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# **The Study of the Antioxidant Activity of Phenolic Components of Manuka Honey**

A thesis

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of

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## **Abstract**

The phenolic compounds of honey have been known to pose significantly antioxidant activity, including iron-binding and free radical scavenging activity. Manuka honey has been widely used in wound treatment and the antioxidant activity of manuka honey is important in that. However, the antioxidant activity of phenolic compounds of manuka honey has been studied in a few of cases. The aim of this study was to identify the molecular structure of phenolic compounds of manuka honey mainly responsible for each type of antioxidant activity (iron-binding and free radical scavenging activity). The measurement of iron-binding type of activity was based on the inhibition of the Fenton reaction using the  $\beta$ -carotene-linoleic acid model system and the measurement of free radical scavenging activity was based on ABTS system.

The phenolic extracts of manuka honey obtained off XAD column was run through Sephadex G-25 column. The elution was pooled to form fractions for assaying of antioxidant activity, so that the fractions with highest antioxidant activity can be detected. The fractions with highest antioxidant activity, including iron-binding and free radical scavenging activity, were re-run through Sephadex G-25 again, and the resulting fractions were assayed. After repeating fourth running through Sephadex G-25 column, 5 pools with highest antioxidant activity were obtained. The elution volumes of these 5 pools were mainly from 105.6 – 115.2 ml, indicating that this volume range had most of the antioxidant activity for phenolic extracts of manuka honey.

Five pools were further separated by Superdex Peptide column on the FPLC system. The results showed that each pool was separated to have several main peaks. Each peak obtained from chromatography of all five pools was taken for activity assay. The peak with highest iron-binding activity was selected for structure identification by UV and mass spectra methods. The conclusion was made that the phenolic compound responsible for iron-binding type of antioxidant activity could be the molecule with molecular weight of 458.

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## Abbreviations

<b>ACN</b>	Acetonitrile
<b>DNA</b>	Deoxyribonucleic acid
<b>DPPH</b>	1, 1-diphenyl-2-picrylhydrazyl
<b>Fe<sup>2+</sup></b>	Ferrous ion
<b>Fe<sup>3+</sup></b>	Ferric ion
<b>FPLC</b>	Fast protein liquid chromatography
<b>GC-MS</b>	Gas chromatography-mass spectrometry
<b>HCl</b>	Hydrochloric acid
<b>H<sub>2</sub>O<sub>2</sub></b>	Hydrogen peroxide
<b>IL-1<math>\beta</math></b>	Interleukin-1 $\beta$
<b>IL-6</b>	Interleukin-6
<b>MIC</b>	Minimum inhibition concentration
<b>MGO</b>	Methylglyoxal
<b>NADPH</b>	Nicotinamide adenine dinucleotide phosphate, reduced form
<b>NF-<math>\kappa</math>B</b>	Nuclear factor kappa B
<b>NMR</b>	Nuclear magnetic resonance
<b>OD</b>	Optical density
<b>O<sub>2</sub><sup>·-</sup></b>	Superoxide
<b>·OH</b>	Hydroxyl radical
<b>ROS</b>	Reactive oxygen species
<b>TNF-<math>\alpha</math></b>	Tumour necrosis factor alpha
<b>UMF</b>	Unique manuka factor

## **Chapter 1: Introduction and Literature Review**

This chapter aims to introduce the research area of this thesis, outlining the traditional and more recent therapeutic properties of honey and demonstrating honey's antibacterial and antioxidant activity. It finally describes the purpose of this study.

### **1.1 Honey: an ancient medicine**

#### **1.1.1 Chemical composition of honey**

Honey is a natural product produced from the nectar and exudation of plants by the honeybees, *Apis mellifera* (Alvarez-Suarez et al., 2010). The natural honey has been reported to contain about 200 substances, which consist of not only highly concentrated solution of sugars, but also the complex mixture of other saccharides, amino acids, peptides, enzymes, proteins, organic acids, polyphenols, carotenoid-like substances, vitamins, and minerals (Gheldof et al., 2002; Sato & Miyata, 2000; White, 1975).

Sugars are the main constituents of honey, comprising about 95% of its dry weight (Alvarez-Suarez et al., 2010). While glucose and fructose are the dominant constituents, about 25 different sugars have been detected (Doner, 1977; Siddiqui, 1970). The view by White (1975) has demonstrated that proteins in honey are mainly enzymes. Honey contains roughly 0.5% proteins (Alvarez-Suarez et al., 2010) and the protein contents in some honeys can be over 1 000 µg/g (Azeredo et

al., 2003). Main enzymes include diastase, invertase, glucose oxidase and catalase. Although the content of amino acids in honey is relatively small, it has been found that almost all of physiologically essential amino acids are present in honey (Cotte et al., 2004; Hermosín et al., 2003). The primary amino acid is proline, contributing 50-85% of the total amino acids (Hermosín et al., 2003). The level of organic acids in honey is relatively low and about 18 organic acids have been detected (Nanda et al., 2003). Most of the acidity present in honey is added by honeybees (Echigo & Takenaka, 1974). Gluconic acid, the predominant honey organic acid, is the product of glucose oxidation, presenting at 50-fold higher levels than other acids (Cherchi et al., 1994). Investigations have shown that a wide range of trace elements are present in honey, including Al, Ba, Bi, Co, Cr, Mo, Ni, Pb, Sn, Ti, as well as minerals (Ca, Cu, Fe, K, Na, Mg, Mn, Zn) (Conti, 2000; Stocker et al., 2005), among them, the main mineral element is potassium while copper presents lowest amount (Nada et al., 2003). Vitamins such as thiamin (B1), riboflavin (B2), pyridoxine (B6), and ascorbic acid (C) have also been reported but their amount is very small in honey (Ball, 2007; Nada et al., 2003). When honey is treated with mild heat or prolonged storage, a compositional change can occur due to caramelization of the carbohydrates, the Maillard reaction, and decomposition of fructose in the acid medium of honey (Villamiel et al., 2001).

Phytochemicals are chemical substances naturally occurring in plants and many of them are now recognized to have health-promoting activity (Apostolidis et al., 2006; Liu, 2003; Liu, 2004; Sun et al., 2002; Vattem et al., 2005). Phenolic substances are the largest group of phytochemicals (King & Young, 1999). The

plants containing phytochemicals might be used as a supply of the bees; thereby bioactive compounds can be transferred to honey. Studies have shown that honey contains great variation in contents of different phytochemicals according to floral sources and climatic conditions, which contribute to different characteristic colors, flavors, aromas, and bioactivities (Abu-Tarboush et al., 1993; Molan, 1996). As herbal medicines are derived from different plants, which can produce different therapeutic properties (Villegas et al., 1997), some honey derived from these specific plants may provide added value for health promotion. Honey produced by bees fed herbal extracts has shown greater antioxidant activity than normal honey (Rosenblat et al., 1997).

### **1.1.2 Antibacterial activity**

Honey has a long history of use as an effective medicine since ancient civilization for a wide range of disease conditions (Molan, 2001). The physiological property of honey has been attributed to production of hydrogen peroxide formed by the enzyme glucose oxidase; antioxidant content, low pH value; osmotic action, and a variety of enzymes (Molan, 2009).

One of the intrinsic features of honey is its antimicrobial property, which allows honey to be stored for a long period without becoming spoiled (Al-Mamary et al., 2002). The antibacterial mechanisms of honey are associated with its high osmolarity, acidity, production of hydrogen peroxide, and non-peroxide antibacterial components such as flavonoids, lysozyme, and the phenolic acids (Molan, 1992; Postmes et al., 1993; Sonwdon & Cliver, 1996; Wahdan, 1998; Willix et al., 1992). Hydrogen peroxide in honey is produced by glucose oxidase

secreted from the hypopharyngeal glands of bees (Molan, 2009). The level of hydrogen peroxide is proportional to relative levels of glucose oxidase and catalase originating from pollen (Weston, 2000). However, not all honey has the same therapeutic effect due to large variation in its antibacterial activity (Molan, 2001). The variable antibacterial activity among honey depends on its floral source (Allen et al., 1991).

A study by Wahdan (1998) has shown that with 21 types of bacteria, including *Escherichia coli*, *Klebsiella sp*, *Pseudomonas sp*, *Staphylococcus sp*, and two types of fungi *in vitro*, honey neutralized more pathogens than sugar control, and undiluted honey completely inhibited the growth of all 21 bacteria. The MIC (minimum inhibition concentration) of honey was found to be from 1.8% to 10.8% (V/V) (Molan, 2001). Although both Gram-positive and -negative bacterial strains are sensitive to honey, some Gram-negative bacteria (*Salmonella dublin* and *Shigella dysenteriae*) being more susceptible than gram-positive strains (*Bacillus cereus*, *Staphylococcus aureus*) (Bogdanov, 1984).

### **1.1.3 Anti-inflammatory activity**

The anti-inflammation properties of honey have been known well (Molan, 2001). Honey has been found to have the involvement of scavenging activity of reactive oxygen species responsible for induction of inflammation (Greten et al., 2004). When honey is applied to wounds, it effectively reduces the inflammation (Burlando, 1978; Subrahmanyam, 1998), as well as reducing oedema around wounds (Dumronglert, 1983; Efem, 1988; Efem, 1993) and exudation from wounds (Burlando, 1978; Efem, 1988; Efem, 1993; Hejase et al., 1996). Honey

has also been observed to relieve the pain that is a feature of inflammation (Burlando, 1978; Keast-Butler, 1980; Subrahmanyam, 1993). The studies of healing animal tissues have indicated that the leucocyte numbers associated with inflammation have been less when the wounds have been treated with honey (Subrahmanyam, 1998). Similar results observed in animal study models have confirmed that the anti-inflammation action of honey cannot be due to removal of bacteria alone (Oryan, 1998; Postmes, 1997). Furthermore, honey has been shown to decrease the stiffness of inflamed wrist joints of guinea pigs (Church, 1954).

One of the anti-inflammatory effects of honey can be attributed to its antibacterial activity since components of the bacterial cell wall are potent stimulators of the inflammatory response (Molan, 2009). The presence of slough in wounds also acts as an inflammatory stimulus, and slough removal by honey application to wounds has shown to help decrease inflammation (Efem, 1988).

#### **1.1.4 Use for wound healing**

A review of honey's use in wound care by Molan (2006) has provided overwhelming evidence that honey is a credible wound treatment option. With regards to wound treatment by honey application, the osmotic action of honey can induce outflow of lymph, which is able to promote extra oxygenation and provide improved supply of nutrients on the wound surface, as well as to flush away proteases that may inhibit the repair process (Molan, 2009). Moreover, honey's osmotic action can create a moist environment that is required for the fibroblasts to contract and pull the margins of the wound together (Molan, 2001). The acidic pH of honey also adds the value to aid wound healing since it can facilitate to

release the oxygen carried by haemoglobin (Molan, 2009). It has been noted that acidification of wounds can improve the speed of the healing process (Kaufman, 1985; Leveen, 1973).

A number of studies have firmly reinforced that honey is an effective medicinal treatment for burns and infected wounds (Molan, 2001; Subrahmanyam, 1996) and it is more effective as a dressing than many other present alternatives (Vermeulen et al., 2005).

#### **1.1.5 Importance of anti-inflammatory activity in wound healing**

The anti-inflammatory action of honey is potentially very important for therapeutic application, as the inflammation can cause major consequences. During the course of inflammation, some mediators called prostaglandins produced by the leucocytes to regulate the activity of surrounding cells can cause the painful symptoms of inflammation, and others can cause blood vessels to dilate and the capillary walls to open up (Molan, 2001). Plasma flows out to cause swelling and to increase the diffusion distance from the capillaries to the cells (Molan, 2001). The opening up of capillaries can cause exudation of serum. If it is prolonged, it would lead to malnutrition (Molan, 2001). The most harmful consequence of inflammation is the production of reactive oxygen species in the tissue. These reactive oxygen species (free radicals) can be very damaging as they are very reactive and are able to break down the proteins, lipids, and nuclei acids (Flohe et al., 1985, Molan, 2001). The continued production of free radicals can lead to localized erosion of body tissue. The free radicals are also involved in stimulating the activity of fibroblasts, which is the basis of repair process (Molan,

2009). The fibroblasts are responsible for synthesis of collagen fibers and other connective tissue components, if inflammation is continued, the over-stimulation can lead to fibrosis and excessive production of collagen fibres (Molan, 2001; Murrell et al., 1990). Thus, there are significant benefits for therapeutic use of anti-inflammatory substances. However, pharmaceutical anti-inflammatory medicines have serious limitations: corticosteroids suppress tissue growth and the immune system (Bucknall, 1984), and the non-corticosteroids are harmful to cells (Brooks, 1985). Honey has been confirmed to possess anti-inflammatory activity without adverse side effects (Molan, 2001).

#### **1.1.6 Antioxidant activity in decreasing inflammation**

Inflammation is part of the non-specific immune reaction occurring in response to any type injury in body (Ferrero-Miliani et al., 2006). A mild short-lived inflammation is essentially required to initiate the healing process, but when the inflammation becomes prolonged, it can slow or prevent the healing (Molan, 2009). Once inflammatory stimulus is extended, the superoxide and hydrogen peroxide are continuously generated as they act to recruit more neutrophils (Flohé et al., 1985, Klyubin, 1996). Hydrogen peroxide activates more neutrophils via the activation of the nuclear transcription factor NF- $\kappa$ B to produce specific cytokines which amplify the inflammatory response by activating leukocytes, and activated neutrophils in turn generate more hydrogen peroxide, which sets up a vicious cycle; hydrogen peroxide from other sources can also trigger this cycle (Baeuerle et al., 1996; Molan, 2009). It has been found that the oxidative species produced from hydrogen peroxide, instead of hydrogen peroxide, are responsible for activating NF- $\kappa$ B, and antioxidants prevent this activation (Grimble, 1994).

The ability of honey to neutralize free radicals has been demonstrated (van den Berg, 2008). A clinical trial of honey dressing on burns has indicated that the way in which honey initiates healing in burns is the control of free radicals by the antioxidant activity of honey (Subrahmanyam, 2003). This antioxidant activity may be partly responsible for the anti-inflammatory action of honey, as oxygen free radicals are involved in various aspects of inflammation. Even if the honey antioxidants do not directly suppress the inflammation, it can at least reduce the amount of damage caused from ROS by scavenging free radicals (Molan, 2001).

In addition to removing free radicals via scavenging, honey has the potential of inhibiting generation of free radicals formed from hydrogen peroxide in the first place through a different mechanism of antioxidants (Molan, 2001). While the superoxide produced in inflammation is relatively unreactive and easily to be converted into less reactive hydrogen peroxide, the generation of peroxide radicals from hydrogen peroxide catalyzed by metal ions, such as iron and copper, can be extremely damaging (Cross et al., 1987). Antioxidant, however, like flavonoids or some polyphenols, are able to sequester these metal ions in a non-catalytic form (Halliwell & Cross, 1994). Although it is important that antioxidants neutralize free radicals when the inflammation has been established, inhibition of the formation of hydroxide radicals would greatly help break the inflammatory vicious cycle.

### **1.1.7 Antioxidant activity of honey**

Honey contains a significantly high level of antioxidants, both enzymatic and non-enzymatic, including catalase, phenolic acids, flavonoids, carotenoids, organic acids, ascorbic acid, amino acids, proteins and Maillard reaction products (Aljadi & Kamaruddin, 2004; Al-Mamary et al., 2002; Frankel, 1998; Gheldof & Engeseth, 2002; Gheldof et al., 2002; Nasuti, et al., 2006; Schramm et al., 2003; Vela, 2007).

Phenolic compounds commonly found in honey include phenolic acids, flavonoids and polyphenols. Honey phenolic acids can be protocatequic acid, p-hydroxibenzonic acid, caffeic acid, chlorogenic acid, vanillic acid, p-coumaric acid, benzoic acid, ellagic acid, cinnamic acid (Estevinho et al., 2008), and flavonoids in honey consist of naringenin, kaempferol, apigenin, pinocembrin, chrysin, galangin, luteolin etc (Beretta et al., 2005; Estevinho et al., 2008). The large and complex flavonoids greatly contribute to honey color, flavor, anti-fungal, and antibacterial activity (Movileanu et al., 2000).

The antioxidant capacity of different honeys depends on the floral sources used by bees to collect nectar, seasonal and environmental factors, as well as processing ways (Al-Mamary et al., 2002; Gheldof & Engeseth, 2002). Although the total antioxidant activity of honey is the combination of a wide range of active substances, the content of phenolic compounds can significantly reflect the total antioxidant activity of honey to some extent (Beretta et al., 2005). However, the level of phenolic compounds present in honey is not always positively proportional to its antioxidant activity (Al-Mamary et al., 2002; Küçük et al.,

2007). The explanation for this activity may be due to the presence of variable types of polyphenols, thereby providing variable scavenging activity (Küçük et al., 2007). Darker honey is likely to have a higher antioxidant contents than light-colored honeys (Estevinho et al., 2008; Gheldof et al., 2002). As well, the antioxidant content is higher in honey with higher water content (Frankel et al., 1998).

In humans, after honey is consumed, an increase in plasma antioxidants has been reported, and the antioxidants give protection in the bloodstream and within cells (Schramm et al., 2003), demonstrating that the bioavailability and bioactivity of honey gives a high efficiency antioxidant transfer from honey to plasma.

### **1.1.8 Manuka honey**

Manuka honey is a unifloral honey derived from the native manuka tree of New Zealand, *Leptospermum scoparium* (Weston et al., 1999). Manuka honey has been recognized to exhibit exceptionally high antibacterial activity (Allen et al., 1991; Russel et al., 1990), including the antibacterial property against *Helicobacter pylori* causing stomach and duodenal cancers (Somal et al., 1994). It is now most widely used in wound healing. A study by Allen et al., (1991) comparing the antibacterial activity of honey from different floral sources indicated that some manuka honeys and viper bugloss honeys can still retain antibacterial activity when tested in the presence of catalase, suggesting the presence of non-peroxide activity. Some works have identified methylglyoxal (MGO) as the component principally responsible for this non-peroxide activity (Adams et al., 2008; Mavric et al., 2008). However, not all manuka honeys possess this non-peroxide

antibacterial activity: this bioactivity present in manuka honey is only from specific localities (Molan, 1995).

Besides the well-known antibacterial activity, manuka honey has shown significant antioxidant activity. The study by Inoue et al., (2005) revealed that a distinctively high level of antioxidant, methyl syringate, posed a specific scavenging activity for superoxide anion radicals, based on DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging systems. The methyl syringate is not only able to neutralize superoxide radicals, but also to bind iron so that the formation of extremely damaging hydroxide radicals generated from hydrogen peroxide is prevented (Brangoulo and Molan, 2010).

## 1.2 Free radicals

Chemically, a free radical is any atom such as oxygen or nitrogen with at least one unpaired electron present, and is able to exist independently (Karlsson, 1997). Free radicals can easily be formed in three ways: 1) by the homolytic cleavage of a covalent bond, generally incurring by high energy input; 2) by the loss of a single electron from a normal molecule; 3) by addition of a single electron to a normal molecule (Cheeseman & Slater, 1993). These free radicals that are highly reactive molecules can be extremely damaging to the lipids, proteins and cellular DNA (Cochrane, 1991), which may lead to many biological complications, including carcinogenesis, mutagenesis, aging, and atherosclerosis (Halliwell & Gutteridge, 1989).

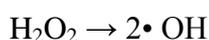
The oxygen-derived free radical is an important group formed during metabolism. One of these reactions found in biological pathways is the respiratory burst process, which result in free radical products termed reactive oxygen species (ROS) (Halliwell & Guteridge, 1999; Winrow et al., 1993). Examples of ROS include superoxide, hydroxyl radicals, and non-oxygen free radical hypochlorites (Conner & Grisham, 1996).

Investigations have suggested that ROS are involved in mediating of certain types of inflammatory tissue injury and the most likely sources of these oxidizing agents are produced via phagocytic leukocytes (Conner & Grisham, 1996). Activation of phagocytes via interaction of certain pro-inflammatory mediators or bacterial components with specific membrane receptors of leucocytes triggers the assembly of the multicomponent flavoprotein NADPH oxidase which catalyzes the

production of superoxide anion radicals (Conner & Grisham, 1996; Klebanoff, 1992). Superoxide will rapidly and spontaneously/enzymatically dismutase to produce hydrogen peroxide and other free radicals (Halliwell et al., 2000).

Besides being produced from superoxide, hydrogen peroxide can also be generated by other oxidase enzymes, such as glycollate and monoamine oxidase, or by the peroxisomal pathway that is for  $\beta$ -oxidation of fatty acids (Chance et al., 1979; Halliwell & Gutteridge, 1999). The production of hydrogen peroxide in human plasma was found to have involvement of an enzyme activity named xanthine oxidase (Lacy et al., 1998); the level of xanthine oxidase has been founded to increase as a result of tissue injury (Friedl et al., 1990).

The main danger of hydrogen peroxide comes from its conversion to reactive hydroxyl radicals ( $\bullet\text{OH}$ ),



either by exposure to UV light (Ueda et al., 1996) or by interaction with some transition metal ions: Ti(III), Cu(II), Fe(II), or Co(II) in vitro (Halliwell & Gutteridge, 1999), and it seems the most important ion among them is iron (Halliwell & Gutteridge, 1990; 1999). Iron exists in two oxidation states: ferrous and ferric ions. The very important reaction of hydrogen peroxide with Fe(II) is the Fenton reaction:



Additional superoxide can also be generated by the reaction of Fe(III) with  $\text{H}_2\text{O}_2$  (Halliwell & Gutteridge, 1986):



This reaction recycles the Fe(III) to the Fe(II) form, which can create more hydroxyl radicals from hydrogen peroxide. The hydroxyl radical, an extremely reactive species, has a short-term life as it immediately reacts with the nearest molecules non-specifically at diffusion-limited rates of reaction ( $\sim 10^{-7} - 10^{-10}$  mol/l/s) (Conner & Grisham, 1996; Schreck et al., 1991). Hydroxyl radicals have been found to peroxidize lipids, oxidize proteins, and enhance DNA scission (Grisham, 1992). Chelating agents may inactivate metal ions; thereby potentially inhibiting the metal-dependent processes.

