contains multiple components with similar sizes which aggregated, tubes 41–59 from Figure 7.12B were pooled and run on size exclusion chromatography using the method described in Section 6.2.2, using water as eluent. The result is shown in Figure 7.14. It shows that a single peak eluted at 17–19 ml, *i.e.* at the elution volume, with absorbance at 220, 280 and 360 nm, indicating that different molecules with similar size were in the pool of the anion exchange fraction. Nevertheless, it is interesting to note that the washing treatment did not affect the activity in the fractions, indicating that what was removed by this treatment was material without iron-binding antioxidant activity.

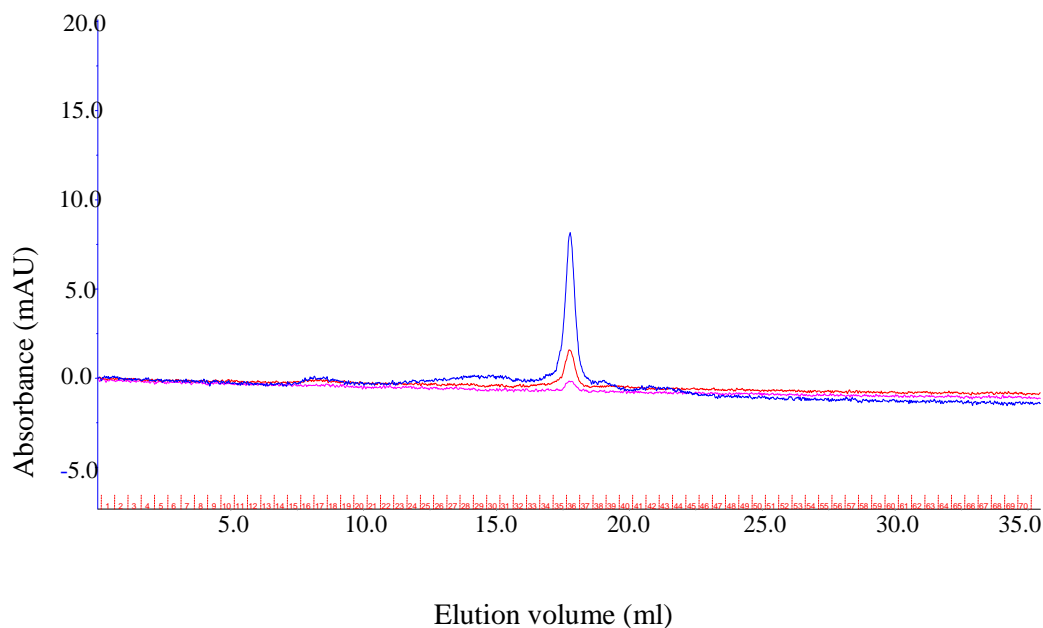


Figure 7.14: Elution profile from the chromatography on Superdex[®] Peptide of the pooled fraction (tubes 41–59) from Figure 7.12B. Deionised water was used as an eluent. Upper, middle and lower chromatograms are absorbance at 220, 280 and 360 nm, respectively.

7.4 Conclusion

The present study has shown that it is possible to isolate the iron-binding antioxidants from honeydew honey by using reversed phase and anion exchange chromatography consecutively. The isolated iron-binding antioxidants in the active fraction of both columns showed higher activity when compared with the activity in the loaded fraction. The diluted fractions from the reversed phase chromatography showed a pro-oxidant effect caused by dilution which is due to the low concentration of antioxidants. It is suggested that the anion exchange column could separate antioxidants from pro-oxidants (*i.e.* the compounds in fraction MQ1). It was aimed to isolate and purify the antioxidants, however, no single compound was able to be isolated in anion exchange chromatography since the activity was distributed over all of the peak MQ3 region. It was concluded at this stage that reversed phase and anion exchange chromatography could isolate the antioxidants, but not successfully purify the compounds. In addition, the aggregation behaviour of the iron-binding antioxidants with other molecules was identified, thus isolation of pure antioxidant compound(s) using the anion exchange column was not feasible.

CHAPTER 8

SALT-FREE ISOLATION AND PURIFICATION OF IRON-BINDING ANTIOXIDANT(S) USING CONSECUTIVE ANION EXCHANGE AND REVERSED PHASE CHROMATOGRAPHY PRIOR TO MASS SPECTROMETRY ANALYSIS

8.1 Introduction

The drawback of performing anion exchange chromatography prior to mass spectrometry (MS) analysis is that the elution often involves a high concentration of salt. The presence of a high salt concentration in the anion exchange fractions obtained is not compatible with electrospray ionization MS (Mohsin, 2000) since there is formation of a salt adduct that suppresses the MS signal (*i.e.* gives a lower response for the analytes) (Gustavsson *et al.*, 2001). This deleterious effect given by the eluent containing salt when integrated with MS often makes it necessary to remove salt prior to MS analysis.

Different techniques have been used for the removal of salt in various sample preparations. The most commonly used technique is with a solid phase extraction (SPE) cartridge which contains hydrophobic silica-C18 particles for retaining the hydrophobic compounds. Other desalting techniques such as ultrafiltration (Chernokalskaya *et al.*, 2004), size exclusion chromatography (Šalplachta *et al.*, 2004) and the use of an online salt trapping column (Yoshida *et al.*, 2006) are also used.

Earlier work attempting to desalt the active fraction from anion exchange chromatography using reversed phase chromatography showed that the antioxidants were not retained on the reversed phase column (data not shown). Therefore, an alternative approach to this situation is to use salt-free eluent(s) in the last step of the chromatographic separations. Thus, the isolation sequence was

switched from the sequence described in Chapter 7 to that of having reversed phase chromatography as the final step in the isolation stages and thus obtaining a salt-free fraction of isolated antioxidants as well as the organic solvent present in the fractions to facilitate the analysis with MS. Since there is no rigid rule in the sequence of the columns needed to be used for isolation and purification, the switching of the chromatography sequence seemed feasible.

The aim of the present work was to isolate and purify the iron-binding antioxidant(s) using salt-free eluents in chromatography techniques prior to electrospray ionization MS analysis.

8.2 Methods

In all cases, the mobile phases were filtered through a 0.22 μm cellulose filter membrane and degassed. The sample was centrifuged at 10 000 $\times g$ for 15 minutes before injecting onto the column. All of the chromatography stages were conducted at 25°C. The columns needed to be cleaned after every 5 to 10 runs to maintain the reproducibility of the peaks. The flow diagram for the isolation and purification process to obtain salt-free fractions of iron-binding antioxidants from fraction SP2 (see Figure 6.4) is shown in Figure 8.1.

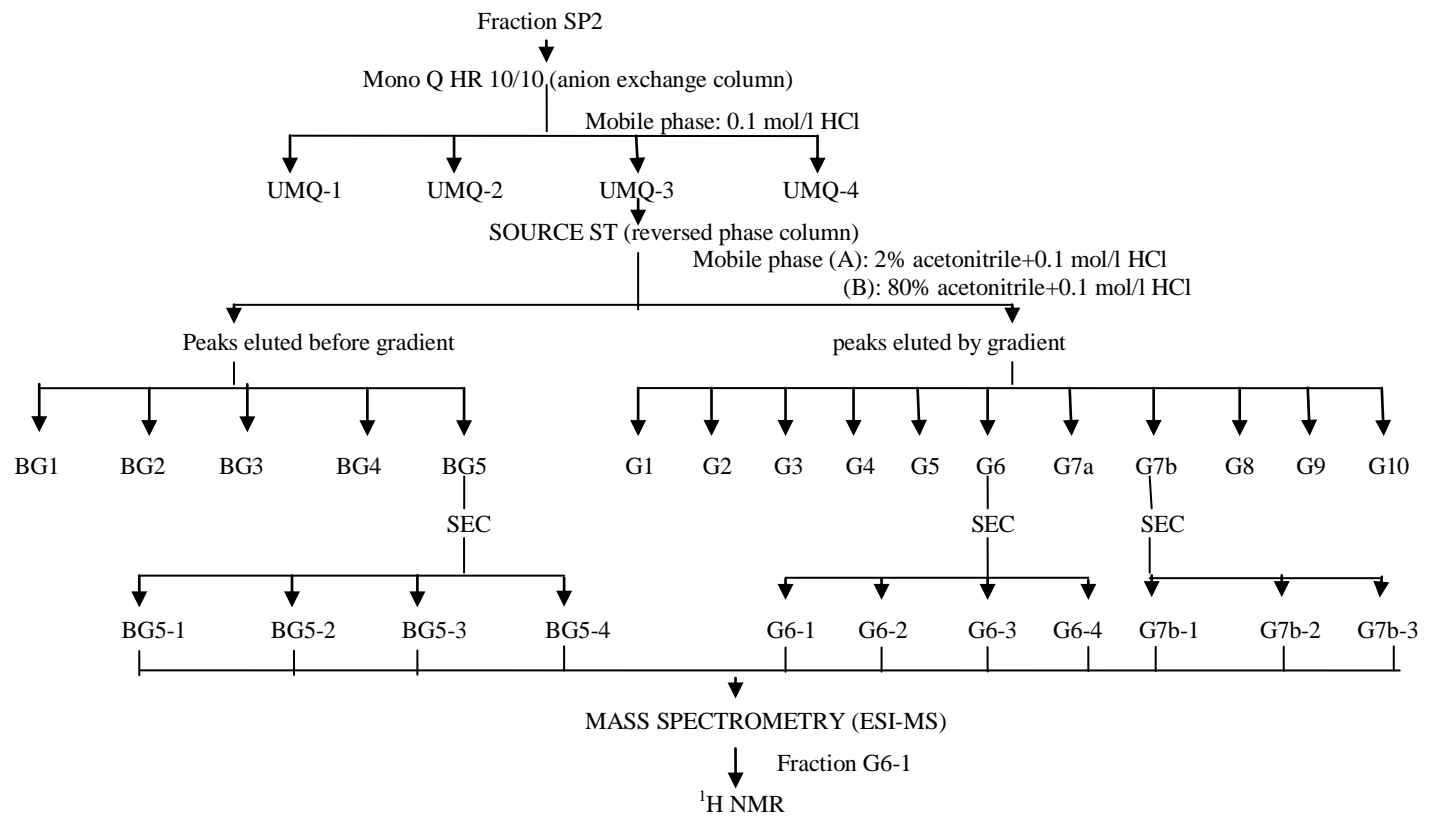


Figure 8.1: Flow diagram of scheme of salt-free purification of fraction SP2. SEC=size exclusion chromatography on Superdex® Peptide HR 10/30, with 30% acetonitrile as the mobile phase.

8.2.1 Chromatography of Superdex[®] Peptide fraction (SP2) on an anion exchange column

The column Mono Q HR 10/10 was first equilibrated with 20 mmol/l ethanolamine-HCl buffer, pH 9.35 for several column volumes until the baseline was stable. One column volume is about 8 ml. Then 250 µl of SP2 fraction (see Figure 6.4) was mixed with 250 µl of 20 mmol/l ethanolamine-HCl, pH 9.5 to ionize the compounds before application onto the anion exchange column (Mono-Q HR 10/10). The equilibration continued with 20 mmol/l ethanolamine-HCl, pH 9.5 for 25 ml to make sure all an ionic analytes bound to the column. The column was then washed with 20 ml of deionised water to remove ethanolamine buffer and unbound compounds before starting the gradient. The removal of ethanolamine buffer before starting the gradient was necessary to avoid buffering preventing the pH from decreasing during the elution gradient of HCl. The analytes were eluted with a linear gradient from water to 0.1 mol/l hydrochloric acid (pH 1) (0–0.1 mol/l) for three column volumes (24 ml), and held at 0.1 mol/l hydrochloric acid for another eight column volumes (64 ml) or until the baseline was stable. The chloride ions (Cl^-) from the eluent are exchanged with anionic compounds, while anions accept protons from the eluent (hydrochloric acid), and thus are converted to their protonated form. A flow rate of 0.8 ml/min was used. Individual fractions of 1.0 ml were collected and pooled based on the respective peaks as shown in Figure 8.2 (in Section 8.3.1), then freeze-dried. During freeze-drying, the aqueous hydrochloric acid in the fractions is evaporated as HCl gas, thus leaving the fractions without any salt ions. All dried fractions then were re-dissolved with 250 µl (the same volume as loaded) of deionised water for quantitative comparison in the iron-binding antioxidant assay.

8.2.2 Further chromatography on a reversed phase column of the active fraction from anion exchange chromatography

The most active fraction from chromatography on Mono Q HR 10/10, fraction UMQ-3 (see Table 8.1 in Section 8.3.1) was diluted to 1.5 ml with deionised water and 500 µl was injected onto the reversed phase column. The reversed phase column used was a SOURCE[™] 5RPC ST 4.6/150 column (4.6 mm x 150 mm) because the previously used reversed phase column (RESOURCE 3 ml) had

become obstructed. The column uses the same matrix (polystyrene divinylbenzene) as the RESOURCE 3 ml column. One column volume is about 7.5 ml. Mobile phase A used was 2% acetonitrile + 0.1 mol/l hydrochloric acid (pH 1) and mobile phase B was 80% acetonitrile + 0.1 mol/l hydrochloric acid (pH 1). The analytes were eluted with a linear gradient of 0–100% B for five column volume (37.5 ml) then the eluent was held at 100% B until the baseline became stable. The individual fractions (0.25 ml/tube) were collected and pooled based on the respective peaks as shown in Figure 8.3 and Figure 8.4A-B (in Section 8.3.2). All pools of fractions were dried with a rotary evaporator at 30°C to remove the acetonitrile and then were each re-dissolved with 250 µl of deionised water for quantitative comparison prior to assay of iron-binding antioxidant assay. The results of the assay are shown in Table 8.2 (in Section 8.3.2).

8.2.3 Size exclusion chromatography of the active fractions from reversed phase chromatography

From each of the selected pooled fractions based on the corresponding peaks that gave high iron-binding antioxidant activity after the reversed phase chromatography (*i.e.* G6, BG5 and G7b) a volume of 250 µl was injected onto a size exclusion chromatography column. The column used was Superdex[®] Peptide HR 10/10. The eluent was 30% acetonitrile with a flow rate of 0.3 ml/min. The fractions that eluted from the column were monitored at 220 nm, 280 nm and 360 nm. Fractions of 0.5 ml were collected and these were pooled based on corresponding peaks in the chromatogram. Then, each pooled fraction was brought up to the same volume as the largest pool, using deionised water. The pools were then assayed for iron-binding antioxidant activity. The results are shown in Figure 8.7A-C. The elution profiles of the fractions are shown in Section 8.3.3 (Figure 8.5A-C).

8.2.4 Characterization of iron-binding antioxidants by electrospray ionization-mass spectrometry (ESI-MS) and ^1H NMR spectroscopy

8.2.4.1: Electrospray-ionization mass spectrometry analysis

The solvent was removed from all the active fractions (G6-1, G6-2, G6-3, G6-4, BG5-1, BG5-2, BG5-3, BG5-4, G7b-1, G7b-2, G7b-3) and selected inactive fractions (G7a, G8 and BG1) using a rotary evaporator at 30°C. Using a four decimal place balance, no mass could be detected in all dried fractions, indicating that there was very little mass in each fraction. The individual fractions were thus assumed to have a weight of 0.1 mg or less. They were then each re-dissolved in 1 ml of 30% acetonitrile for mass spectrometric analysis.

