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Antioxidants in Australian Floral Honeys

–Identification of health-enhancing nutrient components

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by Bruce R D'Arcy

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Foreword

Knowledge of the concentrations and identity of the antioxidant components of Australian floral honeys, including polyphenols such as flavonoids and phenolic acids, will enable consumers and the food industry to make better choices as to the floral type of honey they use for health and nutrition.

This publication considers the extraction of antioxidant flavonoids and other polyphenols from straightline samples of species-specific floral types of Australia honey, namely yapunyah, leatherwood and Salvation Jane honeys, and the identification and quantification of these antioxidants.

This project is part of RIRDC's Honeybee Program which aims to improve the overall image of honey in Australia through increased knowledge of the properties of honey, so that domestic use of liquid honey can be increased.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to RIRDC's diverse range of over 1200 research publications, and forms part of our Honeybee R&D program, which aims to improve methods of extraction, storage and transport of honey, and increase the use of honey in the food industry and for medical and therapeutic uses.

Most of our publications are available for viewing, downloading or purchasing online through our website:

- downloads at www.rirdc.gov.au/reports/Index.htm
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Peter O'Brien
Managing Director
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Researcher	Contribution
Mr Graham Kerven	Technical support for HPLC and LC-MS analysis
Mr Arnaud Robert (visiting research student from Noordelijke Hogeschool Leeuwarden, University of Professional Education, The Netherlands in 2002)	Experimental data on yapunyah honey and recovery study of phenolic standards
Ms Maaike Wilborts (visiting research student from Wageningen University, The Netherlands in 2002)	Experimental data on spotted gum honey and recovery study of phenolic standards
Mr Kok Kin Wong (UQ postgraduate coursework student 2003)	Literature research and experimental data on yapunyah honey
Mr Thibault Bernard (visiting research student from the Institut Universitaire Professionalise (IUP IIA), Quimper, France in 2003)	Experimental data on yapunyah honey
Ms Gwenola Plainvert (visiting research student from the Institut Universitaire Professionalise (IUP IIA), Quimper, France in 2003)	Experimental data on leatherwood honey
Ms Morgane Pelle (visiting research student from the Institut Universitaire Professionalise (IUP IIA), Quimper, France in 2003)	Experimental data on Salvation Jane honey
Ms Marieke Pastink (visiting research student from Wageningen University, The Netherlands in 2003)	Analysis of quantitative data for yapunyah and leatherwood honeys
Dr Kapila Seneviratne (visiting academic researcher from University of Keleniya, Sri Lanka in 2003)	Analysis of quantitative data for Salvation Jane honey
Mr Mehdi Moosavi (visiting PhD research student from Shahid Bahonar University of Kerman, Islamic Republic of Iran in 2003-04)	Analysis of quantitative data for yapunyah and leatherwood honeys
Dr Lihu Yao (PhD student graduated in 2002)	Some literature research and text of Chapter 1, and some discussion.

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Contents

List of Tables

List of Figures

List of Figures (continued)

Executive Summary

Polyphenols in foods are thought to play important roles in human health such as cancer preventative and anti-inflammatory, radical scavenging and antioxidative activities. The most important classes of antioxidant polyphenols are the flavonoids and phenolic acids. It is these substances in tea, wine, fruits and vegetables that are most responsible for the antioxidant characteristics, and thus the healthy image of these foods. However, little data exist on these components in Australian floral honeys, hence the need for this study.

Multiple samples of the three floral types of Australian honey, yapunyah, leatherwood and Salvation Jane, and one sample of spotted gum honey were examined for their antioxidant polyphenol concentrations through an extraction and HPLC/LC-MS analysis of flavonoids and phenolics.

However, before this could occur, a chemical analysis method for the extraction of antioxidant flavonoids and phenolic acids from honey using Amberlite XAD-2 resin was optimised, and recovery studies were done. During the optimisation of the Amberlite XAD-2 extraction method, it was found that, contrary to previous studies (Yao, 2002), the phenolic acids, gallic acid and ellagic acid were not retained on the resin during the extraction under the acidic conditions ideal for such retention. This suggests that these two phenolic acids could not be extracted from honey using Amberlite XAD-2 resin as indicated by Yao (2002). In addition, the percentage recovery of the phenolic acids, caffeic acid, *p*-coumaric acid and ferulic acid, reported by Yao (2002) to be in yapunyah and other honey types, varied between 15.5 and 62%. However, this does not mean that other unknown (not identified) phenolic acids detected in this study were not recovered in higher yields. In contrast, the extraction efficiencies for flavonoid standards such as quercetin, hesperetin and chrysin were much better, with the latter two having recoveries of >83%, in agreement with previous literature studies.

Next, identification and quantification of honey flavonoids and other polyphenols were done using high performance liquid chromatography (HPLC) with diode array detection (DAD) at 290 nm (phenolic acids) and 340 nm (flavonoids), and using liquid chromatography (LC) - mass spectrometry (MS) with negative ionisation, including the use of the sensitive, selective ion recording (SIR) mode. HPLC and LC-MS methodology was developed so that separation of flavonoids and phenolic acids was maximised, enabling accurate quantification.

After LC-MS analysis of extracts from five samples of yapunyah honey using the mass spectral, selected ion recording (SIR) mode with negative ionisation, it was found that the phenolic acids, gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, and ellagic acid were detected in negligible concentrations (<13.2 μg/ 100 g), which suggests that the previous reporting of these phenolic acids in two samples of yapunyah honey in much higher concentrations by Yao (2002) was in serious error. This was because the study of Yao (2002) did not involve LC-MS analysis in the identification of these phenolic acids, only HPLC-DAD analysis. Such an approach is fraught with possible error. However, a number of other phenolic acids were detected in the yapunyah honey samples during the study reported here, but could not be identified, even with the use of LC-MS.

Flavonoids identified and quantified in the five yapunyah honey samples were tricetin, pinobanksin, quercetin, luteolin, quercetin 3-methyl ether, and 8-methoxy kaempferol, in agreement with Yao (2002). These flavonoids have been reported before in Eucalypt honeys, with the three flavonoids, tricetin, quercetin and luteolin being characteristic of Eucalypt honeys. The concentration of flavonoids in yapunyah honey samples ranged from 568.98 to 990.37 μg/100 g honey (mean of 747.64 μg/100 g honey), while the concentrations of phenolic acids ranged from 407.15 to 625.17 μg/100 g honey (mean of 510.82 μg/100 g honey). These were lower than previously reported for two samples of yapunyah honey by Yao (2002). The reason for this difference in concentrations is not known at this time, but may be due to different samples being used.

The study of ten leatherwood honey samples quantified the phenolic acid, caffeic acid, and the flavonoids, tricetin, pinobanksin, luteolin, pinocembrin, and chrysin, as well as many unidentified phenolic acids. The concentration of flavonoids in the ten leatherwood honey samples ranged from 638.7 to 1579.1 μg/100 g honey (mean of 901.2 μg/100 g honey), while the concentrations of phenolic acids ranged from 1177.8 to 2718.8 μg/100 g honey (mean of 2066.6 μg/100 g honey). Leatherwood honey did not contain many flavonoids, but was rich in many phenolic acids. This is a very interesting result when considered in the light of the high concentrations of volatiles (some of which were phenolic compounds) previously found in leatherwood honey by D'Arcy *et al*. (2001) in an earlier RIRDC project.

For the six Salvation Jane honey samples, the flavonoids, pinobanksin, luteolin, kaempferol and pinocembrin, and the phenolic acids, 4-hydroxyphenyllactic acid and α-cyano-4-hydroxycinnamic acid were identified and quantified. In addition, there were a significant number of other flavonoids and phenolic acids quantified, whose identity could not be determined even with the use of LC-MS. The concentration of flavonoids in the seven Salvation Jane honey samples ranged from 245.11 to 484.54 μg/100 g honey (mean of 407.19 μg/100 g honey), while the concentrations of phenolic acids ranged from 344.27 to 954.75 μg/100 g honey (mean of 732.01 μg/100 g honey).

However, even with the use of LC-MS, many polyphenols could not be identified, although they were quantified against a standard polyphenol to give some indication of their relative concentrations. A future more detailed study is needed to identify these polyphenols.

Amongst the yapunyah, leatherwood and Salvation Jane honeys, leatherwood honey has approximately 2-3 times the concentration of total flavonoids/phenolic acids than the other two honeys. This was also the case for the volatile compound concentration, with leatherwood honey containing a larger number and range of volatiles and in much higher concentrations than other Australian floral honeys (D'Arcy *et al.*, 2001).

Finally, the implications of this study are that since only four floral types were studied, a detailed comparison between floral types, to determine which Australian honey type has the highest concentrations of antioxidant flavonoids and phenolic acids, is not possible at this time.

In conclusion, the scientific data generated during this project on the identity and concentration of antioxidant flavonoids and phenolic acids in Australian honey will enable the further marketing of honey as a healthy and nutritious food to the Australian food industry and consumers, in addition to its use as a sweetener.

1. Introduction

This review of the literature mainly concerns the antioxidant properties of natural honeys, particularly the flavonoids and phenolic acids. The antioxidant capacity of honey is discussed extensively. Enzymes, such as glucose oxidase and catalase are also some of the antioxidants in honey, but are discussed only briefly in this review. There is no attempt here to discuss other antioxidants occurring in honey such as Maillard reaction compounds, amino acids, ascorbic acid etc. Honey flavonoids and their antimicrobial properties, authentication of honey using phenolic compounds, and the application of honey as an antioxidant in food have been explored, including the inhibition of lipid oxidation and enzymic browning. The methods for determining honey polyphenols are discussed at the end of the review.

1.1 What is Honey?

Honey is defined as 'the natural sweet substance produced by honey bees from nectar of blossoms or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which honey bees collect, transform and combine with specific substances of their own, store and leave in the honeycomb to ripen and mature', in the *Codex Alimentarius* produced by the Food and Agricultural Organisation of the United Nations (Molan, 1996). Honey is a completely natural product, and raw honey can be used directly from the comb as taken from the beehive (Molan, 1996).

Honey contains about 181 substances (Al-Manary *et al*., 2002), including sugars, proteins, moisture, vitamins, minerals, hydroxymethyfurfural (HMF), enzymes, flavonoids, phenolic acids, volatile compounds, and so on. However, the main constituents of honey are moisture, glucose (dextrose), fructose, maltose, sucrose, mineral matter and proteins (Kirk and Sawyer, 1991).

1.2 Uses of Honey

1.2.1 History

Honeybees make honey to use and store as food, and humans exploit these traits. Honey was probably discovered by humans tasting the sweet substance in honeycombs from the hollows of a tree, log, or cave. Thus, it is one of the earliest forms of sweeteners and long precedes the use of cane and beet sugar (Coulston, 2000). Beekeeping for the purpose of obtaining honey is an ancient art, at least as early as the Egyptians (2000 – 5000 years ago) who used honey in medicine, in embalming, and for food (Coulston, 2000).

1.2.2 Food system and applications

Honey has been a staple of the kitchen for centuries. It absorbs and retains moisture – an important quality in the baking industry to keep breads and cakes moist and fresh (Coulston, 2000). Furthermore, honey improves the browning quality, texture, delays staling and thus increases the shelf life of breads (Caffin *et al*., 1996). Honey can be used as sweetener in canned or frozen fruits, jams, jellies and drinks. One popular drink of Elizabethan times was mead, fermented honey (Coulston, 2000). Wines, known as meads, have been made from honey for thousands of years, including sparkling mead and sherry mead (White, 1992).

There is a growing interest in the reintroduction of healthy bacteria into the colon following diarrheal diseases or extended use of antibiotics. Addition of honey to milk prior to the production of yoghurt results in a significant amount of bifidobacteria surviving processing, because the type of oligosaccharides, with varying degrees of polymerisation, in honey enhances growth, viability and activity of bifidobacteria in milk (Coulston, 2000). Therefore, honey is acceptable as a probiotic and/or prebiotic food (Coulston, 2000).

1.2.3 Medicinal uses

Honey has been used since ancient times as a remedy for burns, cataracts, ulcers and wound healing, simply because it has a soothing effect during its initial application to open wounds (Coulston, 2000). Given its physical properties, honey provides a protective barrier and, owing to its high osmolarity, it creates a moist wound-healing environment in the form of a solution that does not stick to wounded tissues (Coulston, 2000). This moist wound environment is believed to prevent bacterial colonisation. Thus, honey reduces inflammation and also reduces exudate formation more promptly than standard treatments (Coulston, 2000).

A number of studies have been done on the antibacterial properties of honeys. It has been demonstrated that the non-peroxide antibacterial activity of New Zealand manuka honey is effective in inhibiting the growth of *Helicobacter pylori*, the bacterium that is responsible for causing gastritis and duodenal ulcers, whereas the hydrogen peroxide antibacterial activity of other honey does not (Molan, 1996). This is why the consumer demand for manuka honey as an antibacterial substance is increasing.

1.2.4 Honey industry in Australia and worldwide

The honeybee is native to Europe and Africa, and has been domesticated for use throughout the world. In 1998, more than 200 million pounds of honey were produced in the United States (Coulston, 2000). In Australia, it is estimated that an average of at least 30,000 tonnes of honey are produced annually, with nearly 45% of this total coming from New South Wales, and between 9,000 and 12,000 tonnes of honey exported each year (Gibbs and Muirhead, 1998). Honey production in Australia also occurs in Queensland, Victoria, South Australia and Western Australia (Gibbs and Muirhead, 1998).

1.3 Antioxidant Capacity in Honey

Antioxidant activity, or simply antioxidant capacity, is the ability and potential of honey in reducing oxidative reactions within the food systems and human health. Notably, these oxidative reactions can cause deleterious reactions in food products (e.g. lipid oxidation in meat, and enzymic browning in fruits and vegetables) and adverse health effects, such as chronic diseases and cancers (Gheldof and Engeseth, 2002). The antioxidants that naturally occur in honey contribute to antioxidant capacity. These compounds are flavonoids, phenolic acids, and some enzymes (e.g. glucose oxidase, catalase), ascorbic acid, carotenoid-like substances, organic acids, Maillard reaction products, amino acids and proteins (Gheldof *et al*., 2002).

However, Gheldof *et al*. (2002) found that while phenolic compounds contribute significantly to the antioxidant capacity of honey, they are not solely responsible for it. In a recent study, Gheldof and Engeseth (2002) showed that there is a strong correlation ($R^2 = 0.963$, $p < 0.0001$) between the antioxidant capacity of a honey and the concentration of its total phenolic acids. However, the antioxidant capacity varies greatly depending on the honey floral source, possibly due to the differences in content of plant secondary metabolites and enzyme activity (Frankel *et al*., 1998).

1.3.1 Determination of antioxidant capacity

A study was done to determine the water-soluble antioxidant capacity of 19 samples of honey from 14 different floral sources using a spectrophotometric assay (Frankel *et al*., 1998). The results were expressed as antioxidant microequivalents (μeq). One antioxidant microequivalent is the ability to reduce one micromole of a pro-oxidant; because each molecule of ascorbic acid is able to reduce two moles of pro-oxidant, one μmol of ascorbic acid has two antioxidant μeq (Frankel *et al*., 1998). Ascorbic acid, as well as many antioxidant alkaloids, are water soluble, so a higher percentage water

content in honey could conceivably allow for greater amounts of dissolved antioxidants for a given amount of honey (Frankel *et al*., 1998).

Frankel *et al.* (1998) reported that the honey sample with the highest antioxidant capacity measured was 20.3 times that of the lowest unifloral honey samples, with the highest value of 432×10^{-5} μeq for 1995 Illinois buckwheat and the lowest value of 21.3×10^{-5} μeq for 1994 California button sage (Frankel *et al*., 1998). Colour accounted for over 60% of the variance in antioxidant capacity for the honeys examined ($r^2 = 0.634$), with darker colour having greater antioxidant capacity. The regression of water content on antioxidant capacity was significant but accounted for a lower proportion of variance in antioxidant capacity than did colour ($r^2 = 0.366$, $p < 0.005$). Many studies have found that buckwheat honeys provide the greatest antioxidant capacity of all. Moreover, other honeys such as soy, sunflower, clover, Hawaiian Christmas berry, tupelo and lehua also posses reasonably high antioxidant capacities (Frankel *et al*., 1998; Gheldof and Engeseth, 2002; Gheldof *et al*., 2002; Nagai *et al*., 2001).

However, as this analysis only took into account the water-soluble antioxidant components, the variability in the lipid-soluble antioxidant components may affect the actual result (Frankel *et al*., 1998). It is likely that most of the antioxidant honey constituents are water soluble, since nectars, from which honeys derive, have higher water contents, from 30-90%. Moreover, certain water-soluble antioxidants may be degraded during spectrophotometric analysis because they are heat-labile (Frankel *et al*., 1998). It is also reported that the antioxidant capacity of honey is attributed principally to its non-protein constituents, as heating honey did not alter its capacity to prevent β-carotene oxidation (Frankel *et al*., 1998). It was concluded that antioxidant content is positively correlated with both water content and honey colour, suggesting that honey with more pigments or secondary plant metabolites may have a higher antioxidant capacity (Frankel *et al*., 1998). Some recent studies of the antioxidant capacity of honeys conducted in the USA confirmed this finding (McKibben and Engeseth, 2002; Gheldof and Engeseth, 2002).

1.3.2 Dietary significance of antioxidants in honey

It is important to note that antioxidant concentrations in honey tend to be relatively lower than many traditional sources of dietary antioxidants. Notably, the lipid-soluble antioxidants of fruits and vegetables are in much higher concentrations than those of honeys, and the total antioxidant capacity of most fruits and vegetables, including the water and lipid-soluble ones, is many times greater than the average value for the water-soluble antioxidant capacities of honey of 885×10^{-5} μeq/mg (Frankel *et al*., 1998). For instance, sweet orange pulp is rich in antioxidants, containing 5680×10^{-5} μeq/mg, with broccoli being 13630×10^{-5} μeq/mg and sweet peppers being 14150×10^{-5} μeq/mg (Frankel *et al*., 1998). Therefore, honey may not serve as a major source of dietary antioxidants, despite the fact that the antioxidant capacities of some honeys are comparable to fruits and vegetables (Gheldof and Engeseth, 2002). Nonetheless, the pleasing taste of honey makes it readily consumed by individuals who may eat very little antioxidant-containing fruits and vegetables (Frankel *et al*., 1998). Since dietary antioxidants provide health benefits, floral source should be a factor in evaluating the potential of honey as an antioxidant-containing food supplement (Frankel *et al*., 1998) and may be used as a healthy alternative to sugar in many products (Gheldof and Engeseth, 2002). Nevertheless, there is very little understanding of this fact by nutritionists, since only a few studies have examined the profiles of antioxidants in honey from various floral sources (Gheldof *et al*., 2002).

1.4 Flavonoids

While there are various types of antioxidants naturally occurring in honey as mentioned previously, this review focuses only on the flavonoids and other phenolic compounds.

1.4.1 Chemistry of flavonoids

Phenolic compounds or polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom. Notably, flavonoids and simple phenolic derivatives are the most common polyphenols (Brovo, 1998). Flavonoids are the secondary metabolites of plants, with more than 5000 compounds having been identified by 1990 (Brovo, 1998). Structurally, flavonoids are derivatives of 1,3-diphenylpropane (Sivam, 2002) and are low molecular weight polyphenols based on the flavan nucleus, which is characterised by a C_6-C_3-C_6 carbon skeleton (see Figure 1.1) (Peterson and Dwyer, 1998). The three phenolic rings are referred to as A, B and C (pyran) rings (Cook and Samman, 1996). Biogenetically, the A ring usually comes from the acetate pathway, whereas ring B is derived from the shikimate pathway (Brovo, 1998). Flavonoids occur naturally as glycosides (with sugar moieties), but occasionally occur as aglycones (without sugar moieties) (Peterson and Dwyer, 1998).

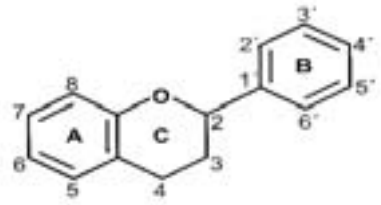


Figure 1.1 Generic structure of flavonoids (Brovo, 1998)

In general, flavonoids can be divided into 13 classes (Figure 1.2). However, Peterson and Dwyer (1998) suggested that biflavans, catechins, proanthocyanidins and tannins can be sub-classified into flavans, subsequently dividing them into six main classes, namely flavanones, flavones, isoflavones, anthocyanins, flavonols and flavans (flavanols). Flavans alone can occur as mono-, bi- and tri-flavans (Figure 1.3). This diverse structure of flavonoids is due to polymerisation and substitutions, such as hydrogenation, hydroxylation, methylation, melonylation, sulphonation and glycosylation (Cook and Samman, 1996). Moreover, flavonoids can be monomeric, dimeric or oligomeric, and vary greatly in size (Cook and Samman, 1996).

1.4.2 Distribution in foods

Flavonoids are plant phytochemicals that cannot be synthesised by animals and humans; even flavonoids found in some animals have a dietary origin in plants (Peterson and Dwyer, 1998). Therefore, flavonoids are present in most plant-derived foods including fruits, vegetables, cereals, grains, nuts, herbs, legumes and honey. In addition, any beverage containing natural flavours and colourings or made from plant material also contains flavonoids, such as white wine, red wine, tea, coffee, juices, cider, cocoa and so on. However, flavonoids are not present in meat, poultry, milk, eggs and seafood (Peterson and Dwyer, 1998).

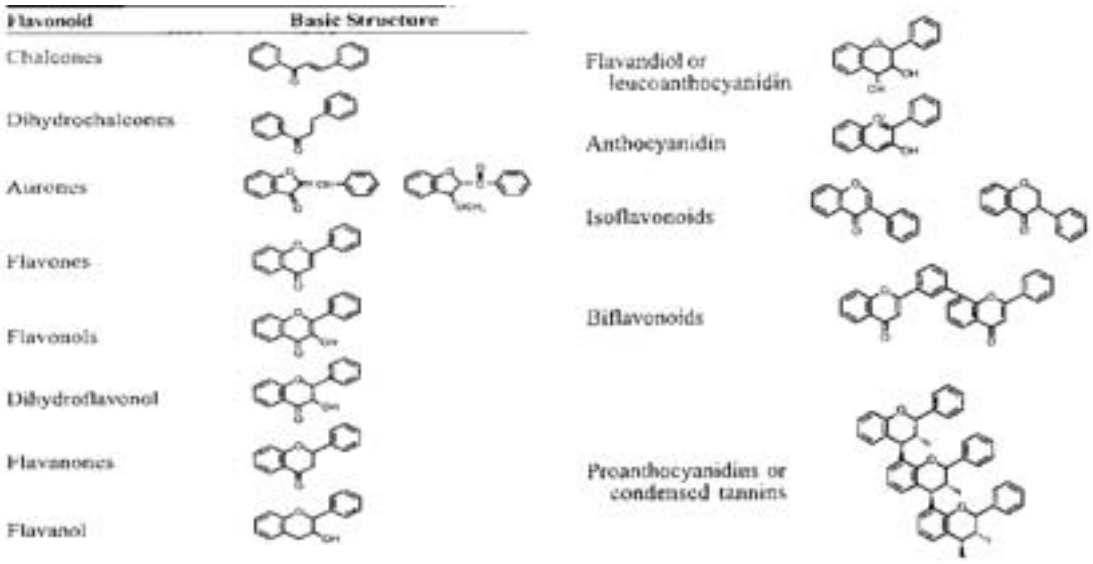


Figure 1.2 The 13 classes of food flavonoids (Brovo, 1998)

The concentration of flavonoids is largely influenced by genetic factors, type of species, environmental conditions (e.g. light), ripeness, variety, processing and storage. In addition, the concentrations vary greatly between cultivars of the same species (Brovo, 1998). For example, the concentrations of flavonoids vary largely in different types of food products from 25 mg/100g in cabbage to 1200 mg/100g in blackcurrant (Brovo, 1998). Flavonoids in foods are responsible for colour, enzymic inactivation, flavour, and inhibition of both vitamin and lipid oxidation. Despite the diversity of flavonoids, only certain classes of flavonoids are present in certain types of plant foods.

Predominantly, flavanones are present in citrus, whereas isoflavonoids occur in legumes, particularly in soybeans. Flavones can be found in grains and herbs (Perterson and Dwyer, 1998). It seems that flavanones and flavones are often present in the same plant, particularly citrus fruits, yet flavones and flavonols are generally not found together, nor are flavanones and anthocyanins (Merken and Beecher, 2000). Though flavonols virtually occur throughout the plant foods, the most common flavonols are quercetin and kaempferol. Beverages, fruits, vegetables, legumes and grains also are a rich source of anthocyanins, because anthocyanins contribute to the colour in plants. Flavans are frequently present in most fruits, grains and teas (Peterson and Dwyer, 1998).

1.4.3 Dietary significance

Dietary intakes of flavonoids are poorly documented. It has been previously estimated that the average intake of all dietary flavonoid in the USA was approximately 1g/day, of which about 170 mg consisted of flavonols. However, this analysis is overestimated due to the less advanced techniques applied previously (Cook and Samman, 1996). Recent publications also showed that the average dietary flavonoid intake in The Netherlands was estimated to be approximately 23 mg/day (Cook and Samman, 1996). However, this analysis was based on the contents of only five flavonoids in the commonly consumed Dutch foods, such as tea, onions and apples (Cook and Samman, 1996). These studies imply that the estimation was only based on some flavonoids, and did not include other phenolic compounds, resulting in inaccurate data.