## **1.3 Antioxidants**

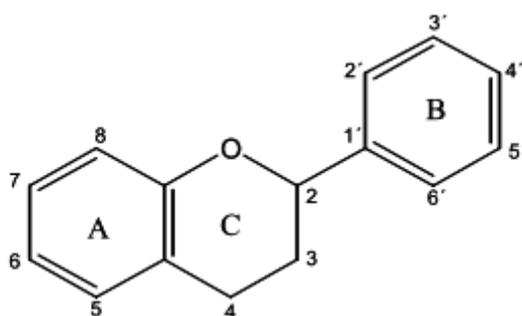
### **1.3.1 Introduction to antioxidants**

The name antioxidant is applied to any substance that significantly delays or prevents oxidation of an oxidizable substrate when present in low concentration, including every type of molecules found in vivo (Halliwell & Gutteridge, 1990). Natural antioxidants can be phenolic compounds (tocopherol, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll substances, amino acids/peptides, and amines), carotenoid derivatives, and ascorbic acid (Hall & Cuppet, 1997; Hudson, 1990; Larson, 1988). Activity of antioxidants is tightly associated with a variety of biological effects, including anti-inflammatory, antibacterial, anti-allergic, anti-thrombotic, and vasodilatory actions (Cook & Sammon, 1996).

### **1.3.2 Classification of phenolic compounds**

While there are various types of antioxidants present in honey, this review only focuses on phenolic compounds. Phenolic compounds or polyphenols are one of the most important groups originating from plants as secondary products (Bravo, 1998). The most important classes of polyphenols are flavonoids and phenolic acids, with more than 5000 compounds already demonstrated (Bravo, 1998). They have been regarded to have effective antioxidant and radical scavenging activities (Takahama, 1998), based on acting in different mechanisms, such as free radical scavenging, hydrogen-donating, metal ion chelating.

Flavonoids are compounds of low molecular weight that commonly occur bound to sugar molecules and they can be categorized as flavonols (the most widely distributed flavonoids, including quercetin, kaempferol, and myricetin), flavanones, flavones, anthocyanidins and isoflavones (genistein and daidzein) (King & Young, 1999). The basic structure of flavonoids is demonstrated in **Figure 1.1**.



**Figure 1.1 Generic structure of flavonoids (Brovo, 1998)**

Flavonoids can act as antioxidants in various ways, including direct trapping of reactive oxygen species, inhibition of enzymes responsible for superoxide anions formation, chelation of transition metals involved in the processes of free radical formation, and prevention of the peroxidation process (Rice-Evans et al., 1996).

Phenolic acids include hydroxybenzoic (the 2 major acids: ellagic and gallic acids) and hydroxycinnamic acids (King & Young, 1999). The hydroxybenzoic acids are mainly found in berries and nuts (Maas et al., 1991). The hydroxycinnamic acids consist of mainly coumaric, caffeic and ferulic acid, which are rarely found in the free form (D'Archivio et al., 2007). Caffeic acid is the most abundant phenolic acid, representing between 75% and 100% of the total hydroxycinnamic acids

contents in most fruits while ferulic acid is the most abundant phenolic acid found in cereal grains (D'Archivio et al., 2007).

Tannins are high molecular weight polyphenols, which can either bind and precipitate or shrink proteins and other organic molecules including amino acids and alkaloids (King and Young, 1999). They are usually divided into two groups: condensed and hydrolysable substances; condensed tannins usually accumulating in outer layers of plants are polymers of catechins or epicatechins and hydrolysable tannins are polymers of gallic or ellagic acids (King and Young, 1999).

### **1.3.3 Antioxidant nature of phenolic compounds**

Different phenolic compounds such as phenolic acids, phenolic diterpenes, and flavonoids have different antioxidative effects (Mayer et al., 1998; Pietta et al., 1998; Shahidi et al., 1992; Vinson et al., 1995). The study by Singleton and Rossi (1965) has indicated that some phenolic compounds may react faster than others under the same conditions.

For monomeric phenolics, the antioxidant property is dependent on extended conjugation, arrangement of phenolic substituents as well as their numbers, and molecular weight (Hagerman et al., 1998). The flavonoids with the most hydroxyl groups can be most easily oxidized (Hondnick et al., 1988). The effectiveness of phenols to scavenge peroxy radicals is due to their molecule structures containing an aromatic ring with hydroxyl groups and association with the activity of reducing and chelating ferrous ion that acts to catalyze lipid peroxidation (Al-

Mamary et al., 2002; Arouma, 1994; Halliwell, 1990). The differences in antioxidant activity are closely related to structural dissimilarities (mainly the degree of hydroxylation and methylation of the compounds) (Mayer et al., 1998).

## **1.4 Objectives of the present project**

Previous studies have shown that honey contains significant level of antioxidants, thereby exhibiting specific antioxidant activity. Manuka honey has been widely used in wound treatment due to its superior antibacterial property and antioxidant activity is important in that. However, there are few studies on reporting antioxidant activity of phenolic components of manuka honey and the contributions of different phenolic components to the iron-binding and free radical scavenging antioxidant activity. The aim of this study was to extract the phenolic compounds from manuka honey and to identify the molecular structures of phenolic compounds responsible for each type of antioxidant activity (iron-binding and free radical scavenging activity). The work will be performed by separating the components using column chromatography to obtain different fractions, assaying these fractions for both types of the iron-binding and free radical scavenging antioxidant activity, and then identifying the molecular structures responsible for each type of antioxidant activity after purifying them.

## Chapter 2 Materials and Methods

This chapter outlines the general materials and methods used throughout this study. The honey selection, chemicals used, chemical solution, and experimental procedures are given in detail.

### 2.1 Materials

#### 2.1.1 Chemicals

All reagents used in this study were analytical grade. Distilled water was used for common chemical solution preparation and double deionized water was used for FPLC system.

ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)

Sigma Aldrich (St Louis, MO, USA)

Acetonitrile

Ajax Finechem Pty Ltd

$\beta$ -Carotene

Sigma Aldrich (St Louis, MO, USA)

Chloroform

BDH Chemicals Ltd (Poole, England)

95% Ethanol

Ajax Finechem Pty Ltd

Ferrozine<sup>®</sup> (3-(2-pyridyl)-5,6-diphenyl-1,2,4-tri-azine-4',4''-disulfonicacid sodium salt)

Sigma Aldrich (St Louis, MO, USA)

Ferrous chloride

BDH Chemicals Ltd (Poole, England)

HCl (hydrochloric acid)

Univar, AR

Linoleic acid

Sigma Aldrich (St Louis, MO, USA)

Methanol	Scharlab S.L Spain
Potassium chloride	BDH Chemicals Ltd., AR
Potassium di-hydrogen phosphate	BDH Chemicals Ltd. (Poole, England)
Potassium hydroxide	BDH Chemicals Ltd. (Poole, England)
Potassium Persulfate	BDH Chemicals Ltd. (Poole, England)
Sodium acetate	May & Baker, AR
Sodium chloride	BDH Chemicals Ltd. (Poole, England)
Sodium formate	Scharlab S.L Spain
Sodium hydrogen carbonate	Prolabo, AR
NaOH (sodium hydroxide)	Scharlab, Reagent grade
Trifluoroacetic acid	May & Baker, AR
Trolox <sup>®</sup> (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid)	
	Aldrich Chemical Company
Tween-40 (Polyoxyethylenesorbitan monopalmitate)	
	Sigma Aldrich (St Louis, MO, USA)

### **2.1.2 Buffers and Solutions**

#### **ABTS stock solution (7 mmol/l)**

The stock ABTS solution was prepared by dissolving 38 mg of ABTS powder in 10 ml of Milli Q water. ABTS<sup>•+</sup> cation radical was produced by adding 6.5 mg of potassium persulfate to have the final concentration of 2.45 mmol/l, and the mixture was allowed to react for 12-16 hours in the dark.

#### **β-Carotene emulsion**

1 ml of  $\beta$ -carotene solution at 4 mg/ml in chloroform was added to the mixture of 100 mg Tween-40 and 10 mg linoleic acid in a 50 ml round bottom flask. The chloroform was evaporated at 40 °C with a rotary evaporator for 10 minutes. Then 25 ml of deionized water was added to form an emulsion, while stirring. The emulsion was completely dissolved off the sides of the round bottom flask.

Blank emulsion was also prepared as above; the 1 ml of  $\beta$ -carotene solution in chloroform was replaced with 1 ml chloroform for blank emulsion solution.

#### **0.25 mmol/l Ferrous chloride solution**

The stock ferrous chloride solution (1 mmol/l) was prepared by dissolving 12.6 mg of ferrous chloride in small amount of deionized water, and making up to 100 ml with deionized water. The 0.25 mmol/l ferrous chloride solution was prepared by diluting the stock solution (1 mmol/l) 1:3 in deionized water.

#### **Ferrozine® standard solution (1.5 mmol/l)**

The 7.5 mmol/l Ferrozine® stock solution was prepared by dissolving 37 mg of Ferrozine® with deionized water, and making the final volume of 10 ml. Ferrozine® standard solution (1.5 mmol/l) was prepared by diluting the stock solution (7.5 mmol/l) 1:4 in deionized water.

#### **0.01 mmol/l Hydrochloric acid**

About 0.86 ml of the concentrated hydrochloric acid (36%) was diluted to 100 ml deionized water.

### **1.2 mmol/l Hydrochloric acid**

About 10.2 ml of the concentrated hydrochloric acid (36%) was diluted to 100 ml deionized water.

### **10 mol/l Sodium hydroxide**

40 g of sodium hydroxide pellets were dissolved in distilled water, and diluted to 100 ml.

### **Trolox<sup>®</sup> standard solution (0.2 mmol/l)**

The Trolox<sup>®</sup> standard solution was prepared by diluting 2.0 mmol/l stock Trolox<sup>®</sup> solution 1:9 with 95% ethanol. A 2.0 mmol/l stock Trolox<sup>®</sup> solution was made by dissolving 25.0 mg of Trolox<sup>®</sup> in 95% ethanol to make a volume of 50 ml.

### **2.1.3 Laboratory instruments**

AKTA FPLC system (GE Healthcare)

BIO-RAD Model550 microplate reader

Condensers

Eppendorf centrifuge

Eppendorf pipettes

Fluostar Optima (BMG Labtechnologies GmbH, Germany)

FPLC system (Pharmacia, Sweden)

Mass spectrophotometer (Bruker Daltonics' micrOTOF™)

Magnetic stirrer

pH Meter

Rotary evaporator

Vortex device

Water-bath

## **2.2 Methods**

### **2.2.1 Honey samples**

The honey used for this study was a composite prepared by pooling 20 different batches of monofloral manuka honeys. It had a non-peroxide antibacterial activity rating of 14 (tested by Honey Research Unit). The manuka honey was provided by Honey Research Unit of The University of Waikato, Hamilton, New Zealand. The composite sample was labeled and stored at 4 °C until analytical processing.

### **2.2.2 Extraction of phenolic compounds from manuka honey**

The procedures for extraction of honey phenolic compounds were performed as described by Weston et al., 1999. XAD-2 resin (54 g, about 100 ml) was soaked with a mixture of water and methanol (100 : 100 ml) in a 500 ml beaker overnight. The mixture was decanted and the resin was washed with water and then packed into a column (2 x 25 cm). The column was washed with 1 liter water.

Precisely 100.00 g of honey sample was dissolved in 500 ml 0.01 mol/l HCl. The dissolved sample solution was filtered through glass filter paper and then added slowly to pass through the column, followed by 50 ml 0.01 mol/l HCl for eluting sugars and polar compounds, and then 150 ml of deionized water was run through the column. The phenolic compounds adsorbed to the XAD resin column were extracted with 150 ml of methanol. The methanol extract was rotary-evaporated to dryness under vacuum at 40 °C. The resulting residue was weighed and dissolved in methanol at a known concentration (40 mg/ml).

### 2.2.3 Antioxidant activity using the $\beta$ -carotene-linoleic acid model system

The  $\beta$ -carotene linoleic acid system used to assay the iron-binding antioxidant activity of sample was performed by the method described by Brangoulo & Molan (2010). The assay is based on the mechanism of bleaching of  $\beta$ -carotene that is a free radical-mediated event resulting from the free radicals formed from the oxidation of the linoleic acid. During this assay,  $\beta$ -carotene is undergoing a rapid discoloration in the absence of an antioxidant. The linoleic free radicals quickly attack the highly unsaturated  $\beta$ -carotene molecules. The loss of characteristic orange color due to double bonds' oxidation can be monitored spectrophotometrically.

The absorbance measurements for antioxidant activity were carried out with a Fluostar optima microtitre plate with one injection system (BMG Labtechnologies GmbH, Offenburg, Germany). The protocol for the assay on the plate reader software was created and used to analyze the plate. Ferrozine® was used as a standard. The results were expressed as Ferrozine® equivalent mmol/l of sample.

The setting up of the 96-well flat-bottomed microtitre plate was shown below (**Table 2.1**): the plate was loaded with 30  $\mu$ l of ferrous chloride solution (0.25 mmol/l) in each well. Then 30  $\mu$ l of deionized water was added to wells (A1, A2, A3, A4) as control solution; Next, 30  $\mu$ l of Ferrozine® standard solution (1.5 mmol/l) was added to B1, B2, B3, and B4. For solutions of honey or fractions, 30  $\mu$ l of sample solution was added to C1, C2, C3 and C4 and corresponding wells in

the following rows. Lastly, 150  $\mu$ l of blank emulsion solution was added to A1 – H1, A5 – H5, and A9 – H9 to serve as blanks (Column1, 5, 9).

**Table 2.1 Setting up of microtitre plate for measuring antioxidant activity using the  $\beta$ -carotene-linoleic acid system.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	Control			B	Sample 7			B	Sample 15		
B	L	Ferrozine®1.5mM			L	Sample 8			L	Sample 16		
C	A	Sample 1			A	Sample 9			A	Sample 17		
D	N	Sample 2			N	Sample 10			N	Sample 18		
E	K	Sample 3			K	Sample 11			K	Sample 19		
F		Sample 4				Sample 12				Sample 20		
G		Sample 5				Sample 13				Sample 21		
H		Sample 6				Sample 14				Sample 22		

The filled plate was transferred to the plate reader. The pump of the plate reader was cleaned first with deionized water, and then loaded with  $\beta$ -carotene emulsion solution. The absorbance at 450 nm was measured for 10 minutes after injecting 150  $\mu$ l of  $\beta$ -carotene emulsion solution into each sample well. The initial measurement at time t=0 was made immediately after the injection. The plate was set up in shake mode after the injection and before each cycle. All determinations were made in triplicate. The assay was performed at 37 °C.

The damage (%) or bleaching of  $\beta$ -carotene was calculated as:

**Damage % = 100 x [1-(A<sub>t</sub>/A<sub>0</sub>)];** where A<sub>t</sub> and A<sub>0</sub> were respectively the absorbance at determined time t=10 minutes and time t=0 minute.

The protective effects of samples were evaluated as:

**Protective effect %= 100 x [(DC-DS)/DC];** where DC and DS were respectively the damage obtained in the control and sample.

The protective effect of Ferrozine<sup>®</sup> standard solution was plotted against its concentration. The protective effect of samples was converted into Ferrozine<sup>®</sup> equivalent in mmol/l of sample from the equation. The dilution factor of samples was taken into calculation.

#### **2.2.4 Free radical scavenging capacity using ABTS assay**

ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) is often used to measure the antioxidant capacity of foods in agricultural and food industry. The ABTS assays used in this study as were carried out as described by Brangoulo and Molan (2010). During the assay, ABTS is converted into its radical cation by addition of sodium persulfate. The resulting blue color ABTS cation is reactive toward most of antioxidants including phenolic compounds. When it reacts with phenolics, ABTS radical cation is converted to colorless neutral form, which can be monitored spectrophotometrically.

The free radical scavenging antioxidant activity of samples was carried out by assaying ABTS cation radical decolonization in a microplate reader, with software that allowed end-point and kinetic measurements to be recorded. Trolox<sup>®</sup> was

used as a standard and the results were expressed as Trolox<sup>®</sup> equivalent mmol/l of sample. For the antioxidant activity evaluation, the stock ABTS • + solution (7 mmol/l) was diluted with deionized water to obtain the absorbance in the range of 2.0-2.4 OD (optical density) at 655 nm. To adjust the absorbance, 15 µl stock ABTS solution was added to 85 µl deionized water, and a further 100 µl deionized water was added to make the final dilution for absorbance reading. When the ABTS was required for the assay, 10 ml of working ABTS radical cation solution was prepared by converting 15 µl to 1.5 ml and 8.5 µl to 8.5 ml.

A standard Trolox<sup>®</sup> solution was prepared at 0.2 mmol/l in 95% ethanol. The samples were diluted to an appropriate degree so that the decrease in absorbance values due to scavenging was in the linear response range of the decolorizing of the radical solution.

To prepare the 96-well flat-bottomed microtitre plate, each column of microtitre plate (eight replicates) was filled with blank, Trolox<sup>®</sup> standard or sample solutions at a volume of 100 µl as shown in **Table 2.2**. After all wells were filled, an initial reading was taken to obtain the absorbance due to color variation of samples, which was termed 'Blank' in the calculations. The Endpoint Protocol was used to take the readings: Absorbance =655 nm, Filter = 405 nm; Shaking time = 9 sec prior to reading.

**Table 2.2 Setting up of microtitre plate for measuring free radical scavenging activity using the ABTS system.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	<b>B</b>	<b>T</b>	<b>S</b>									
B	<b>L</b>	<b>R</b>	<b>A</b>									
C	<b>A</b>	<b>O</b>	<b>M</b>									
D	<b>N</b>	<b>L</b>	<b>P</b>									
E	<b>K</b>	<b>O</b>	<b>L</b>									
F		<b>X</b>	<b>E</b>									
G			<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>
H												

Then 100 µl of ABTS<sup>•+</sup> solution was added to every well on the plate using a multi-channel pipettor in a stepwise manner column by column from left to right side in a 10 second interval. The entire filling of micro plate was 110 seconds and another 10 seconds normally to set the filled plate into the plate reader; the plate reader took 35 seconds to read a plate, based on the BIO-RAD Model 550 microplate reader. The time taken to fill the plate and to start the second reading termed ‘Endpoint’ was recorded. The total time spent from 0 second when filling the first column to completing the reading of ‘Endpoint’ was used for Trolox® equivalent antioxidant activity calculation. The Endpoint Protocol was also employed to take these readings. Immediately, the third reading was taken using Kinetic Protocol to obtain absorbance readings. This was the rate of the ongoing reaction and the negative velocity was obtained as the absorbance obtained was

continuing to decrease. Kinetic Protocol: Abs = 655 nm; No shaking time; 5 readings to be taken at 25 second intervals; Negative velocity to be calculated.

Thus, three sets of data were obtained and saved as 'Blank', 'Endpoint', and 'Velocity report'. To determine the Trolox® equivalent values of samples, the calculations were based on the data obtained during running of the assay, which were: average 'Blank' value for each column; average 'Endpoint' value of each column; average velocity of each column.

Step 1: Determining true zero-time endpoint:

'True Endpoint' = Average 'Endpoint' + [(average velocity) x (Time for slow reaction)];

Step 2: Determining the difference in OD between sample at 'Endpoint' and sample 'Blank', that is, the amount of color due to ABTS remaining after scavenging:

Color due to ABTS remaining = 'True Endpoint' – OD of sample 'Blank';

Step 3: Scavenging capacity of samples or Trolox® standard:

Scavenging = (OD of ABTS at start) – (OD of ABTS at 'True Endpoint')

Step 4: Determination of Trolox® equivalent values: calculated by proportion with reference of the scavenging activity of samples to that of Trolox® standard solution

Trolox® equivalent values = [(Scavenging at 'True Endpoint' by sample)/ (Scavenging by Trolox® standard)] x (Trolox® concentration) x dilution factor

The final concentration of Trolox® in the reaction mixture was 0.1 mmol/l. The final concentration of sample solution was depending on its dilution factor.

### **2.3 Statistical analysis**

All analyses were performed in triplicate. The chromatograms obtained were processed by CorelDRAW X4 software and the data obtained were processed by GraphPad Prism5 software. The results are expressed as mean  $\pm$  SEM (standard error of the mean). The chromatogram shown was from a single experiment, representing 3 same experiments.

## **Chapter 3 Separation of Phenolic Compounds Based on Column Chromatography**

The aim of this chapter was to compare the antioxidant activity of fractions of phenolic extracts obtained in **Section 2.2.2** using Superose 12 column with two eluent systems: 10% methanol and 30% acetonitrile (ACN) with HCl, pH = 2.0. After the eluent system of 30% ACN with HCl was confirmed to be suitable for phenolic compound separation, the phenolic extracts were fractionated using Sephadex G-25 column. The resulting fractions were subject to assaying of antioxidant activity so that the fractions with highest antioxidant activity can be further separated and purified.