An electrospray ionization mass spectrometer (ESI-MS), a MicroTOF instrument (Bruker Daltonics), was used in both positive and negative ion mode. Manual injection was performed using a syringe pump and the scanning mass range of the mass spectrometer was 50–800 m/z . The instrument was operated by the technicians in the Chemistry Department; Pat Gregard and Wendy Jackson. The results are shown in Figure 8.8 (A–D) and 8.9 (A–B) in Section 8.3.4. The formula weight of the selected m/z ions was calculated by Dr Michele Prinsep using a Bruker Data Analysis software.

8.2.4.2 ^1H NMR spectroscopy analysis

A sample for NMR was prepared by dissolving the freeze-dried fraction G6-1 in 1 ml of D_2O , and this solution was then transferred to an NMR tube. The mixture was immediately analyzed by ^1H NMR spectroscopy (Bruker Avance, 400 MHz) with 800 scans acquired. The instrument was operated by Dr Michele Prinsep in the Chemistry Department. The result is shown in Section 8.3.4 (Figure 8.10).

After analysis using D_2O as solvent, the same sample then was subjected to rotary evaporation at 30°C to remove the solvent prior to freeze drying. The freeze-dried fraction G6-1 was dissolved in $\text{DMSO-}d_6$ (Aldrich, 0.5ml) and transferred to an NMR tube. A ^1H spectrum was acquired (Bruker Avance, 400 MHz) with presaturation of water and solvent signals, for 4 hours (2048 scans). The ^1H NMR spectrum of the sample in $\text{DMSO-}d_6$ is shown in Figure 8.11 (in Section 8.3.4).

8.3 Results and Discussion

8.3.1 Isolation of Superdex[®] Peptide active fraction (SP2) on anion exchange column using salt-free eluent

One of the sorption and eluting techniques in ion exchange chromatography which is widely applied is the changing of counter-ions of the columns. In anion exchange chromatography, the counter-ions that replace the analytes are negative ions, commonly chloride, sulphate, acetate and carboxylate ions. The equilibration of the column prior to sample loading with 20 mol/l ethanolamine-HCl, pH 9.5 buffer was to bind chloride ions in the buffer onto the Mono Q resin. Based upon results in Chapter 7, the iron-binding antioxidants will ionize at alkaline pH, thus will replace chloride ions on the matrix to bind onto the column. Hydrochloric acid was used as eluent so that the antioxidant anions would have their ionisation suppressed and thus no longer bind to the column. Also it is a strong acid and can evaporate as a gas when fractions are freeze-dried. Thus, this will yield salt-free fractions.

Few researchers have used acids for elution of antioxidant compounds from an anion exchange column. Zhang *et al.* (2009) used 0.2 mol/l hydrochloric acid to elute the antioxidant peptide from rapeseed protein hydrolysates. Saiga *et al.* (2003) used 1.0 mol/l hydrochloric acid to elute acidic antioxidant peptides from porcine myofibrillar proteins. Others used 1% formic acid (Chaisakdanugull *et al.*, 2007) and acetic acid (Kammerer *et al.*, 2011) as an eluent to elute organic compounds and phenolic compounds from fruits. Previously, this technique has been used by Piez *et al.* (1952) to desalt a solution of amino acids on an anion exchange column.

Figure 8.2 shows the elution profile of compounds from Superdex[®] Peptide SP2 fraction chromatographed on a Mono Q HR 10/10 column. The chromatogram was expected to be different from the chromatogram of reversed phase active fraction (RE11) in Chapter 7 on the same column. This is because different fractions (at a different stage of isolation) were loaded, as well as different eluting conditions being used. There were four fractions obtained, designated as UMQ-1 to UMQ-4 as marked in Figure 8.2. Elution of the compounds in fractions UMQ-3 and UMQ-4 was only observed at 220 nm, while compounds in fraction UMQ-1

and UMQ-2 were detected at 220, 280 and 360 nm. Since the column used was an anion-exchanger, the retention of these peaks until eluted with the gradient signified that fractions UMQ-1 to UMQ-4 are negatively charged compounds, with UMQ-3 and UMQ-4 carrying a lot of negative charges, since they were retained more strongly.

The tubes under the respective peaks were pooled and freeze-dried. Then, each dried pool was re-dissolved in 250 µl of deionised water and assayed for iron-binding antioxidant activity. Table 8.1 shows the results. Fraction UMQ-3 was found to exhibit the highest iron-binding antioxidant activity. The activity in fraction UMQ-3 was 5.6-fold higher than that in fraction UMQ-4. Fractions UMQ-1 and UMQ-2 were pro-oxidants. Unlike in Chapter 7, where the dilution of the fractions would affect the behaviour of the antioxidants to become pro-oxidants, the present result shows that fractions UMQ-1 and UMQ-2 were pro-oxidant in nature since no dilution has been applied to these fractions. The result also indicated that antioxidants (fractions UMQ-3 and UMQ-4) had been separated from pro-oxidants (fractions UMQ-1 and UMQ-2).

The UV absorbance at 220 nm for fractions UMQ-3 and UMQ-4 is characteristic of peptide bonds, which is indicative that proteinaceous components may be in these fractions. However, tannins such as procyanidin (Yanagida *et al.*, 2002) and ellagitannins (Aaby *et al.*, 2005; Lee, 2004) also absorb at 220 nm. The active compounds from willow bark which have a phenol ring, salicin (2-(hydroxymethyl)phenyl-β-D-glucopyranoside) and its hydrolysis product, saligenin (hydroxyl benzyl alcohol), also have been reported to absorb UV at 220 nm (Juntheikki & Julkunen-Tiitto, 2000).

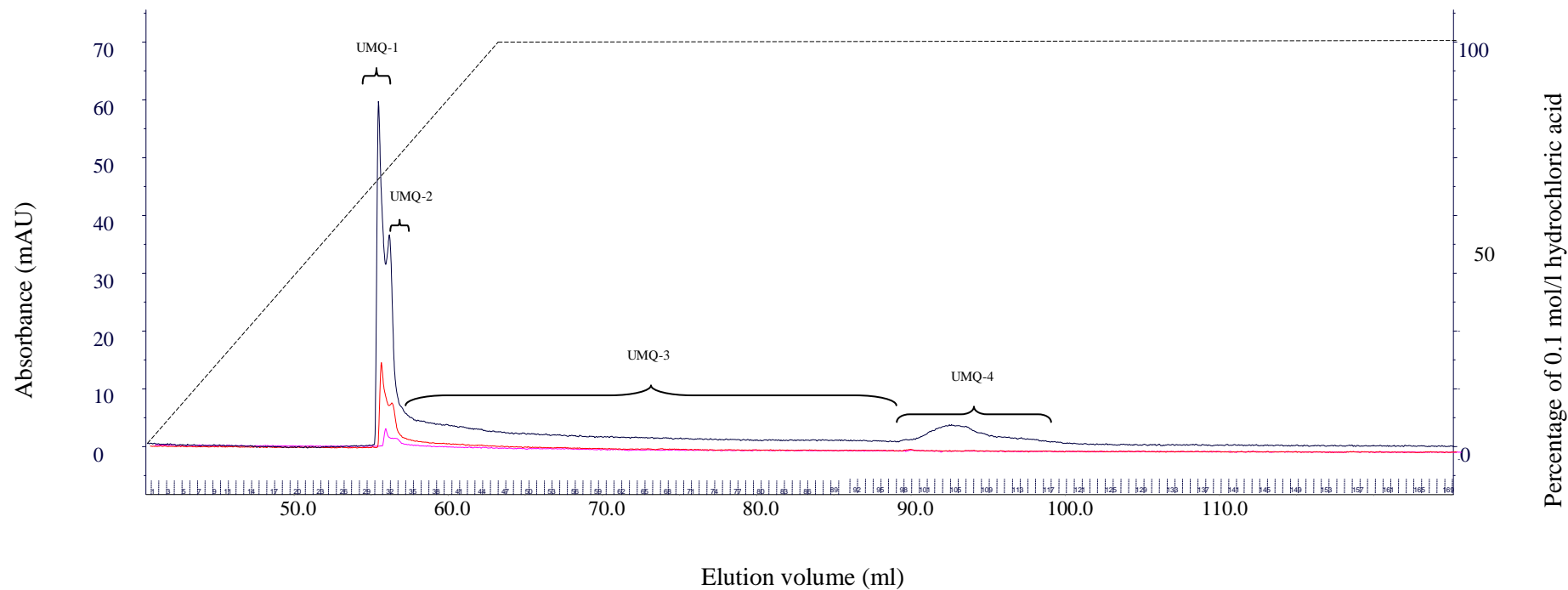


Figure 8.2: Elution profile of fraction from Superdex[®] Peptide (SP2) chromatographed on Mono Q HR 10/10 anion exchange column. The eluting conditions were mobile phase A: deionised water, and mobile phase B: 0.1 mol/l hydrochloric acid. The dotted line indicates the linear gradient at 0-100% B in 24 ml and hold up at 100% B until the baseline was stable. The flow rate was 1 ml/min. Upper, middle and lower lines are absorbance at 220, 280 and 360 nm, respectively.

Table 8.1: Summary of the quantitative estimation of the iron-binding antioxidant activity of the fractions isolated from chromatography on a Mono Q HR 10/10 column. Activity is shown as Ferrozine equivalent ($\mu\text{mol/l}$) \pm standard error of mean ($n=3$).

Activity of material from column (from Figure 6.4)	Expected activity of pooled fraction if in volume loaded	Volume of concentrated pooled fraction from anion exchange chromatography (Figure 8.2)	Dilution of original loaded from pooled fraction	Measured activity in pooled fraction	Loss/gain in activity in pooled fraction
Fraction SP2 = 328.60	1/18 of 328.60 = 18.25		250 μl /250 μl (no dilution)		
		UMQ-1 = 250 μl		UMQ-1 = -545.6 ± 39.6	loss (pro-oxidant effect)
		UMQ-2 = 250 μl		UMQ-2 = -566.1 ± 2.2	loss (pro-oxidant effect)
		UMQ-3 = 250 μl		UMQ-3 = 309.0 ± 77.0	gain
		UMQ-4 = 250 μl		UMQ-4 = 54.9 ± 19.6	gain

It has been reported that tannins generally carry highly negative charges and adsorb tightly onto anion exchange columns (Zhang *et al.* 2009) which is in agreement with the late elution of antioxidants in the present result work. Thus, it could be suggested that UMQ-3 is a mixture of several different components which consists of the compounds having hydroxyl, phenol and/or carboxylic acids. To separate these various components, thus it seemed sensible to use reversed phase chromatography in the next isolation step.

8.3.2 Isolation of the antioxidant component of anion exchange fraction (UMQ-3) by reversed phase chromatography

The retention mechanism of the SOURCE™ 5RPC ST 4.6/150 column is similar to that of the RESOURCE 3 ml column used previously (*i.e.* hydrophobic and/or π - π bonding), as discussed in Section 7.3.1.1. To ensure that all compounds in fraction UMQ-3 existed in a protonated form, 0.1 mol/l hydrochloric acid was added into the mobile phases to suppress any ionization so that the molecules become more hydrophobic and could be retained on the reversed phase column on which retention was not achieved previously with acetonitrile alone (chromatogram not shown). In addition, compounds like phenolic acids would be protonated due to the acidification, which would potentially enhance their retention onto the column matrix (Jeffery *et al.*, 2008).

The elution profile of fraction UMQ-3 on SOURCE™ 5RPC ST 4.6/150 column is shown in Figure 8.3. The zoom-in of parts of chromatogram in Figure 8.3 is shown in Figures 8.4A and B. As seen in Figures 8.4A and B, several peaks were detected at 220 and 280 nm. The result was in agreement with the previous assumption made in Section 8.3.1 that fraction UMQ-3 was a mixture of various different compounds. The chromatogram indicates that components in fraction UMQ-3 were separated well on the SOURCE™ 5RPC ST 4.6/150 column with different polarity and/or π - π bonding behaviour. Fractions marked as BG1–BG5 in Figure 8.4A represent peaks detected before the gradient which indicates hydrophilic compounds since they were not retained on the column. Fractions marked as G1–G10 in Figure 8.4B represent peaks eluted by the gradient, which indicates that they are more hydrophobic.

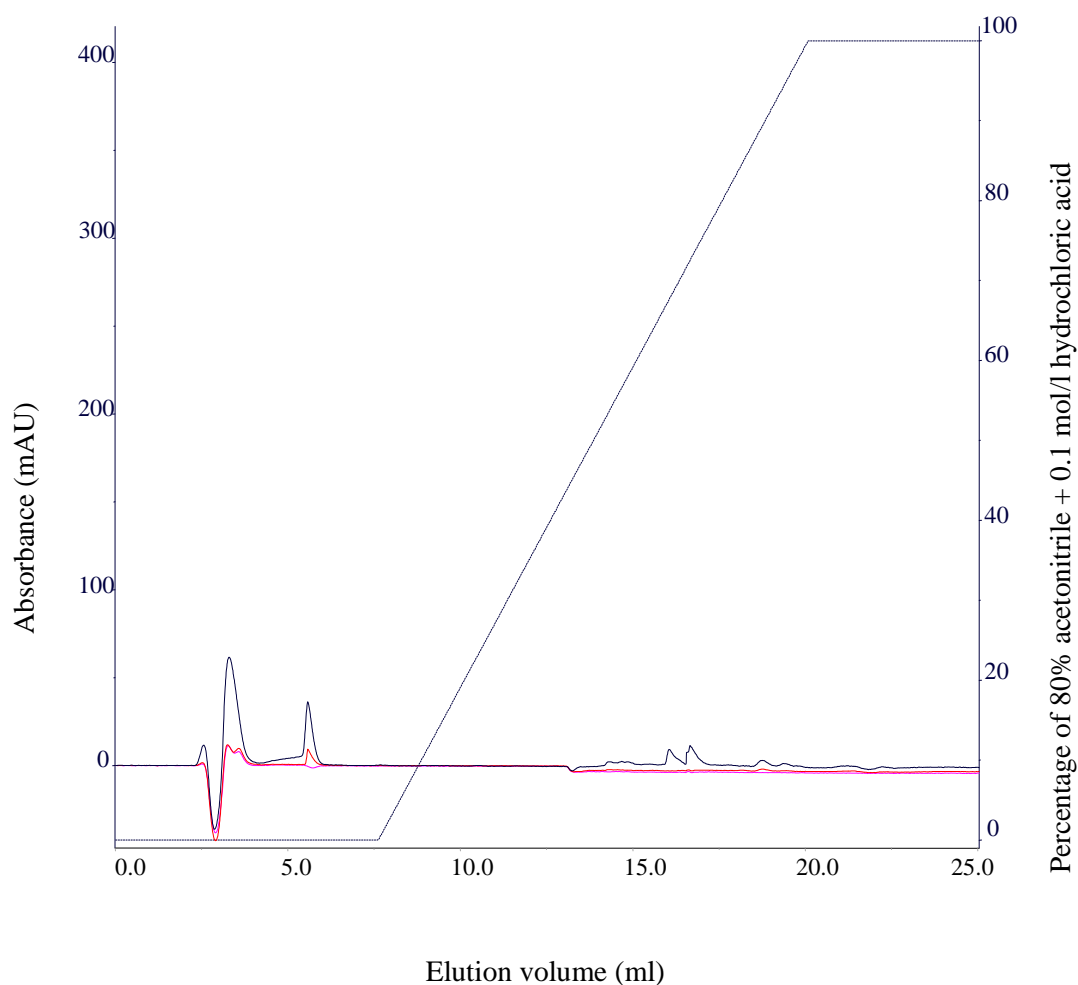


Figure 8.3: Elution profile of UMQ-3 fraction on SOURCE™ 5RPC ST 4.6/150 column. The eluting conditions were mobile phase A: 2% acetonitrile + 0.1 mol/l hydrochloric acid. Mobile phase B: 80% acetonitrile + 0.1 mol/l hydrochloric acid. The dotted line indicates the linear gradient at 0-100% B for 37.5 ml and hold up at 100% B until the baseline is stable. Upper, middle and lower lines are absorbance at 220, 280 and 360 nm, respectively.