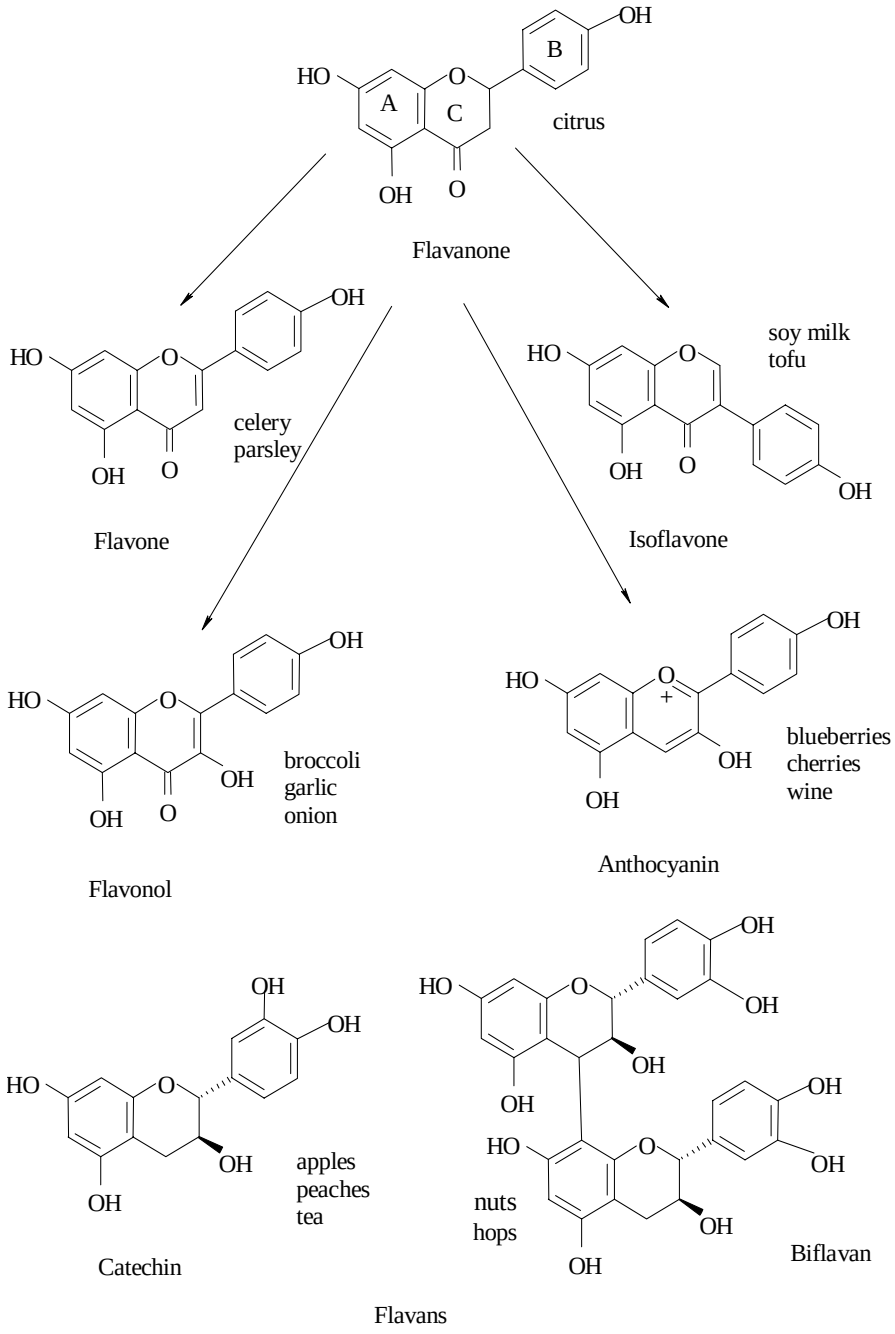


Figure 1.3 Structure and dietary occurrence of the main classes of flavonoids. Arrows indicate biosynthetic path (Peterson and Dwyer, 1998)

1.4.4 Physiological roles in human health

Many health professionals claim that flavonoids exert positive effects on human health, including antibacterial, antiviral, anti-inflammatory (Cook and Samman, 1996), antithrombotic, vasoprotective, hypocholesterolemic and hypolipolenic effects (Brovo, 1998). Therefore, flavonoids may have important applications in the prevention and treatment of cancers, cardiovascular disease, allergies, vascular fragility, gastric ulcers, duodenal ulcers, and bacterial and viral infections (Brovo 1998).

For example, anthocyanins from bilberry or black currants are used to enhance vision and increase capillary resistance (Peterson and Dwyer, 1998). Flavonoids exert their protective effects through the reduction of free radical formation, protection of α-tocopherol in low density lipoprotein (LDL) from oxidation, regeneration of oxidised ascorbic acids, and chelation of divalent cations (Brovo, 1998). Some studies demonstrated that diets high in flavonoids can reduce the development of cardiovascular diseases (Cook and Samman, 1996; Muldoon and Kritchevsky, 1996).

1.4.5 Flavonoid structure and antioxidant capacity

The structure of the B ring (Figure 1.1) is the primary determinant of the antioxidant capacity of flavonoids (Pannala *et al*., 2001). Flavonoids such as quercetin, with 3',4'-OH substituents in the B ring and conjugation between the A and B rings, have antioxidant potentials four times that of Trolox, the vitamin E analogue. Removing the *ortho*-OH substitution or reducing the 2,3-double bond in the C ring decreases the antioxidant capacity by more than 50% (Rice-Evans *et al*., 1995, 1996, 1997). The carbonyl in the central ring (C ring) and the C_2-C_3 double bond could participate in radical stabilisation that increases antioxidant capacity, as occurs with quercetin.

1.4.6 Flavonoid toxicity

Flavonoids can form complexes with metal cations through their carboxyl and hydroxyl groups, and thus interfere with the intestinal absorption of minerals (Brovo, 1998). Flavonoids, such as tannins, strongly inhibit iron absorption and hence reduce iron bioavailability (Brovo, 1998). Although the chelating action of flavonoids on metals such as copper and iron can have negative effects by reducing their bioavailability, this action can be beneficial in certain circumstances. In the native state, copper and iron can be the initiators of hydroxyl radical production by the Fenton and Haber-Weiss reactions; thus, chelation of these metals is one of the ways flavonoids exert their antioxidant effect (Brovo, 1998).

Toxic effects have been documented from doses of 1–1.5 g/day of flavonoid drugs such as cianidanol, including acute renal failure, haemolytic anaemia, thrombocytopenia, hepatitis, fever and skin reactions (Cook and Samman, 1996). However, in diets containing a wide variety of foods, flavonoids are unlikely to be consumed in toxic quantities because foods originating from plants contain many diverse types of flavonoids in varying quantities (Cook and Samman, 1996).

1.5 Flavonoids in Honeybee Products

1.5.1 Nectar flavonoids

Nectar is a secretion of specialised glands of plants called nectaries that are located in or near the flower, or on any aboveground (extrafloral) structures (Gojmerac, 1980). Nectar is a solution that contains a mixture of sugars and other materials. The major constituents of honey originate from nectar, so honey flavonoids should originate primarily from the nectar. The differences in honey flavour and aroma also arise from differences in the components in the nectar of different floral sources (Rowland *et al*., 1995). Seven flavonoids (glycosides) have been identified from honey floral nectars (Table 1.1), of which only the aglycones are detected in honey.

Table 1.1 Flavonoids identified in the floral nectar used to produce honey

Flavonoid (common name)	Reference
Kaempferol 3-sophoroside; quercetin 3-sophoroside	Gil *et al*., 1995
Hesperidin	Ferreres *et al*., 1993
Isorhamnetin 3-rhamnoside; kaempferol 3-rhamnoside; myricetin 3'-methyl ether, quercetin	Ferreres *et al*., 1996a

Analysis of orange tree nectar revealed that the glycoside, hesperidin, is the major flavonoid detected and, therefore, should be the main source of its aglycone, hesperetin in citrus honey (Ferreres *et al*., 1993). Kaempferol 3-sophoroside contributes 93% of the flavonoids in rosemary nectar, and thus, could be the main source of kaempferol found in rosemary honey (Gil *et al*., 1995). Further, some nectar glycosides from other plant sources have not been detected in the corresponding honeys, suggesting that they are also hydrolysed by honeybee enzymes to produce the corresponding aglycones (Ferreres *et al*., 1993, 1996a). However, studies on unifloral honeys from New Zealand have shown that the origin of their aromatic acids may not be directly related to compounds in the flowers (Tan *et al*., 1988).

1.5.2 Honeydew honey flavonoids and other polyphenols

Some honeys are the excretion of plant-sucking aphids, coccids and scale insects (Gojmerac, 1980), and are termed honeydew honey. This type of honey normally contains 5-18% dry matter, including salivary enzymes. Some compounds occurring in this honey are not found in the host plant, suggesting that they are synthesised by insects, honeybees and/or alteration by microorganisms and/or insects (Gojmerac, 1980). Pinocembrin, an antimicrobially active flavanone, has been found in 11 of 12 honey samples during a study of honeys of different origins (Bogdanov, 1989). In addition, pinocembrin has been found in four Swiss honeys of floral and honeydew origins (Bogdanov, 1989; Anklam, 1998). Forest blossom honeys containing a large amount of honeydew honey have a high concentration of 3,4-dihydroxybenzoic acid (> 5 mg/kg). The presence of this compound enables this honey type to be distinguished from other types of honeys (Joerg and Sontag, 1992, 1993). The concentration of free protocatechuic acid is 3.4-6.8 mg/kg in honeydew honeys, higher than honeys from other sources (Steeg, 1987; Steeg and Montag, 1988c). The aromatic acids in New Zealand manuka honey might originate from the sap of the manuka tree rather than from the nectar (Russell *et al*., 1990). It has been observed that the manuka tree is often infested with a scale insect, *Eriococcus orariensis*, which gives rise to a honeydew that could be collected by the honeybees (Russell *et al*. 1990). However, Weston *et al*. (1999) recently showed that manuka honey was derived from nectar and not honeydew.

1.5.3 Pollen flavonoids

Pollen contains 44% carbohydrates, 22% water and 21% protein (Echigo *et al*., 1986). Pollen is the protein source, not the supply of energy, for honeybees (Gojmerac, 1980). Protein in pollen is essential for body growth, tissue repair, and other normal honeybee functions. Newly emerged adults eat the pollen as a source of protein, vitamins, minerals, and fats, which enables the brood food glands to develop normally (Gojmerac, 1980; Winston, 1987). In addition, pollen has versatile uses for humans (Crane, 1996). Honeybee-collected pollen is produced commercially in at least 18 countries, with Australia alone producing between 60 and 130 t a year (Crane, 1996).

Pollen has been considered a very useful indicator of the botanical origin of honeys, and has almost the whole profile of flavonoids found in honeys sourced from the same plant sources (Amiot *et al*., 1989; Tomás-Barberán *et al*., 1989; Campos *et al*., 1990; Gil *et al*., 1995). Flavonoids in pollen occur as both glycosides and aglycones, and are usually highly hydroxylated, such as for kaempferol (Ferreres *et al*., 1991, 1992). Rosemary pollen has similar concentrations of quercetin and kaempferol 3-sophoroside, while sunflower pollen contains mainly quercetin 3-rutinoside. Some honey flavonoids are lipophilic compounds that are not detected in pollen but are common in plant exudates and resins. However, honey flavonoids can arise partly from pollen flavonoids, with the original glycosides being hydrolysed to yield aglycones by the enzymes present in honeybee saliva (Ferreres *et al*., 1991, 1992). A study by Campos *et al*. (1990) revealed that the flavonoid content reached 0.5% in pollen, whereas Serra-Bonvehi *et al*. (2001) showed that total phenols were more than 0.85%, and flavonoids were more than 0.35% of pollen, with flavonol glycosides dominating. Up to 82% of the pollen samples analysed by Serra-Bonvehi *et al*. (2001) contained at least 14 phenolic components, primarily rutin, myricetin, and cinnamic acids.

Studies by Tomás-Barberán *et al.* (1989) showed that jara pollen collected by honeybees mainly contained quercetin and isorhamnetin 3-glycosides, with trace concentrations of myricetin and kaempferol 3-glycosides. This flavonoid pattern is similar to that of the natural jara pollen, suggesting that the flavonoid pattern could be used as a chemical marker (Tomás-Barberán *et al*., 1989). Similarly, the compound, 8-methoxykaempferol 3-glycoside could be the biochemical marker of almond honeybee pollen, since it is present only in almond pollen, and not in the apple, pear, cherry and plum pollens (Tomás-Barberán *et al*., 1989). Thus, the characteristic flavonoid patterns occurring in honeybee pollens could be used as biochemical markers of plant origin (Tomás-Barberán *et al*., 1989, Campos *et al*., 1997). There are twenty-seven flavonoids, including four aglycones and twenty-three glycosides identified from honeybee pollen (Table 1.2), of which the four aglycones are also components of honey.

Table 1.2 Flavonoids identified in honeybee pollens

Flavonoid and glycoside (common name)	Reference[a]
Apigenin 3-glycoside	1
7-methoxyherbacetin 3-diglycoside	2
8-methoxyherbacetin 3-diglycoside	2
8-methoxyherbacetin 3-glycoside	2
7-methoxyherbacetin 3-sophoroside	2
8-methoxyherbacetin 3-sophoroside	2
Herbacetin glycoside	2
Isorhamnetin 3-glycoside	1
Isorhamnetin 3-sophoroside-diglycoside	2
Kaempferol 3-diglycoside	1, 3
Kaempferol 3-glycoside	1, 3
8-methoxykaempferol 3-glycoside	1
Kaempferol 3-neohesperidoside	2
7-methoxykaempferol 3-neohesperidoside	2
Kaempferol 3-sophoroside	2, 4
Luteolin*	2
Myricetin*	2
Myricetin 3-galactoside	2
Myricetin 3-glycoside	1
Quercetin*	4
Quercetin 3-methyl ether	2
Quercetin 3-diglycoside	1, 2
Quercetin 3-glycoside	1
Quercetin 3-rhamnoside	2
Quercetin 3-rutinoside	4
Quercetin 3-sophoroside	2
Tricetin*	2

[a] 1. Tomás-Barberán *et al*., 1989; 2. Campos *et al*., 1997; 3. Ferreres *et al*., 1998; 4. Ferreres *et al*., 1992.
***** Aglycones found in honey.

1.5.4 Propolis flavonoids

Propolis, also referred to as honeybee glue (Grange and Davey, 1990), is the sticky, resinous material produced by the honeybee (Gojmerac, 1980; Bankova *et al*., 1982). It consists of a mixture of beeswax secreted by the honeybee, plant secretions from the buds and bark of trees, plant exudates from tree wounds, and possibly secretions from other vegetation gathered by the honeybees (Gojmerac, 1980). Thus, it is a highly complex mixture of waxes, resins, balsams, oils, and pollen. The composition of propolis is highly variable, probably related to the variation in the species of

plants from which it is collected (Gojmerac, 1980). Propolis is used by honeybees as a glue to seal the opening of the hives, and as a slow release antiseptic (Gojmerac, 1980; Winston, 1987).

Propolis may contain a hundred different substances, including about 80 flavonoids, which are the main source of its antimicrobial action (Bankova *et al*., 1982, 1983; Greenaway *et al*., 1990; Marcucci, 1995; Park *et al*., 1997). Large proportions of phenolic compounds occur in propolis, which may reach nearly one third of the mass, whereas the flavonoids present in propolis could be in a concentrated form (Serra-Bonvehi *et al*., 1994), which might reach 10% (w/w) of propolis (Campos *et al*., 1990). Pinocembrin alone ranges 4.0-4.6% (w/w) of propolis samples sourced from five countries of different temperate geographical areas (Houghton *et al*., 1995). Honeybees modify the composition of the original propolis through the action of β-glucosidase, which they secrete during propolis collection (Park *et al*., 1997). This enzyme hydrolyses the flavonoid heteroglycosides into aglycones. Moreover, significant differences in the contents of phenolic compounds were found between fresh propolis (20.7-24.7%) and aged propolis, which has ca 20% less phenolic content than that of fresh propolis (Serra-Bonvehi and Ventura-Coll, 2000).

Although propolis is very difficult to remove from hive bodies and frames, some countries now take even greater interest in it than previously. This is because of its various antibiotic properties (Grange and Davey, 1990; Popova *et al*., 2001), and its various biological (Bankova *et al*., 1982, 1983; Sun, 1995), therapeutic, antiviral, and anti-tumour activities (Serra-Bonvehi *et al*., 1994; Marcucci, 1995; Boudourova-Krasteva, *et al*., 1997; Matsuno *et al*., 1997; Park *et al*., 1997, 1998). The antimicrobial properties of propolis (Gojmerac, 1980; Bankova *et al*., 1983; Grange and Davey, 1990; Greenaway *et al*., 1990; Marcucci, 1995; Matsuno *et al*., 1997; Park *et al*., 1998) may be attributable to its high flavonoid content. Among those, pinocembrin and galangin have been proven to be responsible for the antibacterial activity, whereas in addition pinocembrin has fungicidal and local anaesthetic activities (Bankova *et al*., 1983).

Eighty-eight flavonoids (including seventeen isoflavonoids) have been identified in propolis, of which twenty-five aglycones are also detected in honey; forty-six aglycones and/or their glycosides are found only in propolis, with pilloin ($C_{17}H_{15}O_5$) recently being identified in propolis and bud exudate from Poland (Maciejewicz, 2001; Maciejewicz *et al*., 2001). The best indicator for the origin of propolis is its chemical composition, compared to the composition of the hypothetical plant source material (Bankova, *et al*., 1999). Studies on propolis by gas chromatography-mass spectroscopy (GC-MS) (Bankova, *et al*., 1992a) revealed that the biological activities of propolis might differ because of the concentrations of phenolic compounds (Bankova, *et al*., 1992b, 1993). Flavonoids are the characteristic components of propolis (Bankova, *et al*., 1987; Sorkun *et al*., 2001). In a recent study, the flavonoid composition of Egyptian propolis was found to be very diverse, suggesting it may have been gathered from more than one plant source (Christov *et al*., 1998).

Propolis flavonoids from tropical regions are found to be lipophilic, methylated 6-oxygenated flavones (Tomás-Barberán *et al*., 1993b). Correlations between the composition and the place of collection or the bee species have not been found. A distinctive characteristic of the flavonoids in New Zealand propolis is the unusually high proportion of dihydroflavonoids that account for about 70% of the total flavonoids (Markham *et al*., 1996). Studies on propolis from Brazil found some similarities in the qualitative composition of the samples (Marcucci *et al*., 1998), which is rich in derivatives of kaempferol, ranging 3.64-191.19 mg/g (Marcucci *et al*., 2000). In Chilean propolis, the phenolic compounds are similar to those identified in propolis from other south American countries (Munoz *et al*., 2001a), with flavonoids being dominated by flavanones, flavones, and flavonols (Munoz *et al*., 2001b). These findings for propolis may provide useful information about the plant sources of a given geographical region.

Koo and Park (1997) showed that the chemical composition of propolis was dependent on the variety of the bee. Bankova *et al*. (1998) suggested that the variations in the chemical composition of propolis collected by three indigenous bee species are derived from differences in the botanical sources being visited by the different species of bees. However, in *Meliponinae* propolis, neither the bee species nor

the geographical locations have been found to determine its chemical composition and plant source (Velikova *et al*., 2000ab). This could be explained by the fact that *Meliponinae* forages over short distances, and uses the first plant exudate encountered during its flights as the propolis source. In addition, the results of Velikova *et al*. (2000ab) confirm that European propolis is definitely of poplar origin and that in border areas, such as Algeria, where poplars are not always available for propolis collection, other plant sources are used; however, this does not affect the antibacterial properties of propolis.

1.5.5 Beeswax flavonoids

As an animal wax, beeswax is a very stable mixture of chemicals with a distinguished history. Beeswax has had multiple uses for thousands of years and is still used in the cosmetic and in candle-making industries (Gojmerac, 1980; Crane, 1996). It is secreted by the honeybees (Crane, 1996), and is composed of hydrocarbons, esters, free acids, monohydric alcohols, and hydroxy acids (Gojmerac, 1980; Winston, 1987). The main flavonoids detected from beeswax are pinocembrin, pinobanksin, pinobanksin 3-acetate, chrysin, galangin and techtochrysin (Tomás-Barberán *et al*., 1993c). In this study, these flavonoids were already present when wax scales were secreted by bees. Further, the same flavonoids were generally present in honey, propolis and *Populus nigra* bud exudates collected in the same geographical region (Tomás-Barberán *et al*., 1993c), suggesting that the flavonoids of beeswax originate from the same source as those of honey and/or propolis. Thus, analysis of beeswax flavonoids could be used as an adjunct in the detection of beeswax adulterations.

1.6 Honey Phenolics

Honey has been used as a medicine, an additive, and a food for thousands of years (Gojmerac, 1980; Molan, 1992). The antibacterial activity of honey has been examined, particularly relating to the effects of low moisture content, acidity (its pH being between 3.2 and 4.5) and enzymic activity. It is generally agreed that the antibacterial activity of most honey is due to hydrogen peroxide (Molan, 1992). However, if the hydrogen peroxide is destroyed, residual non-peroxide antibacterial activity is observed in several honeys. Part of this activity appears to be due to pinocembrin (Bogdanov, 1984, 1989, 1997; Siess *et al*., 1996) and possibly other phenolic compounds (Russell *et al*., 1990; Weston *et al*., 1999, 2000) with antibacterial properties. Further work needs to be done to fully elucidate the factors responsible for this activity.

Although studies on honeys, honeybees, and the basic composition of honeys started a hundred years ago (Gojmerac, 1980; Winston, 1987), interest in honey phenolic compounds has only recently increased. This is because of their potential roles as biochemical markers for authenticating the geographical (Tomás-Barberán *et al*., 1993a; Martos *et al*., 2000b), or botanical origins of honeys (Berahia *et al*., 1993; Tomás-Barberán *et al*., 1989; Gil *et al*., 1995; Andrade *et al*., 1997ab; Tomás-Barberán *et al*., 2001), or both (Martos *et al*., 1997, 2000b; Anklam, 1998; Anklam and Radovic, 2001).

Some of the components in honey are due to maturation of honey, some are added by honeybees, and some are derived from plants (Gojmerac, 1980; Sun, 1995). During the secondary metabolism of plants, various phenolics are formed (Joerg and Sontag, 1992, 1993). The concentration of these substances differs in various plant species and growing seasons. It also differs according to various agricultural techniques and geographical distribution of the plant.

The distribution of three main phenolic families (benzoic acids, cinnamic acids, and flavonoids) show different profiles in honey from different floral origins, with flavonoids being the most common in floral honeys (Amiot *et al*., 1989; Campos *et al*., 1990). Therefore, a characteristic distribution pattern of phenolic compounds should be found in unifloral honeys sourced from the corresponding plant sources.

1.6.1 Honey flavonoids

Honey flavonoids can originate from nectar, pollen or propolis. Propolis, being a natural constituent of honeycombs, has components that are probably distributed between the relatively lipophilic beeswax and the more hydrophilic honey (Ferreres *et al*., 1992). As the flavonoids are relatively lipophilic, their concentration in honey is much lower that that in propolis (Bogdanov, 1989; Ferreres *et al*., 1992). The flavonoid content is approximately 0.5% in pollen, 10% in propolis and about 0.005 – 0.010% in honey (Ferreres *et al*., 1992). Only flavonoid aglycones (without sugar moieties) seem to be present in propolis and honey, while honeybee pollen contains flavonols in herosidic forms (Anklam, 1998). The flavonoids in honey and propolis have been identified as flavanones and flavanones/flavanols (Anklam, 1998). In general, the flavonoid concentration in honey is approximately 20 mg/kg (Ferreres *et al*., 1994c).

Unlike flavonoids in nectar or pollen, some of the flavonoids found in honey are aglycones with an unsubstituted B ring (Campos *et al*., 1990; Ferreres *et al*., 1993). Figure 1.4 shows some flavonoids and simple phenolics identified in honey. Some compounds in Figure 1.5 were also frequently found in honey, such as kaempferol, myricetin, quercetin, gallic acid and chlorogenic acid. The concentration of honey phenolics varies according to the floral origin, ranging from 5.8 mg/100g in acacia honey to 96 mg/100 g in strawberry honey (Amiot *et al*., 1989). The concentration of phenolic acids ranged 0.1-111.4 mg/100 g honey, with phenolic esters ranging 13-50.4 mg/100 g honey (Joerg, 1996). In addition, the flavonoid concentration was found to be 0.02-2.4 mg/100 g honey in Tunisia (Martos *et al*., 1997). Because of these variations, the browning reaction due to the preferential oxidation of *o*-diphenols is less effective in honeys containing a high concentration of total phenolics (Amiot *et al*., 1989; Campos *et al*., 1990; Siess *et al*., 1996).

There have been 33 flavonoids identified in honey (Table 1.3), of which 11 are also found in the floral nectar, 9 in honeybee pollen, and 25 in propolis (Boudourova-Krasteva *et al*., 1997). Moreover, there are over 70 other phenolics identified from honey and propolis (Bankova *et al*., 1987; Joerg and Sontag, 1992, 1993; Sabatier *et al*., 1992; Joerg, 1996; Andrade *et al*., 1997ab; Anklam, 1998; Tazawa *et al*., 1999). Compounds that have been identified include flavones such as chrysin; flavonols such as kaempferol; flavanones such as hesperetin; and phenolic acids.

In the Northern Hemisphere where poplars (the source of propolis) are native, honeys show flavonoid profiles characterised by the presence of propolis flavonoids (Tomás-Barberán *et al*., 2001). Many honeys, but not Australian honeys, show flavonoid profiles that have their origins in propolis (Tomás-Barberán 1993a; Martos *et al*., 2000b). In contrast, honeys sourced from most equatorial regions and Australia are devoid of propolis-derived flavonoids, but are dominated by flavonoids from other parts of the plant such as nectar and pollen. Nevertheless, several honeys from Central and South America, and New Zealand are exceptions. In these cases, non-native poplar trees have been planted and the honey flavonoid profile is reminiscent of a propolis type profile (Tomás-Barberán *et al*., 1993a).