### **3.1 Comparison of antioxidant capacity of phenolic compounds separated using Superose 12 with two eluent systems**

#### **3.1.1 Chromatography**

Superose 12 HR 10/30 is a prepacked column with narrow sized Superose, which is designed for high performance gel filtration of biomolecules, proteins, and peptides. Superose is a cross-linked, agarose-based medium that has enabled high flow rates at low back-pressures. The Superose 12 to be used has a bed volume of appropriately 24 ml, and it has the optimal separation range from 1 000 to 300 000 in molecular weight, based on size exclusion.

Honey has been known to contain various phenolic profiles, particularly including tannins, flavonoids, and flavones. It is preferable to run the phenolic compounds with acidic eluent as some of flavonoids and tannins can autoxidize under the effect of alkali (Bandyukova & Zemtsova, 1970).

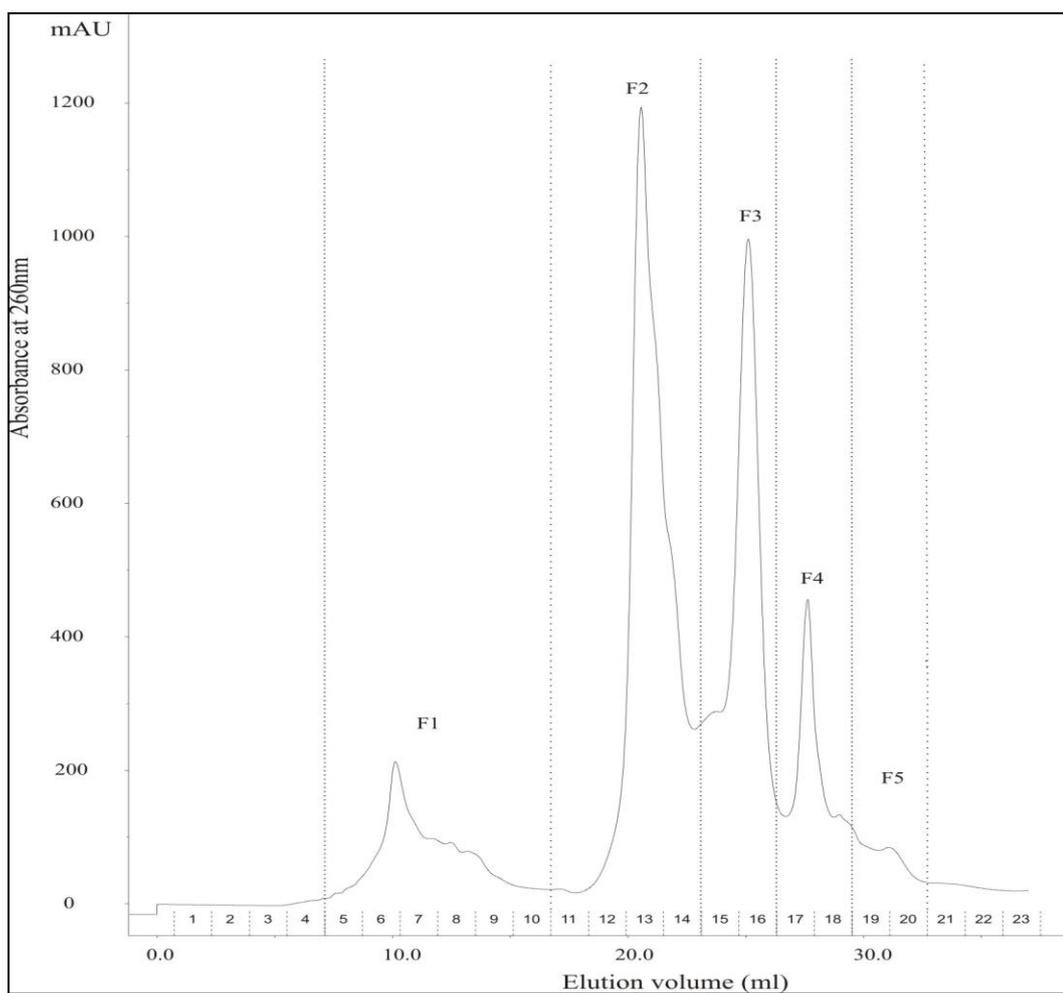
### **(A) Method**

The Superose 12 column was connected to the AKTA FPLC system and the phenolic extract samples were injected to run through the Superose 12 column with two different eluent systems: 10% methanol, and 30% ACN with HCl. The equilibration was carried out first with 5 volumes of eluent at the flow rate of 0.8 ml/min before sample injection.

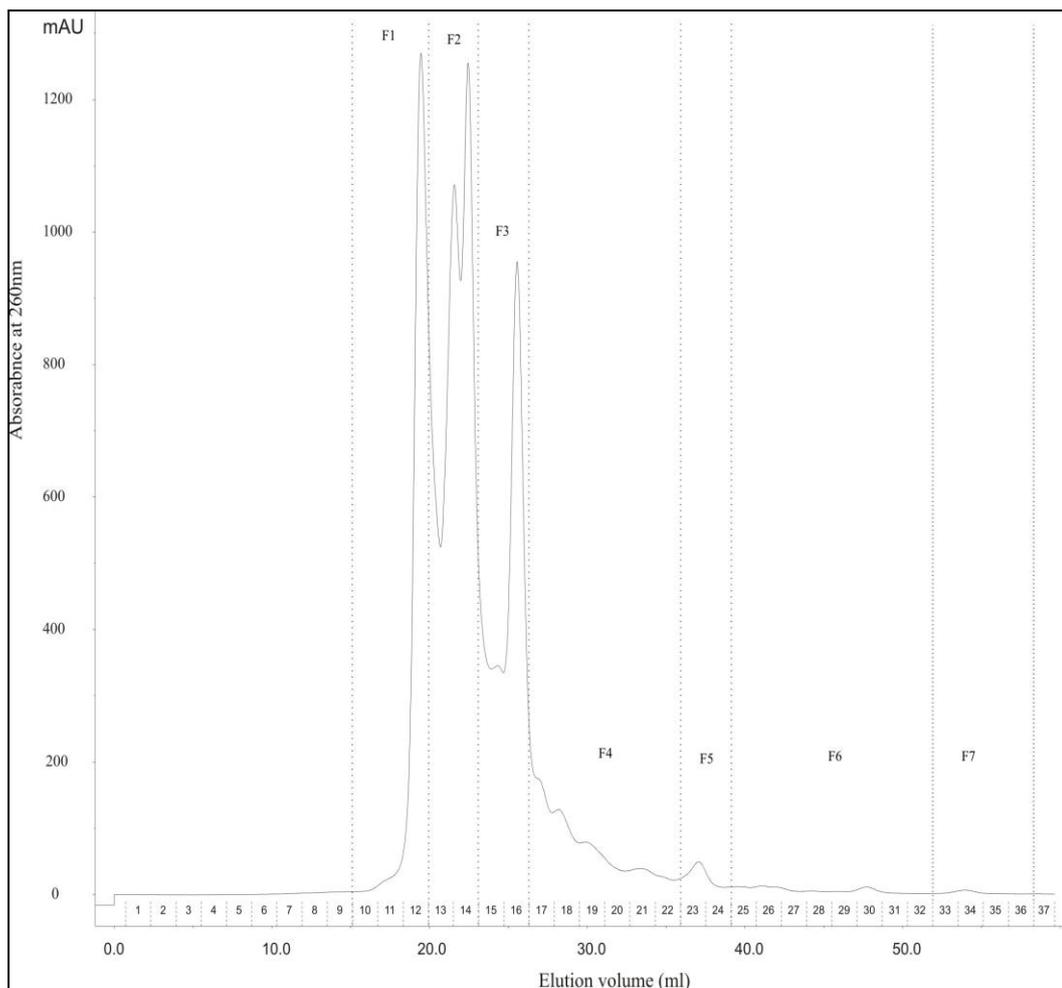
The experimental protocol was performed as below: **Sample injection:** 500  $\mu$ l diluted phenolic extract (4 mg/ml); **Eluent:** 10% methanol; 30% ACN with HCl; **Monitoring:** 260 nm; **Flow rate:** 0.8 ml/ ml; **Fraction collector:** 1.6 ml per tube.

### **(B) Results**

FPLC chromatogram of phenolic extracts of manuka honey, eluted with 10% methanol, indicated that there were several peaks obtained (**Figure 3.1**). The chromatogram of phenolic extracts eluted with 30% ACN with HCl is shown in **Figure 3.2**, indicating that several peaks were also obtained.



**Figure 3.1 Chromatogram of phenolic compounds of manuka honey on Superose 12 column eluted with 10% methanol. Tube numbers are listed on X-axis. Dotted lines mark the groups of tubes pooled for each fraction (F1—F5).**



**Figure 3.2 Chromatogram of phenolic compounds of manuka honey on Superose 12 column eluted with 30% ACN with HCl. Tube numbers are listed on X-axis. Dotted lines mark the groups of tubes pooled for each fraction (F1—F7).**

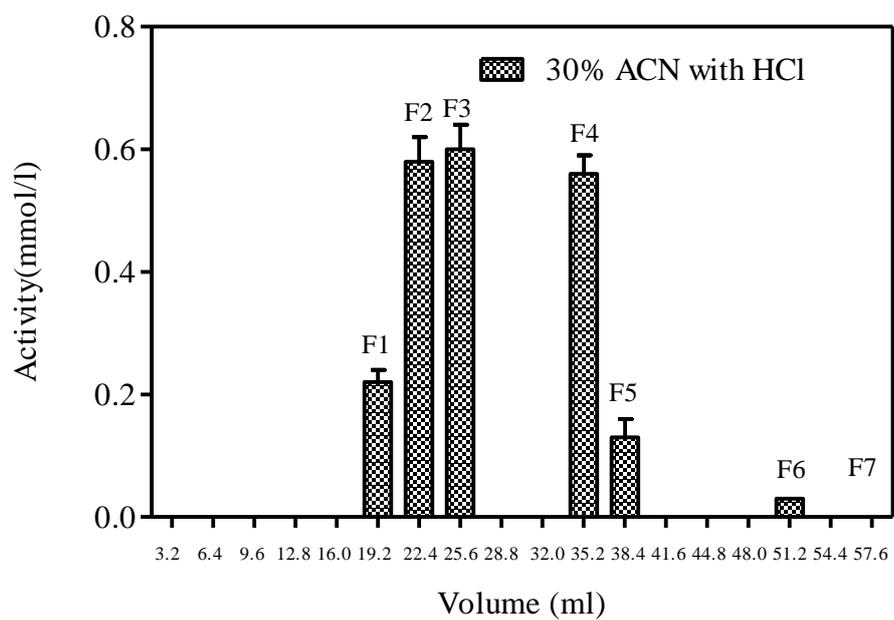
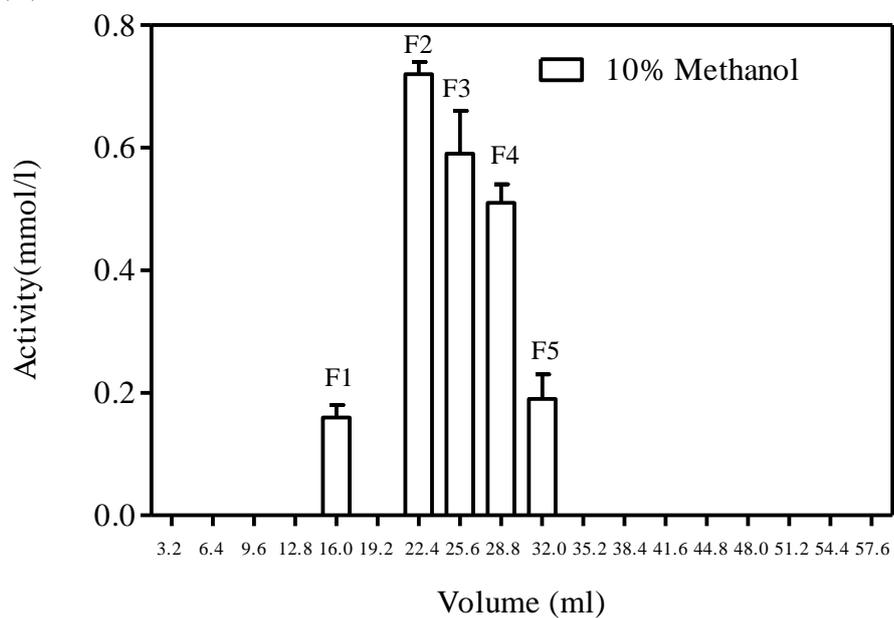
### **3.1.2 Assaying of antioxidant activity of fractions obtained from phenolic compounds**

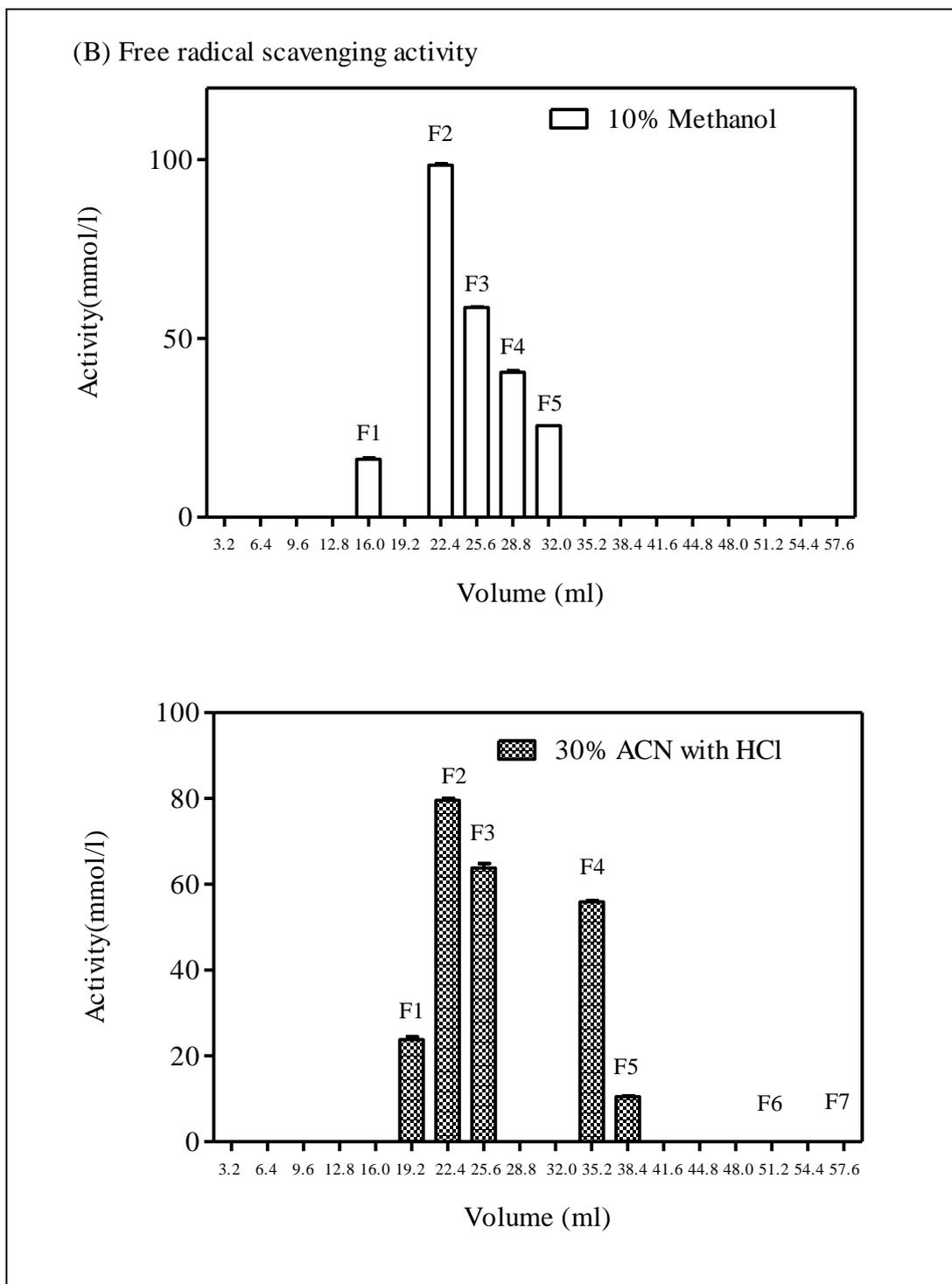
To assay the antioxidant capacities of fractions obtained from the phenolic extracts by chromatography on Superose 12, the tubes containing each fraction were pooled as shown in **Figures 3.1 and 3.2**. Due to the use of highly concentrated phenolic compounds and complexity of phenolic profiles, both the

chromatograms did not show the clear and distinct separation. However, the chromatogram with 10% methanol (**Figure 3.1**) displayed that there was 5 main fractions obtained, and the chromatography with 30% ACN with HCl (**Figure 3.2**) indicated 7 major fractions present.

The 5 fractions obtained from 10% methanol eluent system were concentrated to 0.5 ml using rotary evaporator at 40 °C. The 7 fractions obtained from 30% ACN with HCl were adjusted pH to 7.0 by adding adequate amount of 1 mol/l NaOH, followed by using rotary evaporator at 40 °C to dryness and then dissolved in 0.5 ml water. The assay of the antioxidant activity of phenolic extracts obtained off XAD column was also performed for comparison. The results for iron-binding activity based on inhibition of Fenton reaction using the  $\beta$ -carotene-linoleic model system are expressed as Ferrozine® equivalent (in mmol/l). The results for free radical scavenging activity measured on the ABTS system are expressed as Trolox® equivalent (in mmol/l). The results for both types of antioxidant activity are shown in **Figure 3.3**.

(A) Inhibition of Fenton reaction





**Figure 3.3** Antioxidant capacities of fractions of phenolic extracts obtained by chromatography on Superose 12 column. Activity in (A) represents Ferrozine® equivalent in mmol/l and activity in (B) represents Trolox® equivalent in mmol/l. Values are expressed as mean  $\pm$  SEM (n = 3).

### 3.1.3 Discussion

The phenolic extracts were obtained off XAD column by the method described in **Section 2.2.2**. The extraction was performed in triplicate. The resulting residues were weighed and did not differ more than 5% (261 mg, 258 mg, and 264 mg). The average residue of phenolic compounds of manuka honey samples was 261 mg per 100 g honey sample. The phenolic extracts were dissolved in methanol at a known concentration of 40 mg/ml.

The phenolic extracts (40 mg/ml) had 2.48 mmol/l Ferrozine® equivalent activity and 260 mmol/l Trolox® equivalent activity. The total fractions separated with 10% methanol had 2.17 mmol/l of Ferrozine® equivalent activity, compared with 30% ACN with HCl, 2.12 mmol/l, and had 239.5 mmol/l of Trolox® equivalent activity, compared with 30% ACN with HCl, 233.6 mmol/l. The total antioxidant activity, including iron-binding and free radical scavenging activity, showed acceptable recovery rates: the lowest recovery rate of 85.5% for Ferrozine® equivalent activity obtained with 30% ACN with HCl and the highest recovery rate of 92.1% for Trolox® equivalent activity obtained with 10% methanol. The recovery rate was calculated by adding the activity value of each pooled fraction as the pooled fraction assayed was concentrated to have the same volume as the sample loaded on the column.

The point of trying 30% ACN with HCl as the eluent was to see if this disaggregating solvent would decrease the size of the large (early-eluting) components seen in **Figure 3.1**, as these components could have been polyphenols bound to proteins. In **Figure 3.1**, the molecular weight of the peak at 9.6 ml in F1

was estimated at greater than 12 400 and the peak at 20.8 ml in F2 was around the range of 1 355 [the estimation of molecular weight was based on Superose 12 HR 10/30 Instructions, Pharmacia Biotech: Cytochrome C (molecular weight = 12 400) was eluted at 16 ml and Vitamin B<sub>12</sub> (molecular weight = 1 355) was eluted at 20 ml]. F1 showed some antioxidant activity and F2 showed the highest activity for both types of antioxidant property among 5 fractions. The chromatogram obtained with 30% ACN with HCl (**Figure 3.2**) showed better separation than the eluent with 10% methanol. In **Figure 3.2**, both peaks at 18.4 ml (in F1) and 21.6 ml (in F2) showed estimated molecular weight range of less than 12 400 and around 1 355. With 30% ACN with HCl system, F1 showed some antioxidant activity and F2 showed the highest activity among 7 fractions.

It was concluded that the acid eluent system did not change the total antioxidant capacity of phenolic compounds and it seemed there had been some dissociation of aggregated antioxidant. It is feasible to apply the phenolic extract to acid eluent system for further separation, and thus obtain the better resolution of peaks which this gave.