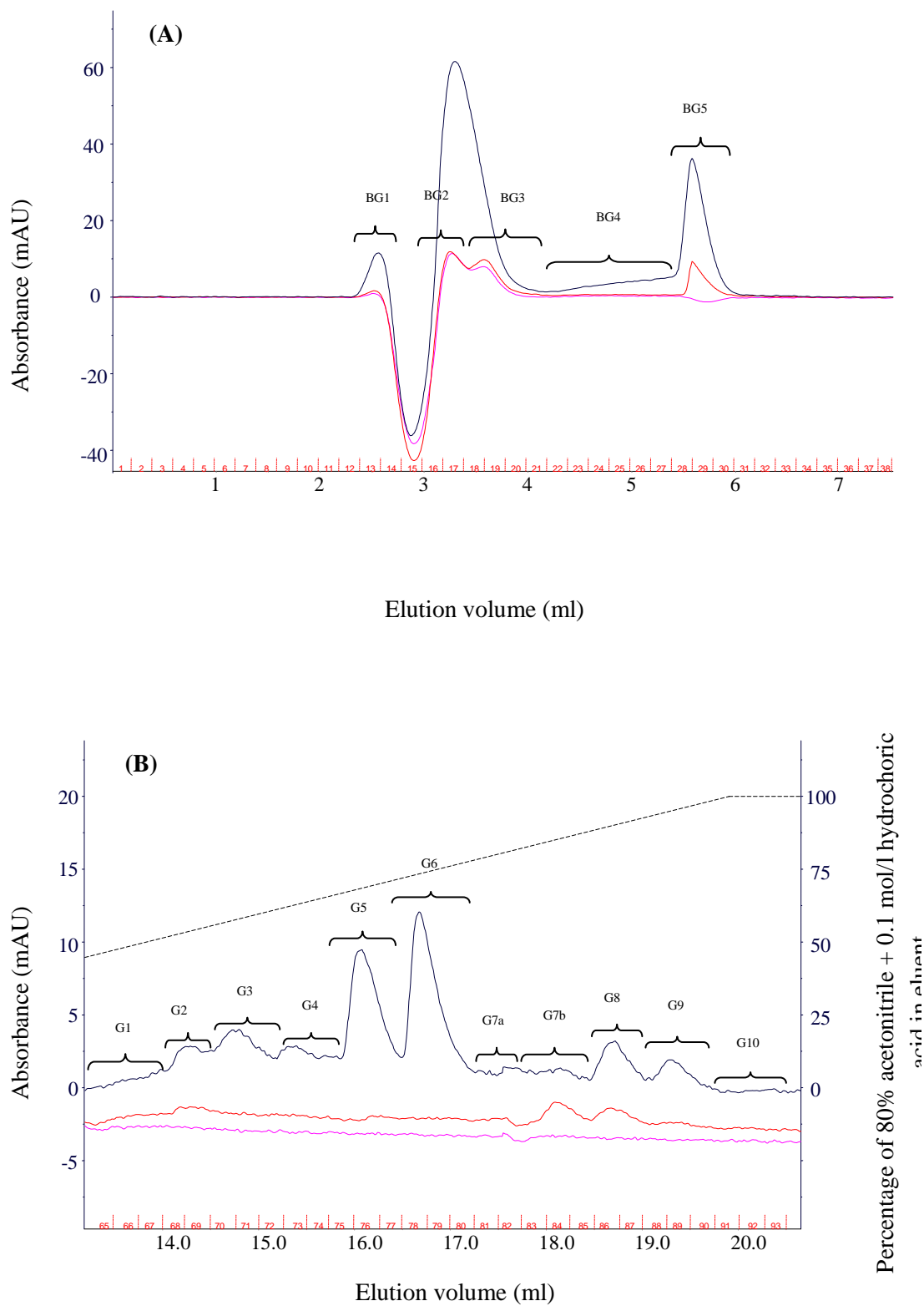


Figure 8.4: A zoom-in of parts of the chromatogram shown in Figure 8.3. (A) Peaks not retained on the column (B) Peaks eluted with the gradient. The dotted line in (B) indicates the gradient of the mobile phase B in the eluent.

The individual tubes under each peak in Figure 8.4A and B were pooled and all pooled fractions of the isolated peaks were subjected to rotary evaporation under reduced pressure at 30°C to remove the acetonitrile and HCl. The dried fractions were then each re-dissolved in deionised water (250 µl) and assayed for iron-binding antioxidant activity. The results are shown in Table 8.2.

Table 8.2 shows the highest activity was given by fraction G6 followed by BG5 and G7b, respectively. The activity given by these fractions was higher than the activity of the fraction loaded on the column. The isolated active fractions from reversed phase (BG5, G6 and G7b) showed a 1.7-fold, 2-fold and 1.6-fold increase in iron-binding activity, respectively, over the estimated activity of the fraction loaded. The result also suggested that antioxidants (fractions with positive activity) have been separated from pro-oxidants (fractions with negative activity).

Since the column matrix contains polystyrene divinyl-benzene resin, fractions G6 and G7b possibly consist of aromatic compounds since the resin preferably interacts with molecules using π - π interaction, hence these fractions possibly contained aromatic molecules. Fraction BG5 which is hydrophilic possibly consists of glycoside ring(s) that make the compounds more polar. It also could consist of peptides since it has been reported that peptides can expose their hydrophilic residues and bury the hydrophobic ones, therefore making the compounds not bind on a reversed phase column (Luders *et al.*, 2005).

Since the chromatogram shows that the active fractions from reversed phase chromatography also absorb UV at 280 nm (peaks BG5 and G7b), these fractions may contain more than one compound. To determine this, size exclusion chromatography (Section 8.2.3) was applied to isolate the different compounds based on their size.

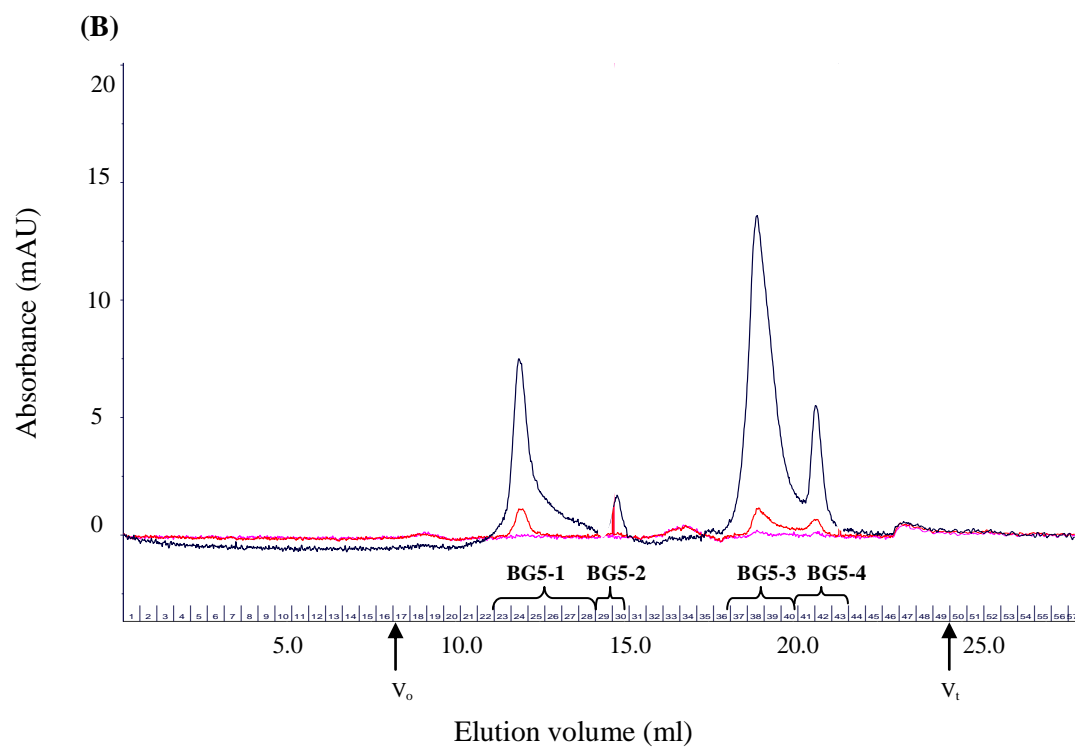
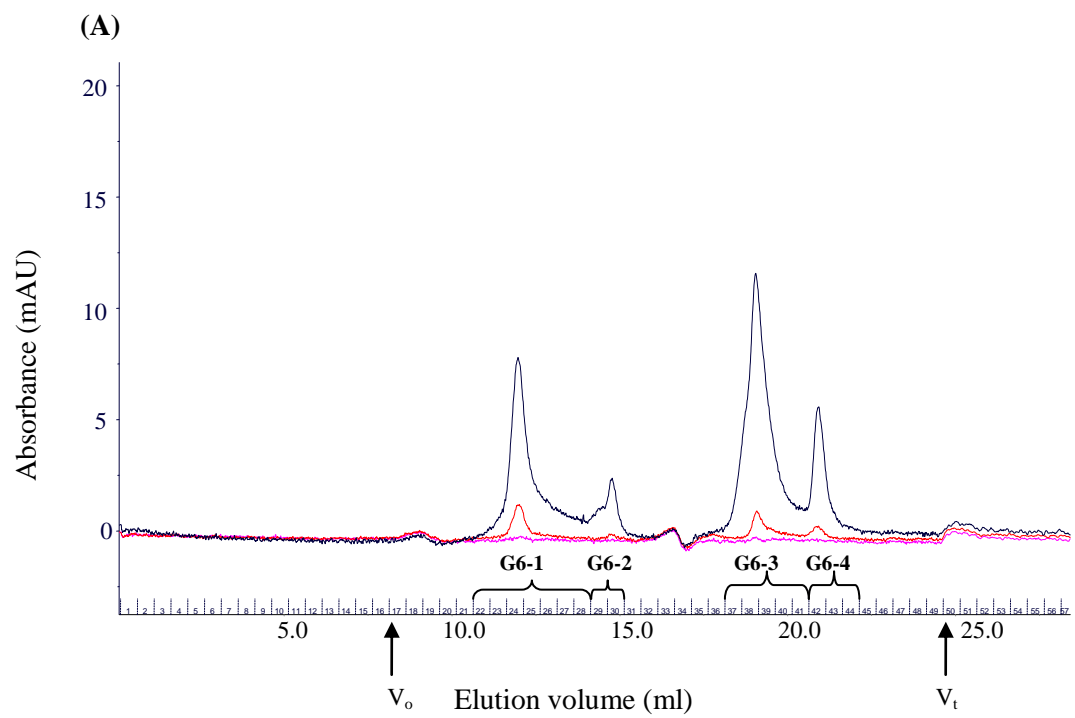
Table 8.2: Summary of the quantitative estimation of the iron-binding antioxidant activity of the fractions isolated from chromatography on a SOURCE 5RPC ST 4.6/150 column. Activity is shown as Ferrozine equivalent ($\mu\text{mol/l}$) \pm standard error of mean ($n=3$).

Activity of material from column (from Figure 8.2)	Expected activity of pooled fraction if in 0.5 ml volume loaded	Volume of concentrated pooled fraction from reversed phase chromatography (ml) (Figure 8.4)	Dilution of original loaded from pooled fraction	Measured activity in pooled fraction	Loss/gain in activity in pooled fraction
Fraction UMQ-3 = 309.0	1/3 of 309.0 = 103.0		0.5 ml/0.25 ml (twice concentrated)		
		BG-1 = 0.25		-2300.5 \pm 6.4	pro-oxidant
		BG-2 = 0.25		-56.2 \pm 44.4	pro-oxidant
		BG-3 = 0.25		-1726.6 \pm 180.6	pro-oxidant
		BG-4 = 0.25		-247.2 \pm 125.7	pro-oxidant
		BG-5 = 0.25		342.6 \pm 20.6	gain
		G-1 = 0.25		-244.6 \pm 57.6	pro-oxidant
		G-2 = 0.25		-651.4 \pm 116.0	pro-oxidant
		G-3 = 0.25		-228.4 \pm 234.7	pro-oxidant
		G-4 = 0.25		25.4 \pm 61.8	loss
		G-5 = 0.25		296.2 \pm 40.2	gain
		G-6 = 0.25		423.0 \pm 47.9	gain
		G-7a = 0.25		-10.9 \pm 0.75	pro-oxidant
		G-7b = 0.25		335.6 \pm 41.7	gain
		G-8 = 0.25		106.6 \pm 52.9	slightly gain
		G-9 = 0.25		198.4 \pm 45.5	gain
		G-10 = 0.25		-179.6 \pm 73.2	pro-oxidant

8.3.3 Isolation of components in the active fractions from reversed phase chromatography on the Superdex® Peptide column

In order to isolate as well as to estimate the molecular size of the iron-binding antioxidants from the active fractions from reversed phase chromatography, aliquots of fractions BG5, G6 and G7b were fractionated on the Superdex® Peptide column. These fractions were chosen since they gave the three highest activities in the order of G6 > BG5 > G7b among the whole fractions collected from reversed phase chromatography.

The chromatograms from fractions G6, BG5 and G7b shown in Figure 8.5 (A-C) showed multiple peaks which were named as designated. The size exclusion chromatography had separated fractions G6, BG5 and G7b (Figure 8.5A-C) into three to four well resolved peaks with estimated molecular weights ranging from 100 to 7000 Da. The chromatogram of BG5 (the unretained fraction on reversed phase chromatography) yielded a similar size exclusion chromatogram in regards to the compounds eluted at the similar elution volume as with fraction G6 (which was a fraction eluted with the gradient on reversed phase chromatography). This could be due to different molecules in both of these fractions having similar size, or due to the same components having been in different aggregated forms which had different characteristics. Each fraction (0.5 ml/tube) was collected and pooled according to the respective peaks as shown in Figure 8.5A-C.