Table 1.3 Flavonoids identified in various honeys

Flavonoid (common name)	Reference
Apigenin	Berahia *et al*., 1993; Ferreres *et al*., 1994ab
Chrysin	Berahia *et al*., 1993; Ferreres *et al*., 1994ab
Eriodictyol	Delgado *et al*., 1994; Ferreres *et al*., 1994b
Flavone; Flavanonol 7-OH	Berahia *et al*., 1993
Galangin	Sabatier *et al*., 1992; Ferreres *et al*., 1996b
Galangin 3-OMe	Berahia *et al*., 1993; Martos *et al*., 1997
Genkwanin	Ferreres *et al*., 1991, 1992, 1993, 1994ab
Hesperetin	Soler *et al*., 1995; Andrade *et al*., 1997b
Isorhamnetin	Ferreres *et al*., 1991; Gil *et al*., 1995
Kaempferol	Amiot *et al*., 1989; Ferreres *et al*., 1998
Kaempferol 8-OMe	Ferreres *et al*., 1994ab; Martos *et al*., 1997
Kaempferol 3-OMe	Ferreres *et al*., 1991, 1992, 1993, 1994d
Luteolin*; Luteolin 7-OMe	Ferreres *et al*., 1991, 1992, 1993, 1994ab
Myricetin*	Delgado *et al*., 1994; Soler *et al*., 1995
Myricetin 3-OMe	Ferreres *et al*., 1994a, 1996a
Myricetin 3'-OMe	Tomás-Barberán, 1993a.
Myricetin 3,7,4',5'-OMe	Martos *et al*., 1997
Naringenin	Amiot *et al*., 1989; Ferreres *et al*., 1994b
Pinobanksin	Ferreres *et al*., 1996b; Siess *et al*., 1996
Pinobanksin 3-acetate	Martos *et al*., 1997
Pinocembrin	Sabatier *et al*., 1992; Ferreres *et al*., 1996b
Pinostrobin	Berahia *et al*., 1993; Martos *et al*., 1997
Quercetin*	Amiot *et al*., 1989; Ferreres *et al*., 1994ab
Quercetin 3-OMe	Ferreres *et al*., 1991, 1992, 1993, 1994ab
Quercetin 3,7-OMe	Gil *et al*., 1995; Martos *et al*., 1997
Quercetin 3,3'-OMe	Ferreres *et al*., 1991, 1992; Gil *et al*., 1995
Quercetin 7,3'-OMe	Ferreres *et al*., 1994ad; Gil *et al*., 1995
Rhamnetin	Amiot *et al*., 1989
Tectochrysin	Berahia *et al*., 1993; Gil *et al*., 1995
Tricetin*	Ferreres *et al*., 1994a

* Aglycones found in honeybee pollen (Yao, 2002)

1.6.2 New Zealand manuka honey flavonoids

Flavonoids were first identified in New Zealand manuka honey by Weston *et al*. (1999b) and are different from those found in European honeys and propolis (Figure 1.4). While most of the identified phenolic components possess antibiotic activity, they did not individually or collectively account for the nonperoxide antibacterial activity of 'active' manuka honey samples.

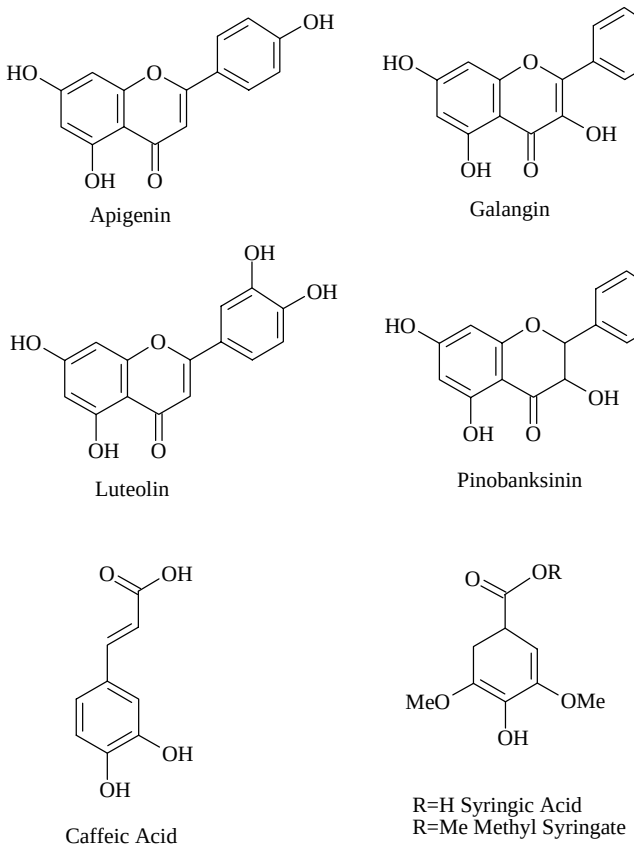


Figure 1.4 Some honey flavonoids and simple phenolic compounds (Weston *et al*., 1999)

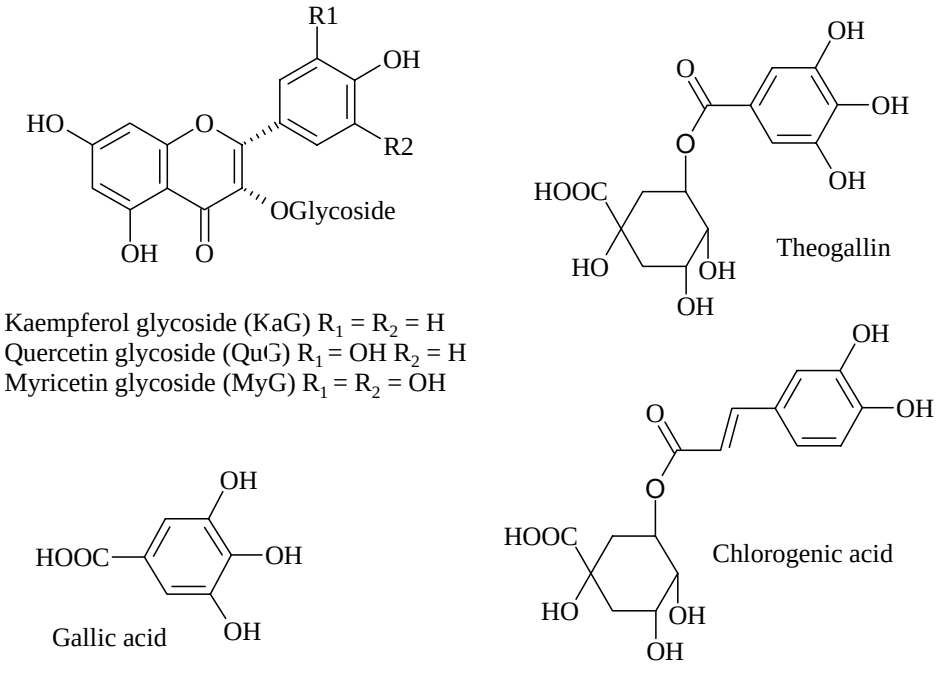


Figure 1.5 Honey flavonoids and phenolic compounds

1.6.3 *Eucalyptus* honey polyphenols

An Australian 'commercial' honey was found to contain three characteristic flavonoids, quercetin, luteolin, and 3-OMe myricetin (Tomás-Barberán *et al*., 1993a), with 3-OMe myricetin being up to 53% of the total flavonoids detected. Moreover, 98% of the flavonoids in this honey were pollen-nectar-derived. Since there was only one Australian honey sample used, there is a need for further investigation of the above compounds as components of Australian honeys.

European *Eucalyptus* honeys show a common and characteristic flavonoid composition, where myricetin, tricetin, quercetin, luteolin and kaempferol are quite constant in their concentrations and relative amounts (Martos *et al*., 2000a). Ellagic acid and the propolis-derived flavonoids pinobanksin, pinocembrin, and chrysin are also present. The contents of these latter nonfloral phenolic compounds were much more variable as would be expected from their propolis origins. Australian *Eucalyptus* honeys show the same flavonoid patterns (myricetin, tricetin, quercetin, luteolin, and kaempferol) as those for the European *Eucalyptus* honeys (Martos *et al*., 2000ab). Of these flavonoids, tricetin is the main marker of river red gum honey, whereas luteolin is a marker of mallee box honey. The main difference between the flavonoid profiles of Australian and European *Eucalyptus* honeys is that in the Australian honey the propolis-derived flavonoids pinobanksin, pinocembrin, and chrysin are seldom found, or are in much smaller concentrations when present (Martos *et al*., 2000b).

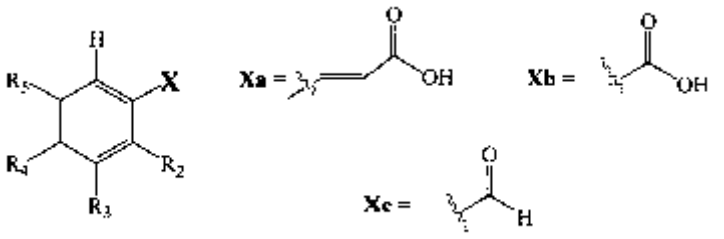
Flavonoids are known as antioxidants in lipid-aqueous and lipid-food systems, thus honey can be used as a source of natural antioxidants in foods (Sabatier *et al*., 1992). It has been shown that higher flavonoid (or total phenolics) concentrations in honey produce better antioxidant capacity (Gheldof *et al*., 2002). Certain flavonoids such as pinocembrin, pinobanksin, chrysin, quercetin, kaempferol, myricetin, and hesperetin are responsible for the antioxidant capacity of honeys (Fahey and Stephenson, 2002). Honey has been used in meat products, yoghurt, bakery, salad dressing and other foods as antioxidants.

1.6.4 Phenolic acids in honey

Phenolic acids (or aromatic carbonic acids) are a subclass of phenolic compounds and arise from the phenyl-propanoid metabolism in plants (Anklam, 1998). Phenolic compounds possessing at least two phenol subunits are the flavonoids, which account for approximately two-thirds of the dietary phenols and have been previously discussed. Those compounds possessing three or more phenol subunits are referred to as the tannins (hydrolysable and non-hydrolysable). In general, phenolic acids are the phenols that possess one carboxylic acid functionality and account for the remaining one-third (Robbins, 2003). However, when describing plant metabolites, phenolic acids refer to a distinct group of organic acids (Figure 1.6) which contain three distinguishing constitutive carbon frameworks: the hydroxycinnamic (Xa), hydroxybenzoic (Xb) structure and aldehyde (Xc) analogue (Robbins, 2003). The basic skeleton remains the same but the numbers and position of the hydroxyl groups on the aromatic ring create the variety (Robbins, 2003).

Like flavonoids, phenolic acids are present in all plants. Notably, cinnamic and benzoic acids derivatives, such as caffeic, *p*-coumaric, vanillic, ferulic and protocatechuic acids exist in virtually all plant foods, including fruits, vegetables and grains, and are physically dispersed throughout the plant in seeds, leaves, roots and stems (Robbins, 2003). Other phenolic acids are found in selected foods or plants such as gentisic and syringic acids (Robbins, 2003). Caffeic acid is one of the most prominent naturally occurring cinnamic acids (Robbins, 2003). Phenolic acids have been associated with the colour, sensory qualities, nutritional and antioxidant properties of foods (Robbins, 2003). Therefore, there is an increasing awareness and interest in the antioxidant behaviour and potential health benefits of phenolic acids. Due to the antioxidant characteristics, the food industry has investigated the concentration and profile of phenolic acids, their effect on fruit maturation, prevention of enzymic browning, and their roles as food preservatives (Robbins, 2003).

Little is known about the absorption and metabolism of phenolic acids despite the increasing interest in their potential health benefits (Robbins, 2003). Only the metabolic fates of caffeic, ferulic, chlorogenic and sinapic acids have been explored (Robbins, 2003). Chlorogenic acid is most likely metabolised by the colonic microflora, while 11-25% of ferulic acid ingested is excreted in urine as free ferulic acid or as glucuronide conjugate (Robbins, 2003). Caffeic acid can selectively block the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma, and allergic reactions (Robbins, 2003). Furthermore, caffeic acid and some of its esters might possess anti-tumour activity against colon carcinogenesis (Robbins, 2003). Currently, caffeic acid derivatives (e.g. dicaffeoylquinic and dicaffeolytartaric acids) are being investigated for their antiviral therapy because they can selectively inhibit HIV-1 integrase which catalyses the integration of viral DNA into the host cromatin (Robbins, 2003).



R_2	R_3	R_4	R_5	X	code	common name
H	H	H	H	a	**1**	cinnamic acid
–OH	H	H	H	a	**2**	*o*–coumaric acid
H	H	–OH	H	a	**3**	*p*–coumaric acid
H	–OH	H	H	a	**4**	*m*–coumaric acid
H	$-OCH_3$	–OH	H	a	**5**	ferulic acid
H	$-OCH_3$	–OH	$-OCH_3$	a	**6**	sinapic acid
H	–OH	–OH	H	a	**7**	caffeic acid
H	H	H	H	b	**8**	benzoic acid
–OH	H	H	H	b	**9**	salicylic acid
H	H	–OH	H	b	**10**	*p*–hydroxybenzoic acid
H	$-OCH_3$	–OH	H	b	**11**	vanillic acid
H	$-OCH_3$	–OH	$-OCH_3$	b	**12**	syringic acid
H	–OH	–OH	H	b	**13**	protocatechuic acid
–OH	H	H	–OH	b	**14**	gentisic acid
–OH	–OH	–OH	–OH	b	**15**	gallic acid
H	$-OCH_3$	$-OCH_3$	H	b	**16**	veratric acid
H	$-OCH_3$	–OH	$-OCH_3$	c	**17**	syringaldehyde
H	$-OCH_3$	–OH	H	c	**18**	vanillin

Figure 1.6 Structure of prominent naturally occurring phenolic aids (Robbins, 2003)

The most common phenolic acids found in honeys are benzoic acids and their esters, and cinnamic acids and their esters (Sabatier *et al*., 1992). Phenolic acids in honey can be analysed by GC after methylation or HPLC with coulometric detection (Anklam, 1998). A careful evaluation of the patterns concerning phenolic acids, phenolic esters and aromatic carbonyl compounds is useful in determining the botanical origin of honeys (Anklam, 1998). Many studies have found that different types of honeys possess distinctive profiles of phenolic acids with different concentrations, varying 10-1000 μg/100g honey (Anklam, 1998). In fact, the two phenolic acids, ellagic and abscisic acids, have been proved to be powerful markers for the floral origin of *Erica*-type heather honey (Ferreres *et al*., 1994a; Ferreres *et al*., 1996). The hydroxycinnamates, caffeic, *p*-coumaric and ferulic acids are dominant in chestnut, sunflower, lavender and acacia honeys.

Most of the studies of phenolic acids in honeys have been explored mainly in European countries, but some similar studies have been done in Australia and New Zealand in recent years (Yao, 2002). Cabras *et al*., (1999) have shown that homogentisic acids, with average concentration of 378 ± 92

mg/kg, were found in strawberry-tree (*Arbutus unedo*) honey but not detected in any of the different monofloral honeys, suggesting that this phenolic acid could be used as a marker of strawberry-tree honey. In addition, rape honeys have been characterised by phenylpropanoic acid and have a high concentration of methyl syringate (Anklam, 1998), whereas buckwheat honey has a higher concentration of 4-hydroxybenzoic acid and no phenylacetic acid (Anklam, 1998). Heather honeys have a high concentration of benzoic acid, phenylacetic acid, mandelic acid and β-phenyllactic acid (Anklam, 1998). In robinia honey, only methyl syringate is present (Anklam, 1998). Even so, while flavonoid profiles of honeys have been extensively investigated, not as much information on individual phenolic acids of honeys (Soler *et al.*, 1995; Tomás-Barberán *et al, 2001*) are presently available.

1.7 Enzymes in Honey

Enzymes naturally occur in honeys, including glucose oxidase, catalase and peroxidase (McKibben and Engeseth, 2002). These enzymes are known to have antioxidant properties. Honey enzymes are currently used in the food industry. However, it is more economical to extract the enzymes from other sources, such as microoganisms (Franklin, 1997).

Glucose oxidase is a highly specific enzyme, commonly extracted from the fungi *Aspergilus niger* and *Penicillium notatum*, which catalyses the oxidation of glucose to glucono-1,5-lactone in the presence of oxygen (Uhlig, 1990), which then spontaneously hydrolyses non-enzymically to gluconic acid using molecular oxygen and releasing hydrogen peroxide (Chaplin and Bucke, 1990).

$$\text{glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{glucono-1,5-lactone}$$

$$\text{glucono-1,5-lactone} + H_2O \longrightarrow \text{gluconic acid}$$

The most important application of glucose oxidase is in the manufacture of gluconic acid and its salt, as well as being particularly useful in medicine, biotechnology, and the detergent, textile, leather, photographic, pharmaceutical, food, feed and concrete industries (Crueger and Crueger, 1990). Glucono-1,5-lactone is more popular in the food products; it is used for baking powders, bread mixes, sausage manufacturing and coagulant of soybean protein in Japan (Crueger and Crueger, 1990).

Glucose oxidase is particularly useful in food preservation for the removal of either glucose or oxygen from foodstuffs in order to prevent off-colour and off-flavour from the Maillard reaction (Uhlig, 1998), and to improve their storage capability (Chaplin and Bucke, 1990). It is generally used in conjunction with catalase which can hydrolyse the toxic by-product, hydrogen peroxide (Woods and Swinton, 1995). These two enzymes are used in the removal of glucose from egg-white before drying for use in the baking industry (Chaplin and Bucke, 1990). Additionally, the glucose oxidase/catalase system can stabilise many food products through removal of oxygen, and thus reduce enzymic browning in beers, fruit juices, wines, cheeses, gluten and mayonnaises (Chaplin and Bucke, 1990; Uhlig, 1998). Through the action of glucose oxidase, milk can be acidified to produce Mozzarella and cottage cheese by the generation of gluconic acid from glucose (Uhlig, 1998). More recently, glucose oxidase can improve the dough strength, particularly in combination with sulphydryl oxidase (Hamer, 1995).

1.8 Glycemic Index of Honey

Australian honeys have a greater range of flavours and colours than honeys from other countries, and vary in the amounts of fructose and glucose they contain (Holt *et al*., 2002). Consequently, it is likely that different types of Australian honey will produce different blood glucose and insulin responses (Holt *et al*., 2002). Holt *et al*. (2002) conducted a study to compare the effects of equal-carbohydrate portions of eight different types of Australian honey on postprandial blood glucose and insulin

responses. The study found that the mean glycemic index (GI) and insulin index (II) are significantly correlated ($r = 0.88$, $p<0.01$), while the glucose content of the honey is significantly associated with their mean GI and II values, with $r = 0.79$ and $r = 0.77$ respectively at $p < 0.05$. However, concentrations of fructose, sucrose and maltose in honeys were not significantly related to the honeys mean GI and II values, nor their organic acid contents and osmolarity (Holt *et al*., 2002).

Table 1.4 summarises the fructose contents and the GI's of different honeys, using glucose as the reference food (glucose GI = 100). The GI's of different honeys vary greatly, from 32 to 87. It is concluded that some substances in honey such as flavonoids and phenolic acids may reduce glycemia (Holt *et al*., 2002).

Table 1.4 Glycemic Index and the fructose contents of 10 honey samples with a serving size of 25 g

Types of honey	Fructose content (%)	Country of Origin	Glycemic Index (glucose = 100)
Locust	na	Romania	32
Unspecified type	na	Canada	87
Bush, sugar bag	na	Australia	43
Pure Capilano	na	Australia	58
Iron bark	34	Australia	48
Yellow box	46	Australia	35
Yapunyah	42	Australia	52
Stringy bark	52	Australia	44
Salvation Jane	32	Australia	64
Red Gum	35	Australia	46

(Holt *et al*., 2002)

1.9 Honey and Lipid Oxidation

Lipid oxidation occurs when oxygen reacts with lipids in a series of free radical chain reactions that lead to complex chemical changes (Fukumoto and Mazza, 2000). Lipid oxidation is known as a major deteriorative factor in the meat system during storage. Lipid hydroperoxides, and their breakdown products have been implicated in some adverse effects, including off-flavour and off-colour development, possible reaction with certain food components such as amino acids and proteins with concomitant losses of nutritional value and functionality, and a variety of health-related problems such as heart disease, cancers (Mckibben and Engeseth, 2002), atherosclerosis, and the aging process (Fukunoto and Mazza, 2000). Therefore, many antioxidants are used extensively in processed meat products to inhibit lipid peroxidation and thus increase shelf-life. However, most of these antioxidants are synthetic and may cause adverse health effect on humans if consumed in large quantities. With today's consumer trends of demanding 'natural food', there is strong interest in the development of natural antioxidants (McKibben and Engeseth, 2002).

One study has shown that honeys from different floral sources are effective at reducing lipid oxidation in cooked, ground turkey patties held at 4 ^{o}C. Buckwheat honey was the most effective of the honeys tested since it provided the highest antioxidant concentration (McKibben and Engeseth, 2002). In this research, honey appears to be a good source of natural antioxidants in addition to its properties of contributing various flavour notes to the meat. Honey is also found to be more effective than α–tocopherol and butylated hydroxytoluene (BHT) at inhibiting lipid oxidation (McKibben and Engeseth, 2002).

1.10 Honey and Enzymic Browning

Some enzymes present in fruits and vegetables have the ability to catalyse oxidation of phenols and lead to enzymic browning of foods (Shahidi and Naczk, 1995). The enzymic reaction occurs during the aging or senescence of fruits and vegetables or as a result of injury to plant products. Polyphenol oxidase (PPO) is activated as a result of disruption of cell integrity, and when the contents of plastid and vacuole are missed (Shahidi and Naczk, 1995). Although PPO action is useful in the production of black tea and raisins, PPO enzymic browning can cause an adverse reaction during the processing of fruit juice and also in fresh fruits and vegetables, often associated with undesirable brown colours, off-flavours, and negative effects on the nutritional value (Chen *et al.*, 2000). In addition, some technological treatments such as crushing, slicing, cutting, extraction, handling, storage conditions, low temperature and thawing cause enzymic browning (Shahidi and Naczk, 1995).

Since consumer demand for natural additives has been increasing, it is desirable to use naturally occurring compounds to inhibit browning (Oszmianski and Lee, 1990). Recently, honey has been investigated as an alternative to the use of chemical preservatives (e.g. sulphites) for browning in the food processing (Chen *et al*., 2000). Honey has been successfully applied to apple slices and grape juice (Martyniuk, 1994; Oszmianski and Lee, 1990) to prevent browning. This is because the presence of substantial amounts of phenolic compounds, particularly, *t*-cinnamic acid and *p*-coumaric acid make a significant contribution to the inhibitory effect of honey on PPO activity and the browning reaction (Martyniuk, 1994). Therefore, in addition to inhibiting browning of fruits and vegetables, honey may contribute to improving appearance, enhancing flavour, and preserving nutritive value (Chen *et al*., 2000).

1.11 Antimicrobial Properties of Honey

Honey flavonoids have been found to produce non-peroxide antibacterial activity, such as for pinocembrin (Sabatier *et al*., 1992). Swiss honeys are known to possess this antimicrobial flavonoid (Anklam, 1998).

The factors responsible for the antimicrobial activity of honey are high osmolarity, acidity, and particularly hydrogen peroxide (Bogdanov, 1997) which is formed from the oxidation of glucose by the enzyme glucose oxidase, during the period when honey is ripening (Weston *et al.*, 1999). Glucose oxidase originates from hypopharyngeal glands of honeybees (Taormina *et al*., 2001). When hydrogen peroxide is removed by adding catalase, some honeys still show significant antibacterial activity (Allen *et al*., 1991), with this activity being referred to as non-peroxide antibacterial activity. The non-peroxide factors of honeys include lysozyme, phenolic acids and flavonoids (Taormina *et al*., 2001). Bogdanov (1997) suggested that the major part of the non-peroxide antibacterial activity may be of honeybee origin, while part may be of plant origin. Wahdan (1998) also suggested that flavonoids and phenolic acids might be a part of the antibacterial activities of honey.

Nevertheless, there are differences in the antibacterial activity of different unifloral honeys (Bogdanov, 1997). Notably, the greatest activity is from manuka honey (*Leptospermum scoparium*), originating from New Zealand, particularly the East Cape region of the North Island. The high antibacterial activity of New Zealand manuka honey is in many cases due entirely to the non-peroxide components. Manuka honey contains several phenolic compounds, including methyl syringate and syringic acid (Russell *et al*., 1990; Weston *et al*., 1999). By examining the antimicrobial activity against *Staphylococcus aureus,* methyl syringate was found to possess significant antibacterial activity.

An Australian honey from a very similar source (*Leptospermum polygalifolium*) has also recently been found to possess a high level of non-peroxide antibacterial activity (Weston, 2000), though the cause of the non-peroxide antibacterial activity is still unclear and requires further investigation.

The non-peroxide antibacterial activity is more heat and light insensitive than the hydrogen peroxide, and remains intact after storage of honey for long periods. Therefore, some authors have found that the non-peroxide antibacterial activity is more important than the hydrogen peroxide in terms of antibacterial effects (Taormina *et al*., 2001). However, the contribution to antibacterial properties of non-peroxide antibacterial activity may be smaller than that of hydrogen peroxide (Weston, 2000).

The flavonoids, such as pinobanksin, pinocembrin, chrysin and galangin, were identified by Weston *et al*. (1999) in manuka honey and were found to be different from those in European honeys and propolis (Weston *et al*., 1999). While most of these flavonoids possessed antibiotic activity, they did not individually or collectively account for the activity of "active" manuka honey (Weston *et al*., 1999). Essentially all of the activity was associated with the carbohydrate fraction of the honey. However, studies on the oligosaccharide compositions showed no differences between the manuka honey samples with residual non-peroxide antibacterial activity and those without this activity (Weston and Brocklebank, 1999). Weston *et al*. (2000) reported that flavonoids and phenolic acids, particularly those derived from propolis, exhibit weak antibacterial activity. Therefore, the flavonoids and other phenolics, whether originating from nectar, pollen or propolis, are only partly responsible for the observed non-peroxide antibacterial properties of "active" samples of New Zealand manuka honey (Weston *et al*., 1999), or may not account for the activity of this honey type at all (Weston *et al*., 2000).

1.12 Authentication of Honey using Phenolic Compounds

1.12.1 Authentication of the geographical origin of honey using phenolic compounds

Most of the work on honey phenolic compounds carried out in Europe involved honeys mainly from European countries (Steeg and Montag, 1987, 1988abc; Speer and Montag, 1987; Hausler and Montag, 1990).

Tomás-Barberán *et al*. (1993a) have compared the flavonoid profiles of various honey samples from different regions in the world. Different types of honeys including propolis, nectar and pollen types of honeys were analysed for the comparison. In general, these honeys from the Northern Hemisphere, where poplars (the source of propolis) are native, show flavonoid profiles characterised by the presence of propolis flavonoids. In contrast, honeys sourced from most equatorial regions and Australia are devoid of propolis-derived flavonoids, but contain flavonoids from other parts of the plant, except for several honeys from Central and South America, and New Zealand (Tomás-Barberán *et al*., 1993a). In these latter cases, non-native poplar trees have been planted and the honey flavonoid profile is reminiscent of a propolis type profile (Tomás-Barberán *et al*., 1993a). These researchers suggested that the imported honeybee colonies might locate poplars in gardens or agro-industrial exploitations. Therefore, studies of honey flavonoid profiles could be related to honey geographical origins.