## **3.2 Separation of phenolic compounds selected using Sephadex G-25**

### **3.2.1 Chromatography**

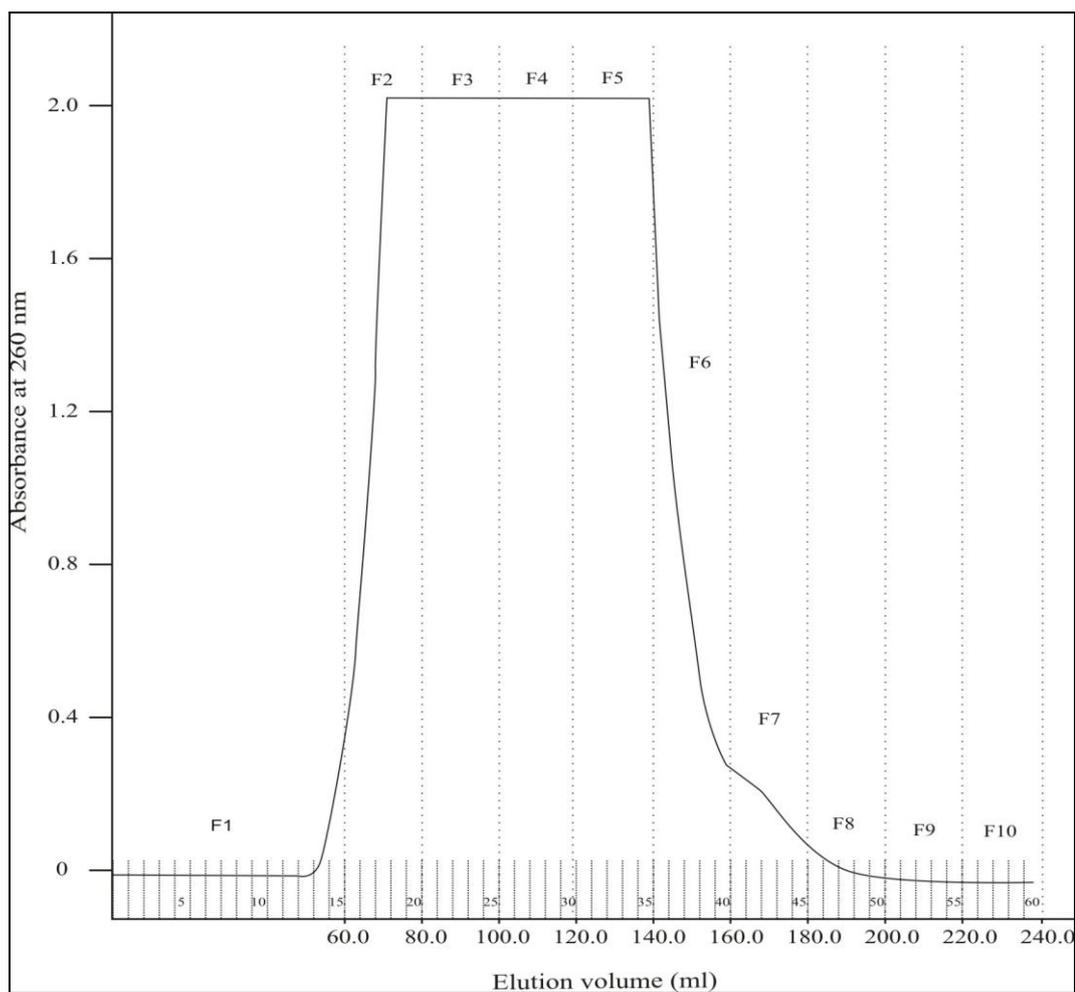
Sephadex has been found to pose a capacity of adsorbing aromatic compounds, especially phenols, and flavonoids and tannins were also found able to be adsorbed by Sephadex (Bandyukova & Zemtsova, 1970). Somers (1966) successfully used G-25 type Sephadex to isolate fractions containing tannins. At present Sephadex has been widely applied to separate various polyphenolic compounds.

#### **(A)Method**

The Sephadex G-25 column was made by filling a 3 x 24.8 cm column with Sephadex G-25 to have a bed volume of appropriately 175 ml. The column was initially washed with water and equilibrated with 400 ml 30% ACN with HCl. Then 10 ml of phenolic extract from the XAD column (**Section 2.2.2**) in 30% ACN with HCl was loaded on the column. A further 250 ml of 30% ACN with HCl was applied to run through the column at a flow rate of 1.6 ml/min. The elution was monitored spectrophotometrically at 260 nm and collected into tubes. The fraction collector was programmed to be 2.5 minutes per tube. The tubes of collected eluate were pooled into fractions, which were subject to assaying both types of antioxidant activity.

## (B) Results

Due to the high concentration of phenolic extract sample used, the elution profile from column chromatogram on Sephadex G-25 (**Figure 3.4**) was above the range of detector absorbance, so individual peaks were not seen.

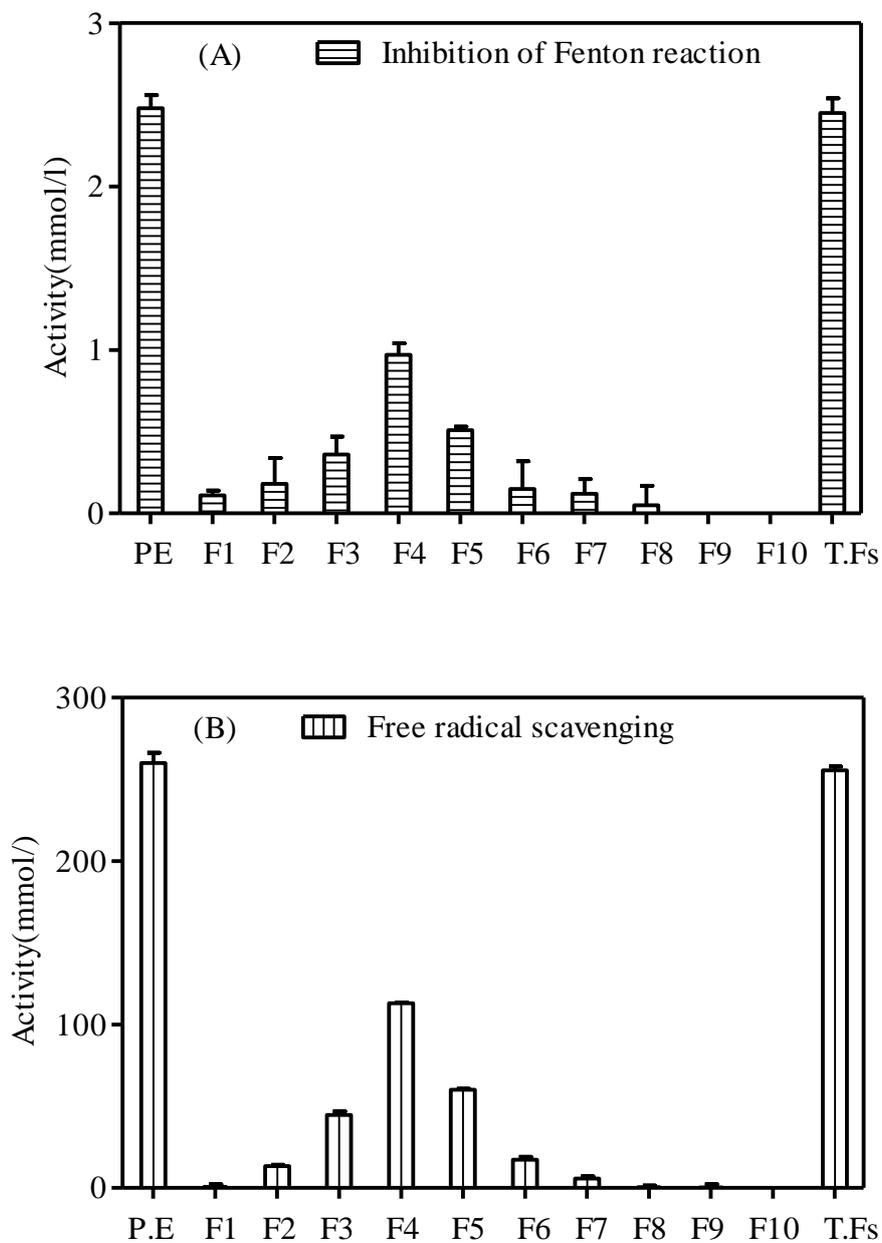


**Figure 3.4** Chromatogram of phenolic compounds of manuka honey on Sephadex G-25 column eluted with 30% ACN with HCl. Tube numbers are listed on X-axis. Dotted lines mark the groups of tubes pooled for each fraction (F1—F10).

In gel filtration through Sephadex, the solute molecules greater than the pores of Sephadex granules in dimension should not be retained by the gel and the small molecules should be penetrated into pores and retained by the gel. Consequently, large molecules or phenolic compounds bound to proteins should go through the column directly and small molecules be eluted. The first 15 tubes were pooled together as fraction1 (F1). The rest of tubes were divided evenly as every 5 tubes were pooled to form a fraction. Thus 10 fractions were obtained, among these fractions; the first fraction was 60 ml and each of the other fractions was 20 ml.

### **3.2.2 Assaying of antioxidant activity of fractions obtained from Sephadex G-25**

The pH of the fraction was adjusted to 7.0. The adjusted fractions were then dried using a rotary evaporator at 40 °C and dissolved in 10 ml deionized water. Samples of each 10 ml fraction solution were taken for both types of antioxidant activity assays. Where any sample solution was required to be diluted for assays the dilution factors were taken for activity calculation. The results for antioxidant activity are listed in **Figure 3.5**.



**Figure 3.5 Antioxidant capacities of the phenolic extracts and its fractions obtained by chromatography on Sephadex G-25 column.** Activity in (A) represents Ferrozine® equivalent in mmol/l and activity in (B) represents Trolox® equivalent in mmol/l. P.E = phenolic extracts loaded on the column; T.Fs = total activity of fractions. Values are expressed as mean  $\pm$  SEM (n = 3).

### 3.2.3 Discussion

According to the data presented in **Figure 3.5**, the total fractions separated from G-25 had 2.45 mmol/l of Ferozine® equivalent activity and 255.6 mmol/l of Trolox® equivalent activity, which was 98.3 % and 98.80 % recovery of the original activity, respectively. The both types of antioxidant activity were mainly attributed to fraction 4, which was eluted from 100 to 120 ml. This fraction 4 was then taken for sequent separation using G-25 again. The fraction 4 was subject to dryness using a rotary evaporator at 40 °C and then concentrated to 2.5 ml 30% ACN with HCl. The resulting solution was labeled F.A for convenience.

### **3.3 Re-chromatography of fraction 4 on Sephadex G-25**

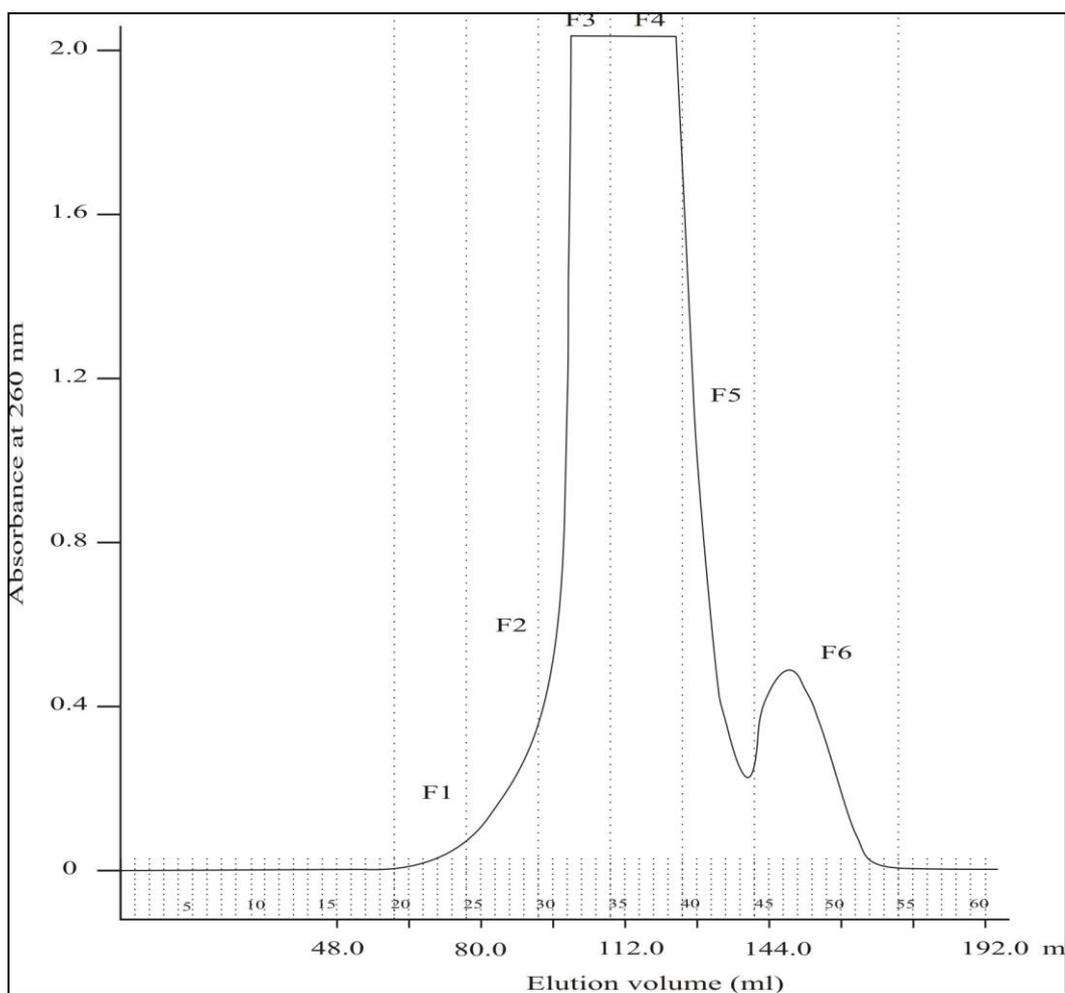
#### **3.3.1 Chromatography**

##### **(A) Method**

The fractionation of F.A using column G-25 was carried out as before (**Section 3.2.1 A**). 2.5 ml of F.A was loaded on the column. The flow rate remained at 1.6 ml/min and the elution was monitored spectrophotometrically at 260 nm. However, the fraction collector was changed to 2 minutes per tube, that is, each tube contained 3.2 ml of eluate. This was so as to have eluted peaks in more tubes, which was able to help divide fraction accurately.

##### **(B) Results**

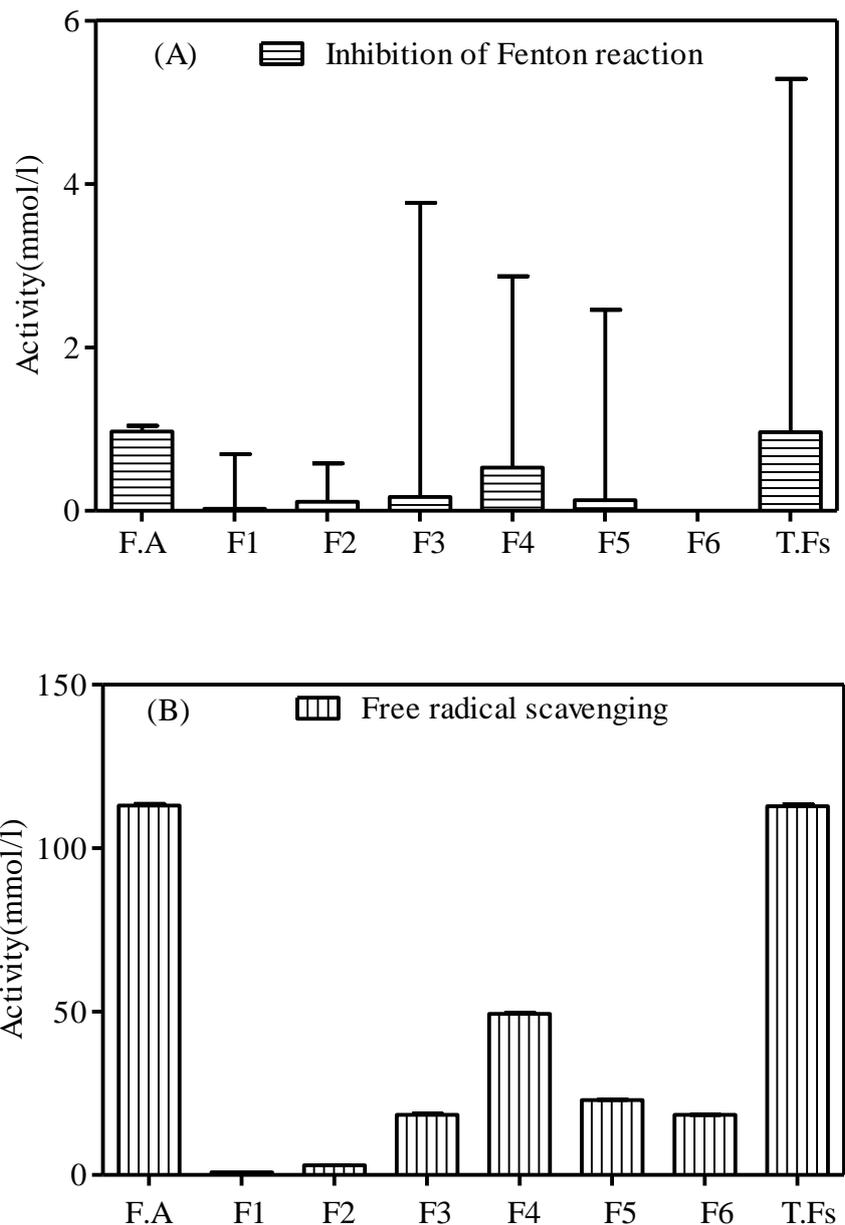
The elution profile from Sephadex G-25 column chromatography is shown in **Figure 3.6**. Due to the high concentration of the material in F.A loaded on the column, the absorbance reading was over the detector range. The tubes collected were pooled into 6 fractions as shown in **Figure 3.6**.



**Figure 3.6 Chromatogram of F.A of phenolic compounds on Sephadex G-25 column eluted with 30% ACN with HCl. Tube numbers are listed on X-axis. Dotted lines mark the groups of tubes pooled for each fraction (F1—F6).**

### **3.3.2 Assaying of antioxidant activity of fractions obtained from F.A**

All of the fractions obtained by pooling were adjusted pH to 7.0 with NaOH first, then dried using a rotary evaporator at 40 °C, and dissolved in 5 ml water. Both types of antioxidant activity were assayed and the results are shown in **Figure 3.7**.



**Figure 3.7 Antioxidant capacities of F.A of phenolic compounds obtained by chromatography on Sephadex G-25 column.** Activity in (A) represents Ferrozine® equivalent in mmol/l and activity in (B) represents Trolox® equivalent in mmol/l. T.Fs = total activity of fractions. Values are expressed as mean  $\pm$  SEM (n = 3).

### **3.3.3 Discussion**

The total 6 fractions separated from G-25 showed 99.5% recovery rate for Ferrozine® equivalent activity and 99.8% recovery rate for Trolox® equivalent activity. However, as can be seen, there was large SEM obtained in the iron-binding assay, this high recovery rate of Ferrozine® equivalent activity might not be very accurate, but the main Trolox® equivalent activity of the F.A was recovered.

Comparing the antioxidant activity of the 6 fractions obtained, the main antioxidant activity was greatly attributed to fraction 4 (tubes 35 to 39), which was eluted from 108.8 to 124.8 ml. The fraction 4 showed 54.9% recovery rate for Ferrozine® equivalent activity and 43.7% recovery rate for Trolox® equivalent.

Fraction 4 was then taken for re-chromatography on Sephadex G-25 column again. As the phenolic compounds are relatively small molecules, compared to other main substances in honey, it would be also valuable to take fraction 5 (tubes 40 to 44) for further investigation.