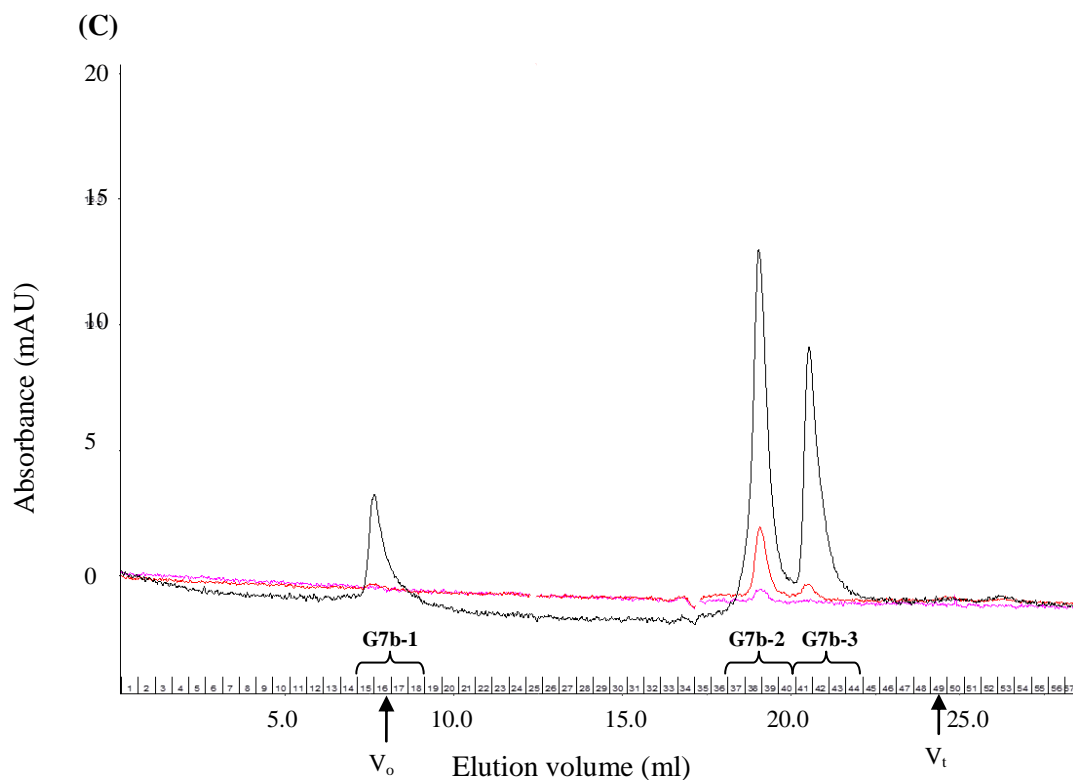


Figure 8.5: The elution profile on Superdex[®] Peptide HR 10/30 of the active fractions from reversed phase chromatography. (A) fraction G6, (B) fraction BG5 and (C) fraction G7b. The eluent was 30% aqueous acetonitrile at a flow rate of 0.3 ml/min. Upper, middle and lower lines are absorbance at 220, 280 and 360 nm, respectively.

All pooled fractions were dried using a rotary evaporator at 30°C, re-dissolved in deionised water (250 µl) and assayed for iron-binding antioxidant activity. Surprisingly, all of the concentrated pooled fractions exhibited a pro-oxidant effect, which created difficulty in identifying the most active peak from the size exclusion chromatography fractions. This raised the possibilities that the antioxidants isolated from SEC were very low in concentration, even after concentrating them, or the concentrated SEC fractions contained pro-oxidant compounds. This current situation is not similar to the case with the pro-oxidant effect that was observed in the fractions from reversed phase chromatography which would be due to dilution, thus lowering the concentration of antioxidants (refer to the discussion in Section 7.3.1.3).

Therefore, based on these literature findings which have been discussed in Section 7.3.1.3, an attempt was made to modify the assay system by lowering the Fe^{2+} concentration in order to increase the assay detection capacity so that the antioxidant activity of the low concentration of antioxidants in the size exclusion chromatography fractions could be detected.

8.3.3.1 *Modification of the iron-binding antioxidant assay*

In theory, lipid peroxidation is associated with the concentration of free Fe^{2+} ion within the environment of cells (Gonzalez *et al.*, 2012; Sevanian & Hochstein, 1985). It has been demonstrated by Repetto *et al.* (2010) and Afanas'ev *et al.* (1989) that iron-induced lipid peroxidation products (*e.g.* thiobarbituric acid-reactive substances and malonaldehyde) are formed in a concentration-dependent manner, that is when free Fe^{2+} ions exist at low concentration in the lipid model system, the level of thiobarbituric acid-reactive substances and malonaldehyde were decreased since less hydroxyl radical was generated and *vice versa*.

Since the hypothesis has been presented in Section 8.3.3 that the pro-oxidant effects observed in the fractions from size exclusion chromatography were due to there being a very low concentration of antioxidants relative to that of Fe^{2+} , it could be assumed that when the concentration of Fe^{2+} ions is reduced to a certain level, the ratio of antioxidants relative to Fe^{2+} ions would be higher, thus will be able to bind approximately 100% of the Fe^{2+} ions (either from the initial

concentration of Fe^{2+} ions and/or those formed from the reducing activity in the assay) and thus protect the lipid against oxidation.

To find out the influence of low Fe^{2+} ion concentration on the overall activity of the antioxidants (*i.e.* at the low concentration they were in after isolation) in the assay, a range of different concentrations of honey solution (2.5, 2.0 and 1.25 % w/v) was tested with a range of different concentrations of Fe^{2+} ions (0.75, 0.375 and 0.1 mol/l). The honey concentrations were chosen based on the range that gave a pro-oxidant effect as shown in Figure 7.6 in Chapter 7. This low concentration of honey was assumed would represent the low concentration of isolated antioxidants in the chromatography fractions. Deionised water was used as a control sample.

As shown in Figure 8.6, the water control allows lipid oxidation in the assay in an Fe^{2+} concentration-dependent manner. This is in agreement with the theory earlier mentioned.

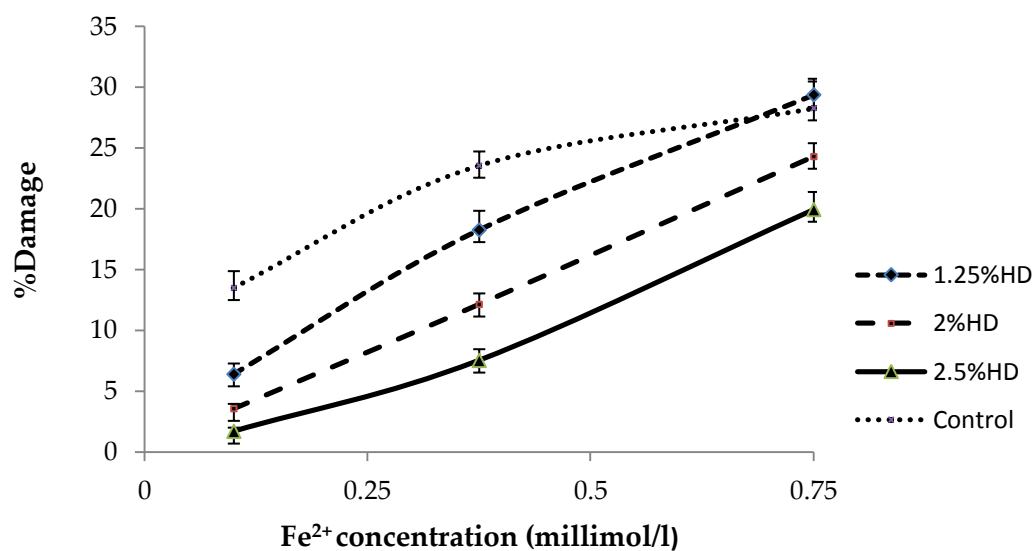


Figure 8.6: Level of damage to the lipid by different concentrations of honey solution catalyzed by Fe^{2+} at different concentration. The values are mean \pm standard error of mean of at least three determinations. Deionised water was used as a control.

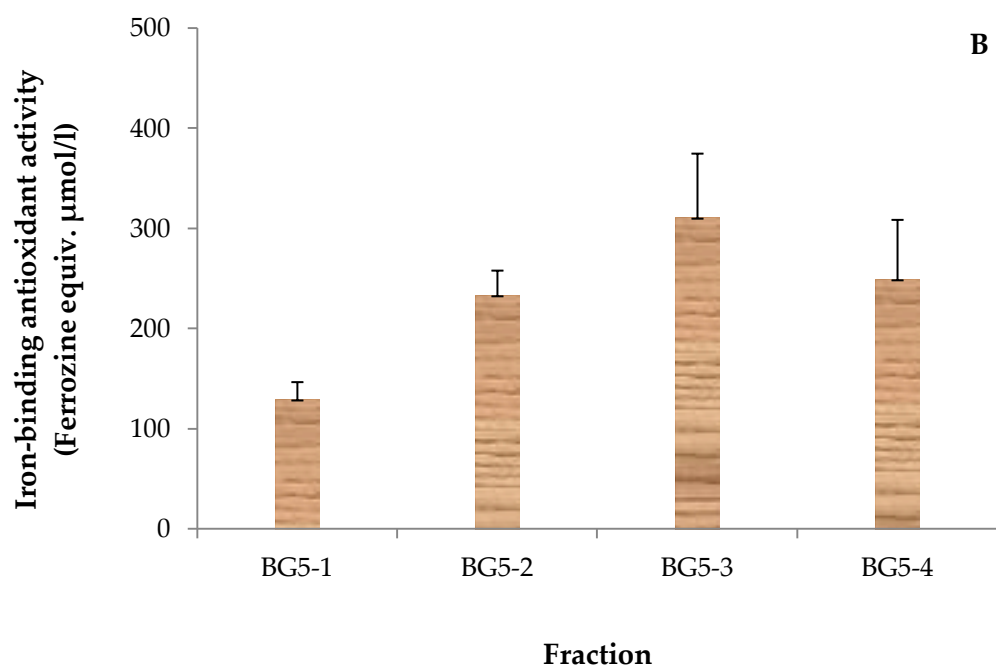
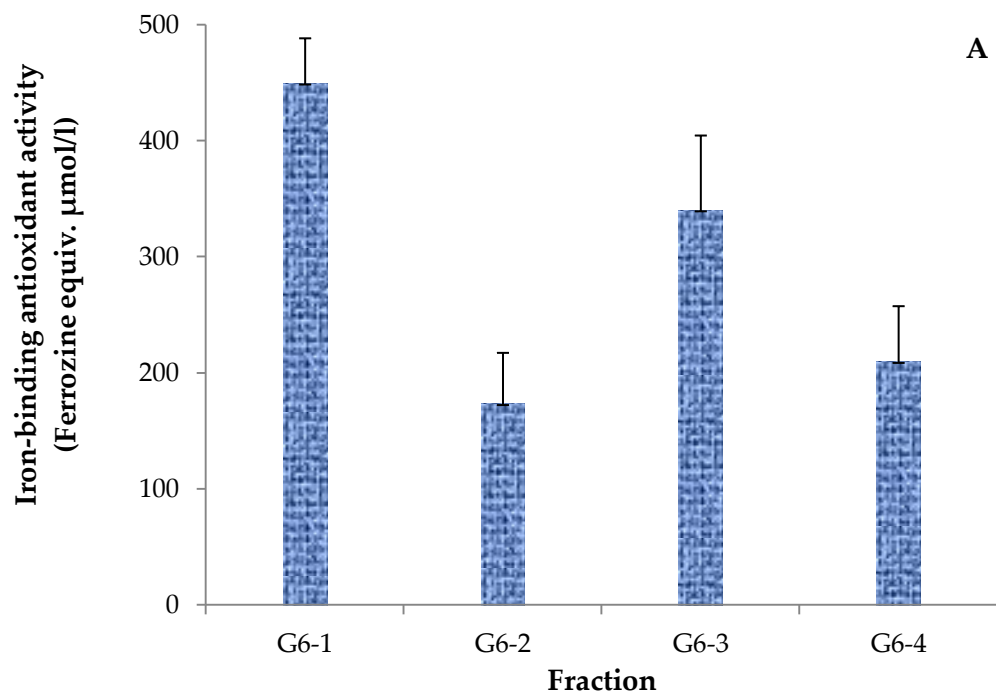
Honey solutions at all concentrations appeared to reduce the damage to lipid at all three Fe^{2+} concentrations compared with the control. However, this was minimal for 1.25% honey solution, where at 0.75 millimol/l of Fe^{2+} , the damage to the lipid was slightly higher than in the control (although the difference was insignificant).

To summarize the results, with a very low concentration of Fe^{2+} used (*i.e.* 0.1 millimol/l) low concentrations of honey solutions showed protection to the lipid since all Fe^{2+} ions were bound to antioxidants. This condition obviously has improved the detection capacity of the assay. Since 0.1 millimol/l of Fe^{2+} gives least damage to the lipid, it was chosen as a new Fe^{2+} concentration for the assay to test the fractions from size exclusion chromatography.

The positive results for low concentration of honey given in the modified assay (low concentration of Fe^{2+}) at least in part agreed with the hypothesis earlier made that the high molar ratio between iron and antioxidants contributed to the pro-oxidant activity in the original assay.

8.3.3.2 Antioxidant activity of the fractions from size exclusion chromatography tested in a modified assay

Each pooled fraction isolated in Section 8.3.3 was brought up to the same volume as the largest pool (3.5 ml) using deionised water, then the pools were re-assayed using the modified iron-binding antioxidant assay as described in Section 8.3.3.1 using 0.1 mmol/l Fe^{2+} . Figure 8.7 shows the activity for each fraction. It is clear that lowering the concentration of Fe^{2+} enabled the assay to pick up the activity from the diluted fractions.



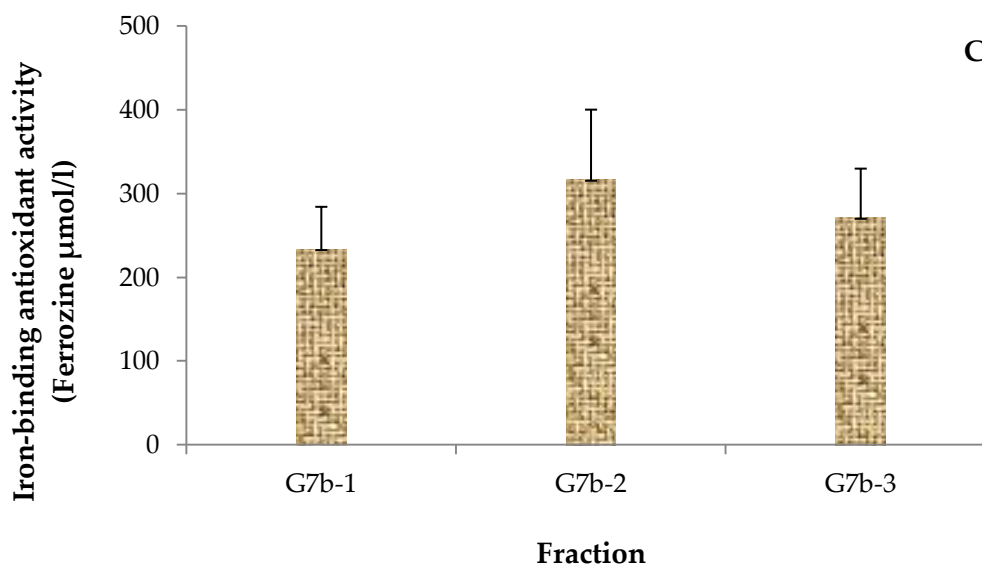


Figure 8.7: The activity measured in fractions from the size exclusion chromatography with 0.1 mmol/l Fe^{2+} in the assay. (A) G6(1-4); (B) BG5(1-4), and (C) G7b(1-3).

8.3.4 Characterization of the iron-binding antioxidants in fraction G6-1 using ESI-MS and ^1H NMR spectroscopy

ESI-MS analysis in both positive and negative ion modes was attempted. The results indicated that all fractions had a better response in the positive ion mode than the negative ion mode (in which no spectrum was detected), therefore, the positive ion mode was used for the entire analysis.

Figure 8.8A-D shows the mass spectra obtained by electrospray ionisation in positive ion mode of fractions of G6 obtained from size exclusion chromatography. The mass spectra of fraction G6-1 and G6-3 contained major ions at m/z 256 and m/z 284 with high intensity. The other less active fractions from the same chromatography (fractions G6-2 and G6-4) also showed the ion m/z 256, but with lower intensity. The intensity of the ions at m/z 256 and m/z 284 in fractions G6-1 and G6-3 was consistent with the antioxidant activity shown by these fractions (Figure 8.7A) compared with that shown by fractions G6-2 and G6-4.

Since the m/z 256 ion was the only major ion in the most active fraction (G6-1), it should be stressed that this indicates the sample is probably pure and that the m/z 256 ion is derived from the active antioxidant.