A study by Bogdanov (1989) has found that the main flavonoid occurring in Swiss honeys is pinocembrin, whereas the concentration of flavonoids in Swiss origin propolis averages 10%, including pinocembrin. This result suggests that flavonoids of Swiss honeys may be propolis derived. Whether pinocembrin could be the marker for the geographical origin has still to be proven, with data from more samples being needed. Similarly, an Australian commercial honey was found to contain three characteristic flavonoids, quercetin, luteolin, and myricetin 3-methyl ether (Tomás-Barberán *et al*., 1993a) amongst the nine flavonoids identified in this honey, with myricetin 3-methyl ether being up to 53% of the total flavonoids detected. Moreover, 98% of the flavonoids in this honey were pollen-nectar-derived. Since there was only one Australian honey used for the comparison, there is a strong need for further investigation of the above compounds as markers of the geographical origins of Australian honeys.

Ferreres *et al*. (1994a) showed that the four characteristic flavonoids found in Portuguese heather honey could be markers of botanical origin for this honey. However, these researchers suggested that further studies on more samples are necessary to confirm whether these flavonoids could also be markers of geographical origins. The flavonoid patterns present in ten Spanish La Alcarria honey samples were found to be very similar, suggesting the possible geographical origin of the honeys (Ferreres *et al*., 1991). Although the concentration and type of individual flavonoids might be different from one honey type to another, the flavonoid profiles or patterns of the honeys from a particular geographical area may be characteristic to that area. Ferreres *et al*. (1992) showed a close correlation between the flavonoid patterns of honey and propolis, and suggested that flavonoid analysis could be more useful in geographical origin determinations than in botanical origin studies, since pollen was not the main source of honey flavonoids.

Concerning the effects of the bee species on honey flavonoid patterns, Vit *et al*. (1997) analysed the flavonoid profiles of honeys from two types of bees to evaluate bee type-dependent differences. Although the number of flavonoid types present in individual samples varied to some extent, the diversity of flavonoids in tropical honeys is lower than that previously measured in temperate honeys. Thus, flavonoids cannot be used as entomological markers to differentiate between the honeys produced by two bee types (Vit *et al*., 1997). A further study by Vit and Tomás-Barberán (1998) on the flavonoids in stingless bee honey showed that honeys from the same geographical origin had very similar flavonoid profiles. Thus, it was proposed that the flavonoid profiles of a therapeutic product called honey eyedrops, made of droplets of stingless bee honey and used for eye treatment, could be used for honey authentication and quality control, and for the determination of the geographical origin of the honey.

A recent study (Martos *et al*., 2000b) on Australian *Eucalyptus* honeys has compared the geographical variations in the flavonoid profiles between Australian and European *Eucalyptus* honeys. This study revealed that the main difference is that in the Australian *Eucalyptus* honeys, the propolis-derived flavonoids, such as pinobanksin, pinocembrin, and chrysin are either seldom found or in very small concentrations (Martos *et al*., 2000b). In contrast, the European *Eucalyptus* honeys are relatively rich in these propolis-derived flavonoids (Martos *et al*., 2000a). Therefore, flavonoid profiles could be used as the geographical markers for monofloral *Eucalyptus* honeys from different regions.

1.12.2 Authentication of the botanical origin of honey using phenolic compounds

Studies on the floral or botanical origins of honeys using flavonoids have been more extensively conducted than the studies on the geographical origins of honeys using flavonoids. This is due to the botanical origin of a honey being one of its main quality parameters, with the price usually related to it (Ferreres *et al*., 1992, 1993). Thus, the determination of the floral origin is recognised as a very important task in honey quality control (Ferreres *et al*., 1992, 1993, 1994c; Guyot *et al*., 1998, 1999). The usual practice is to identify the floral source of honey by pollen analysis (Ferreres *et al*., 1993, 1994c). However, pollen analysis may not apply to some species of plants such as citrus since the amount of pollen present in the honey is generally small and very variable due to the maximal secretion of nectar not coinciding with anther maturation (Ferreres *et al*., 1993, 1994c). Thus, the analysis of the component that is only characteristic to citrus nectar becomes essential in the determination of the botanical origin of citrus honey. A chemical approach to the characterisation of honey floral source might be more accurate and more readily available (Tan *et al*., 1989). The use of the analysis of flavonoids and other phenolic compounds in the identification of honeys has been suggested (Amiot *et al*., 1989). This technique has since been used as a tool for studying the floral origins of various honeys (Ferreres *et al*., 1991, 1992, 1993; Guyot *et al*., 1998, 1999).

Citrus honey. Hesperetin is a possible marker for the floral origin of citrus honey, since it has not been detected in honeys of any other origins (Ferreres *et al*., 1993, 1994c). The volatile compound methyl anthranilate has been suggested as floral marker for both citrus nectar and honey (Ferreres *et al*., 1994c). This may be because the concentration of methyl anthranilate in citrus honey is very high

and it has been found to give a distinctive flavour to citrus honey (Serra-Bonvehi and Ventura-Coll, 1995). However, there is no correlation between the concentrations of this compound and hesperetin (Ferreres *et al*., 1994c). Methyl anthranilate in citrus honey ranges 1.44-3.60 mg/kg, while hesperetin ranges 0.28-0.84 mg/kg (Ferreres *et al*., 1994c). Since flavonoids are stable non-volatile secondary metabolites, they are little affected by environmental factors. Thus, hesperetin could be used as an additional marker in the determination of honey of citrus origin (Ferreres *et al*., 1994c).

Heather honey. Studies by Ferreres *et al*. (1994a) showed that flavonoids found in Portuguese heather honeys contain the common trioxygenated B ring featured structure. This attribute has not been found in flavonoids in any other honeys of different floral origins, and suggests these flavonoid compounds are markers of the botanical origin for heather honeys (Ferreres *et al*., 1994a). However, the nectar sourced from the heather flowers contains a different pattern of flavonoids (Ferreres *et al*., 1996a). The total flavonoids in Portuguese heather honey ranges 0.06-0.5 mg/ 100 g honey, with myricetin, myricetin 3-methyl ether, myricetin 3'-methyl ether, and tricetin being the most characteristic flavonoids (Ferreres *et al*., 1994a; Anklam, 1998). In addition, there are five phenolic acids that appear to be characteristic markers for Portuguese heather honeys: ellagic, *p*-hydroxybenzoic, syringic, *o*-coumaric and *p*-coumaric acids (Andrade *et al*., 1997ab). Ellagic acid is present in all heather honeys in significant concentrations, ranging 0.1-0.6 mg/100 g honey (Ferreres *et al*., 1996a). Also, abscisic acids range 2.5-16.6 mg/100 g in the heather honeys and nectars (Ferreres *et al*., 1996b). Therefore, ellagic and abscisic acids, along with myricetin 3'-methyl ether, have been suggested as potential markers for the floral origin of heather honey because they were not found in any other monofloral honeys (Ferreres *et al*., 1996ab). The only exception is a French honey sample of the botanically related *Calluna Ericaceae* that contains ellagic acid.

Sunflower honey. Studies on French sunflower honey revealed that these unifloral honeys are rich in flavonoids, which account for 42% of total phenolic compounds (Amiot *et al*., 1989, Sabatier *et al*., 1992). Among these phenolic compounds, there are 37% cinnamic acids and 21% benzoic acids (Amiot *et al*., 1989, Sabatier *et al*., 1992). The ratio of the flavonoid content to the content of all phenolic compounds of 44 sunflower honeys from different French regions was not significantly different (Sabatier *et al*., 1992). Thus, the flavonoid profiles and content may be promising indicators of the floral origin of French sunflower honey. Soler *et al*. (1995) suggested that the amount of an individual flavonoid or phenolic acid could be related to a certain floral origin. Sabatier *et al*. (1992) found five main flavonoids, pinocembrin, chrysin, pinobanksin, galangin and quercetin, together with tectochrysin and kaempferol as minor flavonoids in French sunflower honey. Amiot *et al*. (1989) suggested that French sunflower honey is characterised by a higher concentration of quercetin, cinnamic acid and chlorogenic acid. A further study by Berahia *et al*. (1993) identified six flavone-flavonols and four flavanone-flavanols in this type of French honey, with pinocembrin dominating the flavonoid profile. Tomás-Barberán (*et al*., 2001) found quercetin characterised sunflower honey.

Eucalyptus honey. In a recent study, European *Eucalyptus* honeys showed a common and characteristic HPLC profile in which the flavonoids, myricetin, tricetin, quercetin, luteolin and kaempferol are quite constant, suggesting they are characteristic of European *Eucalyptus* honeys (Martos *et al*., 2000a). The propolis-derived flavonoids pinobanksin, pinocembrin and chrysin were also detected in most of these *Eucalyptus* honey samples. The concentrations of these phenolic compounds were much more variable due to their propolis origins. The three main flavonoids, myricetin, tricetin and luteolin, of European *Eucalyptus* honeys have not been identified as floral markers in any other honeys previously analysed or reported, suggesting that these could be useful markers (Martos *et al*., 2000a).

Australian unifloral *Eucalyptus* honeys showed the same flavonoid patterns as those for European *Eucalyptus* honeys (Martos *et al*., 2000ab). The flavonoids myricetin, tricetin, quercetin, luteolin and kaempferol, which were previously suggested as floral markers of European *Eucalyptus* honeys (Martos *et al*., 2000a), have been found in all the Australian unifloral *Eucalyptus* honey samples examined in a recent study (Martos *et al*., 2000b). Thus, flavonoid analysis could be used as an objective method for determining the botanical origin of *Eucalyptus* honeys, no matter the

geographical origin (Martos *et al*., 2000b). Among the flavonoids analysed in Australian *Eucalyptus* honeys, tricetin has been shown to be the main marker for river red gum (*E. camaldulensis*) honey, whereas luteolin has shown to be the main marker for mallee box (*E. pilligaensis*) honey. These results indicate that species-specific differences in samples of Australian *Eucalyptus* honey could be detected using flavonoid analysis (Martos *et al*., 2000b).

Rosemary honey. Rosemary nectar contains kaempferol 3-sophoroside as the only significant constituent (Gil *et al*., 1995). The concentration of kaempferol in rosemary honeys has been found to be 0.4-1.2 mg/kg, with its coefficient of variation being much smaller than those observed for the other flavonoids (Gil *et al*., 1995). This result suggests that kaempferol in rosemary honey may originate from rosemary nectar. However, Soler *et al*. (1995) suggested that the presence of kaempferol in rosemary honey could not be considered as proof of its floral origin because it could originate from other flower nectars. Rosemary pollen has been found to contain a significant amount of kaempferol glycosides, suggesting that the pollen could be an alternate source for the kaempferol found in rosemary honey (Ferreres *et al*., 1998). Further studies have revealed that the concentration of kaempferol in rosemary honey is 0.33-2.48 mg/kg, but no correlation has been found between the kaempferol concentrations present in the honey and pollen samples (Ferreres *et al*., 1998). Therefore, kaempferol derivatives present in rosemary pollen do not contribute significantly to kaempferol concentrations present in rosemary honey, and nectar may be the botanical origin of this compound (Ferreres *et al*., 1998). At least, the absence of kaempferol in rosemary honey or its presence in small concentrations could be considered as evidence of a different floral origin (Gil *et al*., 1995).

Other unifloral honeys. In other unifloral honeys, the concentration of an individual flavonoid can be related to a certain floral origin. For example, *Calluna* honey is characterised by ellagic acid; alder honey by 8-methoxykaempferol (Soler *et al*., 1995); thyme honey by rosmarinic acid (Andrade *et al*., 1997ab; Guyot *et al*., 1998); and lavender honey by luteolin (Ferreres *et al*., 1993, 1994bc; Delgado *et al*., 1998), naringenin and gallic acid (Andrade *et al*., 1997ab; Guyot *et al*., 1998). Phenolic acids are characteristic of various honeys, such as phenylpropanoic acid for rape honey and 4-hydroxybenzoic acid for buckwheat honey (Steeg and Montag, 1988a). In addition, it is also possible to characterise rape honey using methyl syringate and orange blossom honey using methyl 4-hydroxybenzoate (Joerg, 1996). Similarly, fir honey is easily identified by its concentration of methyl ferulate, and strawberry-tree honey by homogentisic acid (Cabras *et al*., 1999).

The characteristic propolis-derived flavonoids pinocembrin, pinobanksin and chrysin are present in most European honeys in variable concentrations (Tomás-Barberán *et al*., 2001). Comparison of the total phenolics in four types of monofloral honeys revealed that the highest concentrations are in lotus honeys and the lowest in sunflower honeys (Vivar-Quintana *et al*., 1999), although pinobanksin and pinocembrin from both propolis and pollen-nectar were found in all four types of honeys. Berahia *et al*. (1993) showed GC-MS analysis provided flavonoid patterns of honeys that could be used in the determination of floral origin. Finally, when the total flavonoid pattern can not be used for authenticating the floral origin of honeys, the concentration of an individual or the total flavonoid content may be considered for the differentiation (Soler *et al*., 1995; Anklam, 1998).

A recent study on European unifloral honeys by Tomás-Barberán *et al*. (2001) revealed five floral markers for lime-tree and chestnut honeys, respectively; one marker for rapeseed honey; three markers for heather honeys; and six markers for *Eucalyptus* honeys. These unidentified specific markers show characteristic UV spectra and are characteristic of the corresponding honeys (Tomás-Barberán *et al*., 2001). In conclusion, individual and/or total flavonoids, their concentrations and/or patterns appear to be very promising biochemical makers in the floral sourcing of honey.

1.13 Analysis of Phenolic Compounds in Honey

Most studies have focused on the analysis of honey flavonoids by using the high performance liquid chromatography (HPLC) method, because this method provides high accuracy and reproducibility for routine work. Yet separation of honey flavonoids by other alternatives, such as capillary electrophoresis, has been developed.

1.13.1 HPLC analysis of polyphenols

Since flavonoid analysis is a very promising technique for the study of botanical and geographical origins (Ferreres *et al*., 1994c), health implications require detailed knowledge of the flavonoid content of the food supply (Merken and Beecher, 2000). HPLC was first used for the determination of flavonoids in 1976 by Fisher and Wheaton (Sivam, 2002).

The main problem in the analysis of flavonoids from honey is the very high sugar content, which makes the extraction of flavonoids and sample preparation for HPLC analysis difficult (Ferreres *et al.*, 1994c). Liquid-liquid partitions produce inconvenient interphases which do not permit the complete recovery of flavonoids. However, this problem has been solved by using the non-ionic polymeric resin Amberlite XAD-2 (Ferreres *et al*., 1994c).

During sample preparation, honey usually requires solid-phase extraction (SPE). Glycosides of flavonoids frequently required hydrolysis to remove the sugar moiety, such as acidic, basic or enzymic hydrolysis (Sivam, 2002). Honey samples can be mixed with 5 parts acidified water (pH 2 with HCl) (Ferreres *et al*., 1994c). HPLC separation systems are usually binary, with an aqueous acidified solvent (solvent A) such as aqueous formic acid, and an organic solvent (solvent B) such as methanol. Duration of the HPLC runs are generally no longer than 1 h, with equilibrium between runs. Flow rate are usually 1.0 – 1.5 mL/min but generally 1.0 mL/min. Columns are usually maintained close to ambient temperature and the injection volume generally is 1 – 100 μL (Sivam, 2002).

UV with photodiode array (PDA) detection is the standard method used for the detection of flavonoids. Since flavonoids are polyphenols, two UV absorption bands are characteristic of this type of compound. Band II, with a maximum in the 240-285 nm range, is believed to arise from the A ring, whereas Band I with a maximum in the 300-550 nm range, presumably arises from the B ring (Sivam, 2002). Quantification of flavonoids is another advantage of HPLC with UV detection – a good estimate of the flavonoid concentration can be determined by comparing integration data for the honey chromatogram with that for a known amount of a readily available standard (Sivam, 2002).

1.13.2 LC-MS analysis of honey polyphenols

Although HPLC is the most common method in determining honey flavonoids, one important technique that has been applied successfully is thermospray ionisation mass spectrometry (TSP-MS). It can be coupled with high performance liquid chromatography (HPLC) to form liquid chromatography-electrospray mass spectrometry (LC-MS) that has been developed to separate flavonoids and flavonoid glycosides, and to obtain molecular weight information (Constant *et al*., 1997; Gheldof *et al*., 2002). LC-MS offers a significant advance not only because of increased stability and efficiency of ionisation of the electrospray interface over the thermospray interface but also because it directly ties the biological activity to both the molecular weight of the active flavonoid glycoside and its aglycoside (Constant *et al*., 1997). Collision-induced dissociation can be used to obtain the molecular masses of the flavonoid aglycones. Thus, the mass of a flavonoid glycoside and its aglycone can now be determined in a mixture, and it is not necessary to isolate the components (Constant et al., 1997).

A number of phenolic compounds (flavonoids and phenolic acids) have been successfully identified from various honeys using LC-MS, including *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-

coumaric acid, *cis*- and *trans*-abscisic acid, pinobanksin, quercetin, pinocembrin, kaempferol, chrysin, galangin and other unknown phenolic compounds (Gheldof *et al*., 2002). The technique of rapidly characterising the biologically active constituents in a mixture to establish if they have been previously identified is known as dereplication (Constant *et al*., 1997). LC-MS provides a powerful tool for the dereplication of a particular sample (Constant *et al*., 1997).

1.13.3 Other methods of analysis

Apart from the HPLC and LC-MS methods, there have been some other alternatives developed for determination of honey flavonoids. Recent publications have shown that capillary electrophoresis (CE) can successfully identify honey flavonoids (Delgado *et al*., 1994; Ferreres *et al*., 1994b). CE was first applied to the separation of biological molecules such as proteins and nuclei acids (Ferreres, *et al*., 1994b). The technique of micellar electrokinetic chromatography (MEKC) or micellar electrokinetic capillary chromatography (MECC) has been developed, which further widened the applications of CE to include separations of neutral (Ferreres *et al*., 1994c) and charged substances (Sivam, 2002).

The MEKC separation of neutral species is accomplished by using surfactants in the running buffer. Partitioning in and out of the micelles formed in the surfactant affects the separation (Sivam, 2002). It has been successfully used for separation of phenolic acids and flavonoids (Sivam, 2002). Perhaps MEKC is the only electrophoretic technique possessing this strength (Sivam, 2002). Therefore, CE is a new tool in the separation sciences which provides high separation efficiency (Delgado *et al*., 1994). Most flavonoid separations have been achieved by MECC, suggesting that CE is a very promising technique for flavonoid separations (Ferreres *et al*., 1994b).

2. Aims and Objectives

2.1 Overall Project Aims

To increase knowledge of the health and nutritional values of Australian honey by determining the identity and levels of antioxidant flavonoids and other polyphenols in straightline samples of three species-specific floral types of Australian honey, with a view to such data being used to promote increased use of honey by consumers and the food industry.

2.2 Project Objectives

- To extract antioxidant flavonoids and other polyphenols from straightline samples of three species-specific floral types of Australia honey, namely yapunyah, leatherwood and Salvation Jane honeys.

- To identify antioxidant flavonoids and other polyphenols from straightline samples of three species-specific floral types of Australia honey, namely yapunyah, leatherwood and Salvation Jane honeys.

- To quantify antioxidant flavonoids and other polyphenols from samples of three species-specific floral types of Australia honey, namely yapunyah, leatherwood and Salvation Jane honeys.

3. Methodology

3.1 Honey Samples

Upon collection, honey samples were stored in the dark in their original containers at –18 °C to –24 °C, to minimise enzymic and chemical activity, until required for analysis.

Species-specific floral honey is the honey that is produced from one species of plant, e.g. one tree, bush or ground cover. This is achieved by the beekeeper pursuing a particular floral species for honey production by hive location and season of production. Careful siting of hives near to one species of plant during its flowering and honey flows of nectar enables beekeepers to control the foraging of their honeybees and, thus, produce high quality 'straightline' samples of species-specific floral types of honey.

In this study, the aroma, taste and colour characteristics, together with information about season, hive location and available floral sources were utilised by supplying beekeepers and honey packers to accurately identify the floral source of the honey samples examined. This procedure is the standard honey-sourcing method utilised by the Australian honey industry.

Individual Australian beekeepers and honey packing companies supplied the following honey samples:

- Five samples of yapunyah (*Eucalyptus ochrophloia*) honey which were sourced from different geographical areas of the flood plains in the 'Channel Country' of Western Queensland (2001-2002). The yapunyah tree flowers from early April until late October and is a major producer of honey in the winter months (150 kg/hive).

- Ten samples of leatherwood (*Eucryphia lucida*) honey which were sourced from different geographical areas of western Tasmania (March 2003). The leatherwood tree is one of the species of tree making up the understorey of cool-climate rainforests found in remote valleys of this mountainous area. The leatherwood tree flowers from mid January until early March.

- Six samples of Salvation Jane (*Echium plantagineum*) honey (1997-1998) were sourced from central NSW. Paterson's Curse or Salvation Jane is a winter annual native to Mediterranean Europe and North Africa, and is a toxic weed (due to alkaloids) of grazed pasture in Australia. However, it is called 'Salvation' Jane because its important to honey production and has been responsible for delivering a large amount of honey every year in Australia.

3.2 Honey Sample Preparation

For the honey preparation, each honey sample was liquefied by heating the entire container containing raw honey in a hot water (70 °C) bath, followed by cooling to room temperature. Next, liquid honey was centrifuged to separate the liquid honey from the beeswax. After centrifugation, the beeswax at the top of the liquid honey was easily removed, and the remaining liquid honey was ready for extraction.

3.3 Chemical Solvents and Standards

The solvents used for the column chromatography were deionised water, and analytical grade hydrochloric acid (HCl) and methanol. The solvents used for re-extraction of flavonoids were deionised water and analytical grade ethyl acetate. The solvents used for HPLC analysis were HPLC grade methanol, deionised water and analytical grade formic acid. The authentic chemical compounds

used for the identification and quantification in this study are listed in Table 3.1, along with their commercial origin.

Table 3.1 Standard compounds for the identification and quantification of honey

Common Name	Chemical name	Source	
8-methoxy-kaempferol	3,5,7,4'-Tetrahydroxy-8-methoxyflavone	1	
Abscisic acid	5-(1-Hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl)-3-methyl pentadienoic acid	2	
Apigenin	5,7,4'-Trihydroxyflavone	1	
Caffeic acid	3(3,4-Dihydroxyphenyl)-prop-2-enoic acid	3	331-39-5
Chlorogenic acid	3-Caffeoyl quinic acid	3	327-97-9
Chrysin	5,7-Dihydroxyflavone	1, 4, 5	480-40-0
p-Coumaric acid	3(4-Hydroxyphenyl)-prop-2-enoic acid	3	501-98-4
Ellagic acid	4,4',5,5',6,6'-Hexahydroxydiphenic acid 2,6,2',6'-dilactone	3	476-66-4
Ferulic acid	3(3-Methoxy-4-hydroxyphenyl)-prop-2-enoic acid	3, 5	1135-24-6
Galangin	3,5,7-Trihydroxyflavone	1	
Gallic acid	3,4,5-Trihydroxybenzoic acid	3	
Hesperetin	5,7,3'-Trihydroxy-4'-methoxyflavanone	3	520-33-2
Isorhamnetin	3,5,7,4'-Tetrahydroxy-3'-methoxyflavone	1	
Kaempferol	3,5,7,4'-Tetrahydroxyflavone	1, 3	520-18-3
Luteolin	5,7,3',4'-Tetrahydroxyflavone	1	491-70-3
Pinocembrin	5,7-Dihydroxyflavanone	4	480-39-7
Quercetin	3,5,7,3',4'-Pentahydroxyflavone	1, 3, 4	117-39-5

1. Laboratory of Phytochemistry, Department of Food Science and Technology, CEBAS (CSIC), Murcia, Spain.
2. Laboratory of Plant Physiology, Department of Botany, The University of Queensland, Brisbane, QLD, Australia.
3. Sigma Chemical Co., St Louis, MO, USA.
4. Extrasynthese, 69726 Genay Cedex, France.
5. Aldrich Chemical Co., Milwaukee, WI, USA.

3.4 Summary of Procedure for the Isolation of Flavonoids and Phenolic Acids

Figure 3.1 summarises methodology for the isolation of flavonoids and phenolic acids from honey using Amberlite XAD-2 resin, followed by the identification and quantification of these compounds using HPLC-diode array detection and LC-MS analysis. This procedure contains some steps based on those reported by Martos *et al*. (1997) and Yao (2002). However, the initial purification procedure of the commercial Amberlite XAD-2 resin was developed during this project. The requirement/procedure for swelling of the resin prior to use was supplied by Dr Tomas-Barberan (pers. comm 2002).