## **3.4 Repeated re-chromatography of fraction 4 and 5 on Sephadex G-25**

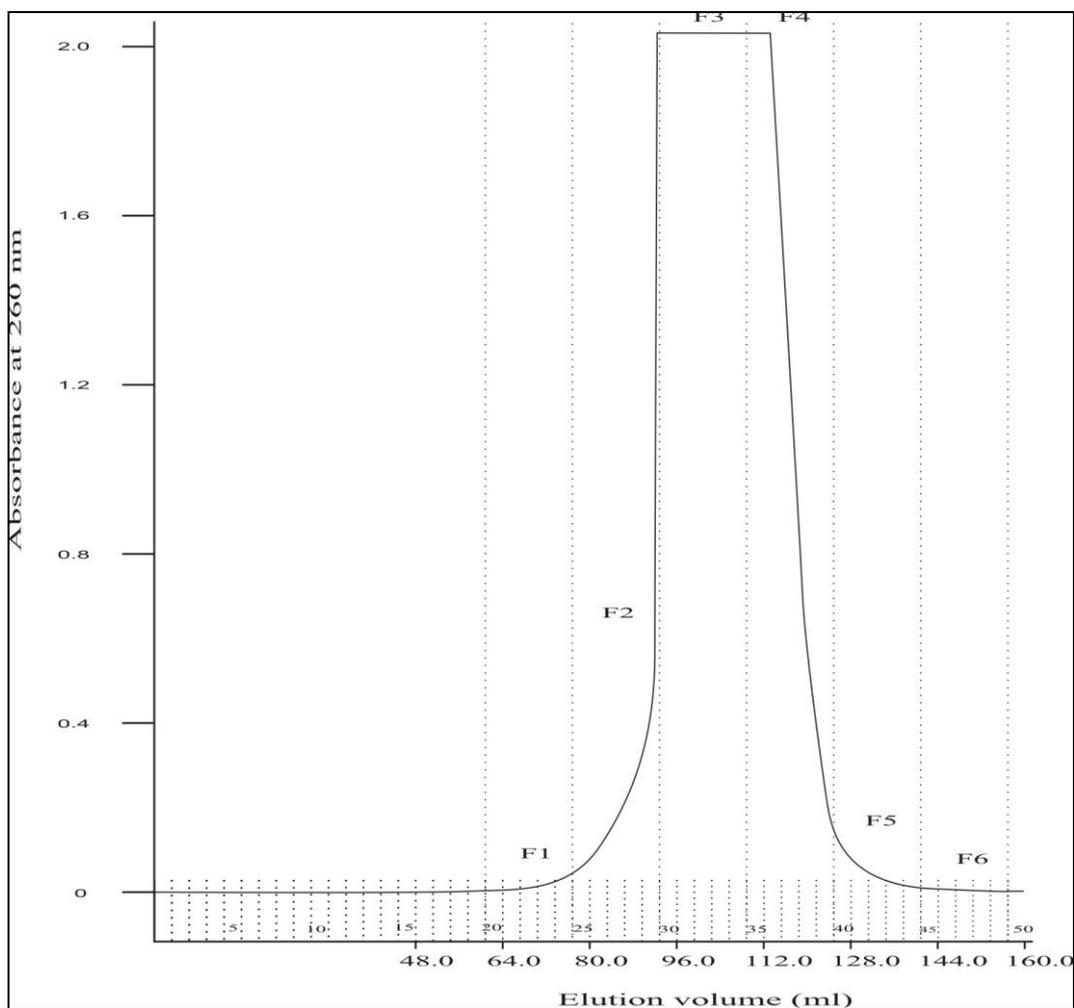
### **3.4.1 Chromatography**

#### **(A) Method**

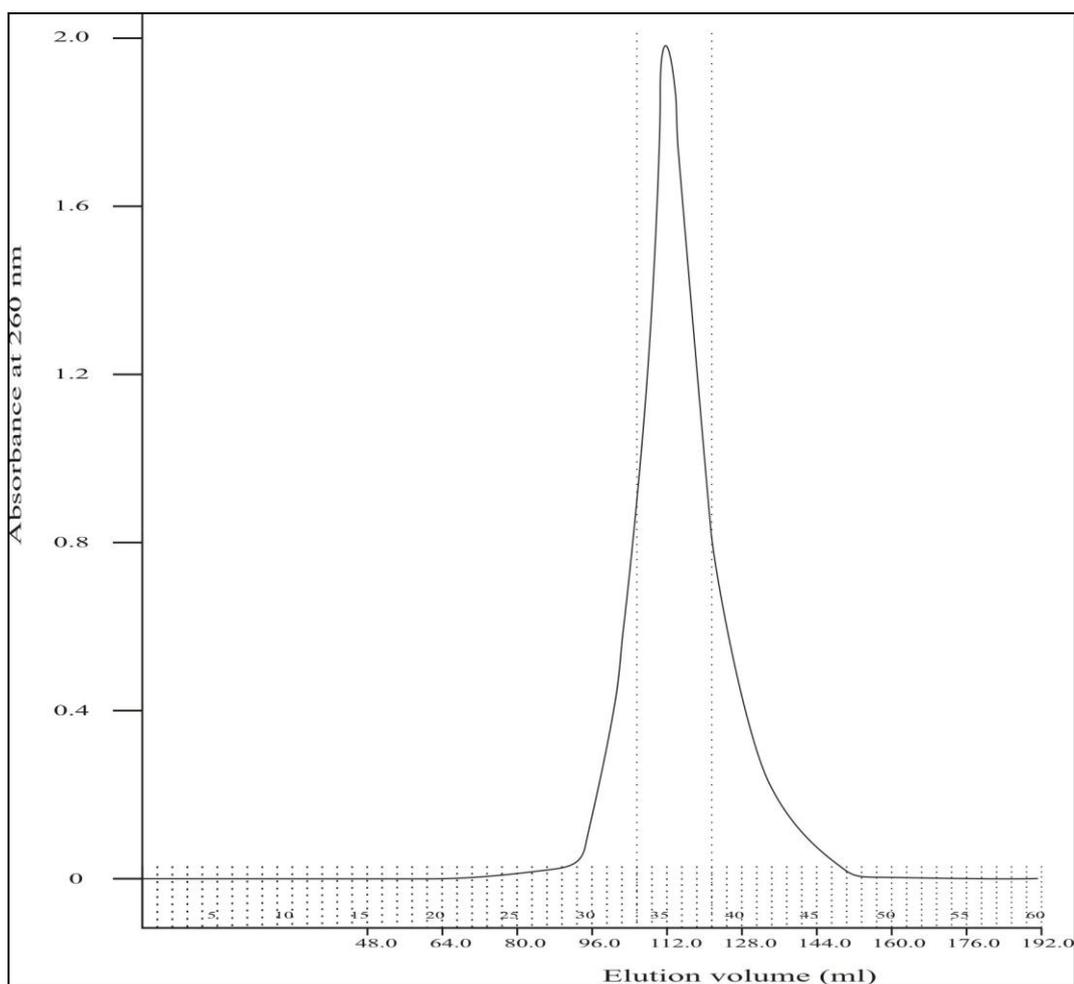
Both fractions 4 and 5 were dried using a rotary evaporator at 40°C and then dissolved in 2.5 ml of 30% ACN with HCl. The resulting solution from fraction 4 was labeled F.B4 and the resulting solution from fraction 5 was labeled F.B5 for convenience. The chromatography of F.B4 and F.B5 was carried out using Sephadex G-25 as previously (**Section 3.3.1 A**). 2.5 ml of the sample was loaded on the column at a flow rate of 1.6 ml/min, and the elution was monitored spectrophotometrically at 260 nm. The elution was collected as 3.2 ml per tube and the tubes of eluate were pooled into fractions.

#### **(B) Results**

Due to the high concentration of the material in F.B4, the elution profile from column chromatography on Sephadex G-25 was above the detector range of absorbance, so individual peaks were not seen. The fractions obtained were pooled into 6 fractions as shown in **Figure 3.8**.



**Figure 3.8 Chromatogram of F.B4 of phenolic compounds on Sephadex G-25 column eluted with 30% ACN with HCl. Tube numbers are listed on X-axis. Dotted lines mark the groups of tubes for each fraction (F1—F6).**

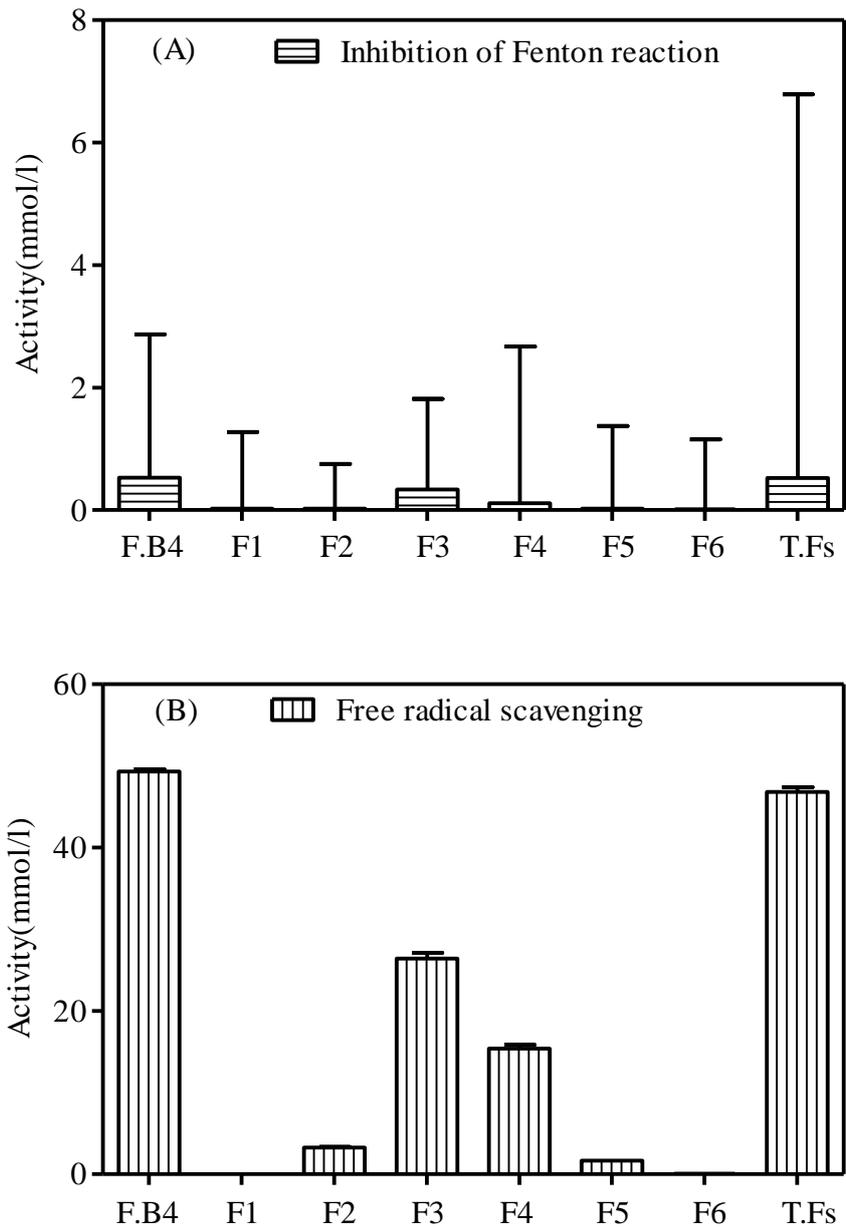


**Figure 3.9 Chromatogram of F.B5 of phenolic compounds on Sephadex G-25 column eluted with 30% ACN with HCl. Tube numbers are listed on X-axis. Dotted lines mark the fraction to be taken for further investigation.**

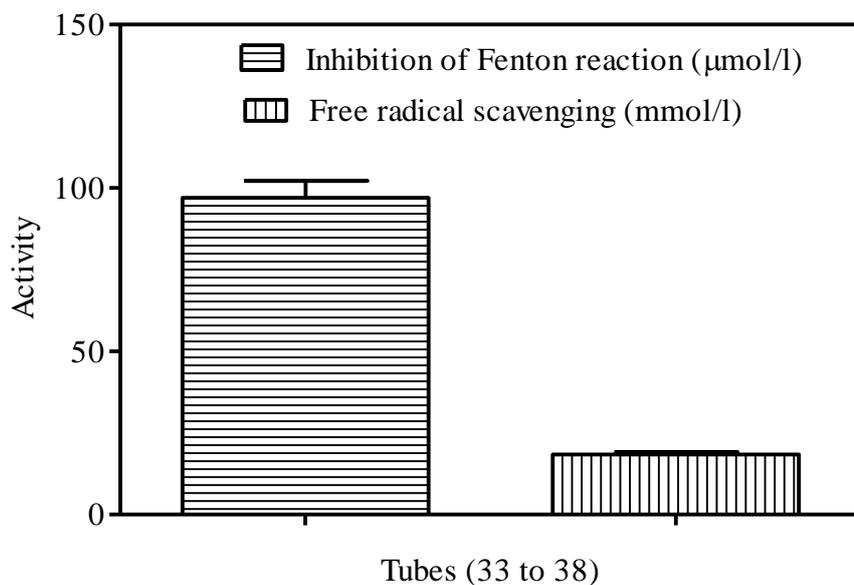
The elution profile of F.B5 from column chromatography on Sephadex G-25 (**Figure 3.9**) showed a major peak obtained. As fractions with higher absorbance had been found to have higher antioxidant activity in the preceding chromatograms, the tubes from 34 to 38 were taken for assaying of antioxidant activity and for further investigation using Superdex Peptide.

### **3.4.2 Assaying of antioxidant activity of fractions obtained from F.B4 and F.B5**

To assay the antioxidant activity, the fractions pooled from F.B4 were adjusted pH to 7.0 first, dried using a rotary evaporator at 40 °C, and then dissolved in 2.5 ml water. The fraction obtained from pooling tubes 34 to 38 of F.B5 was evaporated and re-dissolved in 2.5 ml water after pH adjustment to 7.0. The results obtained from F.B4 are shown in **Figure 3.10** and the results obtained from F.B5 are shown in **Figure 3.11** .



**Figure 3.10 Antioxidant capacities of fractions from F.B4 obtained by chromatography on Sephadex G-25 column.** Activity in (A) represents Ferrozine® equivalent in mmol/l and activity in (B) represents Trolox® equivalent in mmol/l. T.Fs = total activity of fractions. Values are expressed as mean  $\pm$  SEM (n = 3).



**Figure 3.11 Antioxidant capacity of the fraction (tubes 34–38) from F.B5 obtained by chromatography on Sephadex G-25 column.** Activity for inhibition of the Fenton reaction represents Ferrozine® equivalent in µmol/l and for free radical scavenging activity represents Trolox® equivalent in mmol/l. Values are expressed as mean ± SEM (n = 3).

### 3.4.3 Discussion

The 6 fractions obtained from F.B4 had the total of 99.2% recovery rate for Ferrozine® equivalent activity and 94.9% recovery rate for Trolox® equivalent activity. However, there was large SEMs obtained in assay for iron-binding type of activity of 6 fractions, the total recovery rate might not be accurate.

Comparing the activity of the fractions obtained from F.B4, both types of antioxidant activity were mainly retained in fraction 3 (tubes 30 to 34) and 4 (tubes 35 to 39) from F.B4, which were eluted from 92.8 to 124.8 ml. In order to

find out the phenolic compound responsible for highest antioxidant activity including the iron-binding and free radical scavenging activity, fraction 3 and 4 from F.B4 were taken for sequent separation using G-25 sephadex again.

The fraction pooled from tubes 34 to 38 of F.B5 showed the 74.6% recovery rate F.B5 for Ferrozine® equivalent activity and 80.3% for Trolox® equivalent activity.

## **3.5 Chromatography of selected fractions on Sephadex G-25**

**(fourth)**

### **3.5.1 Chromatography**

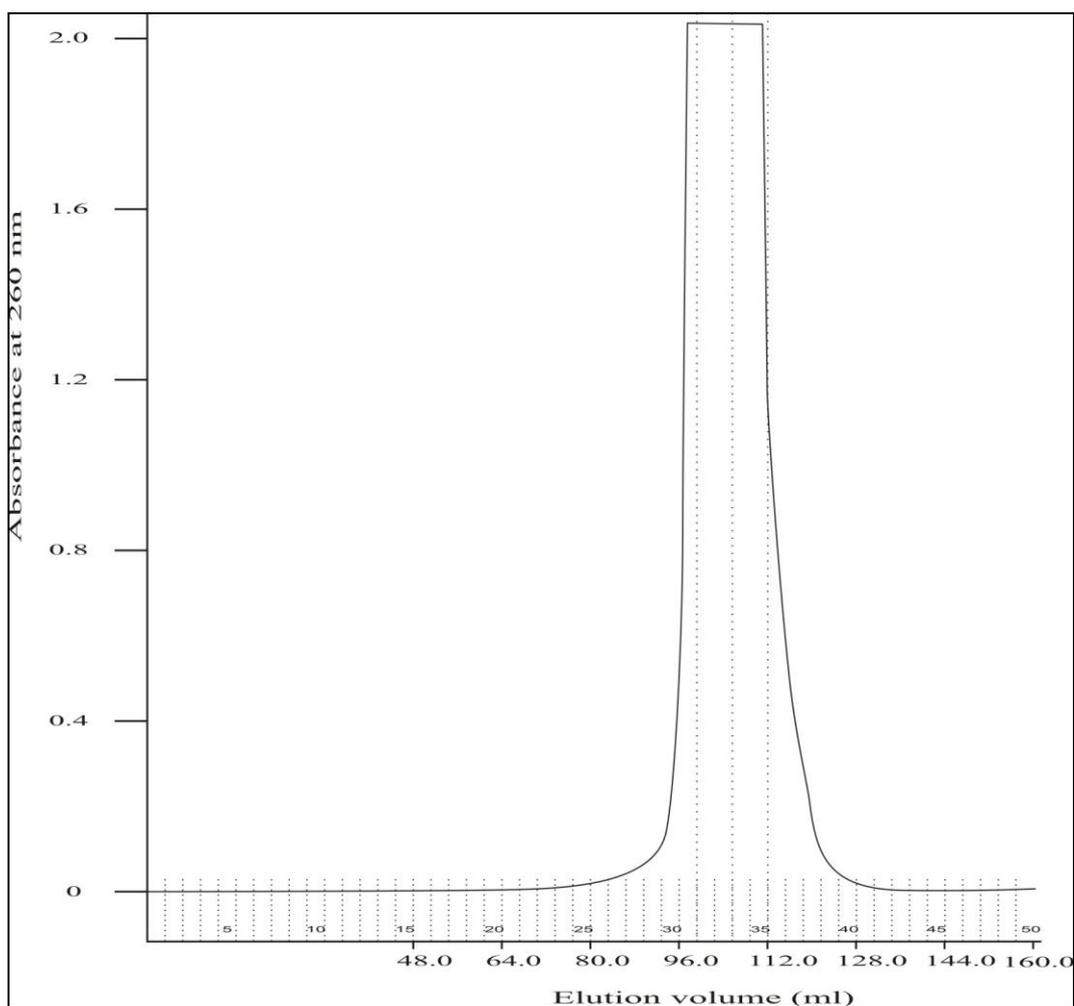
#### **(A) Method**

Each of fractions 3 and 4 from F.B4 was dried using a rotary evaporator at 40°C and then dissolved in 2.5 ml of 30% ACN with HCl. The resulting solution from fraction 3 was labeled F.C and the resulting solution obtained from fraction 4 was labeled F.D for convenience. The combined solution from F.B5 was similarly dried and re-dissolved in 2.5 ml 30% ACN with HCl. This resulting solution was labeled as Pool 5 and was stored frozen in the dark for future separation with Superdex Peptide.

The re-chromatography of separation of F.C and F.D using Sephadex G-25 column was performed as before (**Section 3.3.1 A**). The flow rate remained at 1.6 ml/min and the elution was monitored spectrophotometrically at 260 nm. The elution was collected as 3.2 ml per tube.

#### **(B) Results**

A total 50 tubes was obtained for F.C and F.D each. The chromatography results are shown in **Figure 3.12** for F.C and **Figure 3.13** for F.D.

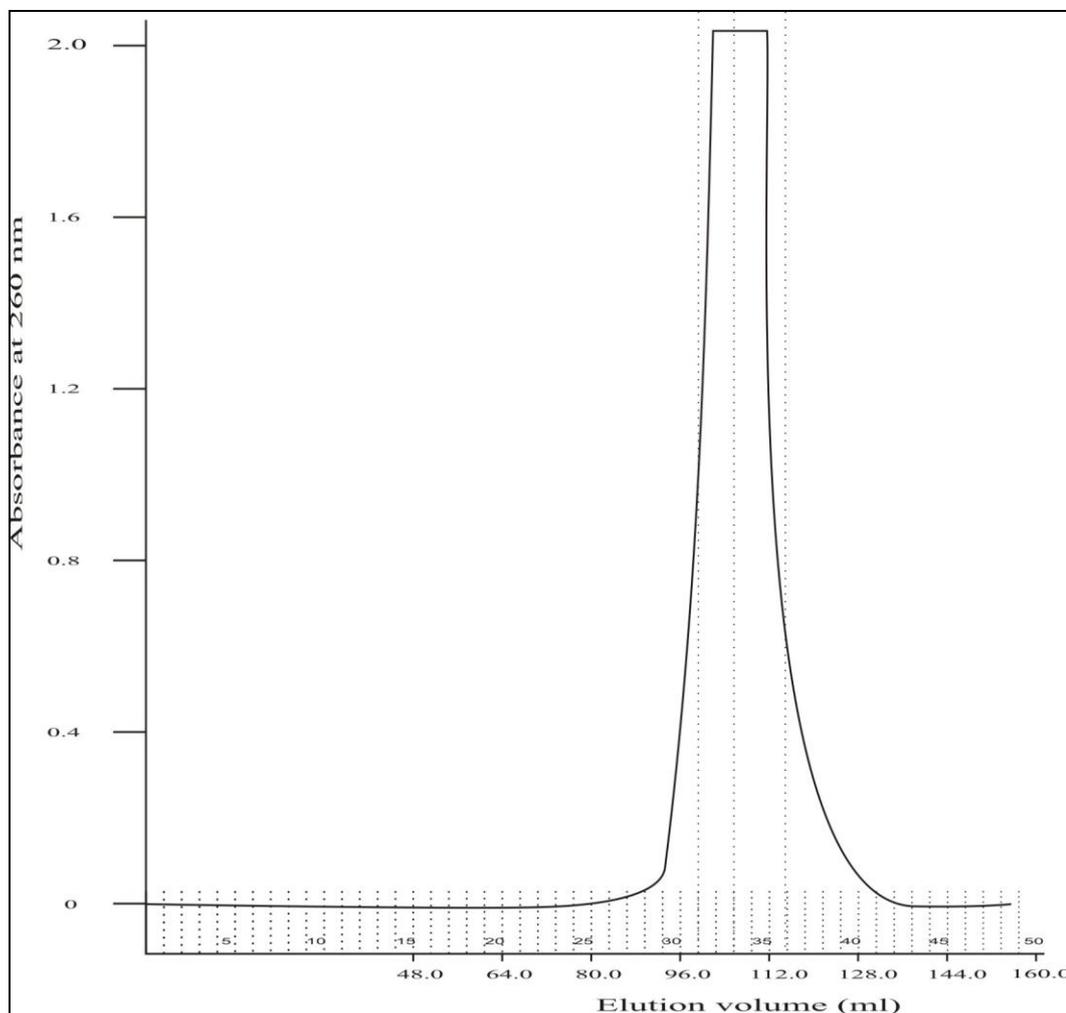


**Figure 3.12 Chromatogram of F.C obtained with Sephadex G-25 column.**

Tube numbers are listed on X-axis. Dotted lines mark the groups of fractions to be taken for further investigation.

Due to the high concentration of F.C, the absorbance reading was over the detector range. The F.C showed highest absorbance range from tubes 32 to 35, which were eluted from 99.2 to 112 ml. This volume range was matching the main antioxidant activity retained in elution from about 100 to 120 ml in the preceding chromatograms. The tubes 32 and 33 were pooled together for assaying

of antioxidant activity as well as for further separation, similarly, the tubes 34 and 35.