Similarly, the mass spectra of fractions BG5-3 and G7b-2 (see Figure 8.9A-B), fractions which exhibited higher iron-binding antioxidant activity than their respective fractions from size exclusion chromatography (Figure 8.7B-C) also contained ions of high intensity at m/z 256 and m/z 284. It was unlikely that m/z 256 and m/z 284 ions were the fragments of a larger molecule since electrospray is a soft ionization technique and would not be expected to produce fragmentation. It is interesting to note that none of these ions were observed in the inactive fractions (*i.e.* fractions G7a, G8 and BG1) as shown in Appendix 2-4.

Potential molecular formulae for the ions m/z 228, 256 and 284 were calculated using the Bruker Data Analysis software. Table 8.3 shows the proposed formula for the ions. It is important to note that the error values for the molecular formulae were relatively large, thus they may not be reliable.

Table 8.3: Proposed formula weight of the selected ions.

m/z ion in positive ion mode	Proposed molecular formulae	Error	Molecular weight
228	C ₁₁ H ₂₆ N ₅	-11.1 ppm	227
	C ₁₄ H ₃₀ NO	12.5 ppm	
256	C ₁₃ H ₃₀ N ₅	-43.5 ppm	255
	C ₁₆ H ₃₄ NO	10.8 ppm	
284	C ₁₅ H ₃₄ N ₅	-40 ppm	283
	C ₁₈ H ₃₈ NO	9.0 ppm	

The proton NMR spectrum of fraction G6-1 is presented in Figure 8.10. The NMR study revealed no significant peaks except for the HOD solvent peak in fraction G6-1. However, there were two very weak signals in the range 1.23-1.86 ppm. These signals could be representative of antioxidants in fraction G6-1, however, these weak signals (in trace level) were likely the result of very low concentration of compounds in the fraction. The signals represent protons in aliphatic environments.

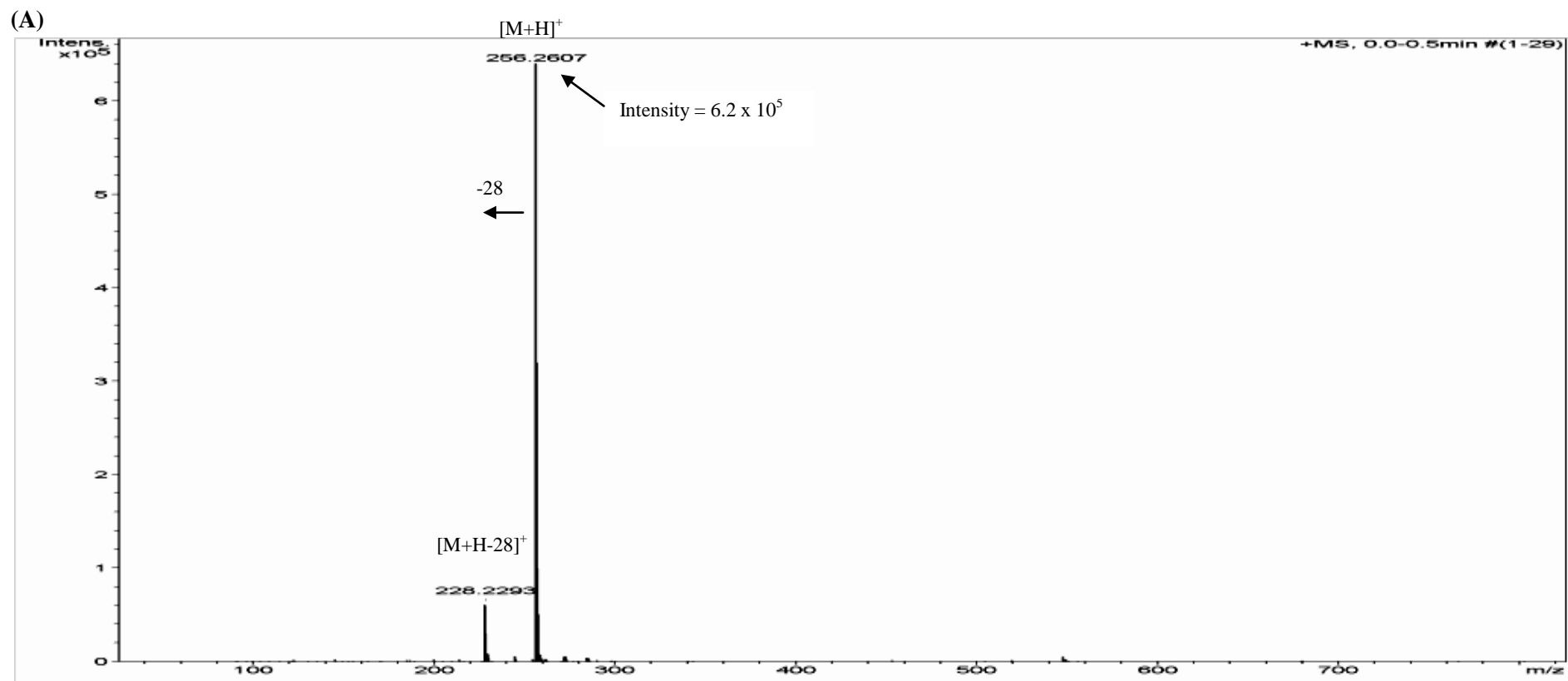


Figure 8.8A: The electrospray ionization-mass spectrum of fraction G6-1 in positive ion mode.

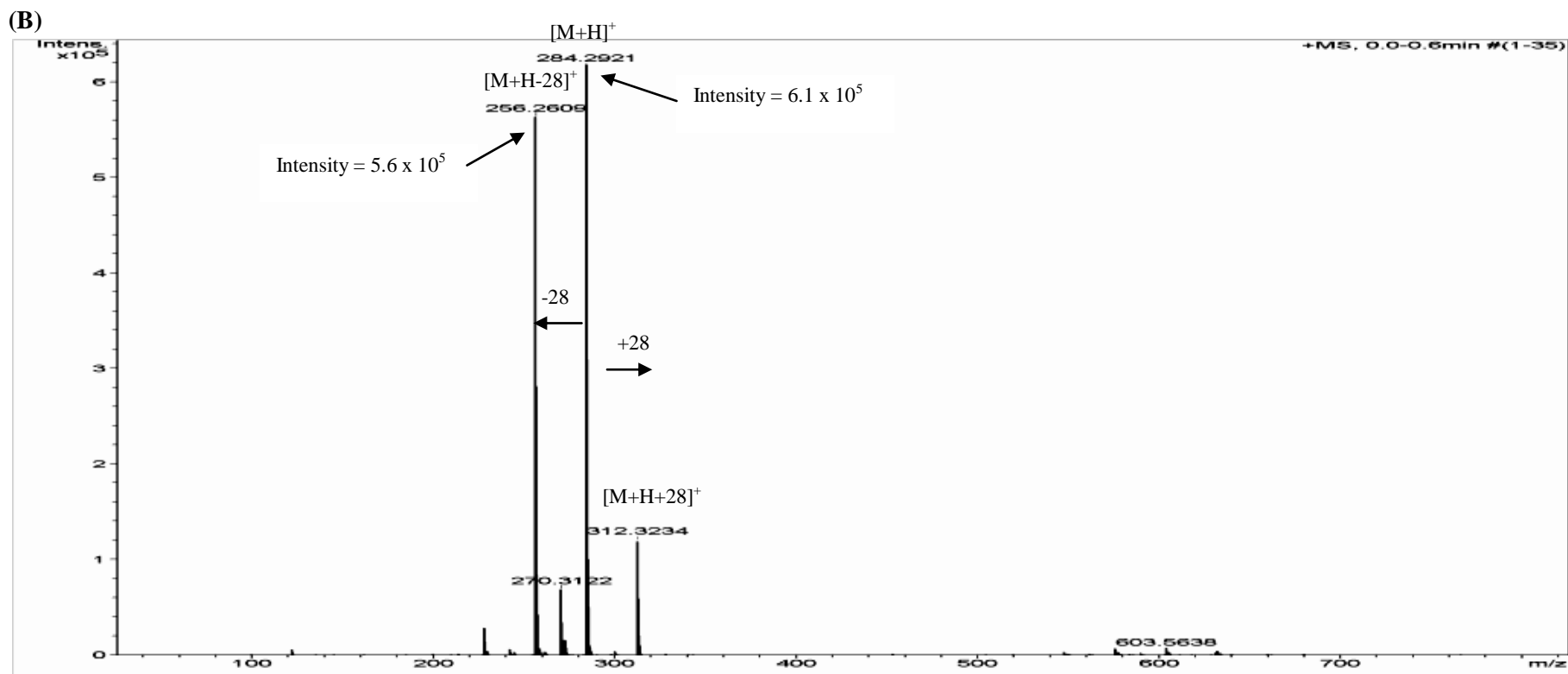


Figure 8.8B: The electrospray ionization-mass spectrum of fraction G6-3 in positive ion mode.

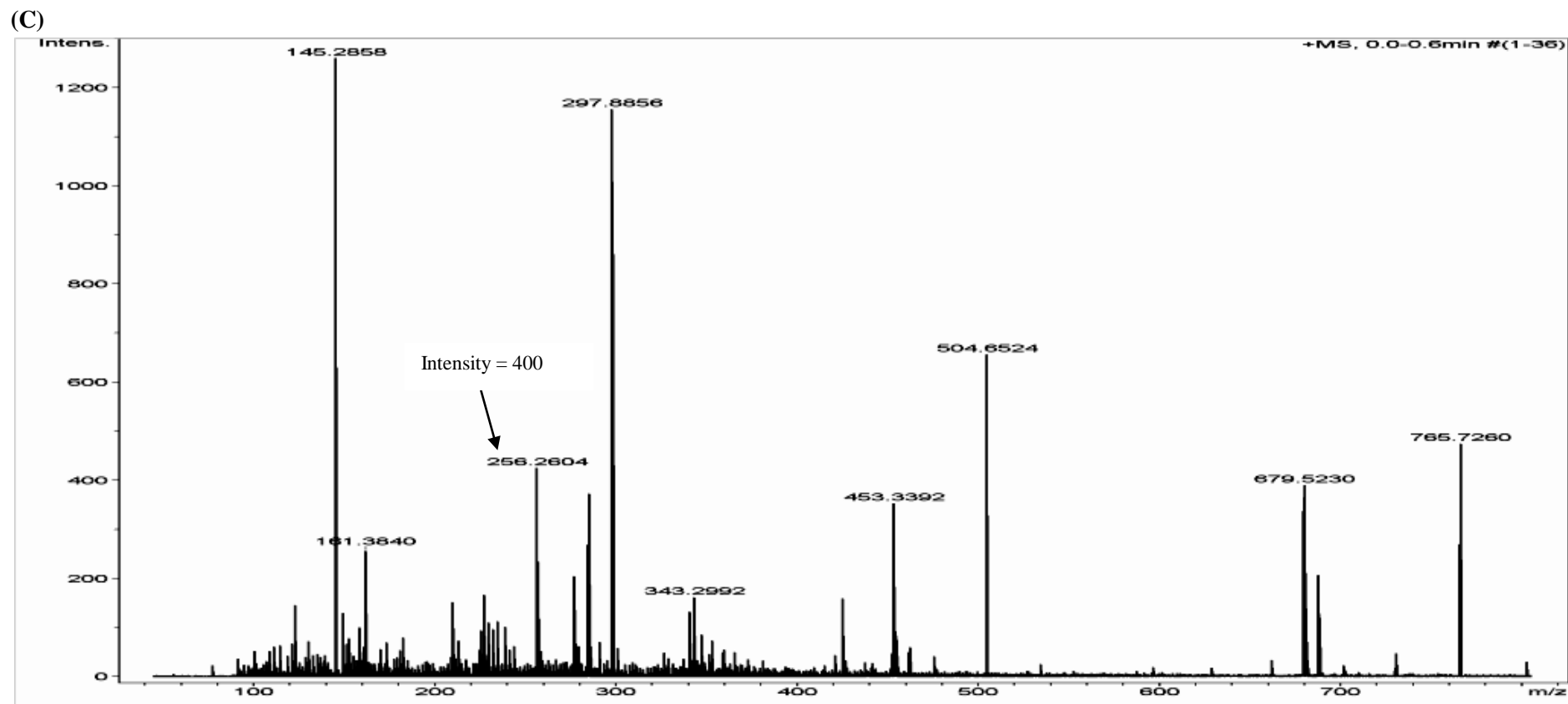


Figure 8.8C: The electrospray ionization-mass spectrum of fraction G6-2 in positive ion mode.

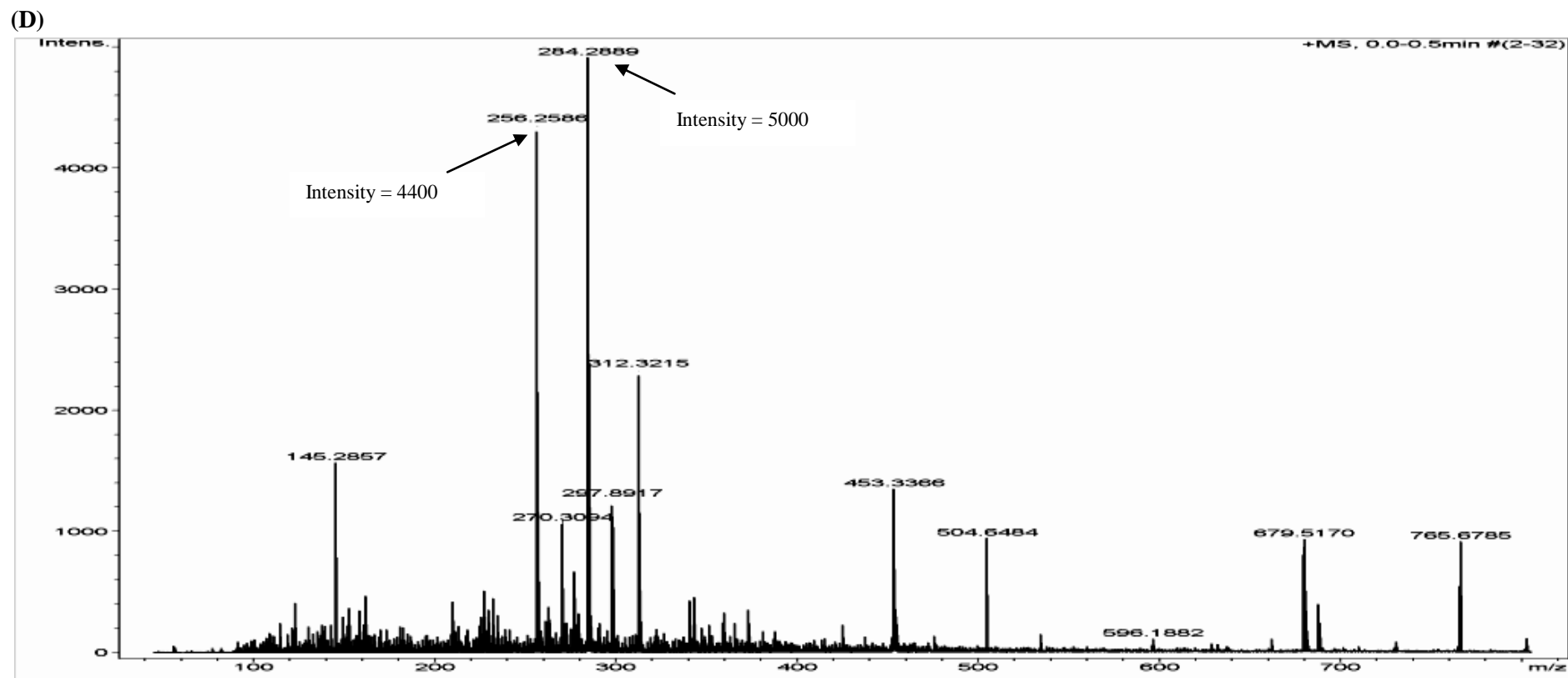


Figure 8.8D: The electrospray ionization-mass spectrum of fraction G6-4 in positive ion mode.