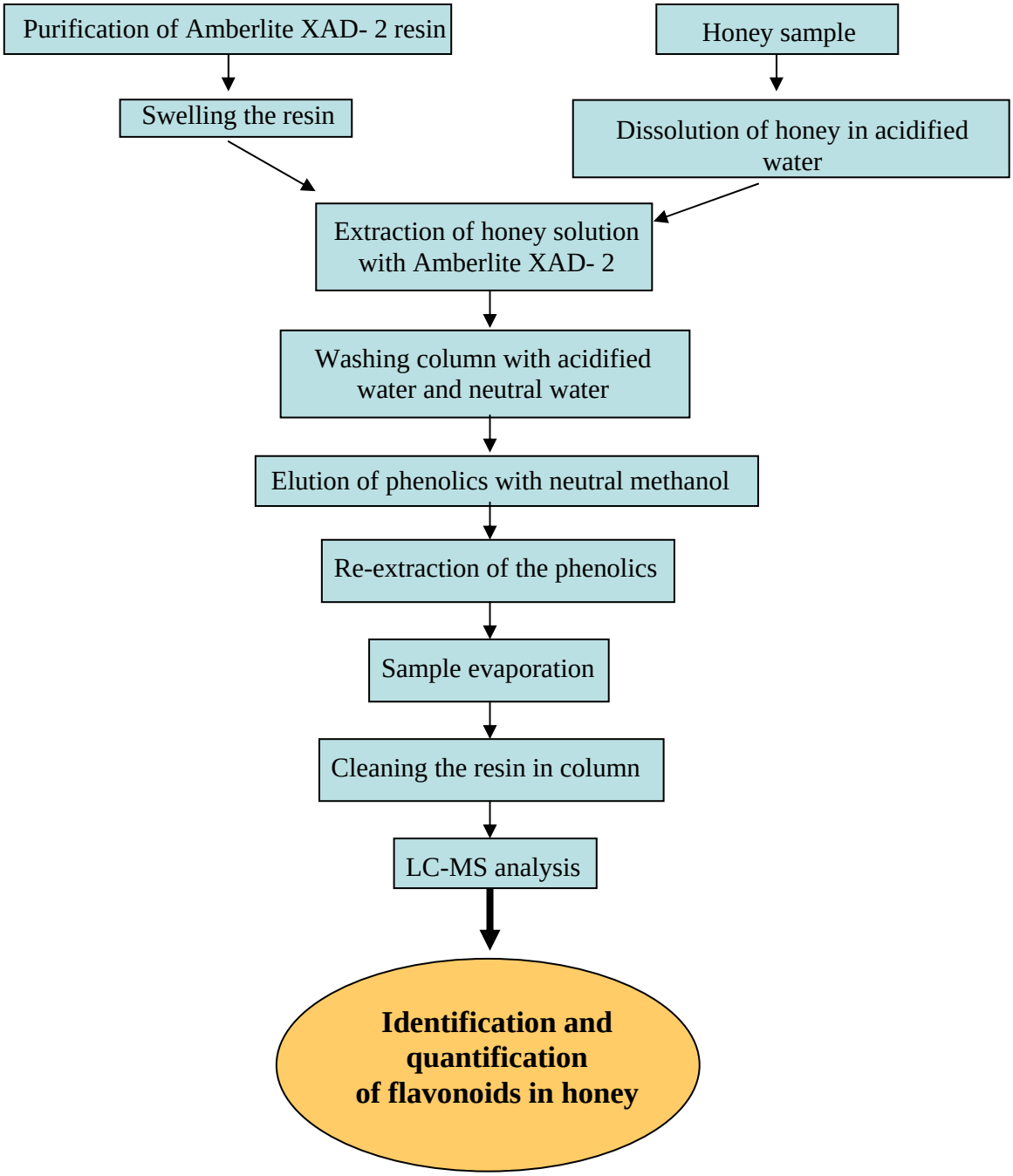


Figure 3.1 Extraction and analysis of flavonoids and phenolic acids from honey

3.5 Purification of the Amberlite XAD-2 Resin

Prior to use, the commercial Amberlite XAD-2 (Supelco, Bellefonte, PA, USA, pore size 9 nm, particle size 0.3-1.2 mm) resin was cleaned with acetone and methanol by soxhlet extraction to ensure it was free from contamination. If the resin had already been cleaned using these solvents and a soxhlet (as below), this intensive cleaning procedure was not repeated.

Soxhlet extraction thimbles were filled with Amberlite XAD-2 resin (25 g) and covered with cotton wool. There were 12 soxhlet apparatus used at a time during the extraction. The extraction with acetone was done for 4 h. In the second stage of the soxhlet extraction, the soxhlet extraction was repeated with methanol for 4 h.

When the soxhlet extraction was completed, the Amberlite XAD-2 resin (300 g) from the thimbles was emptied into a 1000 mL beaker, and then this beaker was filled with methanol to cover the Amberlite XAD-2 resin. A stirring rod was used to stir the slurry gently for a short while and the methanol was decanted off. This was repeated 5–10 times until the methanol was clear. The purpose of this rinsing was to wash away all the residual acetone to ensure minimal contamination with foreign particles from the Amberlite XAD-2 resin. Next, a UV spectrophotometer was adjusted to a wavelength of 400 nm to check the turbidity. One pure methanol sample was prepared for a blank and a sub-sample from the last methanol washings was tested. The washing was repeated until the absorption of the methanol washings was <0.1 abs. The remaining methanol in the beaker with the cleaned Amberlite XAD-2 resin was removed using a Buchner funnel and filter paper. The Amberlite XAD-2 resin does not need to be completely dry before use. All the clean Amberlite XAD-2 resin was kept in a screw-capped plastic bottle and stored in the fridge because the resin is susceptible to microbial growth.

3.5.1 Washing of Amberlite XAD-2 resin (previously soxhlet cleaned as above) after use for every 10 honey extractions

Solid Amberlite XAD-2 resin (200 g) was placed in a 500 mL beaker, covered with methanol, stirred with a stirring bar on a magnetic stirred (with no heating) for 10 min, and filtered through a Buchner funnel. This process was repeated four more times.

3.6 Swelling of the Amberlite XAD-2 Resin

The original method for extraction was carried out as described previously by Martos *et al*. (1997) and Yao (2002). A solution of methanol (200 mL) and deionised water (200 mL) (equivalent to 1:1 volume) was added to cover 150 g of Amberlite XAD-2 resin (Supelco, Bellefonte, PA, USA, pore size 9 nm, particle size 0.3-1.2 mm) in a flask, which was then stoppered to avoid contamination or loss of solvents. The flask was left to stand overnight to ensure complete swelling of the resin. No mixing or stirring was required as this disturbs the resin structure. The solvent was filtered off on a Buchner funnel using filter paper. This solid Amberlite XAD-2 resin was then washed with water (300 mL) on the Buchner funnel. Finally, the solid resin (150 g) was immediately added to the acidified standard solution or honey solution as described below**.** The resin was not filtered after swelling until the honey had been liquefied and centrifuged as above, and the honey was dissolved in acidified water as detailed below.

3.7 Recovery and Repeatability Study of Polyphenol Standards Extracted/Filtered using Amberlite XAD-2 Resin

A mixture of authentic samples of 9 polyphenols was extracted/filtered through Amberlite XAD-2 resin in a column to determine the recovery of these compounds and the repeatability of the extraction/filtration methods for polyphenols.

3.7.1 Preparation of the mixture of authentic standards

A mixture of authentic compounds in methanol (5 mL), at a concentration of 100 μg/mL for each of the following 9 phenolic compounds (previously found in honeys) was prepared:

> gallic acids, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, ellagic acid, quercetin, hesperetin, and chrysin.

3.7.2 Experiment to optimise the method for extracting polyphenols using Amberlite XAD-2 resin – 'mixing' or 'elution' methods

Two identical standard solutions (5 mL of 100 μg/mL for the 9 polyphenols) were prepared as described in Section 3.7.1.

One of these solutions was mixed ('mixing method') with the Amberlite XAD-2 resin (150 g) prior to adding to the glass column and eluting. The other solution was added to the Amberlite XAD-2 resin already loaded in the glass column ('elution method') prior to eluting.

For the 'mixing method', it was based on a modification of the method of Yao (2002), which was based on the method of Martos *et al*. (1997, 2000ab). Here, a standard mixture (5 mL of 100 μg/mL for each compound) was thoroughly mixed with acidified deionised water (400 mL, adjusted to pH 2 with concentrated HCl). The solution was then mixed with 150 g (larger than the amount of resin used by Yao, 2002 and Martos *et al*., 1997, 2000ab) of pure Amberlite XAD-2 (Supelco, Bellefonte, PA, USA, pore size 9 nm, particle size 0.3-1.2 mm) and stirred with a magnetic stirrer for 10 min, which was considered enough time to absorb honey phenolics with a recovery > 80% (Tomás-Barberán *et al*., 1992, Martos *et al*., 1997). The slurry of Amberlite XAD-2 resin and honey was then packed in a glass column (42 × 3.2 cm).

For the 'elution method', a column of pure Amberlite XAD-2 (Supelco, Bellefonte, PA, USA, pore size 9 nm, particle size 0.3-1.2 mm) in acidified water was prepared by packing a slurry of the pure Amberlite XAD-2 resin (150 g) in acidified deionised water (adjusted to pH 2 with concentrated HCl) into a glass column (42 × 3.2 cm). Next, the standard mixture (5 mL of 100 μg/mL for each compound) was thoroughly mixed with acidified deionised water (400 mL, adjusted to pH 2 with concentrated HCl) and then eluted through the resin in the column to absorb the phenolic compounds onto the Amberlite XAD-2 resin.

The columns prepared by the 'mixing method' or the 'elution method' were then washed with acidified water (pH 2 with HCl, 250 mL), followed by deionised water (300 mL). The phenolic compounds absorbed on the column were then eluted with neutralised methanol (900 mL; larger volume than used previously by Yao, 2002 and Martos *et al*., 1997, 2000ab). This extract was concentrated to dryness on a rotary evaporator under reduced pressure (40 °C). Next, the residue was redissolved in deionised water (5 mL) and extracted with ethyl acetate (3 x 5 mL) (instead of diethyl ether as for Martos *et al*., 1997, 2002 a,b; ethyl acetate was used because it is more polar than diethyl ether, indicating that more flavonoids and other phenolic compounds can be extracted). The ethyl acetate extracts were then combined, and the ethyl acetate was removed by flushing with nitrogen while being warmed on a hotplate. The dried residue was then redissolved in 5 mL of methanol (HPLC grade) to ensure the same dilution of phenolic compounds added to the Amberlite XAD-2

extraction/filtration. The solution was filtered through a 0.45 μm membrane filter prior to HPLC analysis.

3.7.3 Experiment to determine the recovery of polyphenol standards extracted/filtered using Amberlite XAD-2 resin, and the repeatability of the extraction method

The recovery/repeatability for the mixing method was determined using the final method below.

A standard mixture (5 mL of 100 μg/mL for each compound) was thoroughly mixed with acidified deionised water (400 mL, adjusted to pH 2 with concentrated HCl). The solution was then mixed with 150 g of purified Amberlite XAD-2 (Supelco, Bellefonte, PA, USA, pore size 9 nm, particle size 0.3-1.2 mm) and stirred with a magnetic stirrer for 10 min. The slurry of Amberlite XAD-2 resin and honey was then packed (poured) in a glass column (42 × 3.2 cm).

Next, the column was washed with acidified water (pH 2 with HCl, 250 mL), followed by deionised water (300 mL). The phenolic compounds absorbed on the column were then eluted with neutralised methanol (900 mL). This extract was concentrated to dryness on a rotary evaporator under reduced pressure (40 °C). Next, the residue was redissolved in deionised water (5 mL) and extracted with ethyl acetate (3 x 5 mL). The ethyl acetate extracts were combined, and the ethyl acetate was removed by flushing with nitrogen while being warmed on a hotplate. The dried residue was then redissolved in 5 mL of methanol (HPLC grade) to ensure the same dilution of phenolic compounds added to the Amberlite XAD-2 extraction/filtration. The solution was filtered through a 0.45 μm membrane filter prior to HPLC analysis.

The above procedure was repeated a further 2 times on identical standard mixtures (5 mL of 100 μg/mL for each compound). The concentrations of each of the nine polyphenols before and after elution through the Amberlite XAD-2 resin were compared and a percentage recovery obtained. In addition, the percentage coefficient of variance [%CV; (standard deviation/mean) x 100] for the concentration of each eluted polyphenol was determined as part of these three trials.

3.7.4 HPLC-PDA and LC-MS analysis of the standard phenolic compounds

The standard mixtures in methanol before (5 mL of 100 μg/mL for each compound) and after (dried extract diluted to 5 mL) extraction/filtration through the Amberlite XAD-2 resin were analysed using HPLC on a Waters 2690 HPLC with a computer-controlled system. Samples (20 μL) were injected using a Waters 2690 Auto Injector. The phenolic compounds were detected using a Waters 996 photodiode array (PDA) detector to obtain the UV spectra of the various phenolic compounds. For analysis by PDA detection, UV spectra were recorded from 220-420 nm at a rate of 1 spectra/s and at a resolution of 2.4 nm. In particular, the chromatograms were monitored at 290 nm and 340 nm, since the majority of the honey flavonoids and phenolic acids show their UV absorption maxima around these two wavelengths (Martos *et al*., 1997).

The standard mixtures in methanol (5 mL of 100 μg/mL for each compound) were analysed for compound identification and quantification using LC-MS on a Waters 2690 HPLC coupled to a Micromass ZMD Mass Spectrometer (MS) with an electrospray ionisation (ESI) and a computer-controlled system operated by MassLynx v 3.5 software. The samples were scanned at m/z values of 120 to 620 using electrospray negative ionization, with the settings listed in Table 3.2.

Table 3.2 Conditions of electrospray ionisation-mass spectrometry (ESI-MS)

Parameter	Value / Unit
Capillary voltage	2.50 V
Cone voltage	25 V
Extractor voltage	5 V
Rf lens	0.5
source temperature	100 °C
desolvation temperature	350 °C

The column used was a reversed phase C-18 column, Merck LiChroCART 125-4 Cartridge (15 cm × 4 cm, particle size 5 µm). The mobile phases were 0.25% formic acid and 2% methanol in ultra high quality (UHQ) water (solvent A) and methanol (solvent B), at a constant solvent flow rate of 1 mL/min.

The method used by Yao (2002) was adapted at the following two points:

- Gallic acid eluted together with the void peak using the method of Yao (2002), which impeded a good quantification of gallic acid. Thus, the starting concentration of methanol for the gradient elution was lowered from 30% as used by Yao (2002) to 10%, to produce a good separation of gallic acid and the void peak.

- For LC-MS analysis, the high acid concentration used in the mobile phase (5% formic acid by Yao, 2002) greatly reduced the ionization and thus the sensitivity of the MS. Therefore, 4 different acid concentrations were tested and the best (0.25% formic acid) was chosen.

Thus, the mobile phases were 0.25% formic acid and 2% methanol in ultra high quality (UHQ) water (solvent A) and methanol (solvent B), at a constant solvent flow rate of 1 mL/min.

- Solvent A: 0.25% formic acid and 2% HPLC grade methanol in UHQ water.
- Solvent B: 100% HPLC grade methanol.

The following gradient was used:

10% methanol (B) flowed through the column isocratically with 90% solvent A for 15 min; and then was increased to 40% methanol (B) by 20 min; to 45% methanol (B) by 30 min; to 60% methanol (B) by 50 min; to 80% methanol (B) by 52 min; to 90% methanol by 60 min; followed by isocratic elution with 90% methanol (B) until 65 min. Finally the gradient was changed to 10% methanol by 68 min, and this composition was held until 73 min. This gradient program is summarised in Table 3.3.

A 15% isopropanol solution in triple deionised water (TDI) was used to wash the column between HPLC runs.

Table 3.3 Solvent gradient for HPLC

Time (min)	Solvent A (%)	Solvent B (%)
0 – 15	90	10
20	60	40
30	55	45
50	40	60
52	20	80
60 – 65	10	90
68 – 73	90	10

Solvent A: 0.25% formic acid and 2% HPLC grade methanol in UHQ water.
Solvent B: 100% HPLC grade methanol.

3.7.5 Identification of phenolic compounds in the standard mixture

UV spectra, retention times and selected ion recording (SIR) mass spectra (with negative ionisation) of the peaks for the authentic standards were recorded. In this study, based on previous research (Martos *et al*., 1997, 2000ab), the flavonoid profiles were the HPLC chromatograms recorded at 340 nm, because most of the flavonoids have their maximum absorption around this wavelength. Similarly, the profiles of phenolic acids were the HPLC chromatograms recorded at 290 nm, where most of the phenolic acids and flavanones have their maximum absorption. However, UV-PDA is not suitable for detection of gallic acid and chrysin either at 290 nm or 340 nm. LC-MS analysis using the molecular weights (M) of polyphenols (Tables 3.4 and 3.5) was more suitable. Here, the mass spectral selected ion recording (SIR) mode is more suitable for these compounds, and was applied based on their molecular weight (M) – 1 $[M-H]^-$, since negative ionization was used (Table 3.6).

Table 3.4 Polyphenols previously found in honey

Name of compound	Mol. Weight (M)	Mol. Formula
Caffeic acid	180	$C_9H_8O_4$
Chlorogenic acid	354	$C_{16}H_{18}O_9$
Chrysin	254	$C_{15}H_{10}O_4$
Ellagic acid	302	$C_{14}H_6O_8$
Ferulic acid	194	$C_{10}H_{10}O_4$
Galangin	270	$C_{15}H_{10}O_5$
Gallic acid	170	$C_7H_6O_5$
Isorhamnetin	316	$C_{16}H_{12}O_7$
Kaempferol	286	$C_{15}H_{10}O_6$
Kaempferol- 8-methylether	316	$C_{16}H_{12}O_7$
Luteolin	286	$C_{15}H_{10}O_6$
Myricetin	318	$C_{15}H_{10}O_8$
o-Coumaric acid	164	$C_9H_8O_3$
p-Coumaric acid	164	$C_9H_8O_3$
Pinobanksin	272	$C_{15}H_{12}O_5$
Pinobanksin 5-methylether	286	$C_{16}H_{14}O_5$
Pinocembrin	256	$C_{15}H_{12}O_4$
Quercetin	302	$C_{15}H_{10}O_7$
Quercetin-3-methylether	316	$C_{16}H_{12}O_7$
Tricetin	302	$C_{15}H_{10}O_7$

Table 3.5 Phenolic acids and flavonoids found in foods

M.W. (M)	Formula	Name
138	$C_7H_6O_3$	*p*-Hydroxybenzoic acid
138	$C_8H_{10}O_2$	Tyrosol
142	$C_6H_6O_4$	Dihydroxybenzoic acid
148	$C_9H_8O_2$	Cinnamic acid
152	$C_8H_8O_3$	Vanillin
152	$C_8H_8O_3$	4-Hydroxyphenylacetic acid
154	$C_8H_{10}O_3$	Hydroxytyrosol
164	$C_9H_8O_3$	*o*/*m*/*p*/-Coumaric acid
168	$C_8H_8O_4$	Homogentisic
168	$C_8H_8O_4$	Vanillic acid
170	$C_7H_6O_5$	Gallic acid
180	$C_9H_8O_4$	Caffeic acid
182	$C_9H_{10}O_4$	DL-Hydroxyphenyllactic acid
189	$C_{10}H_7NO_3$	α-Cyano-4-hydroxycinnamic acid
194	$C_{10}H_{10}O_4$	Ferulic acid
198	$C_9H_{10}O_5$	Syringic acid
224	$C_{11}H_{12}O_5$	Sinapic acid
242	$C_{11}H_{14}O_6$	Elenolic acid
254	$C_{15}H_{10}O_4$	Chrysin
256	$C_{15}H_{12}O_4$	Pinocembrin
268	$C_{16}H_{12}O_4$	Tectochrysin
270	$C_{15}H_{10}O_5$	Apigenin
270	$C_{15}H_{10}O_5$	Galangin
270	$C_{16}H_{14}O_4$	Pinocembrin-7-methylether
272	$C_{15}H_{12}O_5$	Pinobanksin
272	$C_{15}H_{12}O_5$	Naringenin
284	$C_{16}H_{12}O_5$	Genkwanin
284	$C_{16}H_{12}O_5$	Galangin-3-methylether
286	$C_{15}H_{10}O_6$	Luteolin
286	$C_{15}H_{10}O_6$	Kaempferol
288	$C_{15}H_{12}O_6$	Eriodictyol
290	$C_{15}H_{14}O_6$	Catechin
302	$C_{16}H_{14}O_6$	Hesperetin
302	$C_{15}H_{10}O_7$	Tricetin
302	$C_{15}H_{10}O_7$	Quercetin
302	$C_{14}H_6O_8$	Ellagic acid
316	$C_{16}H_{12}O_7$	Quercetin-3-methylether
316	$C_{16}H_{12}O_7$	Isorhamnetin
316	$C_{16}H_{12}O_7$	8-Methoxy-kaempferol
318	$C_{15}H_{10}O_8$	Myricetin
330	$C_{17}H_{14}O_7$	Quercetin-3-3-dimethylether
330	$C_{17}H_{14}O_7$	Quercetin-3-7-dimethylether
354	$C_{16}H_{18}O_9$	Chlorogenic acid
362	$C_{18}H_{18}O_8$	Rosmarinic acid
374	$C_{19}H_{18}O_8$	Myricetin-3,7,4',5'-tetramethylether

Table 3.6 Identification of honey polyphenols

Phenolic acids	Name of Compounds	Trace source
	Gallic acid	SIR-169
	Chlorogenic acid	PDA 290 nm SIR 353
	Ferulic acid	PDA 290 nm SIR 193
	Caffeic acid	PDA 290 nm SIR 179
	p-Coumaric acid	PDA 290 nm SIR 163
	Ellagic acid	PDA 340 nm SIR 301
Flavonoids	Quercetin	PDA 340 nm SIR 301
	Quercetin-3-methylether	PDA 340, SIR 315
	Chrysin	SIR 253
	Hesperetin	PDA 340 SIR 301
	Myricetin	PDA 340 SIR 317
	Tricetin	PDA 340, SIR 301
	Pinobanksin	PDA 340, SIR 271
	Pinobanksin 5-methylether	PDA 340, SIR 285
	Kaempferol	PDA 340, SIR 285
	Kaempferol- 8-methylether	PDA 340, SIR 315
	Pinocembrin	PDA 340, SIR 255
	Isorhamnetin	PDA 340, SIR 315
	Galangin	PDA 340, SIR 268
	Luteolin	PDA 340, SIR-285

*SIR involves $[M-H]^-$

3.8 Final Extraction Method of Honey Samples with Amberlite XAD-2 Resin

Based on the above experiments involving extraction of standard solutions of polyphenols, a final optimised method was selected for analysis of the honey samples.

Liquefied honey (80 g) was thoroughly mixed with acidified deionised water (400 mL, adjusted to pH 2 with concentrated HCl) for 30 min (with no heating) until completely dissolved. The resulting honey solution was then filtered through filter paper under vacuum to any remove solid particles.

Next, the filtrate was mixed with 150 g of clean, swelled Amberlite XAD-2 (Supelco, Bellefonte, PA, USA, pore size 9 nm, particle size 0.3-1.2 mm) (larger than the amount of resin used by Yao, 2002 and Martos *et al*., 1997, 2000ab) and stirred slowly with a magnetic stirrer for 60 min. The slurry of the Amberlite XAD-2 resin and solvent was then packed (poured) in a glass column (42 × 3.2 cm), and the resin was washed (10 mL/min) with acidified water (pH 2 with HCl, 250 mL), followed by rinsing with deionised water (300 mL at 10 mL/min) to remove all sugars and other polar constituents of honey.

The phenolic compounds absorbed on the column (Ferreres *et al*., 1991) were then eluted with neutralised methanol (1000 mL; adjusted pH 6-7 with 0.1 M NaOH) (larger volume than used previously by Yao, 2002 and Martos *et al*., 1997, 2000ab). This extract was concentrated to dryness on a rotary evaporator under reduced pressure (40 °C).

Next, the residue was redissolved in deionised water (5 mL) and extracted with ethyl acetate (5 mL × 3; instead of diethyl ether as used by Yao, 2002 and Martos *et al*., 1997, 2000ab). The ethyl acetate extracts were combined and then evaporated to dryness by flushing with nitrogen while being warmed on a hotplate.

3.9 Cleaning the Resin While Still in the Glass Column

When the above elution was completed, the Amberlite XAD-2 resin in the glass column was then washed with of methanol (250 mL) to clean the resin (this methanol was discarded). The resin was then left in the column until required for extraction of the next honey sample.

Then, immediately prior to the next honey extraction, 400 mL of a solution of methanol/deionised water (1:1) was used to wash the column (the washings were discarded), prior to removing the resin from the column into a beaker, followed by subsequent swelling overnight. The column was kept wet at all times, ready for the next sample.

3.10 HPLC-PDA and LC-MS Analysis of the Honey Extracts

3.10.1 Preparation of standards

A series of four mixed standard solutions were prepared with concentrations of 2.5 μg/mL, 10 μg/mL, 50 μg/mL and 100μg/mL for each of caffeic acid, quercetin and chrysin in methanol.

3.10.2 Preparation of honey extracts prior to HPLC and LC-MS analysis

The dried residue was redissolved volumetrically with 1 mL of methanol (HPLC grade) and filtered through a 0.45μm nylon membrane filter into a 2 mL glass vial, ready for HPLC analysis.

3.10.3 HPLC-PDA and LC-MS conditions

The solutions of the honey extracts in methanol (1 mL) were analysed using HPLC on a Waters 2690 HPLC with a computer-controlled system. Samples (20 μL) were injected using a Waters 2690 Auto Injector. The phenolic compounds were detected using a Waters 996 photodiode array (PDA) detector to obtain the UV spectra of the various phenolic compounds. For analysis by PDA detection, UV spectra were recorded from 220-420 nm at a rate of 1 spectra/s and a resolution of 2.4 nm. In particular, the chromatograms were monitored at 290 nm and 340 nm, since the majority of the honey flavonoids and phenolic acids show their UV absorption maxima around these two wavelengths (Martos *et al*., 1997).

The column was a reversed phase column, Merck LiChroCART 125-4 Cartridge (15 cm × 4 cm, particle size 5 μm). The mobile phase consisted of solvents A and B as detailed below, at a constant flow rate of 1 mL/min.

- Solvent A: 0.25% formic acid and 2% HPLC grade methanol in UHQ water.
- Solvent B: 100% HPLC grade methanol.

The following gradient was used:

10% methanol (B) flowed through the column isocratically with 90% solvent A for 15 min; and then was increased to 40% methanol (B) by 20 min; to 45% methanol (B) by 30 min; to 60% methanol (B) by 50 min; to 80% methanol (B) by 52 min; to 90% methanol by 60 min; followed by isocratic elution with 90% methanol (B) until 65 min. Finally the gradient was changed to 10% methanol by 68 min. and this composition was held until 73 min. This gradient program is summarised in Table 3.3.

A 15% isopropanol solution in triple deionised water (TDI) was used to wash the column between HPLC runs.

In addition, the solutions of honey extracts in methanol (1 mL) were analysed for compound identification and quantification using LC-MS on a Waters 2690 HPLC coupled to a Micromass ZMD

Mass Spectrometer (MS) with an electrospray ionisation (ESI) and a computer-controlled system operated by MassLynx v 3.5 software. The samples were scanned at m/z values of 120 to 620 using electrospray negative ionization with the settings listed in Table 3.2.

3.10.4 Identification and quantification of polyphenols in honey extracts

The phenolic compounds were identified and quantified according to the method reported by Martos *et al*. (1997, 2000ab) and Yao (2002), and through the use of ESI-MS with negative ionisation. In order to identify each peak in the chromatograms of the honey extracts, UV spectra, retention times and selected ion recording (SIR) of the mass spectra of all peaks were compared with those of the authentic samples listed in Table 3.1. In this study, the polyphenols were quantified using the external standard method and authentic compounds (four working standards of caffeic acid, quercetin and chrysin) (Martos *et al*., 1997; Yao, 2002). The concentration of a specific compound was calculated using Microsoft Excel 2000 by plotting the peak areas against the concentrations on a linear calibration curve.