**Figure 3.13 Chromatogram of F.D obtained with Sephadex G-25 column.**

Tube numbers are listed on X-axis. Dotted lines mark the groups of fractions to be taken for further investigation.

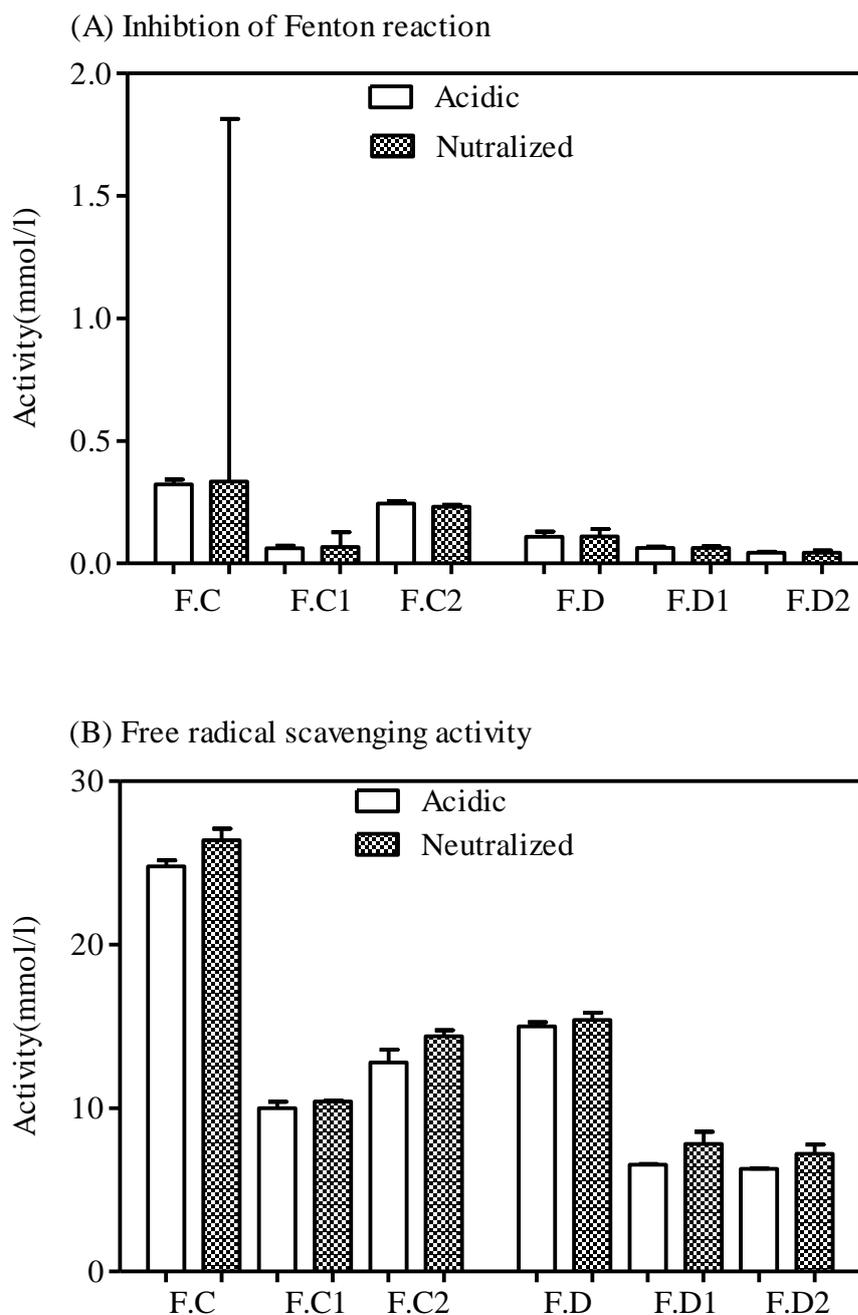
Due to the high concentration of F.D, the absorbance reading was over the detector range. The F.D showed highest absorbance range from tubes 32 to 36, which were eluted from 99.2 to 115.2 ml. This volume range was matching the main antioxidant activity retained in elution from about 100 to 120 ml in the

preceding chromatograms. The tubes 32 and 33 were pooled together; similarly, the tubes 34, 35 and 36 were pooled together for activity assaying and further separation.

### **3.5.2 Assaying of antioxidant activity of fractions obtained from F.C and F.D**

Both the pools made from tubes 32 and 33 of F.C and from tubes 34 and 35 of F.C were 6.4 ml. The pool from tubes 32 and 33 of F.D was 6.4 ml, and the pool made from tubes 34, 35, and 36 of F.D was 9.6 ml. Because their volumes were relatively small, it became difficult to adjust pH for assaying of antioxidant activity, and these pools were present in acid form, the antioxidant function could be affected by the state of ionization of phenolic and carboxylic groups.

Thus, it was necessary to check if the low pH value altered the antioxidant activity. The way to do this would be pooling small samples of the various fraction pools, and diluting them as much as the activity can be still measured. Besides, a sample for assaying activity in the acidic form was also prepared for comparison before neutralization. In this way, a large enough volume can be obtained for pH adjustment to 7.0. The results of the antioxidant activity of the combined solutions in acid form and pH 7.0 are showed in **Figure 3.14**.



**Figure 3.14 Antioxidant capacities of the fractions obtained from F.C and F.D in acid and neutralized form.** F.C1= tubes 32 and 33 of F.C; F.C2 = tubes 33 and 34 of F.C; F.D1 = tubes 32 and 33 of F.D and F.D2 = tubes of 34, 35 and 36. Activity in (A) represents Ferrozine® equivalent in mmol/l; Activity in (B) represents Trolox® equivalent in mmol/l. Values are expressed as mean  $\pm$  SEM (n = 3).

### **3.5.3 Discussion**

It seemed that the low pH value did not alter the antioxidant activity of fractions selected. Since the fractions to be taken were in small volumes in sequent experiments, the assaying of antioxidant activity was performed in acid form without pH adjustment.

The 4 pools obtained from F.C and F.D showed significant iron-binding and free radical scavenging types of antioxidant activity. Comparing the antioxidant activity of F.C, the tubes 34 and 35 in 105.6 – 112 ml had the highest activity; similarly, the tubes 34, 35, 36 of F.D in 105.6 – 115.2 ml had the highest activity, indicating that the elution from 105.6 to 115.2 ml had a lot of antioxidant activity. The next step was necessary to establish a method that was to separate the compounds responsible for each type of antioxidant activity.

## 3.6 Separation of phenolic compounds using Superdex Peptide

### 3.6.1 Chromatography

Superdex Peptide HR 10/30 is a pre-packed column for size exclusion chromatography of peptides and other small biomolecules. It is made of the covalent bonding of dextran to highly cross-linked porous agarose beads. It has a separation range of 100 to 7 000 in molecular weight. The column has an internal diameter of 10 mm and about 30 cm in length, which gives a bed volume of appropriately 24 ml.

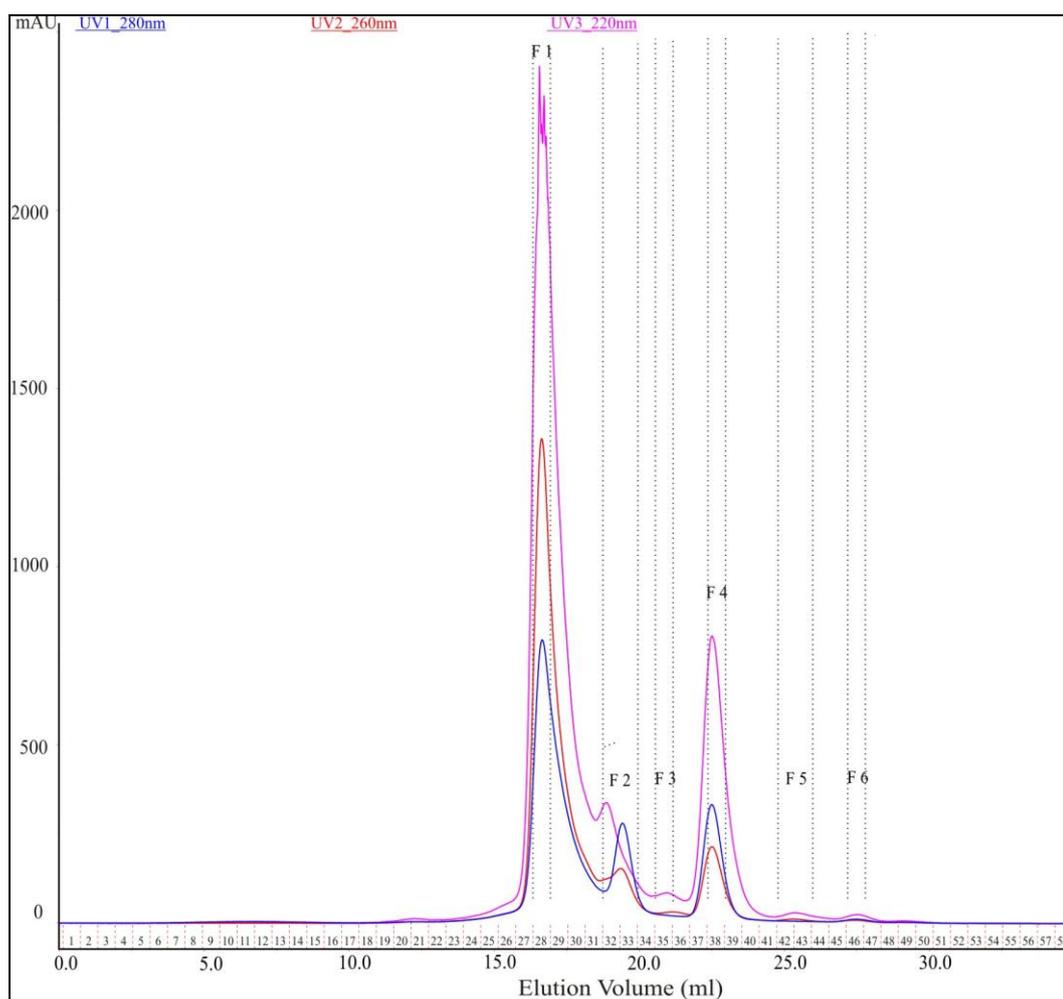
#### (A) Method

The 4 pools obtained from F.C and F.D in **Section 3.5.1** were concentrated to 1/10 of their original volume using a rotary evaporator at 40 °C to dryness then re-dissolved in 30% ACN with HCl. The resulting pool from tubes 32, 33 of F.C was labeled as Pool 1, and the pool from tubes 34, 35 of F.C was labeled as Pool 2. The pool from tubes 32, 33 of F.D was labeled as Pool 3, and the pool from tubes 34, 35, 36 was labeled as Pool 4. The pool from F.B5 obtained in **Section 3.4.1** was labeled as Pool 5. These five pools were subject to running through Superdex Peptide HR 10/30.

First, the column connected to the AKTA FPLC system was equilibrated with 30% ACN with HCl for 5 column volumes of solvent at a flow rate of 0.6 ml/min. The experiment protocol was then set up as below: **Sample injection:** 100 µl; **Eluent:** 30% ACN with HCl; **Spectrometer:** 220, 260, and 280 nm simultaneously; **Flow rate:** 0.6 ml/ ml; **Fraction collector:** 0.6 ml per tube.

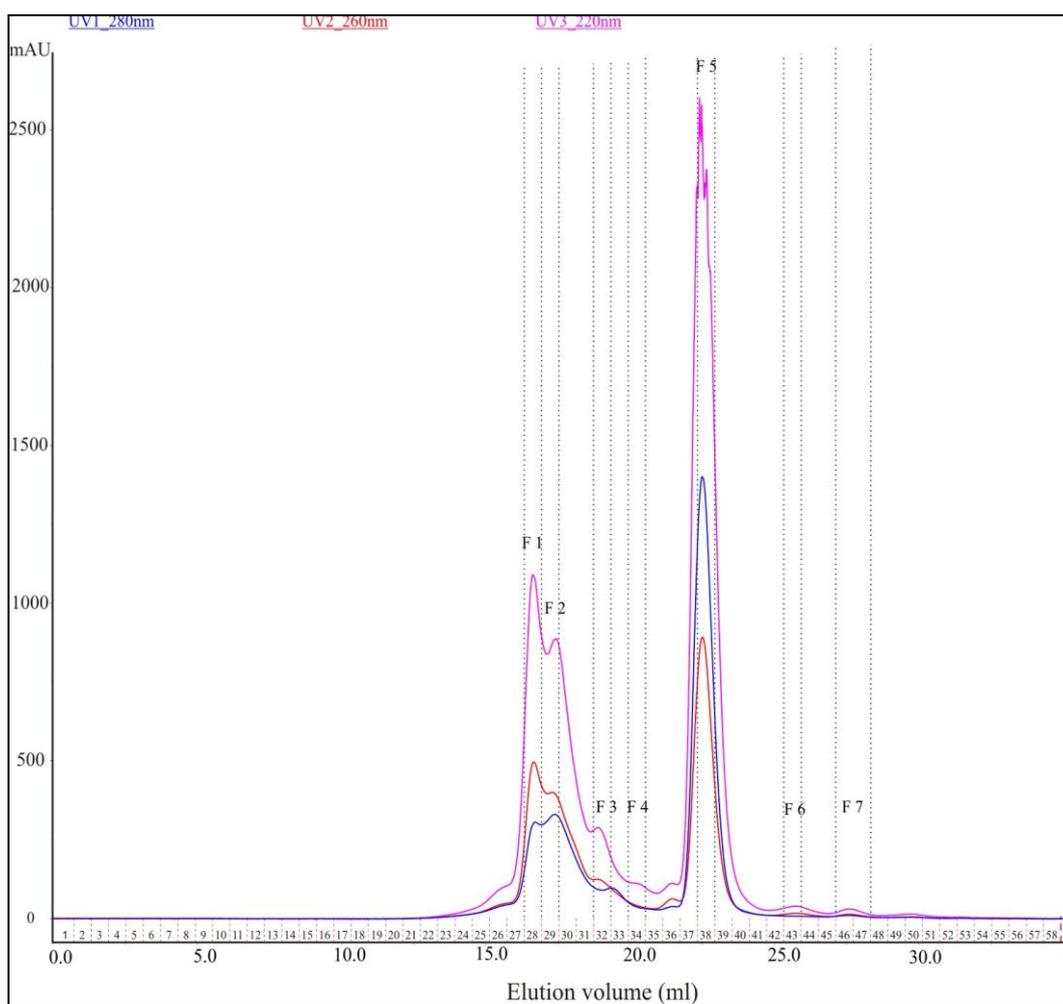
## (B) Results

The results from FPLC chromatogram for each pool on Superdex Peptide are shown in **Figures 3.15 – 3.19**. For each pool, there clearly were several peaks obtained, showing that successful separation was achieved.



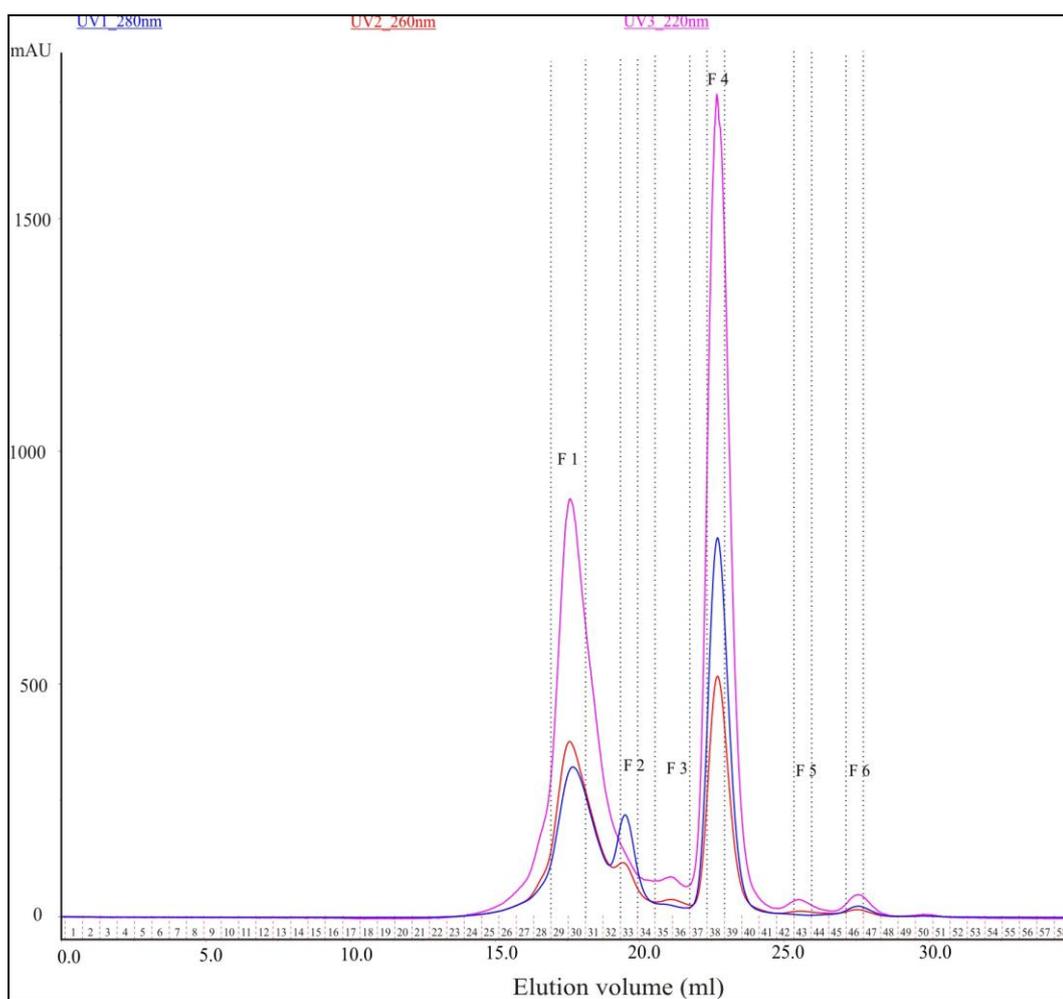
**Figure 3.15 FPLC chromatogram of Pool 1 on Superdex Peptide column.**

Tube numbers are listed on X-axis. Dotted lines mark the tubes selected for each fraction (F1—F6).