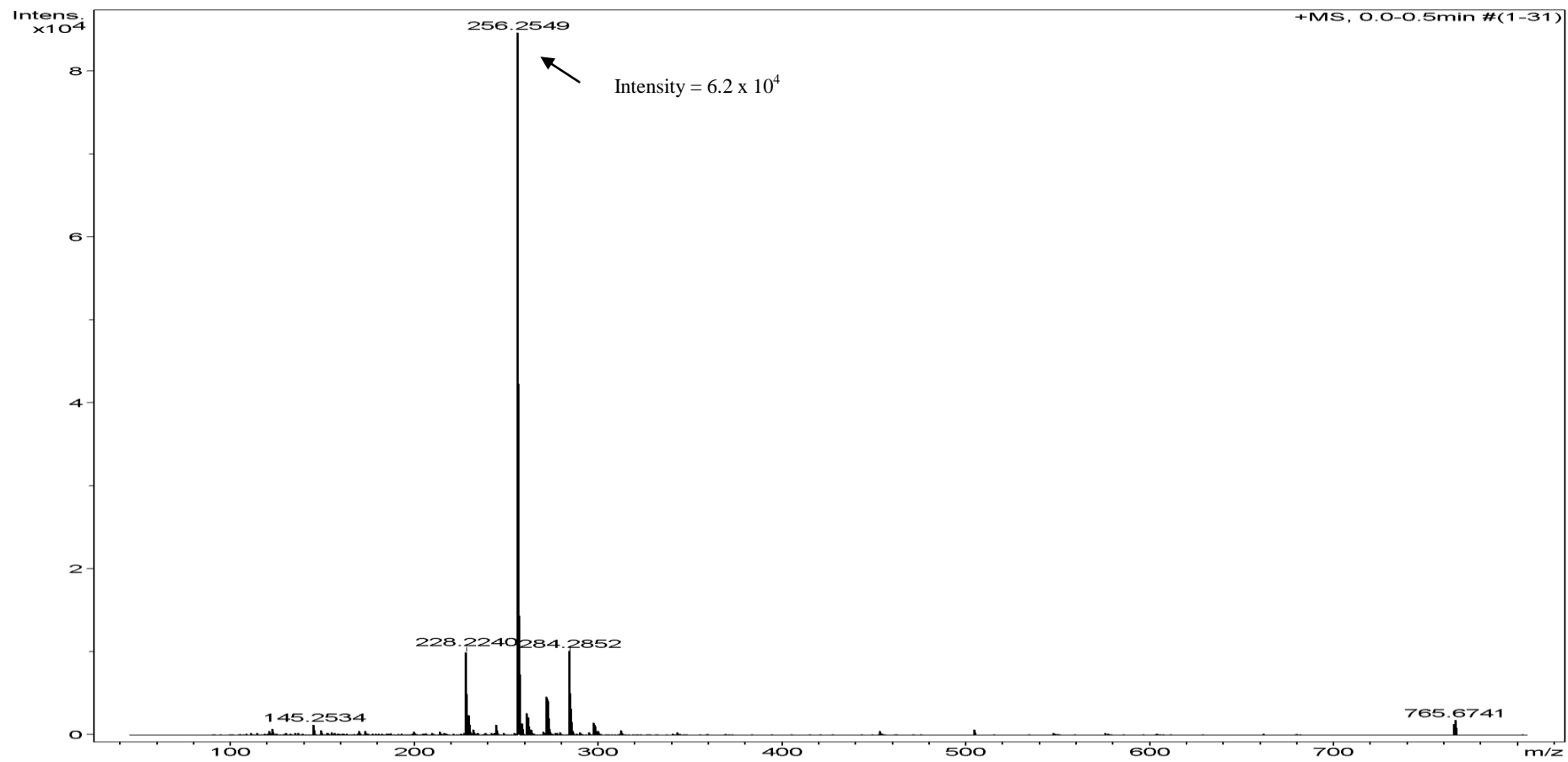


Figure 8.9A: The electrospray ionization-mass spectrum of fraction BG5-3 in positive ion mode.

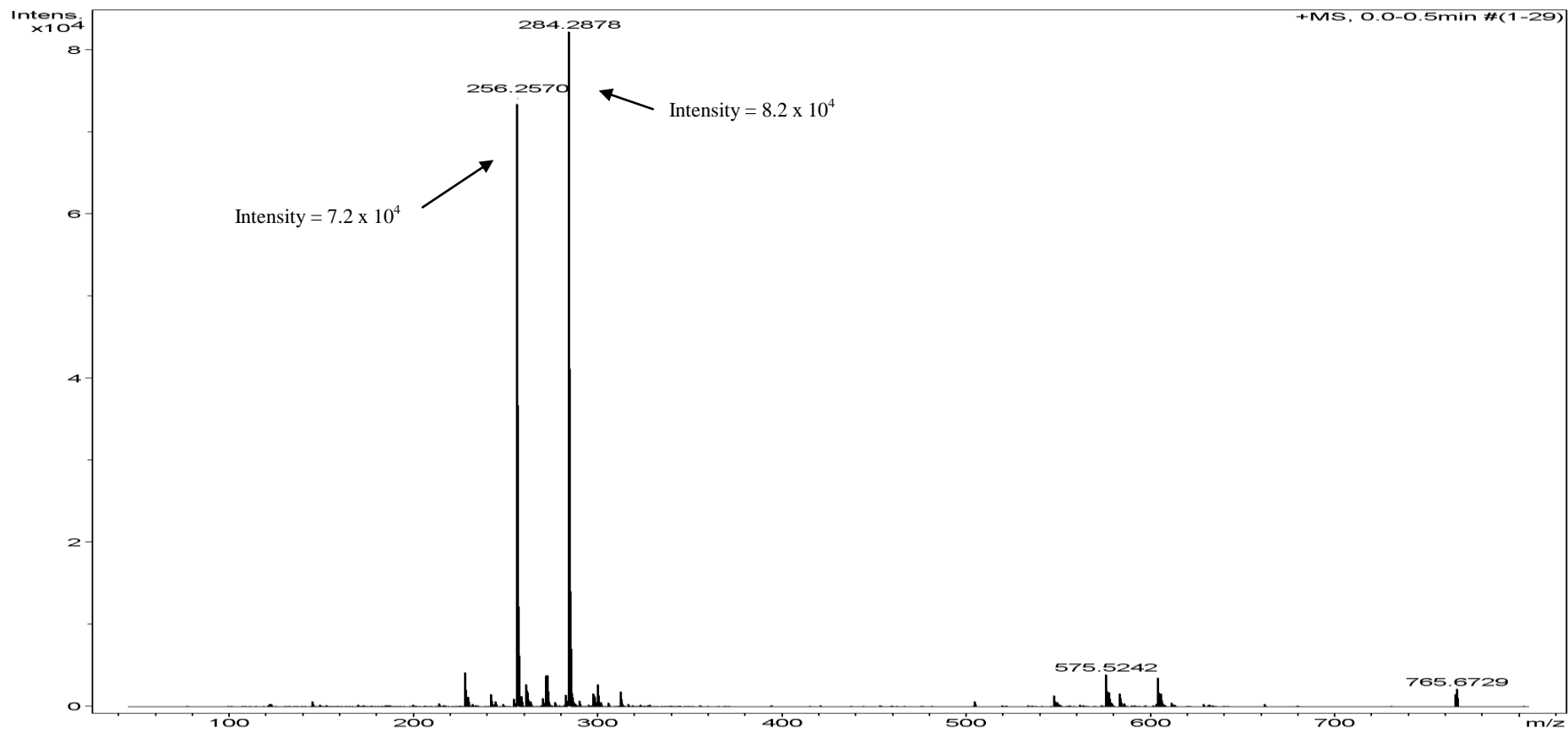


Figure 8.9B: The electrospray ionization-mass spectrum of fraction G7b-2 in positive ion mode.

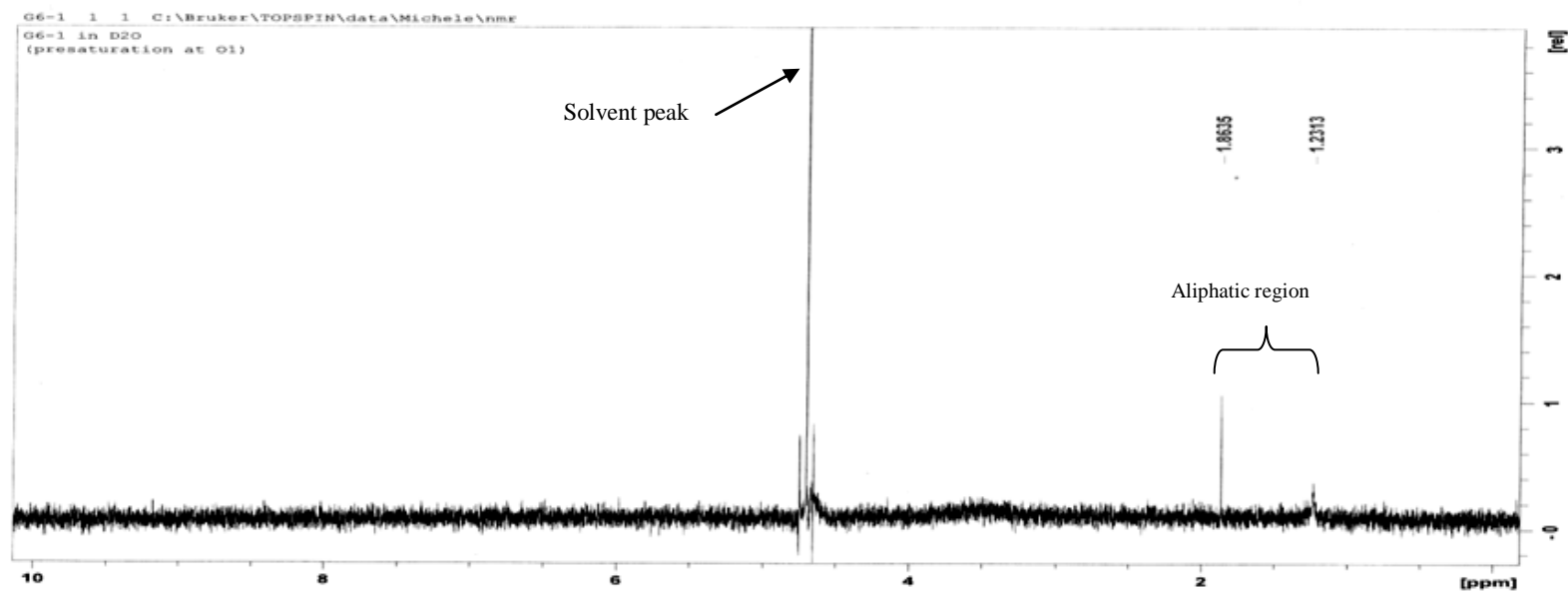


Figure 8.10: ^1H NMR spectrum of fraction G6-1 in D_2O .

It has been reported that ^1H NMR spectra of honeydew honeys from Italy exhibited aliphatic resonances in the range of 0.85-1.6 ppm and this was suggested to be a marker of honeydew honey (Beretta *et al.*, 2008).

The ^1H NMR spectrum of fraction G6-1 in $\text{DMSO-}d_6$ (Figure 8.11) contains a doublet of doublet peaks between 7.0-7.5 ppm which is characteristic of a *para*-disubstituted aromatic ring. A sharp peak appearing at 8.53 ppm could possibly be attributed to the presence of NH. However, whether the aromatic ring and NH are related in one compound is uncertain. There were notable peaks in the alkyl region of the spectrum between 0.8-1.2 ppm.

There were no peaks in the spectrum that could readily be attributed to oxygen-containing groups. The empirical formulae calculated by the mass spectrometry software contained one oxygen atom for the antioxidants (*i.e.* m/z 256 = $\text{C}_{16}\text{H}_{35}\text{NO}$ and m/z 228 = $\text{C}_{14}\text{H}_{30}\text{NO}$). However, the error values for the molecular formulae were relatively large so may not be reliable.

The difficulty in observing any easily interpretable data in the NMR spectrum is probably due to the very low mass of the fraction. The spectrum obtained in DMSO did look somewhat better than that obtained in D_2O , and contained more signals but still not much information could be gained. Thus, to gain more information from NMR spectroscopy, it is recommended to use a more concentrated sample for the structural elucidation of the antioxidants. NMR spectroscopy is a relatively insensitive technique compared to many other analytical methods such as mass spectrometry.

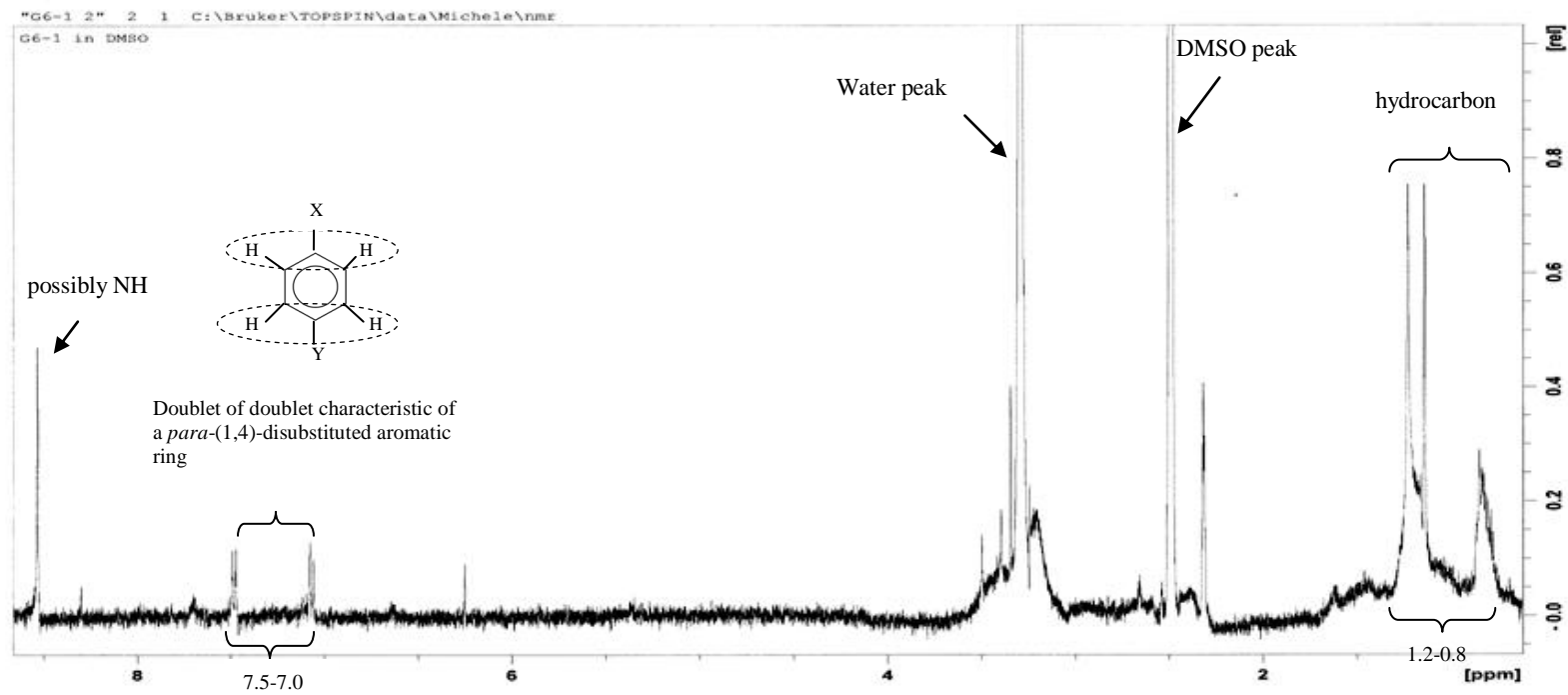


Figure 8.11: ^1H NMR spectrum of sample G6-1 in $\text{DMSO-}d_6$ with suppression of water and solvent peaks.