In this study, also according to previous studies (Martos *et al*., 1997, 2000ab), the flavonoid profiles were the HPLC chromatograms recorded at 340 nm, because most of the flavonoids have their maximum absorption around this wavelength. Similarly, the profiles of phenolic acids were the HPLC chromatograms recorded at 290 nm, where most of the phenolic acids and flavanones have their maximum absorption. However, PDA is not suitable for detection of gallic acid and chrysin either at 290 nm or 340 nm. The mass spectral selected ion recording (SIR) mode is more suitable for these compounds and was applied based on their m/z values (molecular weights).

Unknown flavonoids were quantified against quercetin at 340 nm, while unknown phenolic acids were quantified against caffeic acid at 290 nm.

4. Results and Discussion

4.1 Identification of the Polyphenol Standards

The compounds in the authentic standard solutions were detected under UV spectrophotometry using photodiode array (PDA) detection at 290 nm, 340 nm or by the mass spectral (MS) selected ion recording (SIR) mode, depending the types of polyphenols. Figure 4.1 shows the peaks of the polyphenols detected by UV-PDA, whereas Figure 4.2 displays the peaks for gallic acid and chrysin detected by the mass spectral SIR mode using a 100 µg/mL standard solution. The peak areas were integrated from the chromatograms of the UV-PDA at 290 nm or 340 nm (Figure 4.1 and Figure A1.1), or the SIR chromatograms (Figure 4.2). The retention times of the compounds of the standard solutions are summarised in Table 4.1. The full UV spectra and negative ionisation electrospray ionisation-mass spectra for eight of the standard compounds listed in Table 4.1 are displayed in Figures A1.2-1.7, Figure A1.13 and Figure A1.17.

In general, most phenolic acids were detected using chromatograms recorded at 290 nm, namely chlorogenic acid, caffeic acid, *p*-coumaric acid, and ferulic acid. However, flavonoids are detected better using chromatograms at 340 nm, such as for quercetin. Although ellagic acid is phenolic acid, it is better detected at 340 nm due to its stronger absorbance at this wavelength. However, neither UV-PDA at 290 nm nor 340 nm can detect gallic acid and chrysin accurately. These two polyphenols were better detected using the mass spectral SIR mode based on their negative ionisation m/z values ($[M-H]^-$), such as SIR 169 for gallic acid and SIR 253 for chrysin. All the standard compounds were detected within 50 min.

Table 4.1 Retention times and the identification parameters of the identified polyphenols of a 100 µg/mL standard solution

Standard Compound	RT^+ (min)	UV Quantification λ (nm)	ESI-MS* $[M-H]^-$	UV Bands (nm)
Gallic acid	2.67	290	169	219, 271
Chlorogenic acid	6.37	290	353	244, 300 (sh), 327
Caffeic acid	7.17	290	179	220, 242, 295 (sh), 325
p-coumaric acid	9.94	290	163	232, 310
Ferulic acid	11.09	290	193	242, 300 (sh), 323
Hesperetin	19.17	340	301	284, 335 (sh)
Ellagic acid	21.07	340	301	253, 305 (sh), 365
Quercetin	28.09	340	301	254, 270 (sh), 305 (sh), 367
Chrysin	47.66	340	253	267, 313

$^+$RT Retention Time
*****ESI-MS Electrospray ionisation-mass spectrum (negative ionisation)

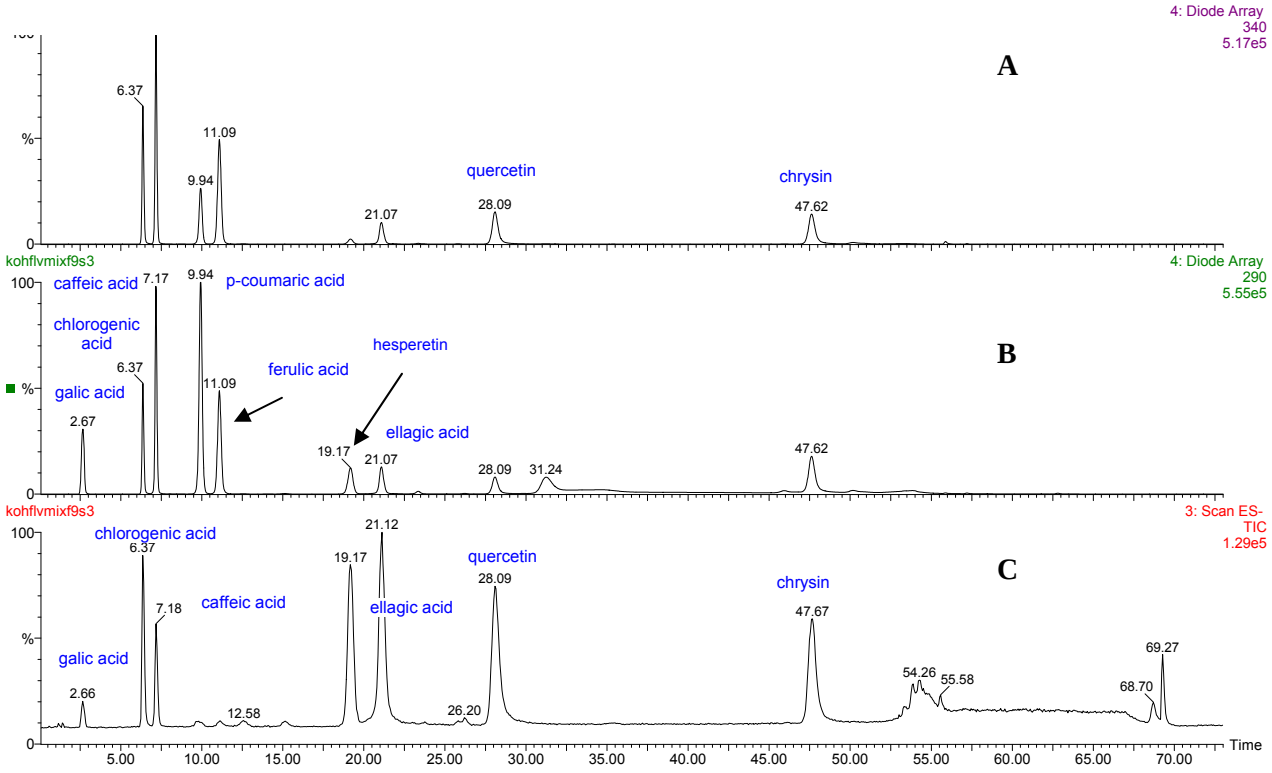


Figure 4.1 **Chromatograms of the 100 µg/mL standard solution detected by UV absorption at 340 nm (A), 290 nm (B), and by mass spectral total ion current (TIC) (C)**

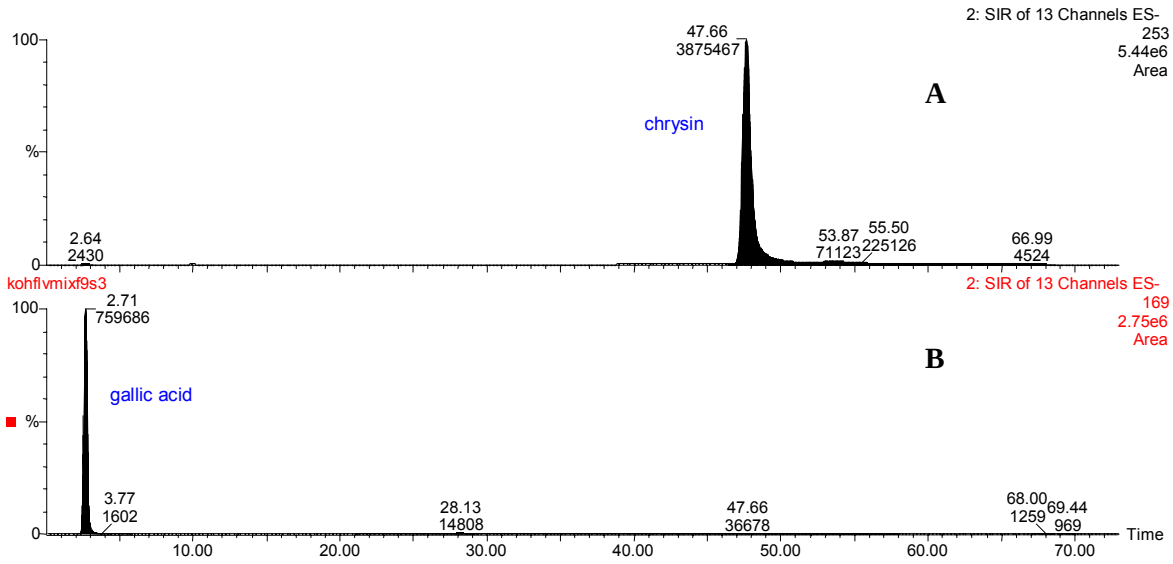


Figure 4.2 **Mass spectral selected ion recording (SIR) chromatograms of chrysin (A) and gallic acid (B) for the 100 µg/mL standard solution.**

4.2 Recovery Study of Flavonoid and Phenolic Acid Standards from Amberlite XAD-2 Resin

4.2.1 Effect of extraction/filtration through Amberlite XAD-2 resin on the composition of the polyphenol standards

The UV absorption chromatograms at 340 nm (Figure 4.3) for the 100 μg/ml standard solution before and after absorption and elution from Amberlite XAD-2 resin suggest that during the extraction/filtration, new peaks are formed and some disappear. Peaks 10 and 14 (Figure 4.3B) are newly formed peaks, not present in the chromatogram of the original standard solution. These peaks may be due to compounds formed from the degradation of chlorogenic acid and ferulic acid. Such results suggest that there may be some chemical reaction of chlorogenic and ferulic acids with the Amberlite XAD-2 resin. Further studies of this are required before a more definitive conclusion can be made.

Ellagic acid was not detected in the extracted/filtered methanol solution suggesting it was not retained on the Amberlite XAD-2 resin at the acid pH used. Gallic acid is not visible at a wavelength of 340 nm but is at 290 nm. A gallic acid peak was detected in the initial standard solution chromatogram recorded at 290 nm, but is not present in the extracted/filtered solution chromatogram recorded at 290 nm (not shown below), suggesting it was also not retained on the Amberlite XAD-2 resin at the acid pH. These findings were further examined in a subsequent experiment detailed below.

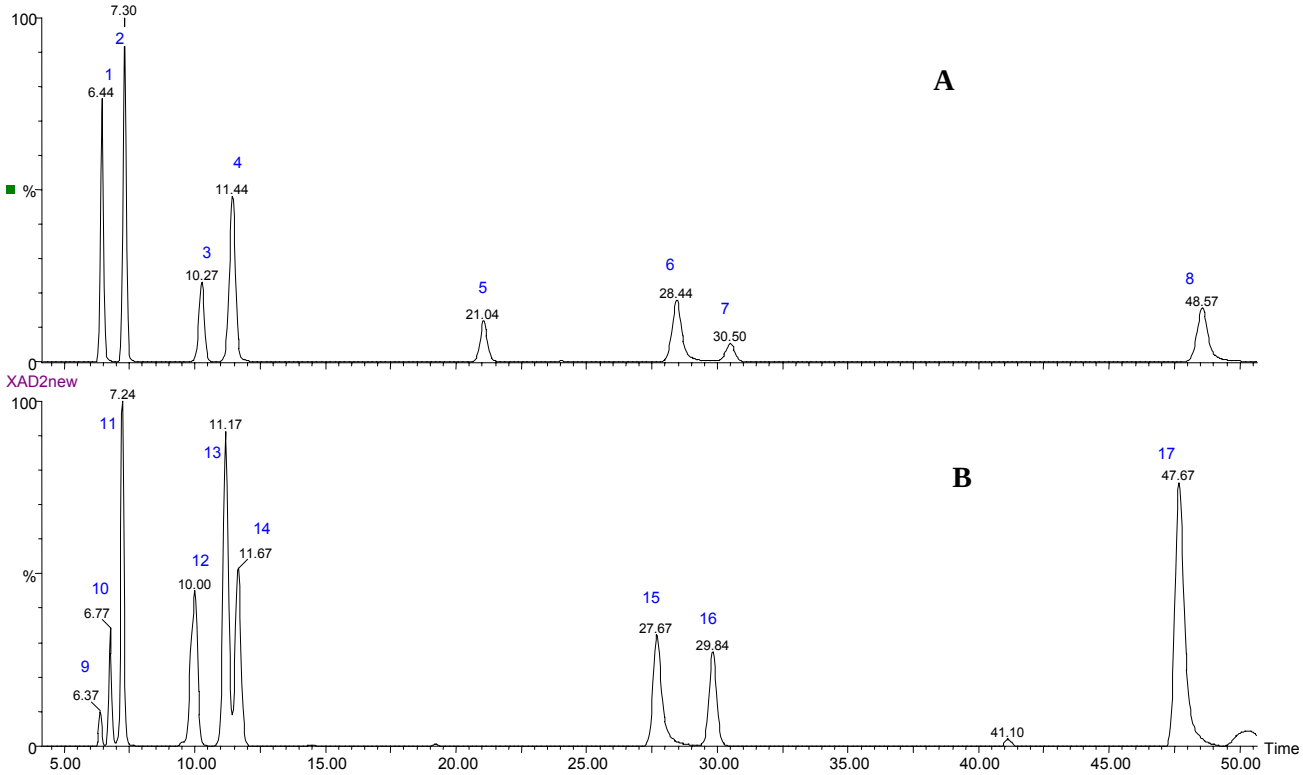


Figure 4.3 HPLC chromatograms of a standard mixture of polyphenols measured on a diode array detector at 340 nm

A: standard mixture (100 μg/ml) of phenolic compounds: 1=chlorogenic acid, 2= caffeic acid, 3= coumaric acid, 4= ferulic acid, 5= ellagic acid, 6= quercetin, 7=hesperetin, 8= chrysin;

B: standard mixture (100 μg/ml) extracted/filtered on column of Amberlite XAD-2 resin: 9=chlorogenic acid, 10= chlorogenic acid reactant, 11= caffeic acid, 12= coumaric acid, 13= ferulic acid, 14= ferulic acid reactant, 15=quercetin, 16= hesperetin, 17= chrysin. Note, gallic acid does not absorb at 340 nm but does at 290 nm (results not shown).

4.2.2 Experiment to optimise the method for extracting polyphenols using Amberlite XAD-2 resin – 'mixing' or 'elution' methods

Two experiments were done to determine the difference in recovery between: (1) mixing the standard polyphenol solution with the Amberlite XAD-2 resin prior to packing into a glass column (called the 'mixing method'); and (2) adding the standard polyphenol solution to the Amberlite XAD-2 resin already packed into the glass column (called the 'elution method').

The recoveries of all phenolic compounds (Table 4.2) are higher when using the 'mixing method' than using the 'elution' method.

Table 4.2 Recoveries of the polyphenols in a standard mixture using the 'mixing' and 'elution' methods with Amberlite XAD-2 resin

	Peak Area in original standard solution		**Peak Area for extracted solution using the 'mixing' method**	**% recovery for the 'mixing' method**		**Peak Area for extracted solution using the 'elution method**	**% recovery for the 'elution method**
290 nm							
Gallic acid	128921		nd	0		nd	0
Chlorogenic acid	186509		nd	0		nd	0
Caffeic acid	313471		99148	**32**		92555	**30**
p-Coumaric acid	461796		285450	**62**		220204	**48**
Ferulic acid	286389		155171	**54**		131163	**46**
Ellagic acid	95501		nd	**0**		nd	**0**
Quercetin	103103		67499	**65**		38379	**37**
Hesperetin	275442		260858	**95**		65714	**24**
Chrysin	196891		195543	**99**		162296	**82**
340 nm							
Gallic acid	-*		-	-		-	-
Chlorogenic acid	194953		nd	**0**		nd	**0**
Caffeic acid	261113		79047	**30**		81389	**31**
p-Coumaric acid	101207		62732	**62**		48083	**48**
Ferulic acid	252662		132647	**52**		120500	**48**
Ellagic acid	63945		nd	**0**		nd	**0**
Quercetin	165914		113258	**68**		37538	**23**
Hesperetin	40660		38320	**94**		9981	**25**
Chrysin	148797		141403	**95**		114030	**77**

***** Gallic acid does not absorb UV at 340 nm
nd Not detected

4.2.3 Experiment to determine the recovery of polyphenol standards extracted/filtered using Amberlite XAD-2 resin, and to determine the repeatability of the extraction method

Three trials (Table 4.3) showed that definitely gallic acid and ellagic acid (and possibly chlorogenic acid) were not present in the methanol eluents from the Amberlite XAD-2 resin column (0% recovery), suggesting these phenolic acids are not initially retained on the Amberlite XAD-2 resin at the acid pH. Further analysis of the acid fraction, in which the sample was originally dissolved, after elution through the Amberlite XAD-2 resin column, revealed a recovery of 12% gallic acid and 19% chlorogenic acid. This confirms that the gallic acid and chlorogenic acid molecules were not initially bound to the Amberlite XAD-2 resin column. In addition, there was no indication that these compounds were eluted from the Amberlite XAD-2 resin during the subsequent acid and water washings, again suggesting these compounds do not bind to the Amberlite XAD-2 resin at the acid pH. These results do not agree with the findings of Yao (2002) who reported that Australian honey contained gallic, chlorogenic and ellagic acids, suggesting some error in the identifications of Yao (2002). It is noted that Yao (2002) did not do a recovery study for the phenolic acids extracted by Amberlite XAD-2 resin. If this had been done, then mis-identification of these phenolic acids in Australian honeys would not have occurred.

The other phenolic acid standards had percentage recoveries of 16-62% suggesting that this method which uses Amberlite XAD-2 resin to extract phenolic acids from acidified solutions is not a good method for determining the concentrations of these particular phenolic acids in honey. However, the recoveries of other phenolic acids (including unidentified or unknown) in honey may be higher, but authentic standards were not available to test this hypothesis.

Table 4.3 Recoveries (%) of the phenolic acids and flavonoids from a standard solution

	Recoveries (%)					
	Trial 1	**Trial 2**	**Trial 3**	**Mean**	**Std Dev**[+]	**%CV***
Gallic acid	0	0	0	0	0	0
Chlorogenic acid	0	11	0	4	6	173
Caffeic acid	15	20	32	22	9	39
p-Coumaric acid	60	48	62	57	8	13
Ferulic acid	60	53	54	56	4	7
Ellagic acid	0	0	0	0	0	0
Quercetin	16	33	65	38	25	65
Hesperetin	84	86	95	88	6	7
Chrysin	85	92	99	92	7	8

[+] standard deviation

***** %CV is (standard deviation/mean) x 100

The recoveries for the flavonoids such as hesperetin and chrysin were as high as previously reported (i.e. > 80% recovery for hesperetin) for the Amberlite XAD-2 extraction method for honey flavonoids (Tomás-Barberán *et al*., 1992, Martos *et al*., 1997). This confirms the method used in this project is optimised for extracting flavonoids efficiently from honey.

4.3 Composition of the Polyphenols in Extracts of Yapunyah (*Eucalyptus ochrophloia*) Honey

4.3.1 Identification of the polyphenols in yapunyah honey

The chromatograms of the extract from the yapunyah honey sample 36345 recorded at the UV wavelengths of 340 nm and 290 nm, and using mass spectrometric analysis show a number of flavonoids that absorb most strongly at 340 nm and phenolic acids that absorb preferentially at 290 nm (Figure 4.4).

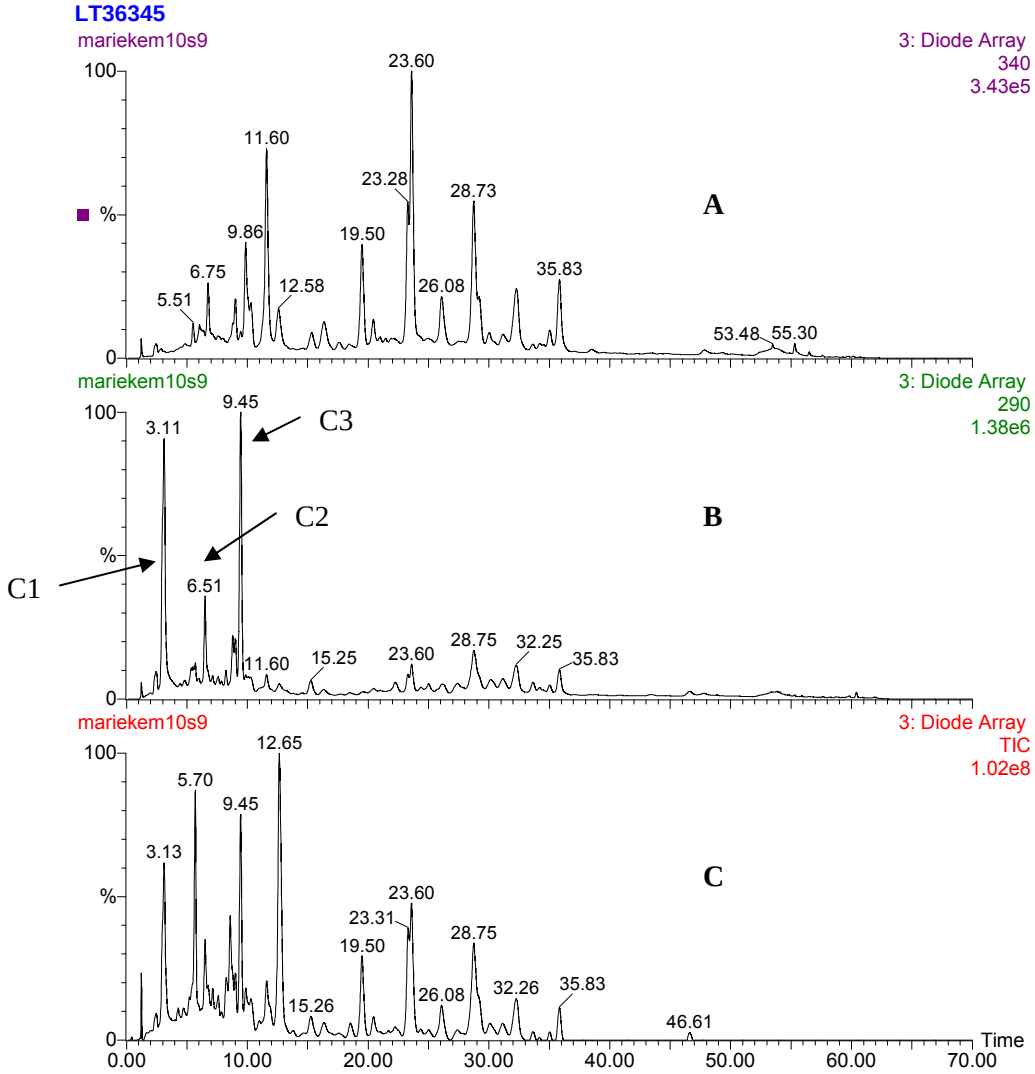


Figure 4.4 **Chromatograms for the flavonoids and phenolic acids of the yapunyah honey sample 36345 recorded at UV wavelengths of 340 nm (A) and 290 nm (B), and using mass spectrometric total ion current (C)**

4.3.1.1 Identification of the phenolic acids in yapunyah honey

A previous study by Yao (2002) reported that three samples of yapunyah honey contained the following phenolic acids:

gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and ellagic acid.

Because some of these (gallic acid, chlorogenic acid and ellagic acid) were found in this project to not be retained by the Amberlite XAD-2 resin during a recovery study involving the extraction of authentic standards (Section 4.2.3), it was necessary to confirm that these and other phenolic acids reported by Yao (2002) to be components of yapunyah and other honey types were in fact present in yapunyah honey.

While the samples of yapunyah honey studied in this project were different to those studied by Yao (2002), it is clear that the HPLC polyphenol fingerprint for yapunyah honey is fairly similar between samples in this project and those studied by Yao (2002). Thus, the same compounds detected by Yao (2002) were also detected in this project. But are the identities assigned to these compounds by Yao (2002) in fact correct?

Clearly, most doubt arises for the identities of the phenolic acids that can be observed in the HPLC chromatogram recorded at 290 nm (Figure 4.4B). To absolutely confirm the presence of the phenolic acids reported to be in yapunyah honey by Yao (2002), mass spectral selective ion recording (SIR) chromatograms were obtained using LC-MS (a method not used by Yao, 2002). The concentrations (2.5-13.3 μg/100 g honey) of gallic acid, chlorogenic acid, caffeic acid and *p*-coumaric acid in two yapunyah honey samples are very low compared to the concentrations found by Yao (2002) of 250 - 3,160 μg/100 g honey. The concentrations are so low (close to the limit of detection of the mass spectral detector) as to suggest that these compounds are in fact not present at all in yapunyah honey, or are not able to be extracted using the analysis method, as was found for gallic acid and chlorogenic acid in the recovery study detailed earlier in Section 4.2.3. Further, no ellagic acid was detected in yapunyah honey when using the SIR 301 as part of LC-MS analysis. This is what was expected since the recovery study described earlier in Section 4.2.3 found that ellagic acid is not retained on the Amberlite XAD-2 resin under acidic conditions, which are conditions that favour attachment of phenolic acids to Amberlite XAD-2 resin sites. In addition, the retention time reported by Yao (2002) for ellagic acid of 9.9 min clearly cannot be correct as the retention time at which authentic ellagic acid eluted in this study was 21 min. Thus, the compound identified and quantified as ellagic acid in yapunyah honey by Yao (2002) is definitely not ellagic acid.

In addition, the three unknown compounds C1, C2 and C3 that elute close to gallic acid, caffeic acid and *p*-coumaric acid respectively (Figure 4.4), are present in much higher concentrations of 18.29-305.72 μg/100 g honey when determined at 290 nm (Table 4.4) relative to the concentration of gallic acid, caffeic acid and *p*-coumaric acid respectively determined using the mass spectral SIR mode (Table 4.4). Also, the UV spectral bands for these three unknown compounds are similar to those for gallic acid, caffeic acid and *p*-coumaric acid respectively (Table 4.5), and confusion with identification could occur. Thus, because the retention times and UV spectra for C1, C2 and C3 are similar to gallic acid, caffeic acid and *p*-coumaric acid respectively (Table 4.4; Figures 4.5-4.7), mis-identification is possible without mass spectral data. This was the case for Yao (2002). The mass spectral data of this project, including unknowns C1, C2 and C3 (Table 4.5; Figure A1.18 in Appendix 1) proved conclusively that these three unknown compounds were not gallic acid, caffeic acid and *p*-coumaric acid respectively. In conclusion, these three unknown compounds (C1, C2 and C3) are probably phenolic acids since their absorption at 340 nm was much lower than at 290 nm where phenolic acids absorb maximally.