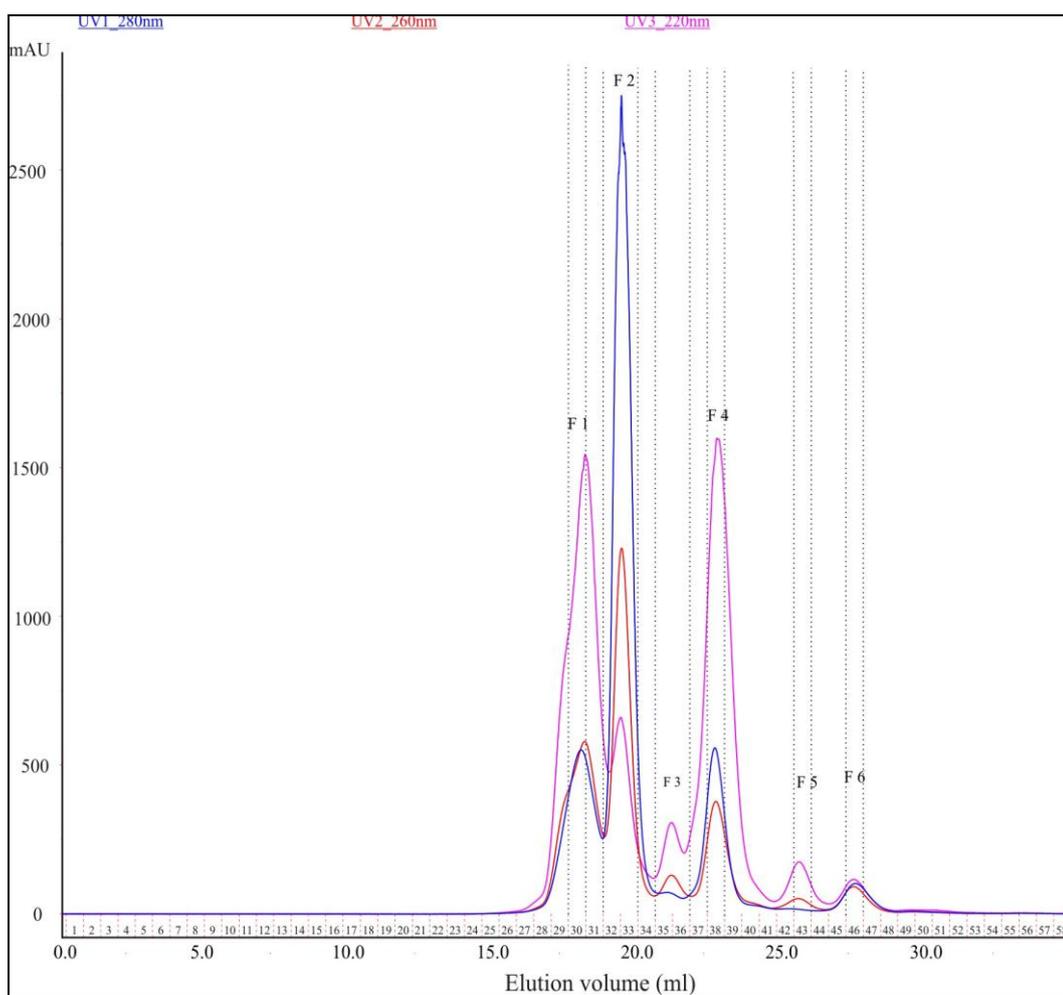


**Figure 3.16 FPLC chromatogram of Pool 2 on Superdex Peptide column.**

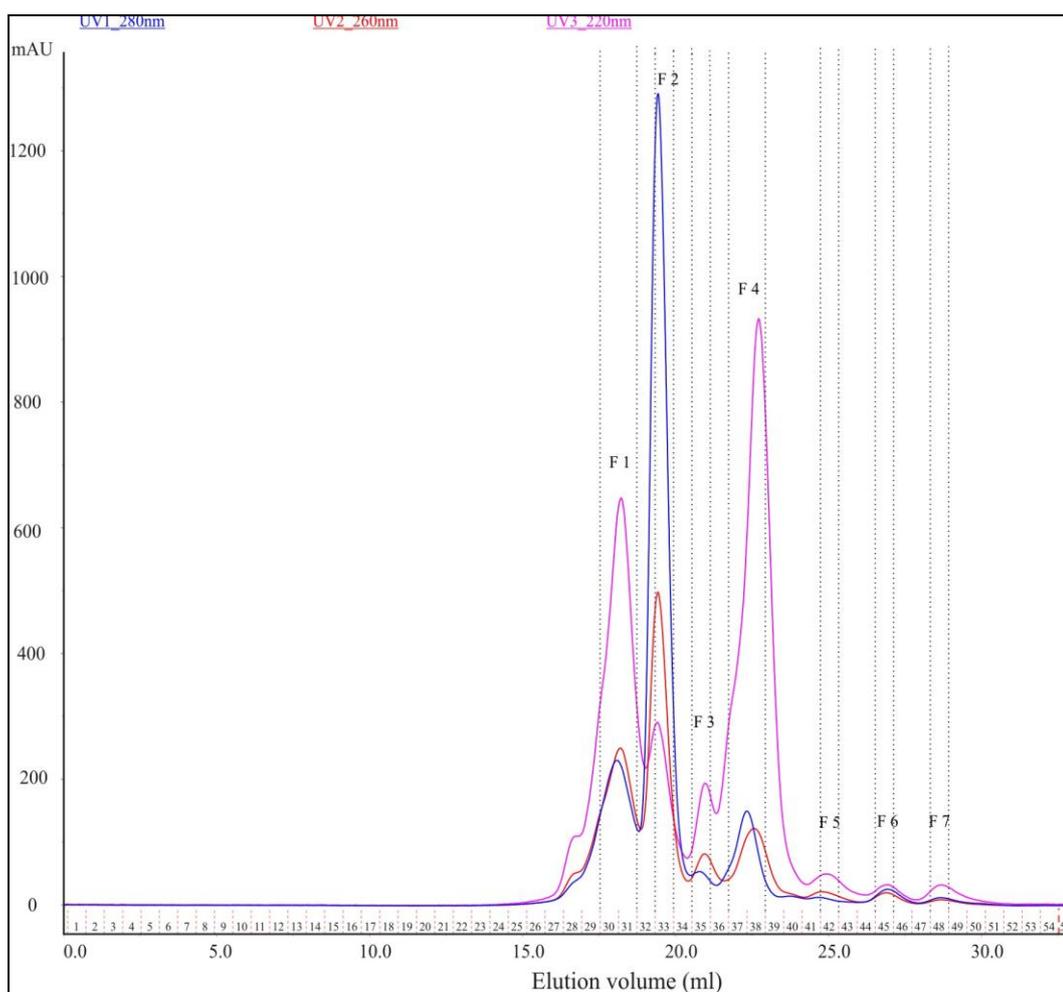
Tube numbers are listed on X-axis. Dotted lines mark the tubes selected for each fraction (F1—F7).



**Figure 3.17 FPLC chromatogram of Pool 3 on Superdex Peptide column.**  
 Tube numbers are listed on X-axis. Dotted lines mark the tubes selected for each fraction (F1—F6).



**Figure 3.18 FPLC chromatogram of Pool 4 on Superdex Peptide column.** Tube numbers are listed on X-axis. Dotted lines mark the tubes selected for each fraction (F1—F6).



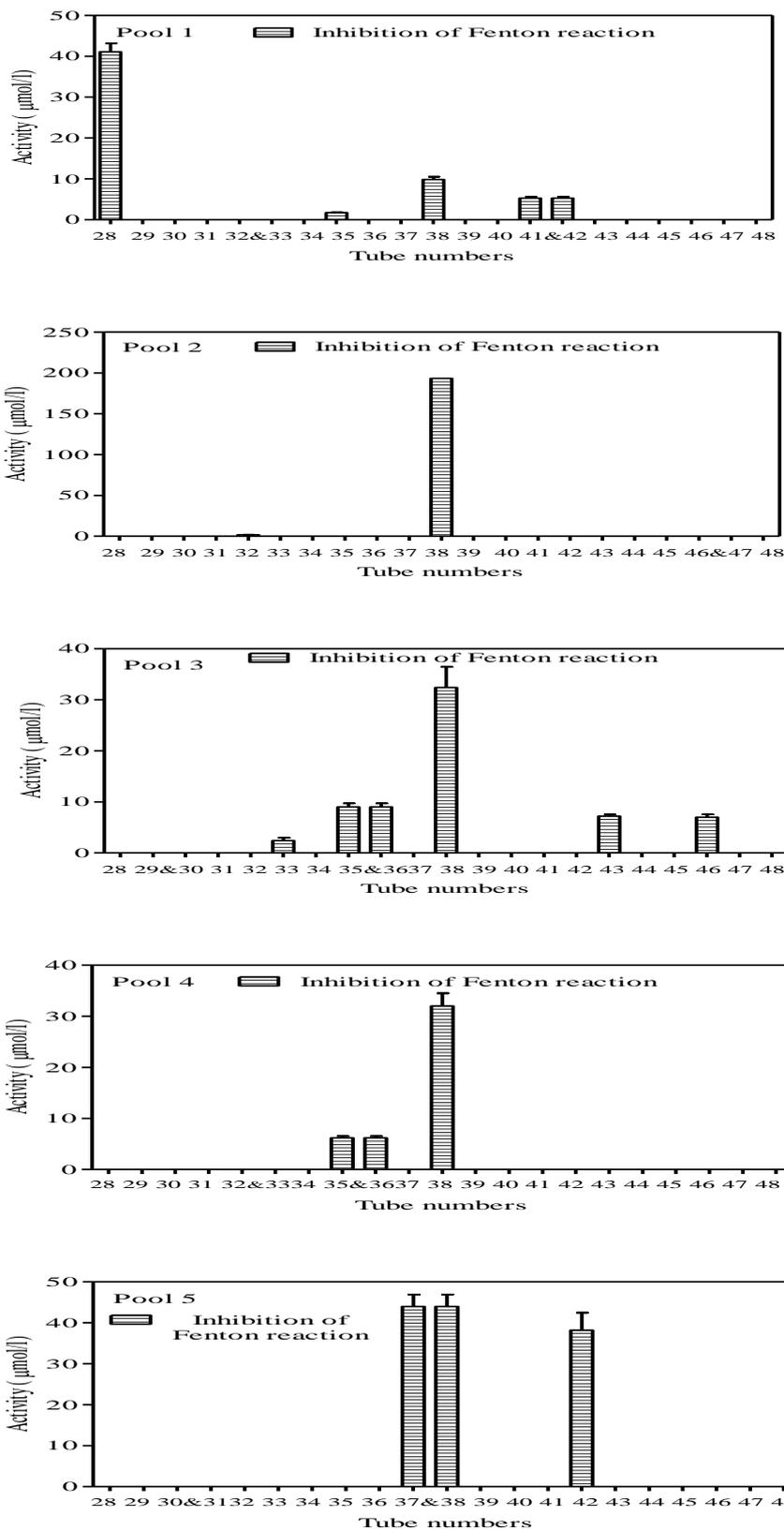
**Figure 3.19 FPLC chromatogram of Pool 5 on Superdex Peptide column.**

Tube numbers are listed on X-axis. Dotted lines mark the tubes selected for each fraction (F1—F7).

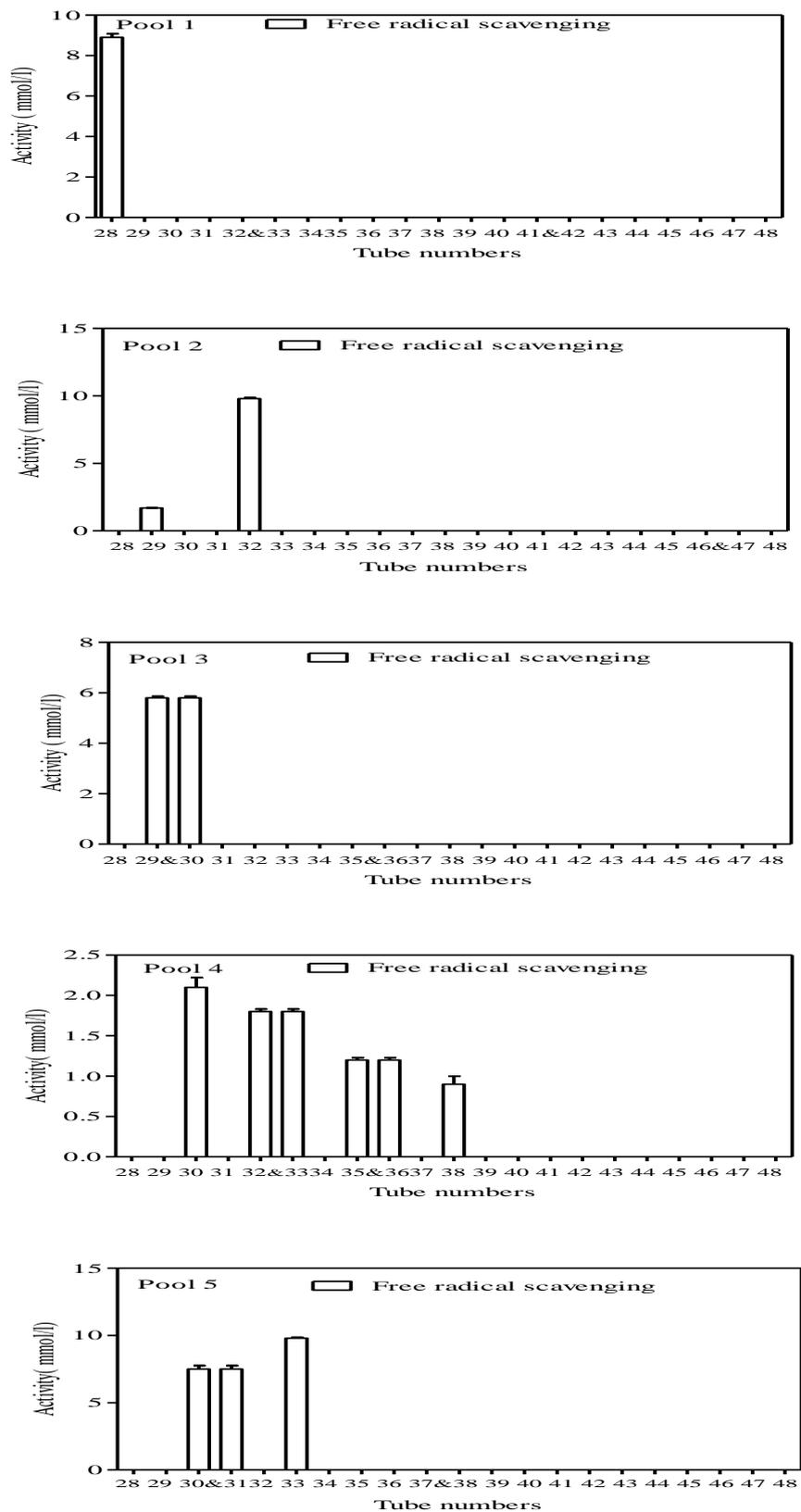
### **3.6.2 Assaying of antioxidant activity of fractions of the five pools obtained from chromatography on Superdex Peptide**

Each peak obtained from chromatography of all five pools was taken for activity assay. The tube corresponded to the highest absorbance in each peak was taken, as shown in **Figures 3.15 – 3.19**. Where any elution of central peak spanned the two tubes, both tubes were pooled together.

The contents of the tubes taken from the peaks from the 5 pools were concentrated to 0.5 ml and assayed directly without pH adjustment. The results of the antioxidant activity of fractions of the 5 pools are expressed in **Figures 3.20 and 3.21**. Ferrozine® equivalent activity for all pools was expressed in  $\mu\text{mol/ml}$  due to the iron-binding activity getting smaller and smaller. Tube numbers are used in figures in order to compare the activity of each pool easily. The activity of the peak assayed by taking two tubes is expressed as tube number & tube number with double bars. The recovery rate of antioxidant activity of the pools loaded on the column and the total of fractions is also listed in **Table 3.1**.



**Figure 3.20** Inhibition of Fenton reactions of the fractions obtained from the 5 pools on Superdex Peptide. The activity represents Ferrozine® equivalent in µmol/l.



**Figure 3.21 Free radical scavenging activity of the fractions obtained from the 5 pools on Superdex Peptide. The activity represents Trolox® equivalent in mmol/l.**

**Table 3.1 Comparison of antioxidant capacities of the 5 pools loaded and the fractions separated on Superdex Peptide.** Inhibition of the Fenton reaction is expressed as Ferrozine® equivalent in  $\mu\text{mol/l}$  and free radical scavenging activity is expressed as Trolox® equivalent in  $\text{mmol/l}$ . Values are expressed as mean  $\pm$  SEM (n = 3).

Pool Number	Ferozine® equivalent ( $\mu\text{mol/l}$ )			Trolox® equivalent ( $\text{mmol/l}$ )		
	Activity of pool loaded	Total activity of fractions	Recovery rate (%)	Activity of pool loaded	Total activity of fractions	Recovery rate (%)
1	62.0 $\pm$ 0.01	58.0 $\pm$ 2.68	93.5	10.0 $\pm$ 0.38	8.9 $\pm$ 0.17	89.0
2	244 $\pm$ 0.01	194.9 $\pm$ 0.01	79.9	12.8 $\pm$ 0.78	11.5 $\pm$ 0.06	89.8
3	63.0 $\pm$ 0.005	58 $\pm$ 4.2	92.1	6.54 $\pm$ 0.03	5.8 $\pm$ 0.06	88.7
4	43.0 $\pm$ 0.004	38.2 $\pm$ 2.23	88.8	6.28 $\pm$ 0.02	6.0 $\pm$ 0.20	95.5
5	97.0 $\pm$ 5.17	82.2 $\pm$ 2.78	84.7	18.4 $\pm$ 0.69	17.3 $\pm$ 0.28	94.0

### **3.6.3 Discussion**

Separations of the pools containing the phenolic compounds by FPLC chromatograms and subsequent assay of antioxidant activity of the fractions obtained have indicated that all of the pools had similar, but quantitatively different phenolic profiles.

The phenolic compounds responsible for free radical scavenging activity in five pools fractionated were distributed in different tubes, mainly in tubes 28, 29, 30, 32 and 33, indicating that several phenolic components were the potential to serve as antioxidants responsible for free radical scavenging.

The iron-binding type of antioxidant activity in Pool 1 when fractionated was mainly distributed in tube 28 and 38 and the other pools (Pool 2, 3, 4, and 5) when fractionated had this type of antioxidant activity mainly in tube 38. In particular, pool 2 presented a clear separation for an unknown phenolic profile, and this separated fraction was dominantly responsible for the iron-binding type of antioxidant activity. The reference elution volume of glycyl – glycyl - glycine with a molecular weight of 189 was at 18 ml (Superdex Peptide Instructions, Pharmacia Biotech). The fraction 5 in Pool 2 was eluted at 22.8 ml, suggesting that it was eluted later than it was expected for its molecular size. This can happen with gel permeation chromatography media because substances can bind to the gel polymer, rather like in RPC. This happens particularly with media for separating a low molecular weight range, as there is a lot of polymer to get small pore sizes in the gel.

The purpose of this study was to characterize the antioxidant components from phenolic extracts of manuka honey. Due to the importance of the property of preventing free radical formation by antioxidants, and because of time constraint, only fraction 5 (tube 38) of Pool 2, representing the highest iron-binding antioxidant activity in pools, was considered for structure identification.

## **Chapter 4 Identification of the Phenolic Compounds Responsible for Iron-binding Type of Antioxidant Activity**

The aim of this chapter was to identify by UV and mass spectra methods the structure of the major antioxidant compound obtained in **Section 3.6.1**.

### **4.1 Identification of the phenolic compounds by UV spectrum**

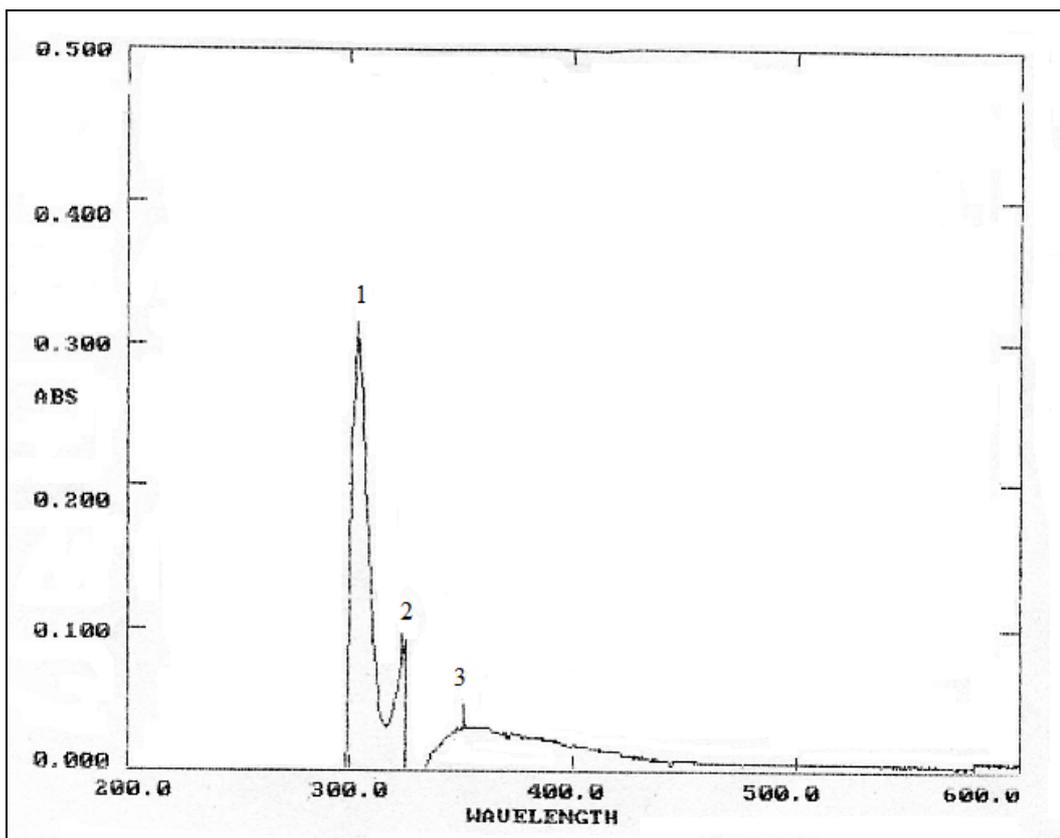
#### **4.1.1 Method**

The comparison of UV spectra of flavonoids in methanol and in the presence of a number of spectral shift reagents is always useful to provide information on their structures (Markham & Mabry, 1975). The spectra comparison method has been used for examining the UV spectra in methanol or methanol + aluminium chloride + HCl for more than 140 flavonoids (Voirin, 1983).

As the sample, fraction 5 (in tube 38) of Pool 2 obtained on Superdex Peptide, had relatively small amount at unknown concentration, it became difficult to add the adequate amount of reagents: aluminium chloride and HCl. For this reason the UV absorption spectrum was found without the addition of spectral shift agents. The sample was dried using a rotary evaporator and directly dissolved in 1 ml of methanol. The resulting sample was scanned from the wavelength of 200 to 600 nm.

#### 4.1.2 Results

The result of UV spectrum is shown in **Figure 4.1**. Three main peaks were present: the first peak was at 304 nm; the second peak was at 325 nm, and the third one was at 351 nm.



**Figure 4.1** Spectrum of the fraction 5 (in tube 38) of Pool 2 obtained by chromatography on Superdex Peptide at wavelength ranged from 200 to 600 nm.

### **4.1.3 Discussion**

Because phenolic compounds show chemical complexities and similarities, the isolation and identification have been challenging. For flavonoids, they make their UV spectra very characteristic, which has two main absorption bands observed: Band I (300 – 380 nm) is due to absorption of ring B and Band II (240 – 280 nm) is due to absorption of ring A (Stafilov et al., 2010). The UV spectrum of the sample exhibited no absorption in the region of 240 – 280 nm, which suggested that it was unlikely to be a flavonoid.

## 4.2 Identification of the phenolic compounds by mass spectrometry

### 4.2.1 Method

A mass spectrophotometer is usually applied to convert the individual molecules to ions so that the individual molecules can be moved and manipulated by external electric and magnetic fields, which enables the ions detected electronically. The mass spectrum is normally presented as a vertical bar graph. The resulting data are stored and analyzed for characterization of the individual molecules. The ion formation and manipulation are conducted in a vacuum, as the ions are very reactive and short-lived.