It is interesting to note that the location of the antioxidants (fraction G6-1) in the size exclusion chromatography (Figure 8.5A) was similar with the location during the first isolation of antioxidants from crude honey with the same column (see Figure 6.3 in Section 6.3.2). Further to that, in this later size exclusion chromatography purification, a single peak was observed, indicating that the antioxidants had been separated from other compounds or impurities, while the UV absorbance of the antioxidants did not change in both separating stages.

However, the information revealed by mass spectrometry regarding the molecular weight of the antioxidants (*i.e.* low molecular weight) did not match with the molecular weight estimated by size exclusion chromatography, which showed that the antioxidants are a large molecule (*i.e.* 2126-6500 Da). As discussed in Section 6.4.1, this large molecular weight estimation given by size exclusion chromatography could be the manifestation of the aggregation of the various molecules in crude honey solution, thus it also could be happening in this later stage as peak G6-1 had a little tailing. In addition, there were two molecules in G6-1 (m/z 256 and m/z 228) which could contribute to the aggregation. Another possibility is that the shape of the antioxidant molecules has prevented the molecules from diffusing fully into the size exclusion chromatography medium.

8.4 Conclusion

The switching of the separation sequence and the modification of the anion exchange and reversed phase eluents used in the present study indicates that the iron-binding antioxidants from the complex mixture obtained from chromatography on Superdex[®] Peptide can be isolated into more diverse groups. However, the elution profile in each type of chromatography was not the same as in the elution profile presented in Chapter 7. However, this could be due to different eluents being used and to the columns being used in a different sequence.

The isolation techniques showed that antioxidants, regardless of the dilution effect, could be separated from the pro-oxidants in both columns. It has been proved in the present study that low molar concentration ratio between antioxidants and Fe²⁺ ions led to the pro-oxidant effect given by fractions from size exclusion chromatography and diluted honey solutions. Even though the molar

concentration of the antioxidants was unknown, it was clear that the antioxidant property of extracts indicated that the activity was concentration-dependent.

The empirical formulae calculated by mass spectrometry suggested that the antioxidants are nitrogen-containing compounds with low molecular weight. However, due to the relatively large error values, this may not be reliable. Information from ^1H NMR spectroscopy in $\text{DMSO-}d_6$ solvent suggested that the antioxidants in fraction G6-1 contained an aromatic compound and possibly NH. However, the trace level concentration of the antioxidants in the isolated fraction prevented the structural elucidation of these compounds.

CHAPTER 9

GENERAL DISCUSSION AND FUTURE WORK

9.1 General discussion

This PhD research was conducted because of the high degree of concern about the deleterious effect of free Fe^{2+} ion which gives rise to $\cdot\text{OH}$ radicals via the Fenton reaction, contributing to the pathogenesis of certain degenerative diseases. These diseases include atherosclerosis, myocardial infarction, stroke, ischemia/reperfusion injury, chronic and acute inflammatory conditions such as non-healing wounds, central nervous system disorders such as forms of familial amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's dementia and a number of age-related disorders and certain cancers (Kell, 2009).

It has been widely accepted that the chelation of Fe^{2+} is an important approach in order to prevent the reaction of the $\cdot\text{OH}$ radical with biological macromolecules. Thus, the primary aim of this study was to isolate and identify the compounds responsible for iron-binding antioxidant activity in honeydew honey which has shown an exceptionally high level of iron-binding activity in the assay. As honey is considered to contain a large variety of compounds, a few potential antioxidant components have been discussed in Chapter 2.

In order to achieve the goal of the research, the study was divided into three parts. The first part of the research was to determine the stability of the iron-binding antioxidants in crude honeydew honey to heat and pH. This was to plan suitable isolation work which would not affect the stability of the antioxidants that might be thermo-labile and pH-sensitive. This has been described in detail in Chapter 4. It was found that the iron-binding antioxidants in crude honeydew honey were stable in alkaline pH (pH 8-11) and were not stable at temperatures higher than 40°C . The stability of the antioxidants in honeydew honey at alkaline pH allowed further isolation with anion exchange chromatography.

The second part of the research was to develop suitable isolation methods for iron-binding antioxidants in honeydew honey. It has been hypothesised that phenolic compounds, especially flavonoids, are responsible for part, or in most of the cases for all, of the antioxidant activity observed in honeys. The potential of the flavonoids to be the iron-binding antioxidant has been reviewed in Chapter 2. Due to the assumption that phenolic compounds could be the iron-binding antioxidants in honey, the widely used method for isolating phenolics in honey using XAD-2 resin was tried. The finding of the study described in Chapter 5 was that the phenolics fraction extracted with XAD-2 did not demonstrate iron-binding antioxidant activity. It should be noted that the hypothesis that phenolics are the main contributor of antioxidant activity in honeys was based on the strong correlation established between total phenolic content and radical scavenging antioxidant activity (Al *et al.*, 2009; Aljadi & Kamaruddin, 2002; Alvarez-Suarez *et al.*, 2010b; Baltrusaityte *et al.*, 2007; Estenvinho *et al.*, 2008; Gheldof & Engeseth, 2002a; Lachman *et al.*, 2010; Pichichero *et al.*, 2009; Socha *et al.*, 2009; Zalibera *et al.*, 2008). As the molecular structural requirements for the radical scavenging and iron binding mechanism are different, not all phenolic compounds which exhibit good radical scavenging activity are also good iron chelators. Note should be taken when using XAD-2 resin that not all phenolic compounds (which are thought to be hydrophobic) or other non-ionic compounds will adsorb onto the resins. This condition had been proved earlier by Deadman (2009) who found that flavonoids from Manuka honey were poorly adsorbed on XAD-2 resin. The chemical nature of the compounds which have become hydrophilic due to glycosylation, or the presence of an abundance of free sugars in the crude sample, would limit the usage of this technique. It is recommended for the user of this technique to test the effluent of the sample to make sure that the compounds of interest do not escape during the isolation process (Alvarez-Suarez *et al.*, 2010a).

The composition of honey is very complex and it contains a high number of organic and inorganic constituents, but only a few of them may have iron-binding antioxidant activity. Therefore, efficient and selective methods are required for isolating and identifying the iron-binding antioxidant components from honey. The isolation work was continued by using various chromatography columns sequentially (*i.e.* size exclusion, reversed phase, anion exchange) as described in

detail in Chapter 6, 7 and 8, using an assay-guided fractionation approach. The sequential chromatography technique has been widely applied in isolating antioxidants from other natural sources. However, to date this technique is not known to have been used in isolating antioxidants in honey.

In the fractionation using size exclusion chromatography (Chapter 6) it was clearly shown that the free sugars in honey were successfully separated from the main antioxidant fraction, which was not achieved with XAD-2 resin previously. Size exclusion chromatography could be an alternative technique as a primary tool in separating the free sugars from antioxidants in the crude extract, especially in samples with high sugar content. It may be because the antioxidants in honeydew honey aggregated into larger molecules that were able to be separated from sugars molecules by size exclusion chromatography. To date, none of the isolation work described in the literature has used size exclusion chromatography to separate free sugars from antioxidant compounds in honey.

The complexity and the wide range of components in the main antioxidant fraction obtained by size exclusion chromatography required further isolation of the antioxidants using a different kind of chromatography. Sufficient separation of the antioxidants was achieved by using reversed phase and anion exchange chromatography, regardless of their sequence, as described in Chapter 7 and Chapter 8.

The assay-guided fractionation clearly led to a successful separation of iron-binding antioxidants from other compounds without any loss of activity of the main antioxidant fractions.

The difference between both isolation procedures was that the isolation procedure described in Chapter 7 was able to isolate the antioxidants, but was not in a form suitable for subsequent analysis by mass spectrometry due to the high content of salt being used as a mobile phase additive in the anion exchange fraction. This procedure could be useful if mass spectrometry is not attempted for the identification of the antioxidants. The isolation procedure described in Chapter 8 gave salt-free isolation of the antioxidants. This method was purposely developed to accommodate the necessity of the analysis with mass spectrometry which is not compatible with any kind of salt. However, due to the different eluents used in

both isolation procedures, it is presumed that the composition of the main antioxidant fractions should be different. This was evident when elution profiles of reversed phase and anion exchange chromatography obtained in both procedures were also different.

The mass spectrometry data was not sufficient in providing the chemical structures of the main antioxidants. The successful identification of the compounds by mass spectrometry was complicated by the fact that a low quantity of compounds were isolated in the final fractions. Even though the empirical formulae calculated by mass spectrometry indicated that the compounds might carry nitrogen and oxygen atoms, this was uncertain due to large error values. On the other hand, NMR spectroscopy revealed that the iron-binding antioxidants isolated from Beech honeydew honey using the salt-free isolation method may contain a *para*-disubstituted aromatic ring. This finding suggests that flavonoids can be ruled out as the main compounds responsible for the iron-binding antioxidant activity in this type of honey, but this is not definite. The presence of a nitrogen atom also might be possible as shown by NMR spectroscopy. However, to confirm these ambiguous results given by mass spectrometry and NMR spectroscopy, a larger quantity of sample needs to be used. There was insufficient time available for repeated purification on a larger scale to be done in the present study.

Honey sometimes can contain peculiar compounds and the composition differs between types of honey. For example, it has recently been found that there is kynurenic acid in Chestnut honey (Beretta *et al.*, 2009; Turski *et al.*, 2009) which was not detected previously in the same type of honey. In addition, kynurenic acid has not been found in other honeys. Kynurenic acid is a tryptophan metabolite which is the only known endogenous antagonist of ionotropic glutamate receptors (Turski *et al.*, 2009). In another study, Truchado *et al.* (2010) identified glucosinolates (*i.e.* β -thioglucoside *N*-hydroxysulfates containing a β -D-glucopyranosyl moiety at a side chain) in Argentinean *Diplotaxis* honey, which had never been found in honeys previously.

The basic core structure of a 1,4-disubstituted aromatic ring that may be in the antioxidant isolated in the present study has also been found in other honeys.

Tan (1989) identified the compounds with this characteristic in White Clover type honey (*i.e.* methyl trans-3-(4-methoxyphenyl)-prop-2-enoate), Viper Bugloss honey (*i.e.* 1,4-dihydroxybenzene) and an unidentified compound in which a hydroxyl group was substituted to methyl 3-phenylprop-2-enoate in Manuka honey. Senanayake (2006) also found compounds with this characteristic (*i.e.* 4-methoxybenzoic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, and 4-methoxyphenylacetic acid). The 4-hydroxyphenylacetic acid and 4-methoxyphenylacetic acid were existed in higher levels in Beech honeydew honey (Senanayake, 2006). To note, the m/z value (m/z 192, 166 and 110) identified by Tan (1989) and the molecular weight of the compounds identified by Senanayake (2006) were not the compounds with m/z 256 and 284 or molecular weight of 255 and 283 isolated in the present study. However, since there was incomplete structural elucidation of the antioxidant isolated in the present study, it was uncertain to conclude that the antioxidant was similar to the compounds found by Tan (1989) and Senanayake (2006). The compound could possibly be conjugated with other functional groups at the 1- and 4- positions which remain unknown at this point of study.

The main problem encountered during the isolation processes was the aggregation behaviour of the antioxidants. It was found that the iron-binding antioxidants seemed to bind with other components in honey. This was evident in the isolation work with size exclusion chromatography (Chapter 6) and reversed phase and anion exchange chromatography (Chapter 7). The aggregation of the honey components has been reported to occur in Canadian honey (Alvarez, 2010). Alvarez (2010) confirmed the composition of aggregates by using mass spectrometry and found that glycosylated phenolic compounds and proteins were responsible for the aggregation condition. In the present study, the compounds that form the aggregates were unknown, however based on the size exclusion chromatography elution profile in Chapter 6, the aggregates consisted of compounds with molecular weight estimated to be 2126 – 6500 Da which rules out protein Maillard reaction products that are known antioxidants in honey.

9.2 Implications of the study

The important finding of this study was that the iron-binding antioxidants were concentration-dependent in exerting their antioxidant effect and would change their behaviour from being antioxidant to pro-oxidant when their concentrations were low relative to that of Fe^{2+} . Dilute antioxidant fractions as well as highly diluted honey solution clearly exhibited a pro-oxidant effect. However, this pro-oxidant effect could possibly be due to the different components which had not been completely separated from the antioxidants. This was evident when a lot of different fractions were isolated by reversed phase chromatography, some having a pro-oxidant effect and some an antioxidant effect at the end of the isolation procedures (see Section 8.3.2, Figure 8.4A and B).

The initial concentration of the Fe^{2+} used in this study (*i.e.* 1.5 mmol/l) might not represent the physiological level of free Fe^{2+} . However, this concentration partly represent the condition of the high concentration of free Fe^{2+} in haemochromatosis (Houglum *et al.*, 1997), thalassemia (Livrea *et al.*, 1996) and in localised areas where inflammation is causing the release of Fe^{2+} from iron-binding proteins (Biemond *et al.*, 1984). Therefore, the pro-oxidants effect given by the diluted honey components at these elevated levels of Fe^{2+} could lead to a toxic effect for anyone who is suffering from these health conditions.

On the other hand, the consumption of Beech honeydew honey in low risk condition (*i.e.* non-overloaded Fe^{2+}) in preventing the oxidative stress mediated by free Fe^{2+} is considered safe, as free Fe^{2+} is kept at low concentration in the body, and tightly regulated, and is released only in small amounts in certain cases such as inflammation or disfunctional of antioxidant enzymes as discussed in Chapter 1. This was evident when the low concentration of the antioxidant fractions exhibited an iron-binding antioxidant activity when low concentration of Fe^{2+} (*i.e.* 0.1 mmol/l) was used in the assay. Honey also can be applied topically in high concentration on inflamed wounds.

Due to the potential implications that could arise, it is not known whether eating honeydew honey will protect or harm the health because the level of free Fe^{2+} ions is not known and will vary in different sites in the body. This, it is proposed to study which markers of damage from the Fenton reaction are measured in people

given different amount of honeydew honey to consume by doing this with healthy people and those with conditions where there is excess of Fe^{2+} likely to be present in the body.

9.3 Limitations of the study

The main limitation of the iron-binding antioxidant assay is its poor stability, due to fast oxidation of Fe^{2+} to Fe^{3+} ions caused by dissolved oxygen in the buffer. This oxidation reaction would decrease the level of free Fe^{2+} ions in the assay. It is also evident in many publications, as discussed in Chapter 7, that Fe^{3+} can be reduced to Fe^{2+} by antioxidants, specifically phenolics, therefore indirectly will increase the level of free Fe^{2+} ions in the assay system. This condition would underestimate the activity of the antioxidants as well as complicate the interpretation of the assay result, especially when the concentration of antioxidants is low. Therefore, the assay must be run immediately once the ferrous chloride solution is prepared, to minimise the formation of Fe^{3+} ions through Fe^{2+} oxidation.