Table 4.4 Concentrations (μg/100g) of the phenolic acids in two yapunyah honey samples determined using the selected ion recording (SIR) mode of LC-MS

Compound	ESI-MS* $[M-H]^-$	Quantification detection conditions	RT (min)	36409 μg/100g	36132 μg/100g
Gallic acid	169	SIR 169	2.4	5.3	3.5
C1		290 nm	3.1	46.84	26.41
Chlorogenic acid	353	SIR 353	5.7	7.5	4.9
C2		290 nm	6.5	36.77	70.07
Caffeic acid	179	SIR 179	6.7	10.1	12.3
***p*-coumaric acid**	163	SIR 163	9.3	13.3	13.1
C3		290 nm	9.5	298.89	305.72
Ferulic acid	193	SIR 193	10.7	45.3	40.5

^RT = Retention Time

*ESI-MS Electrospray ionisation-mass spectrum (negative ionisation)

Table 4.5 Some phenolic acids in yapunyah honey

D'Arcy (2004) sample								Yao (2002) sample
RT (min)^	**Compound**	**UV (nm)**	**ESI-MS* $[M-H]^-$**	**RT (min)**	**Unknown Compound**	**UV (nm)**	**ESI-MS* $[M-H]^-$**	**RT (min)**
2.4	Gallic acid	219, 271	169	3.1	C1	231, 283	198	~ 2.0
6.7	Caffeic acid	220 (sh), 242, 295 (sh), 325	179	6.5	C2	222, 265, 285 (sh)	211	~ 6.2
9.3	*p*-Coumaric acid	232, 310	163	9.5	C3	230, 285	175, 195, 209	~ 6.5

^RT = Retention Time

*****ESI-MS Electrospray ionisation-mass spectrum (full negative ionisation mass spectra in Figure A1.18 of Appendix 1)

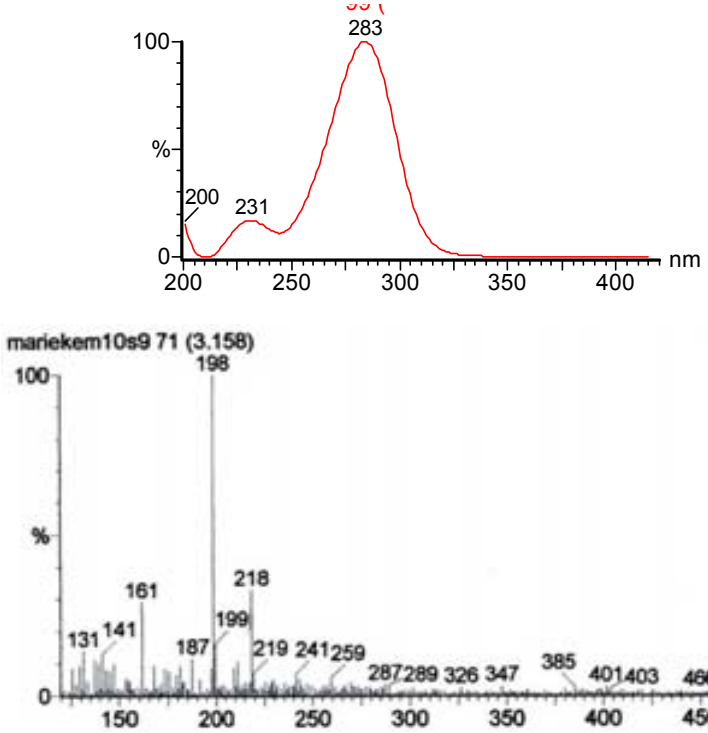


Figure 4.5 **UV and electrospray ionisation-mass spectra (negative ionisation) for the unknown phenolic acid C1 in yapunyah honey**

C1 - 3.1 min [identified incorrectly by Yao (2002) as gallic acid]

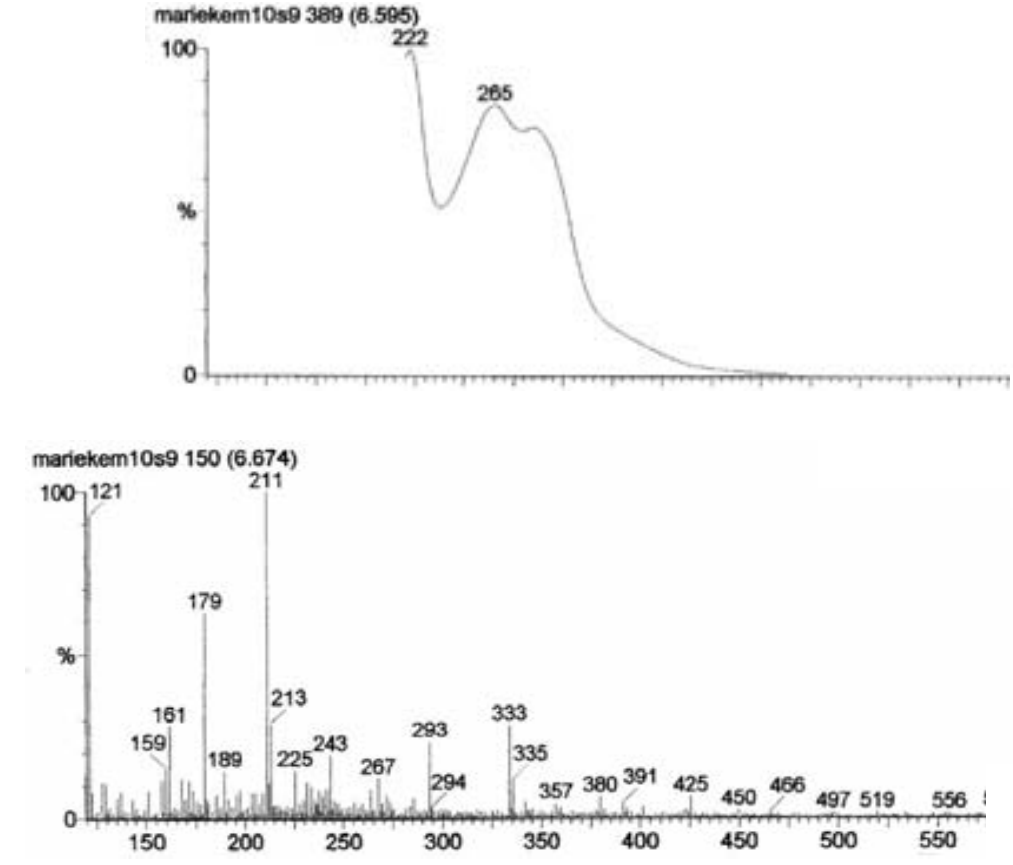


Figure 4.6 **UV and electrospray ionisation-mass spectra (negative ionisation) for the unknown phenolic acid C2 in yapunyah honey**

C2 – 6.5 min [identified incorrectly by Yao (2002) as caffeic acid]

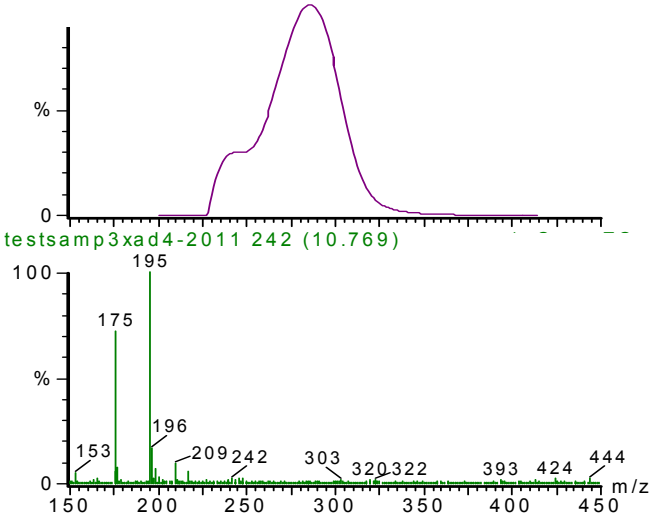


Figure 4.7 UV and electrospray ionisation-mass spectra (negative ionisation) for the unknown phenolic acid C3 in yapunyah honey

C3 - 9.5 min [identified incorrectly by Yao (2002) as *p*-coumaric acid]

4.3.1.2 Identification of the flavonoids in yapunyah honey

The flavonoids identified in yapunyah honey (Table 4.6) were tricetin, pinobanksin, quercetin, luteolin, quercetin 3-methylether, 8-methoxy kaempferol. The full negative ionisation electrospray ionisation-mass spectra for tricetin (Figure A1.10), pinobanksin (Figure A1.11), luteolin (Figure A1.12) and quercetin 3-methylether (Figure A1.14) are displayed in Appendix 1. The detection of these compounds is in agreement with Yao (2002) who identified tricetin, quercetin, luteolin, quercetin 3-methyl ether, kaempferol (in 2 of 3 samples), 8-methoxy-kaempferol (in 1 of 3 samples), pinocembrin (in 2 of 3 samples), and chrysin (in 1 of 3 samples).

4.3.2 Quantification of the polyphenols in yapunyah honey

The concentrations of flavonoids and phenolic acids found in this study (Tables 4.6-4.8) were lower than the concentrations reported by Yao (2002), both individually and in total.

One other study of Australian honeys using HPLC (Martos *et al*., 2000b) reported concentrations of flavonoids lower than that found by Yao (2002), but not as low as found in this study. For example, two yellow box honey samples reported by Martos *et al*. (2000b) showed concentrations of flavonoids about 20% of those reported by Yao (2002) for two different yellow box honey samples. In this study of five yapunyah honey samples, concentrations are about 20% of those reported by Yao (2002) for three different yapunyah honey samples. This common difference (20% lower) suggests two possibilities: incorrect quantification methods used by Yao (2002) or the yapunyah and yellow box honey samples analysed by Yao (2002) contained higher concentrations of flavonoids that those samples analysed in this study and by Martos *et al*. (2000b). The full details of the quantification method used by Yao (2002) is not stated. While the type of authentic standards used are detailed, the concentration of the standard solutions used to calculate the sample concentration is not stated.

This study also detected and quantified many unknown flavonoids (which absorbed more strongly at 340 nm) in yapunyah honey that were not reported by Yao (2002). Finally, there have been few studies of the phenolic acids in honey to compare concentrations, and none using the Amberlite XAD-2 resin extraction method, other than that of Yao (2002). Since it has been shown above that Yao (2002) incorrectly identified many of the phenolic acids detected in yapunyah honey, it is also possible that these were incorrectly quantified, hence the higher concentrations reported by Yao (2002) relative to those found in this study.

Table 4.6 Concentration (μg/100 g) of the flavonoids in yapunyah honey samples

Honey		**36345**	**36132 (MP)**	**36132 (TB)**	**36134**	**36409**	**36135**	**Mean**	**SD**	**%CV**
Compound	**RT(min)^**									
Unknown 6	9.9	64.69	90.13	2.70	121.03	99.25	29.84	67.94	44.74	65.9
Unknown 7	11.6	134.19	38.95	205.14	180.19	219.54	45.71	137.29	79.09	57.6
Unknown 8	12.6	28.86	3.25	5.43	6.28	15.05	41.03	16.65	15.23	91.5
Unknown 8a	15.3	13.79					13.91	13.85	0.09	0.6
Unknown 8b	16.4	30.73					6.34	18.53	17.24	93.0
Unknown 8c	17.6	5.81					6.68	6.24	0.62	9.9
Unknown 9	19.5	72.12	187.04	11.94	17.10	11.33	5.41	50.82	71.11	139.9
Unknown 10	20.5	15.94	13.82	8.12	6.31	19.46	39.95	17.27	12.14	70.3
Unknown 10a	22.1	5.18					26.93	16.05	15.38	95.8
Unknown 11	23.3	77.05	34.01	43.43	17.60	43.48	18.89	39.08	21.79	55.8
Tricetin	23.6	185.12	64.66	105.04	38.77	105.55	32.98	88.69	56.61	63.8
Unknown 11a	24.5						18.77	18.77		
Pinobanksin	25	5.27	4.84		0.71		4.49	3.83	2.10	54.9
Quercetin	26.1	43.46	47.46	24.24	20.72	41.87	12.57	31.72	14.37	45.3
Luteolin	28.7	154.58	72.65	85.98	66.10	128.26	51.18	93.13	39.93	42.9
Quercetin 3-methylether	30.1	9.95	4.77	4.41	1.19	4.90	14.71	6.66	4.84	72.8
Unknown 11c	31.2	12.10					9.61	10.86	1.76	16.2
Unknown 13	32.3	66.56	15.22	13.60	30.47	36.34	41.61	33.97	19.52	57.5
8-Methoxy kaempferol	35	10.25	1.56	18.69	4.26	5.72	9.48	8.33	6.03	72.4
Unknown 15	35.8	53.19	6.77	4.45	54.32	77.66	54.49	41.81	29.52	70.6
Unknown 15a	39.2						15.69	15.69		
Unknown 15b	43.3						14.26	14.26		

Table 4.6 Continued

Honey		**36345**	**36132 (MP)**	**36132 (TB)**	**36134**	**36409**	**36135**	**Mean**	**SD**	**%CV**
Compound	**RT^**									
Unknown 16*	47.8	0.21	1.76	23.38	14.24	0.04	18.60	9.71	10.33	106.4
Unknown 16a*	49.3	0.14						0.14		
Unknown 16b	53.1						58.07	58.07		
Unknown 16c	53.3						50.07	50.07		
Unknown 17*	53.5	0.81	0.69	12.42	14.80	0.11	40.11	11.49	15.44	134.3
	54.3						47.24	47.24		
	54.7						15.78	15.78		
Unknown 17a*	55.3	0.38					62.66	31.52	44.04	139.7
	55.6						129.19	129.19		
Total		**990.37**	**587.58**	**568.98**	**594.08**	**808.56**	**936.25**	**747.64**	189.38	25.3

*calculated at 340 nm using the calibration curves of chrysin; compounds 6-15 were calculated at 340 nm using the calibration curves of quercetin
^Retention Time

Table 4.7 Concentration (μg/100 g) of the phenolic acids in yapunyah honey samples

Honey		**36345**	**36132 (MP)**	**36132 (TB)**	**36134**	**36409**	**36135**	**Mean**	**SD**	**%CV**
Compound	**RT (min)^**									
Unknown 1 (C1)	3.1	234.53	35.21	26.41	53.70	46.84	18.29	69.16	82.04	118.6
Unknown 2	5.7	22.28	16.40	10.19	12.08	102.21	15.80	29.83	35.71	119.7
Unknown 2a	6.1						27.10	27.10		
Unknown 3 (C2)	6.5	78.07	119.57	70.07	111.25	36.77	95.98	85.28	30.34	35.6
Unknown 3a	7.1	5.57						5.57		
Unknown 3b	7.6	5.95					20.09	13.02	10.00	76.8
	7.9						19.99	19.99		
Unknown 3c	8.2	8.77					14.58	11.68	4.11	35.2
Unknown 4	8.9	35.47	31.86	1.28	1.74	77.59	31.54	29.92	28.02	93.7
p-Coumaric acid*	9.3			13.10		13.30	45.99	24.13	18.93	78.5
Unknown 5 (C3)	9.5	234.53	204.10	305.72	343.78	298.89	218.31	267.55	56.20	21.0
Total		**625.17**	**407.15**	**426.77**	**522.55**	**575.61**	**507.67**	**510.82**	83.94	16.4

*Quantified at 340 nm relative to quercetin. All other compounds quantified at 290 nm relative to caffeic acid
^RT Retention Time

Table 4.8 Total concentration (μg/100 g) of the flavonoids and phenolic acids in yapunyah honey samples

	Honey Sample								
	36345	**36132 (MP)**	**36132 (TB)**	**36134**	**36409**	**36135**	**Mean**	**SD**	**%CV**
Flavonoids	990.37	587.58	568.98	594.08	808.56	936.25	747.64	189.4	25.3
Phenolic Acids	625.17	407.15	426.77	522.55	575.61	507.67	510.82	83.9	16.4
Flavonoids + Phenolic Acids	**1615.54**	**994.73**	**995.75**	**1116.63**	**1384.17**	**1443.92**	**1258.46**	**259.4**	**20.6**

4.4 Composition of the Polyphenols in Extracts of Leatherwood (*Eucryphia lucida*) Honey

4.4.1 Identification of the polyphenols in leatherwood honey

The chromatograms of the extract from the leatherwood honey sample 1A recorded at UV wavelengths of 340 nm and 290 nm, and using mass spectrometric analysis show a number of flavonoids that absorb most strongly at 340 nm and phenolic acids that absorb preferentially at 290 nm (Figure 4.8). Chromatograms for the leatherwood samples L28 and L3 are displayed in Figure A1.19 in Appendix 1.

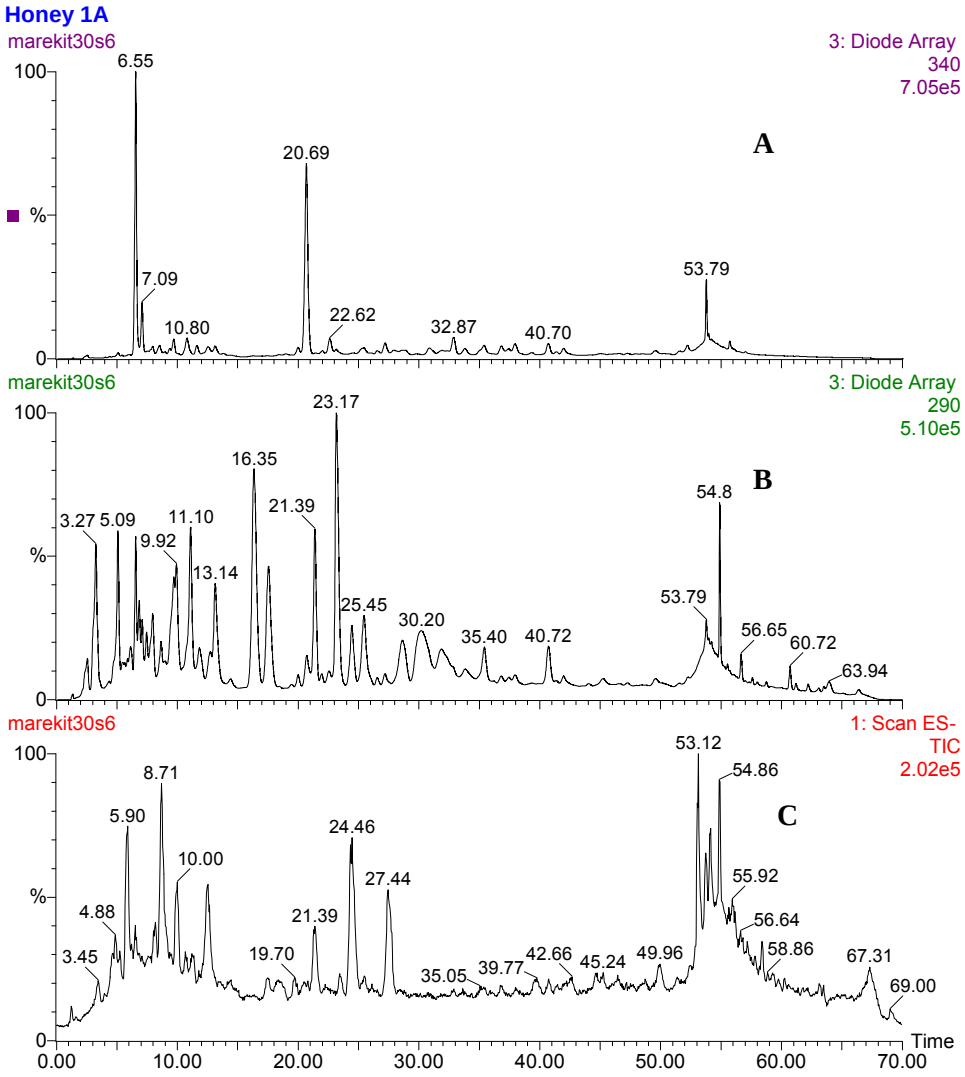


Figure 4.8 **Chromatograms for the flavonoids and phenolic acids of the leatherwood honey sample (1A) recorded at UV wavelengths of 340 nm (A) and 290 nm (B), and mass spectrometric total ion current (TIC) (C)**

In these leatherwood honey samples, some compounds were identified, namely caffeic acid, tricetin, pinobanksin, luteolin and pinocembrin. The full electrospray ionisation-mass spectra (with negative ionisation) for pinocembrin (Figure A1.16) and chrysin (Figure A1.17) are displayed in Appendix 1.

4.4.2 Quantification of the polyphenols in leatherwood honey

Initially, mass spectral selected ion recording (SIR) was used to determine the concentrations of some known flavonoids and phenolic acids. The results (Table 4.9) suggest gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, quercetin and chrysin were present in only trace concentrations, while ferulic acid was not detected.

Table 4.9 Concentration (µg/100 g)* of the phenolic acids and flavonoids in leatherwood honey samples L28 and L30 using mass spectral selected ion recording (SIR) mode

	Compound	RT^ (min)	ESI-MS^{+} [M-H]$^{-}$	L30	L28
Phenolic acid	Gallic acid	2.66	SIR 169	2.65	1.59
	Chlorogenic acid	6.17	SIR 353	5.49	1.81
	Caffeic acid	7.10	SIR 179	8.89	13.21
	p-coumaric acid	9.78	SIR 163	6.16	8.35
	Ferulic acid		SIR 193	nd	nd
Flavonoid	Hesperetin		SIR 609	nd	nd
	Quercetin	28.4	SIR 301	4.76	7.57
	Chrysin	47.1	SIR 253	4.72	5.09

*Quantified using mass spectral selected ion recording (SIR) mode relative to a 25 µg / mL mixed standard of the above authentic compounds

^RT = Retention Time

$^{+}$ESI-MS Electrospray ionisation-mass spectrum (negative ionisation)

The calibration data used to quantify flavonoids (using quercetin at 340 nm) and phenolic acids (using caffeic acid at 290 nm) are detailed in Table 4.10.

Table 4.10 Retention times (RT) and the corresponding peak areas of the standard solutions

			Standard Area			
Compound	Detector	RT (min)	2.5 ug/ml	10 ug/ml	50 ug/ml	100 ug/ml
Caffeic acid	290 nm	7.2	3499	12931	63963	144601
Quercetin	340 nm	28.6	1462	6146	33906	69597
Chrysin	340 nm	48.1	1620	6527	34324	72192

Caffeic acid: area = 1411.7 x conc
Quercetin: area = 691.73 x conc
Chrysin: area = 714.31 x conc

The concentrations of the identified and unknown compounds are shown in Tables 4.11 - 4.13. The concentrations of the unknown compounds 3 and 17 were calculated using the calibration curve of quercetin, since these compounds had a bigger peak area at 340 nm. The concentrations of the other compounds were calculated using the calibration curve of caffeic acid, because these compounds had a bigger peak at 290 nm. One should expect that the flavonoids, pinobanksin, luteolin and pinocembrin would have a bigger peak area at 340 nm, but this was not the case. Unknown compounds 3 and 17 seem to be flavonoids since they absorb more strongly at 340 nm. The other unknown compounds seem to be phenolic acids since they absorb more strongly at 290 nm.

Leatherwood honey does not contain many flavonoids, but is rich in many phenolic acids (mean of 2066.6 μg/100 g honey) (Table 4.13). This is a very interesting result when considered in the light of the high concentrations of volatiles (some of which are phenolic compounds) previously found in leatherwood honey by D'Arcy *et al*. (2001) in an earlier RIRDC project.

Table 4.11 Concentrations (µg/100 g) of the flavonoids in leatherwood honey samples

	Compound							
	Unknown 3*	**Unknown 17***	**Tricetin**	**Pinobanksin**	**Luteolin**	**Pinocembrin**	**Chrysin**	**Total**
Retention Time (min)	6.6	20.7	24.5	25.5	30.2	40.7	47.1	
Honey Sample								
L2	507.84	338.71	8.31	37.95	26.15	13.41	0.99	933.36
L28	263.77	280.83	11.82	39.24	6.02	10.4	1.3	613.38
L30	340.6	221.15	11.94	46	19.62	13.27	3.05	655.63
1A	212.79	258.85		48.98	92.12	25.99		638.73
2A	354.91	239.26		5.62	49.03	19.89		668.71
3A	601.94	455.5		33.32	10.33	28.48		1129.57
4A	297.19	418.39		30.92	12.71	36.12		795.33
L5	859.36	635.12		9.94	32.93	41.77		1579.11
L12 (MP)	315.77	295.79		66.79	135.36	21.63		835.34
L12 (GP)	394.38	464.54		36.34	6.50	34.39		936.16
L13	552.26	545.9		2.00	3.54	33.51		1137.21
Mean	426.53	377.64	10.69	32.46	35.85	25.35	1.78	901.23
SD	188.84	136.09	2.06	19.71	41.94	10.49	1.11	292.50
%CV	44.3	36.0	19.3	60.7	117.0	41.4	62.4	32.5

*Quantified at 340 nm using the calibration curve of quercetin. All other compounds were quantified at 290 nm using the calibration curve of caffeic acid.