The sample, fraction 5 (in tube 38) of pool 2 obtained from chromatography on Superdex Peptide in **Section 3.6.1**, was identified as characteristic mass fragment ions using Bruker Daltonics' micrOTOF™ spectrophotometer in positive ionization mode. The standard settings were used and sodium formate was used as the calibration standard. The sample dissolved in methanol was infused via syringe pump at 180 ml/hour.

The system parameters were listed below:

Source type: ESI; Ion Polarity: Positive; Set Corrector Fill: 47 V

Scan begin: 200 m/z; Set Capillary Exit: 100.0 V; Set Pulsar Pull: 398 V

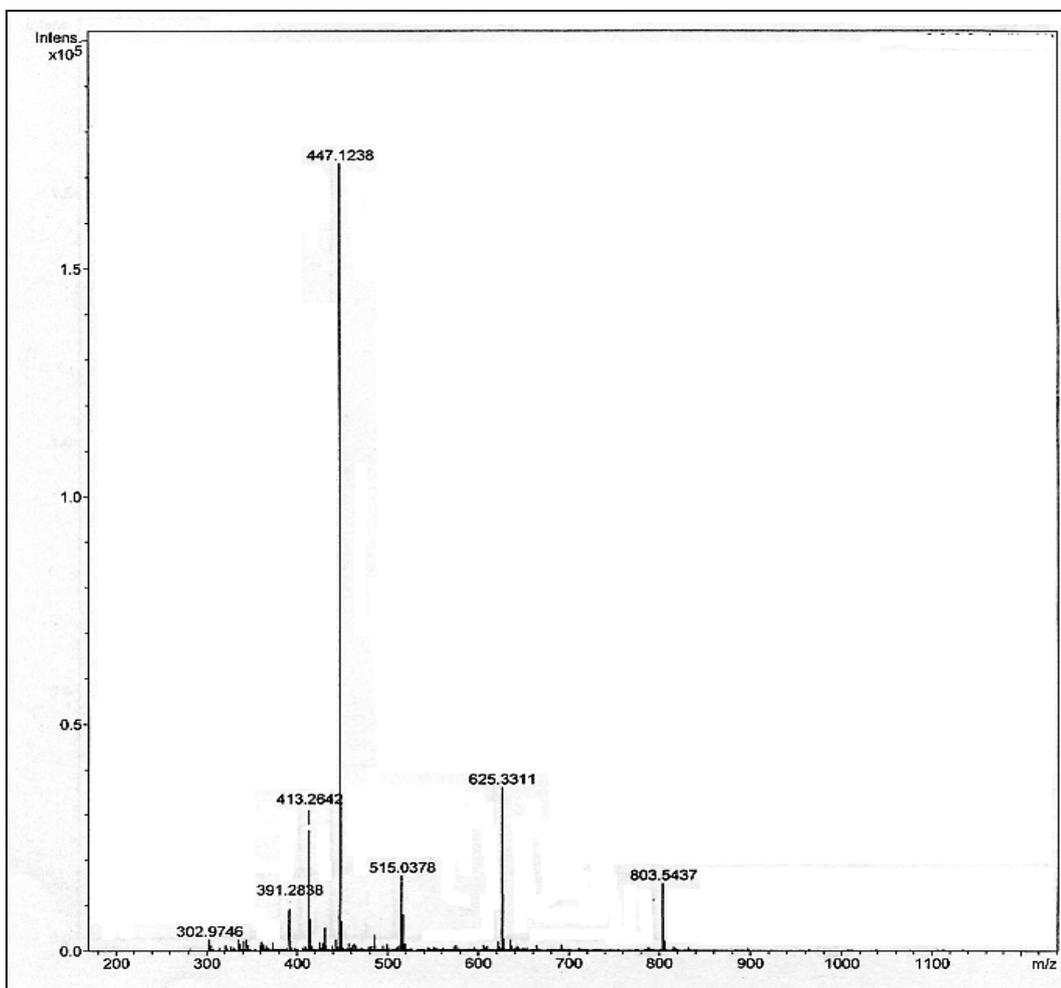
Scan end: 1200 m/z; Set Hexapole RF: 400.0 V; Set Pulsar push: 398 V

Set Skimmer 1: 33.3 V; Set Hexapole 1: 25.0 V; Set Reflector: 1300 V

Set Flight Tube: 9000 V; Set Detector TOF: 1976 V.

#### 4.2.2 Results

The sample was ionized by electron bombarding to present its spectrum (**Figure 4.2**).



**Figure 4.2 Positive ion spectrum of the fraction 5 (in tube 38) of Pool 2 obtained by chromatography on Superdex Peptide.**

### 4.2.3 Discussion

It was detected that the difference between the peaks at  $m/z$  803, 625 and 447 was 178 in **Figure 4.2**. Sakushima et al. (1988) studied the positive and negative ion mass spectra of flavonoid glycosides, and their results showed that the positive ion spectrum of flavonol 3,7-O-diglucosides exhibited fragment ions due to the loss of the terminal sugar of glycosides. The structure they had of  $m/z$  611 (molecular weight 610 +  $H^+$ ) got broken by the loss of a glucose ring side of the linking oxygen to  $m/z$  449. The part that broken off had a molecular weight of 163, but the  $m/z$  decreased by only 162 ( $611 - 449 = 162$ ). There was also a peak at  $m/z$  443 present, and this was expected to be from the break occurring on the other side of the linking oxygen of glucose, which gave the broken part with a molecular weight of 179, decreasing the  $m/z$  by 178.

In this study, the largest molecule detected was  $m/z$  803. The loss of a 178 fragment would take the 803 peak down to  $m/z$  625 ( $803 - 178 = 625$ ), and the loss of another 178 fragment would take it down to  $m/z$  447 ( $625 - 178 = 447$ ). Given the findings of Sakushima et al. (1988) mentioned above, this 178 fragment could be a sugar molecule. If the two 178 fragments were sugars, the smallest fragment after breaking off these two sugar units would be  $446 + H^+$  or  $424 + Na^+$ . As the original substance before sugars added would have had at least two hydroxyl groups to which the sugar units attached, it should have the molecular weight of 446 plus the addition of two oxygen atoms (molecular weight. 32), which was 478, or  $424 + 32 = 456$ .

By looking up all types of structures for phenolics, it seemed that only flavonoids would match the molecular weight and types of fragments. However, this outcome was contrary to the conclusion reached in **Section 4.1**. In order to confirm that the fragment with molecular weight of 179 that broke off were sugars or some other components attached by an ester linkage, the fraction obtained from chromatography on Superdex Peptide was hydrolyzed by refluxing in acid, and then fractionated using a RPC (reversed phase column) on the FPLC system.

## 4.3 Hydrolysis of the phenolic compounds for FPLC analysis

### 4.3.1 Hydrolysis

The hydrolysis of the sample was carried out as described by Nuutila et al. (2002). Briefly, 1 ml of the sample was hydrolyzed by refluxing at 80 °C for 2 hours in 1.2 mol/l HCl in 50% aqueous methanol. Then the hydrolyzed sample was concentrated to 500 µl by rotary evaporator at 40 °C and then centrifuged for 5 minutes at RPC 5000.

### 4.3.2 Chromatography

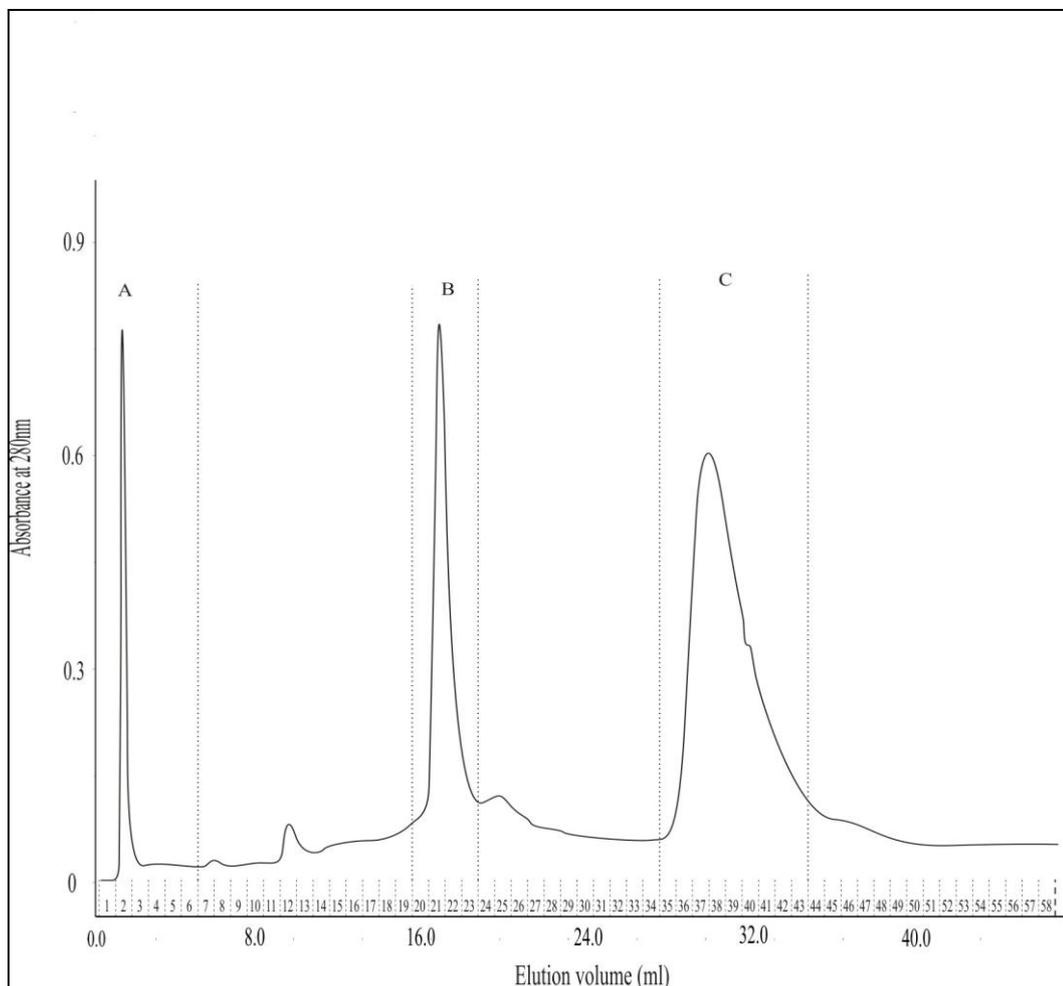
The hydrolysate was analyzed using a Source 15RP Column connected to the FPLC system at room temperature. The Source15 RPC (3 ml) is a polymeric, reversed phase chromatography medium based on rigid, mono-disperse polystyrene/divinylbenzene.

The running program was as follows:

**Sample injection:** 200 µl; **Eluent:** methanol; H<sub>2</sub>O with 300 µl/l trifluoroacetic acid; **Monitoring:** 280 nm; **Flow rate:** 1.3 ml/min; **Fraction collector:** 1.3 ml per tube.

From time 0 to 20 minutes, the sample was run at 20-60% gradient of methanol in water with 300 µl/l trifluoroacetic acid. After 20 minutes, the system remained running with 60% methanol in water with 300 µl/l trifluoroacetic acid till the end. Once each run was done, the column was washed with 100% methanol for 2

minutes, then returned to 20% methanol and re-equilibrated for next analysis. The results from FPLC are shown in **Figure 4.3**. A total of 58 tubes were obtained.



**Figure 4.3 FPLC chromatogram of the hydrolyzed sample on Source 15RPC.** Tube numbers are listed on X-axis. Dotted lines mark the tubes pooled for each fraction (A, B, and C).

### 4.3.3 Discussion

There were three main peaks obtained with the column. As the study by Nuutila et al. (2002) stated that the hydrolyzed standard mixture of flavonoids and phenolic acids from onion and spinach was completely detected with clear peaks at 280 nm, only the three main peaks obtained by hydrolysis were taken for mass spectra identification. Thus, each peak was pooled as shown in **Figure 4.3** and the resulting pools were dried using a rotary evaporator at 40 °C, then re-dissolved in 0.5 ml methanol, which were named A, B and C for convenience. However, sugars or isoprenoids released by hydrolysis would not have been detected by absorbance at 280 nm.

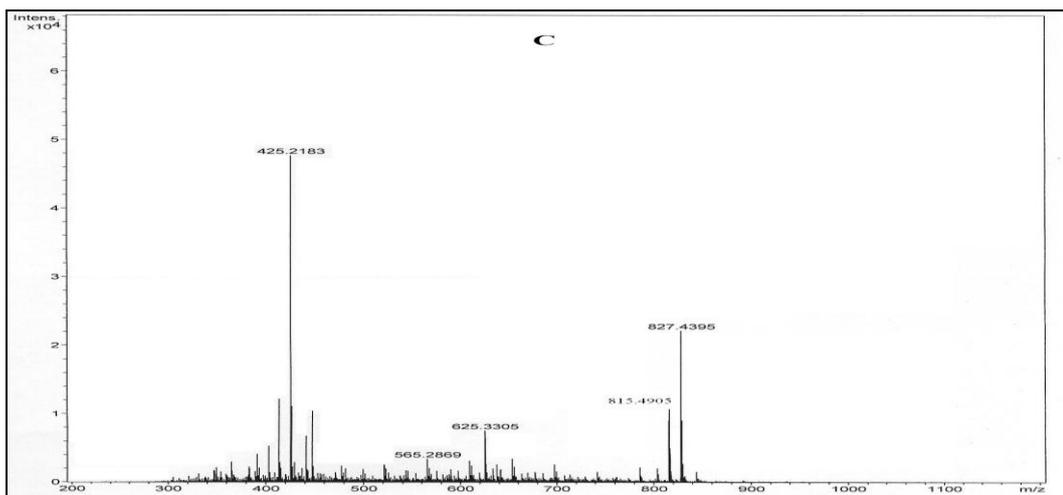
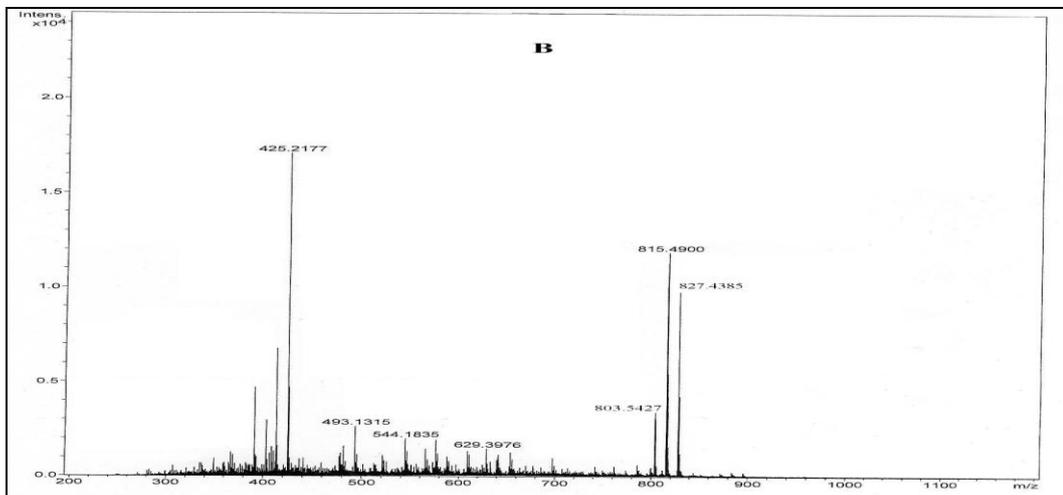
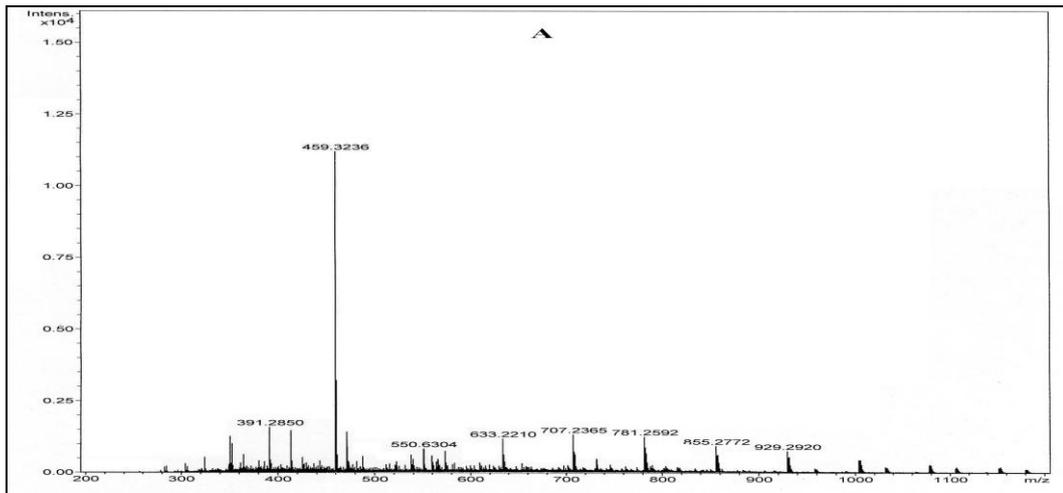
## **4.4 Mass spectrometry of the components obtained from hydrolysis**

### **4.4.1 Method**

The MS identification of the components released by hydrolysis was performed as previously (**Section 4.2.1**).

### **4.4.2 Results**

The mass spectra for A, B and C are showed in **Figures 4.4**.



**Figure 4.4 Positive ion spectra of A, B and C from hydrolysis of the fraction 5 (in tube 38) of Pool 2 on Superdex Peptide.**

#### 4.4.3 Discussion

Fraction B in **Figure 4.4** had the peak at  $m/z$  803 (same peak as in **Figure 4.2**), and B and C both had peaks greater than  $m/z$  800, it demonstrated that B and C were molecules that did not hydrolyze. The mass spectra of B and C also showed that was possibly fragmentation of these, or possibly contained components released by hydrolysis.

The peak at  $m/z$  447 obtained in the first mass spectra run was possibly the same fragment as the peak at  $m/z$  425 in B and C obtained after hydrolysis, the 447 peak being with a  $\text{Na}^+$  added on, but the 425 peak in B and C with an  $\text{H}^+$  added on.

Fraction A was probably the component of the molecule that was released by hydrolysis. The two 178 fragments broken off to get the 625 and 447 peaks in the original mass spectra run (**Figure 4.2**) would not have been detected in the FPLC monitoring at 280 nm if they were sugar molecules or might be other non-phenolic molecules. These two fragments would be eluted between the fractions collected.

The difference in  $m/z$  between 459 in A and 425 in B and C would be because when hydrolysis occurred (rather than fragmentation in MS) the elements of water would have been added on. When the two 178 components were released they were replaced each with OH ( $2 \times 17$ ), adding 34 to 425 to give 459. As the charge in  $m/z$  459 was due to addition of  $\text{H}^+$ , the molecular weight of the non-sugar component would be 458. The phenolic compound responsible for iron-binding type of antioxidant activity could be the molecule with molecular weight of 458.

There was another consideration, A was much more polar than B and C, as it was eluted early in the gradient of methanol. Removal of sugars would make molecules less polar, so the fragments of mass 178 were probably not sugars. Another possibility was compound prenylation (de Freitas & Mateus, 2001). If the fragments removed were prenyl groups, it would be a flavonoid. Tannins if hydrolyzed would release phenolic compounds which would have been detected in UV monitoring of reverse phased FPLC system of the hydrolysate. Similarly, if the components of mass 458 were esterified with a phenolic acid (Hong et al., 2001), then the fragment of mass 178 removed by hydrolysis would have been detected in the monitoring on the RPC FPLC system.

## **4.5 Summary and future work**

### **4.5.1 Summary**

This study examined the antioxidant activity of phenolic extracts from manuka honey and separated the phenolic compounds mainly responsible for antioxidant activity. The results have shown that manuka honey had antioxidant activity of both the iron-binding and free radical scavenging types of activity. The phenolic extracts from manuka honey partially contributed to the total antioxidant activity of manuka honey.

A separated unknown phenolic compound was significantly responsible for the iron-binding antioxidant activity measured by the  $\beta$ -carotene-linoleic acid system. This compound was taken for molecular identification. The other phenolic components of manuka honey were responsible for most of free radical scavenging activity. The identification of the phenolic compound selected by UV and mass spectra methods suggested that it could be the molecule with molecular weight of 458.

### **4.5.2 Recommendation for future work**

In order to accurately identify the molecular structure of the phenolic compound obtained on Superdex Peptide, the work that should have been done included:

- (1) Run the fraction obtained from Superdex Peptide on Source15 RPC before hydrolysis, this was important to see if there was more than one substance present. Some of the components in the peaks after hydrolysis may have been extra substances in the peak from Superdex Peptide;

- (2) Collect all components from hydrolysis (including those not absorbing UV) to characterise these by finding out their absorption spectrum as well as their molecular weight on MS;
- (3) Test any component of the hydrolysate of molecular weight 180 with colorimetric reagents that detect sugars, and if found to be a sugar then use an enzymic glucose test to find if the sugar is glucose;
- (4) If it is not glucose but is a sugar, then run FPLC to identify which sugar it is;
- (5) If it is not a sugar then run GC-MS (gas chromatography-mass spectrometry), as a volatile derivative if necessary, to find what it is;
- (6) Find the UV absorption spectrum of the peak from Superdex Peptide, and of the components from hydrolysis, with and without aluminium and other additives, to see if the compound has a spectrum matching that of other known polyphenols;
- (7) Prepare a larger quantity of the peak samples from Superdex Peptide, followed by RPC if RPC shows more than one substance present, so that NMR (nuclear magnetic resonance) spectroscopy can be run on the purified antioxidant so its structure can be identified.

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