9.4 Recommendations for future work

This research was an exploratory study in finding the iron-binding antioxidants in Beech honeydew honey. Although the isolation methods have been successfully developed and there has been a preliminary characterisation of the antioxidant components, several areas would be of interest for further research studies. Future work should isolate the iron-binding antioxidants in large scale so that the structural elucidation of the antioxidants could be easily made. As NMR is a less sensitive spectroscopy technique, the isolation of a substantial amount of compounds should be prioritised. This also possibly could improve the error values gained by mass spectrometry, thus would give more reliable molecular formulae for the antioxidants.

Using a low concentration of Fe^{2+} , representative of the physiological level of iron, should be considered in order to relate the mechanism of action of antioxidants *in vivo*, especially when the antioxidants have a concentration-dependent type of

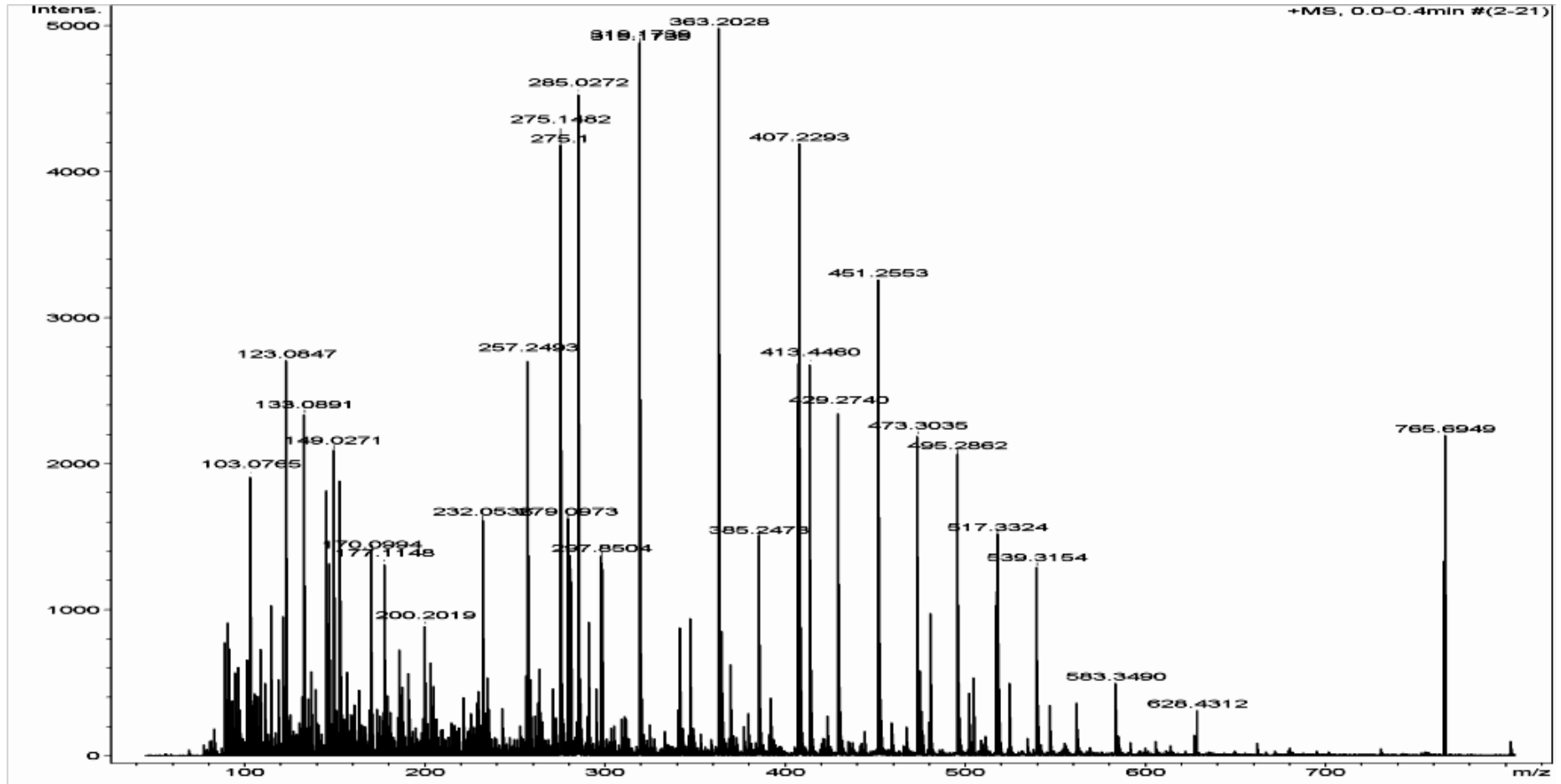
action. The isolated antioxidant should be tested on cells to confirm that there is the expected iron-binding antioxidant activity which could be further investigated for preventing iron-mediated damage in diseases.

Appendices

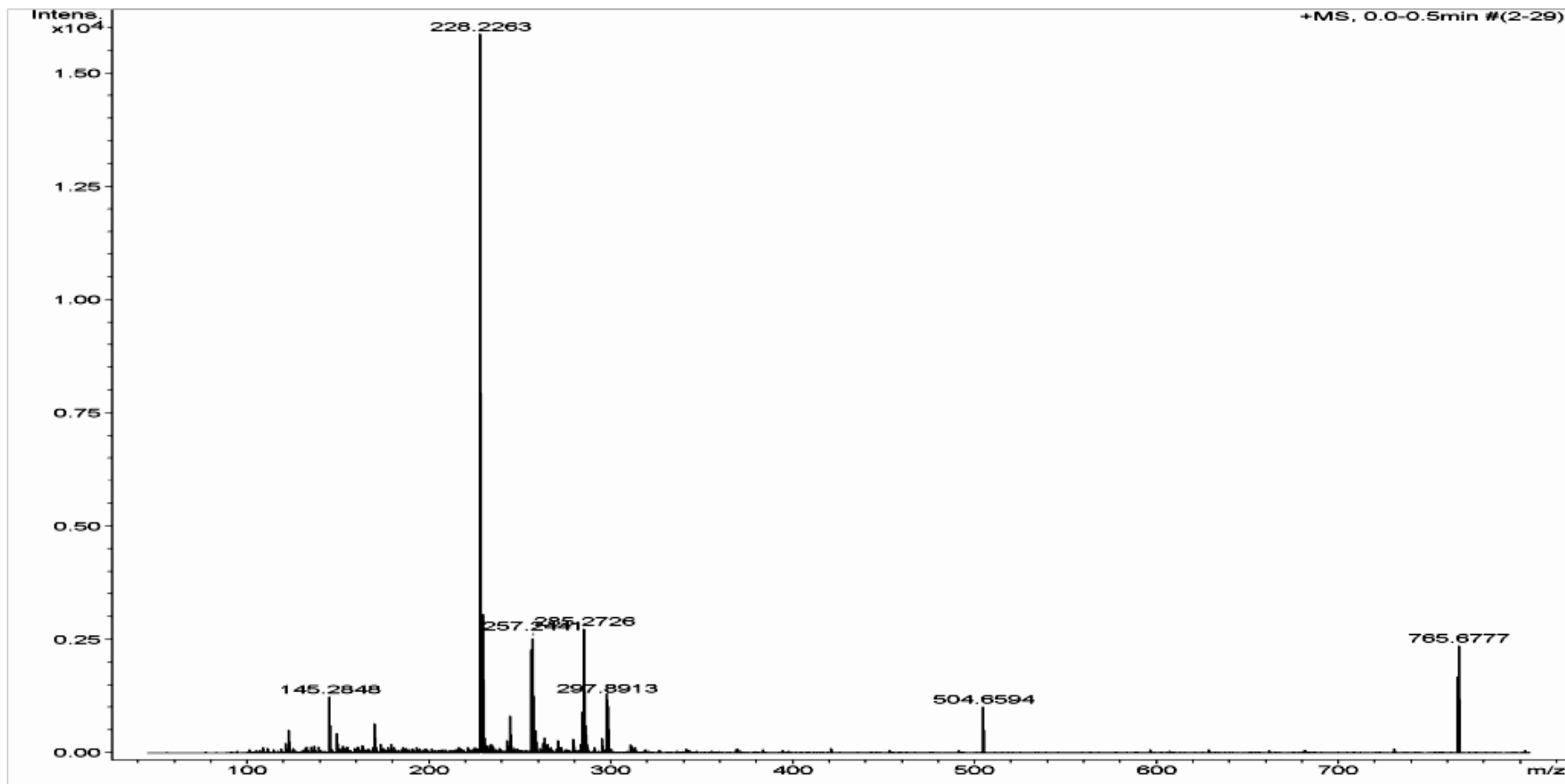
Appendix 1 (continued)

Sample 18 blank	0.2041	0.1953	.	.
Sample 18	0.678	0.3776	0.668966667	0.344
	0.6607	0.3298	.	.
	0.6682	0.3246	.	.
Sample 3 blank	0.2025	0.1906	.	.
Sample 3	0.7032	0.6618	0.706733333	0.6639
	0.6952	0.6567	.	.
	0.7218	0.6732	.	.
Sample 11 blank	0.1755	0.1677	.	.
Sample 11	0.6418	0.3066	0.6357	0.275833333
	0.6279	0.2626	.	.
	0.6374	0.2583	.	.
Sample 19 blank	0.6939	0.6888	.	.
Sample 19	1.215	1.1928	1.202733333	1.174666667
	1.1973	1.1664	.	.
	1.1959	1.1648	.	.
Sample 4 blank	0.2225	0.21	.	.
Sample 4	0.7055	0.6248	0.705233333	0.624333333
	0.7035	0.6233	.	.
	0.7067	0.6249	.	.
Sample 12 blank	0.3392	0.3125	.	.
Sample 12	0.6903	0.5567	0.6857	0.553966667
	0.6847	0.5512	.	.
	0.6821	0.554	.	.
Sample 20 blank	0.1568	0.152	.	.
Sample 20	0.6372	0.4793	0.636366667	0.4798
	0.6321	0.4768	.	.
	0.6398	0.4833	.	.
Sample 5 blank	0.2172	0.2116	.	.
Sample 5	0.7072	0.6713	0.707433333	0.671466667
	0.7047	0.6709	.	.
	0.7104	0.6722	.	.
Sample 13 blank	0.1572	0.1535	.	.
Sample 13	0.6079	0.3584	0.622833333	0.370833333
	0.6426	0.3839	.	.
	0.618	0.3702	.	.
Sample 21 blank	0.1467	0.1447	.	.
Sample 21	0.6157	0.4124	0.617133333	0.4155
	0.6149	0.4123	.	.
	0.6208	0.4218	.	.
Sample 6 blank	0.2228	0.2147	.	.
Sample 6	0.6645	0.6015	0.663933333	0.601933333
	0.6614	0.5977	.	.
	0.6659	0.6066	.	.
Sample 14 blank	0.2569	0.2491	.	.
Sample 14	0.7241	0.6024	0.728433333	0.6126
	0.7375	0.6252	.	.
	0.7237	0.6102	.	.
Sample 22 blank	0.1391	0.1341	.	.
Sample 22	0.596	0.3473	0.600433333	0.357933333
	0.6032	0.3558	.	.
	0.6021	0.3707	.	.

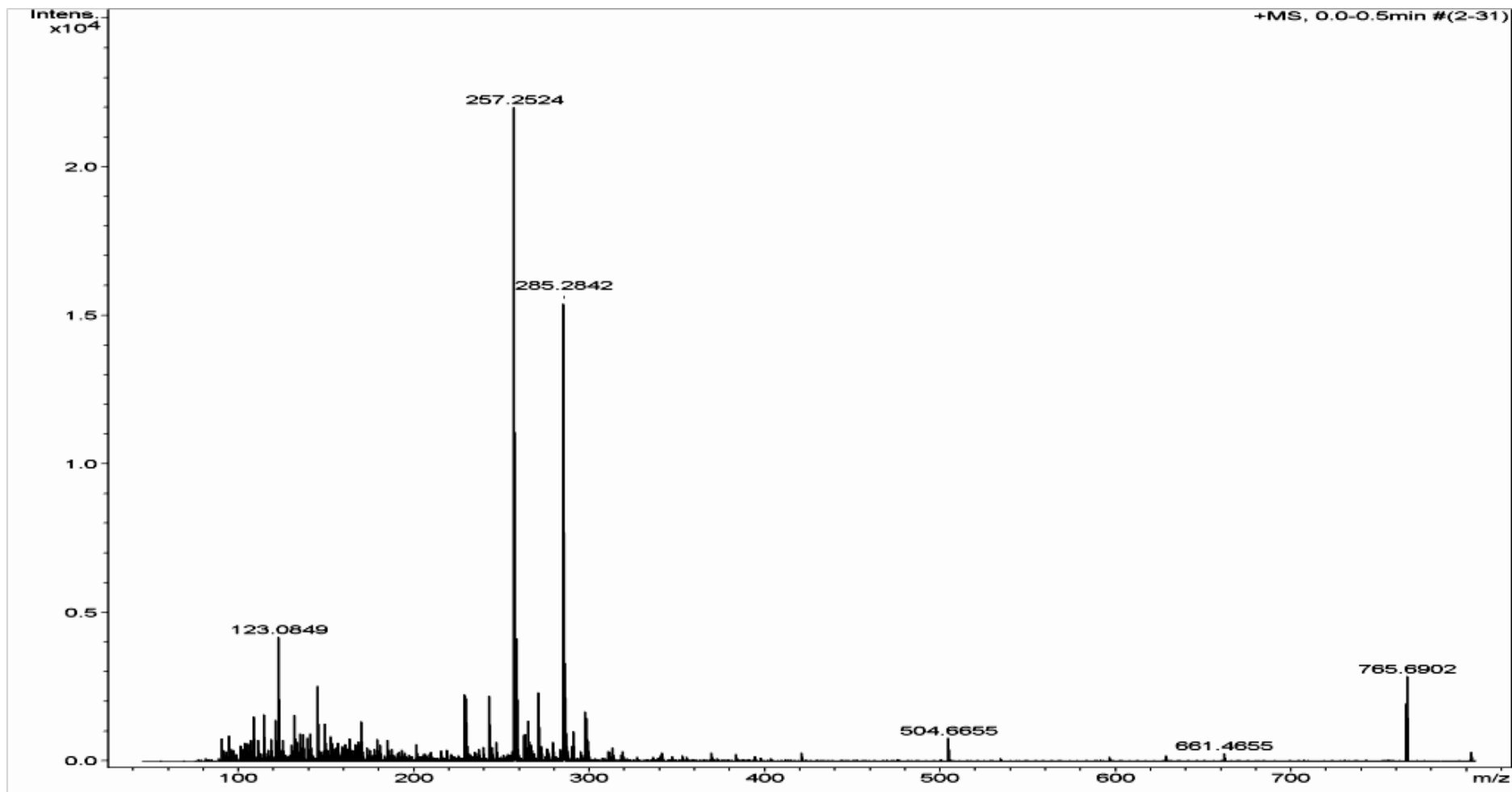
Appendix 2: The electrospray ionization-mass spectrum of fraction G7a in positive ion mode.



Appendix 3: The electrospray ionization-mass spectrum of fraction G8 in positive ion mode.



Appendix 4: The electrospray ionization-mass spectrum of fraction BG1 in positive ion mode.



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