Table 4.12 Concentration (μg/100 g) of the phenolic acids in leatherwood honey*

Honey	RT^	L2	L28	L30	1A	2A	3A	4A	L5	L12 (MP)	L12 (GP)	L13	Mean	%CV
Compound														
Unknown 0	**2.4**	31.5	62.32	59.27	19.79								43.22	48.32
Unknown 1	**3.3**	97.85	32.51	96.36	74.87	69.25	117.74	501.23	70.33	76.69	58.50	40.82	112.38	116.84
Unknown 2	**5.1**	97.18	50.99	27.18	46.16	111.79	77.43	54.58	164.61	46.39	58.34	98.54	75.74	52.21
Unknown 2a	**5.9**	18.24	19.96	15.6	7.30								15.27	36.75
Unknown 3a	**6.9**	18.45	69.13	58.05	19.39								41.26	63.47
Caffeic acid	**7.1**	14.77	13.02	9.59	17.35	20.77		18.94	24.90	42.72	44.94		23.00	54.88
Unknown 3b	**7.4**	32.19	20.2	21.97	13.91								22.07	34.37
Unknown 3c	**8.0**	40.38	38.71	31.51	13.02								30.90	40.54
Unknown 3d	**8.7**	17.95	370.63	35.42	28.87								113.22	151.71
Unknown 3e	**9.6**	26.97	67.34	58.31	18.76								42.85	55.09
Unknown 4	**9.9**	107.52	9.05	1.02	42.99	52.16	68.77	104.92	101.62	165.46	35.70	7.70	63.35	81.77
Unknown 5	**11.1**	134.88	115.53	102.19	74.96	66.78	113.49	128.61	88.95	149.04	113.52	162.05	113.64	25.97
Unknown 6	**13.1**	158.75	194.25	137.95	57.35	84.68	114.19	80.25	161.50	132.77	128.60	101.98	122.93	32.85
Unknown 7	**16.4**	908.87	203.17	226.97	150.71	146.07	246.68	515.75	448.30	659.57	713.06	502.55	429.24	59.85
Unknown 8	**17.7**	100.63	40.49	154.15	83.65	119.78	207.45	155.27	226.30	203.51	201.98	219.11	155.67	40.07
Unknown 8a	**18.2**		80.47		17.51								48.99	90.86
Unknown 9	**21.4**	142.36	207.53	136.15	65.89	97.92	144.96	91.71	126.27	124.25	136.29	146.26	129.05	28.33
Unknown 10	**23.2**	495.57	457.67	294.33	136.76	215.63	341.41	290.71	414.36	306.77	315.19	390.36	332.61	31.32
Unknown 11	**24.5**	144.26	102.93	79.75	32.74	61.89	145.76	45.24	161.27	101.94	148.40	146.32	105.11	45.81
Unknown 12	**28.7**				44.86	31.11	50.26	41.86	13.33	51.46	52.38	40.25	40.69	32.23
Unknown 13	**31.9**	10.37	7.75	6.86	49.30	31.07	51.82	16.25	59.44	33.77	36.65	37.12	30.95	59.77

*Compounds were quantified at 290 nm using the calibration curve of caffeic acid.
^RT Retention Time (min)

Table 4.12 Continued

Honey	RT^	L2	L28	L30	1A	2A	3A	4A	L5	L12 (MP)	L12 (GP)	L13	Mean	%CV
Unknown 13a	**33.4**	8.23	3.95	7.45	7.59								6.81	28.41
Unknown 14	**35.5**	15.05	5.22	6.15	20.91	4.61	12.05	16.72	16.67	32.31	24.53	20.98	15.93	54.30
Unknown 14a	**36.7**	6.72	5.89	7.01	3.93								5.89	23.59
Unknown 14b	**37.5**	12.26	7.63	6.83	3.59								7.58	47.21
Unknown 14c	**38.0**	18.11	13.68	18.43	5.90								14.03	41.61
Unknown 14e	**42.0**	12.60	11.09	15.93	7.40								11.76	30.10
Unknown 14f	**45.7**	7.64	9.22	7.05	7.62								7.88	11.83
Unknown 14g	**49.6**	24.23		0.33									12.28	137.62
Unknown 14h	**52.0**	14.27		4.55									9.41	73.04
Unknown 15	**53.8**				72.83	16.22	287.81	285.21	390.01	70.86	184.63	484.64	224.03	74.38
Unknown 16	**54.9**				31.86	106.38					124.50		87.58	56.06
Total		**2717.8**	**2220.3**	**1626.4**	**1177.8**	**1236.0**	**1979.8**	**2332.9**	**2467.9**	**2197.5**	**2377.2**	**2399.0**	2066.6	24.6

*****Compounds were quantified at 290 nm using the calibration curve of caffeic acid.

^RT Retention Time (min)

Table 4.13 Total concentration (μg/100 g) of the flavonoids and phenolic acids in leatherwood honey samples

Honey Sample	L2	L28	L30	1A	2A	3A	4A	L5	L12 (MP)	L12 (GP)	L13	Mean	%CV
Total Flavonoids	933.4	613.4	655.6	638.7	668.7	1129.6	795.3	1579.1	835.3	936.2	1137.2	901.2	32
Total Phenolic Acids	2718.8	2220.3	1626.4	1177.8	1236.0	1979.8	2332.9	2467.9	2197.5	2377.2	2399.0	2066.6	25
Flavonoids + Phenolic Acids	**3652.2**	**2833.7**	**2282.0**	**1816.5**	**1904.7**	**3109.4**	**3128.2**	**4047.0**	**3032.8**	**3313.4**	**3536.2**	**2967.8**	24

4.5 Composition of the Polyphenols in Extracts of Salvation Jane (*Echium plantagineum*) Honey

4.5.1 Identification of the polyphenols in Salvation Jane honey

Six samples of Salvation Jane honey, P8728, P8684, P8774, P0120, P8676, and P8932 were analyzed in this project. These samples showed similar chromatographic profiles. Out of these samples, P8728 showed the best chromatograms, and this sample was used for the identification of phenolic acids and flavonoids (Figure 4.9).

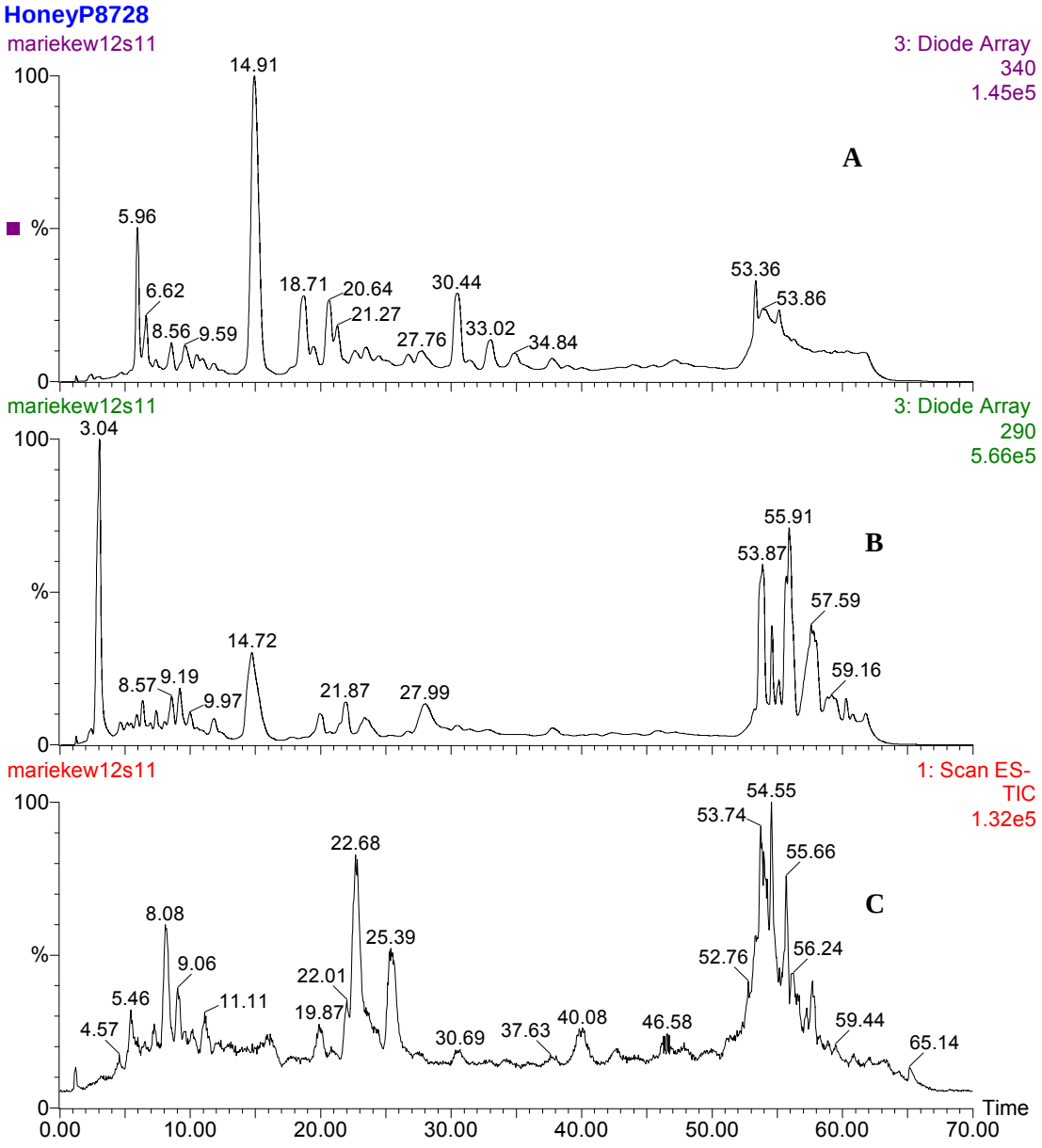


Figure 4.9 **Chromatograms for the flavonoids and phenolic acids of the Salvation Jane honey sample P8728 recorded at UV wavelengths of 340 nm (A) and 290 nm (B), and mass spectrometric total ion current (TIC) (C)**

The first identifications were done using the available standards for flavonoids and phenolic acids. Using the mass spectral SIR mode to analyse two of the Salvation Jane honey samples (P0729 and P8932) (Table 4.14), it was found that a number of phenolic acids and flavonoids were present in very

low concentrations (or not at all). These polyphenols were chosen for SIR analysis as they were previously reported by Yao (2002) to be components of Australian honeys, and authentic standards were available.

Table 4.14 Concentration (μg/100 g) of the phenolic acids and flavonoids quantified in two Salvation Jane honey samples using LC-MS in the selected ion recording (SIR) mode

	Compounds	Quantification detection conditions	P0729	P8932
Phenolic Acids	Gallic Acid	SIR 169*	nd	nd
	Chlorogenic Acid	SIR 353*	2.74	2.20
	Caffeic Acid	SIR 179*	7.01	7.21
	p-Coumaric Acid	SIR 163*	3.09	6.35
	Ferulic Acid	SIR 193*	9.41	9.36
	Ellagic Acid	SIR 301*	0.10	nd
Flavonoids	Quercetin	SIR 301*	6.17	4.07
	Chrysin	SIR 253*	4.95	6.39
	Hesperetin	340 nm	nd	nd

nd Not detected

*SIR Selected ion recording for electrospray ionisation-mass spectrum with negative ionisation-ESI-MS $[M-H]^-$

In the analysis of mass spectra for the polyphenols in samples of Salvation Jane honey, molecular ions of these compounds were compared to those of the many phenolic compounds found in honey (Table 3.4) and other foods (Table 3.5). The identified compounds are listed in the Table 4.15.

Among the flavonoids, luteolin, kaempferol and pinocembrin were identified correctly by their retention times, UV spectra and mass spectra. The UV spectra and full negative ionisation electrospray ionisation-mass spectra of luteolin (Figure A1.12), kaempferol (Figure A1.15) and pinocembrin (Figure A.1.16) are displayed in Appendix 1. α-Cyano-4-hydroxycinnamic acid and 4-hydroxyphenyllactic acid were identified by their mass spectra in the SIR mode. The full negative ionisation electrospray ionisation-mass spectra for these two compounds are displayed in Figures A1.8 and A1.9 (Appendix 1) respectively. Even though these compounds were well separated in HPLC chromatograms, the signals in the total ion chromatograms of these compounds were very weak.

Table 4.15 Phenolic acids and flavonoids identified in Salvation Jane honey

Compound	Detected wavelength(nm)	Retention time (min)	ESI-MS $[M-H]^-$	UV Absorbance (nm)
4-hydroxyphenyllactic acid[a]	290	5.46	181	230, sh
α-cyano-4-hydroxycinnamic acid[a]	290	5.91	188	241, 331
Pinobanksin[b]	340	23.46	271	248, 291
Luteolin[c]	340	27.76	285	241, 303
Kaempferol[c]	340	31.41	285	244, 303
Pinocembrin[c]	340	37.74	255	246, 295

*ESI-MS $[M-H]^-$ Electrospray ionisation-mass spectrum (negative ionisation)
[a]Confirmed by LC-MS (SIR) only, UV(standard) not available
[b]Confirmed by LC-MS (SIR) and UV
[c]Confirmed by LC-MS (SIR)and UV

4.5.2 Quantification of the polyphenols in Salvation Jane honey

Phenolic acids in the honey samples were quantified using a calibration curve based on concentrations of 2.50, 10.00, 50.00 and 100.00 μg/mL for caffeic acid (Figure 4.10). Flavonoids in the honey samples were quantified using a calibration curve based on concentrations of 2.50, 10.00, 50.00 and 100.00 μg/mL for quercetin (Figure 4.11). The concentrations of identified flavonoids and phenolic acids present in the Salvation Jane honey samples P8728, P8684, P8774, P0120, P8676, and P8932 are listed in Table 4.16, while those for the unknown phenolic acids and flavonoids are listed in Table 4.17. Total concentrations of phenolic acids and flavonoids are listed in Table 4.18. Sample P0120 showed an unusually high phenolic acid concentration compared to the other samples. This is due to the very high intensity of the peak at 3.04 min (unknown 3) in P0120.

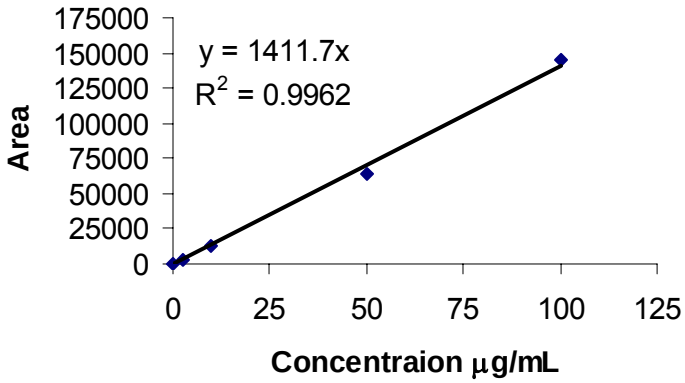


Figure 4.10 Calibration curve of caffeic acid

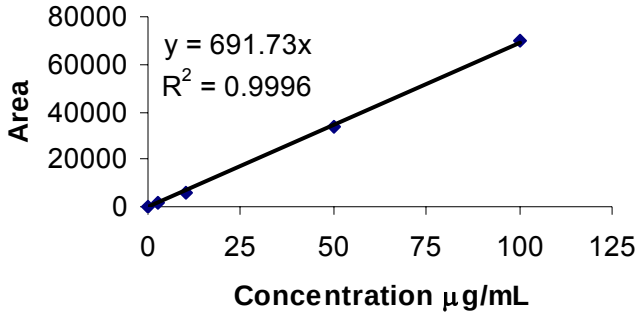


Figure 4.11 Calibration curve of quercetin

Table 4.16 Concentrations (μg/100 g) of the identified flavonoids and phenolic acids in Salvation Jane honey samples

	Honey Sample										
Compound	**RT^**	**P8728**	**P8684**	**P8774**	**P0120**	**P8676***	**P8676a***	**P8932**	**Mean**	**SD**	**%CV**
290 nm											
4-hydroxyphenyllactic acid	5.46	3.78		6.18		2.67			4.21	1.79	42.6
340 nm											
α-cyano-4-hydroxycinnamic acid	5.96	29.23	0.72		49.2	20.06	5.7		20.98	19.42	92.6
pinobanksin	23.46	8.57		10.07	3.52	5.03			6.80	3.04	44.7
luteolin	27.76	12.92	15.89	16.92	6.33	15.53	3.15	3.22	10.57	6.13	58.1
kaempferol	31.41	4.75	11.14	9.82	10.23	11.47	8.74	4.2	8.62		
pinocembrin	37.74	7.58	1.04	4.78	4.12	7.45	6.79	6.06	5.40		

^ RT Retention Time (min)
***** Two separate extracts of the same Salvation Jane honey sample P8676

Table 4.17 Concentrations (μg/100 g) of all flavonoids and phenolic acids in Salvation Jane honey samples

	Honey Sample										
Compound	**RT^**	**P8728**	**P8684**	**P8774**	**P0120**	**P8676[#]**	**P8676a[#]**	**P8932**	**Mean**	**SD**	**%CV**
λ 290 nm											
Unknown 1	1.26	1.18	1.18	1.13	1.99	2.05	3.66	4.35	2.22	1.29	58.3
Unknown 2	2.41	6.37					9.58		7.98	2.27	28.5
Unknown 3	3.04	188.02	400.19	560.94	1324.2	451.86	709	412.69	578.13	365.42	63.2
Unknown 3a	4.72						21.92	4.54	13.23	12.29	92.9
Unknown 3b	5.34						15.04	24.82	19.93	6.92	34.7
Unknown 4	4.66	7.1	0.9	5.36	5.24	3.27			4.37	2.37	54.2
Unknown 5	5.17	5	6.23	5.83	4.17	2.13			4.67	1.63	34.8
4-hydroxyphenyllactic acid	5.46	3.78		6.18		2.67			4.21	1.79	42.6
α-cyano-4-hydroxycinnamic acid*	5.96	29.23	0.72		49.2	20.06	5.7		20.98	19.42	92.6
Unknown 8	6.36	13.99	11.6	14.25	123.3	18.48	56.86	21.96	37.21	41.03	110.3
Unknown 8a+	6.62	10.49	6.23	11.67	21.15		16.93	23.32	14.96	6.62	44.2
Unknown 9	6.94	2.27	2.29	1.23	5.36	1.29	2.45	4.83	2.82	1.64	58.1
Unknown 10	7.39	6.55	13.63	12.19	5.57	17.95	8.6	11.77	10.89	4.32	39.7
Unknown 10a	8.04						5.35	7.51	6.43	1.53	23.8
Unknown 10b	8.57						14.05	17.05	15.55	2.12	13.6
Unknown 10c	9.27						33.63	18.03	25.83	11.03	42.7
Unknown 11	8.04	2.56	3.29	2.98	3.69	5.74			3.65	1.24	33.9
Unknown 12	8.57	20.18	13.26	11.21	22.88	17.96			17.10	4.82	28.2
Unknown 13	9.19	27.79	7.71	14.42	24.2	18.11			18.45	7.94	43.0
Unknown 14	9.97	12.11			11.71	6.46	9.28	7.12	9.34	2.57	27.6
Unknown 15	10.51	3.5	1.52	1.12	8.11	2.39	4.68	3.23	3.51	2.36	67.4
Unknown 16	11.81	14.64	1.16		11.6	2.89	1.16	2.32	5.63	5.92	105.2
Unknown 17	12.8			2.48		3.87			3.18	0.98	31.0
Unknown 17a	13.1						4.95	3.41	4.18	1.09	26.1
Unknown 17b	14.41						1.67		1.67		0.0
Unknown 17c	15.26						47.17		47.17		0.0
Total phenolic acids (1-17c at 290 nm)		**344.27**	**463.68**	**639.32**	**1601.22**	**577.18**	**954.75**	**543.63**	**732.01**	427.3	58.4

Table 4.17 Continued

Compound	RT^	P8728	P8684	P8774	P0120	P8676[#]	P8676a[#]	P8932	Mean	SD	%CV
λ 340 nm											
Unknown 18	14.91	183.06	183.48	211.71	49.87	252.28	246.85	267.12	199.20	73.78	37.0
Unknown 19	18.71	43.35		7.98	54.76	19.3			31.35	21.47	68.5
Unknown 20	19.44	8.1	28.86	15.93		21.59	15.59	10.51	16.76	7.56	45.1
Unknown 21	20.64	28.51	12.62	21.32	29.79	30.12	11.33	17.96	21.66	8.03	37.1
Unknown 22	21.27	14.88	3.17	17.74		14.62	12.43	2.4	10.87	6.49	59.7
Unknown 22a	22.12						5.86	7.06	6.46	0.85	13.1
Unknown 23	22.61	6.43	8.98	12.87	10.01	8.83	68.08	29.88	20.73	22.31	107.6
pinobanksin	23.46	8.57		10.07	3.52	5.03			6.80	3.04	44.7
Unknown 25	24.44	5.97		4.52	2.85	1.64			3.75	1.90	50.6
Unknown 25a								6.35	6.35		0.0
Unknown 25b	25.25						8.82	3.73	6.28	3.60	57.4
Unknown 26			2.21	3.41	1.47	2.86			2.49	0.84	33.7
Unknown 27	26.71	6.33	4.09	8.93	5.54	3.22			5.62		0.0
luteolin	27.76	12.92	15.89	16.92	6.33	15.53	3.15	3.22	10.57	6.13	58.1
Unknown 27a	27.90						23.06	16.76	19.91	4.45	22.4
Unknown 27b	29.15						9.47		9.47		0.0
Unknown 28	30.44	42.83	5.98	34.72	4.28	30.22	4.86	2.44	17.90	17.29	96.5
kaempferol	31.41	4.75	11.14	9.82	10.23	11.47	8.74	4.2	8.62	2.97	34.5
Unknown 28a								4.28	4.28		0.0
Unknown 29	33.02	16.8	3.34	17.84	4.34	17.24	7.56		11.19		
Unknown 29a	34.15						2.62		2.62		0.0
Unknown 30	34.84	11.09	4.47	12.05	9.24	15.59	3.82		9.38	4.55	48.6
Unknown 30a	36.07						1.95	1.13	1.54		0.0
Unknown 30b	36.69						2.74	6.64	4.69	2.76	58.8
pinocembrin	37.74	7.58	1.04	4.78	4.12	7.45	6.79	6.06	5.40		
Unknown 31	38.91	2.58			2.45	0.93		1.98	1.99	0.75	37.7

Table 4.17 Continued

Compound	RT^	P8728	P8684	P8774	P0120	P8676[#]	P8676a[#]	P8932	Mean	SD	%CV
Unknown 32	40.02	1.42		1.06		1.74		1.74	1.49	0.32	21.7
Unknown 33	42.91	2.92	1.32		2.47	4.31			2.76	1.24	44.9
Unknown 33a	43.7						2.12	2.49	2.31	0.26	11.4
Unknown 34	43.94	4.4	9.77	3.3	3.19	6.9	2.02	4.36	4.85	2.65	54.6
Unknown 35	45.47	3.15		1	3.57	1.71		0.66	2.02	1.29	64.0
Unknown 36	47.14	15.69	9.77	8.06	15.93	9.68	19.75	19.76	14.09	4.91	34.8
Unknown 37				4.49					4.49		0.0
Total flavonoids (18-37 at 340 nm)		**441.82**	**312.36**	**440.19**	**245.11**	**482.26**	**484.54**	**444.05**	**407.19**	91.77	22.5

* Quantified at 340 nm not 290 nm as for all other phenolic acids
+ Quantified at 340 nm as for the flavonoids
^ RT Retention Time (min)
[#] Two separate extracts of the same Salvation Jane honey sample P8676

Table 4.18 Total concentrations (µg/100 g) of the flavonoids and phenolic acids in Salvation Jane honey samples

		Honey Sample									
Compound		**P8728**	**P8684**	**P8774**	**P0120**	**P8676***	**P8676a***	**P8932**	**Mean**	**SD**	**%CV**
Total phenolic acids (290 nm)		344.27	463.68	639.32	1601.22	577.18	954.75	543.63	732.01	427.3	58.4
Total flavonoids (340 nm)		441.82	312.36	440.19	245.11	482.26	484.54	444.05	407.19	91.77	22.5
Flavonoids + Phenolic Acids		**786.09**	**776.04**	**1079.51**	**1846.33**	**1059.44**	**1439.29**	**987.68**	**1139.20**	382.87	33.6

***** Two separate extracts of the same Salvation Jane honey sample P8676

4.6 Composition of the Polyphenols in Extracts of Spotted Gum Honey

4.6.1 Identification of the polyphenols in spotted gum honey

The chromatogram for an extract of spotted gum honey is displayed in Figure 4.12. There were 9 major compounds detected.

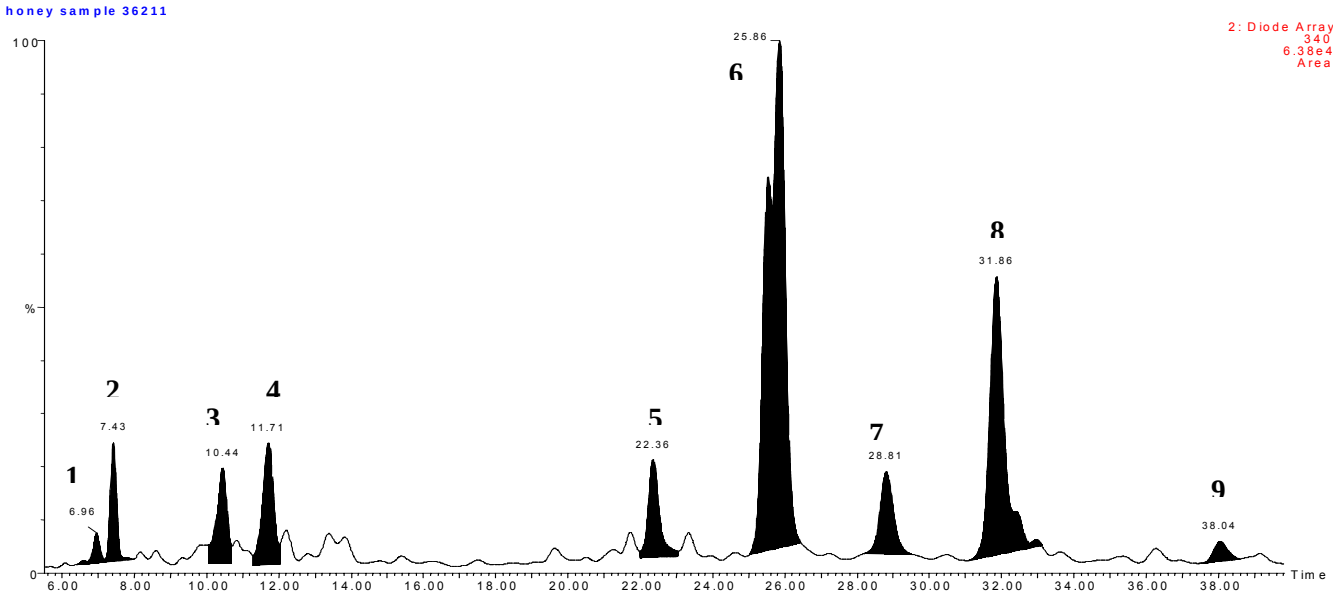


Figure 4.12 The UV absorption HPLC chromatogram of spotted gum honey measured at 340 nm with a photodiode array detector: 1, 3, 4, 9 = unidentified compounds; 2 = caffeic acid; 5 = myricetin; 6 = tricetin; 7 = quercetin; 8 = luteolin

Compounds 6, 7 and 8 were identified as tricetin, quercetin and luteolin respectively (Table 4.19). Compound 2 had the same mass spectrum as caffeic acid, but its UV spectrum was not. The other compounds could not be identified. Yao (2002) also found the characteristic flavonoids tricetin, quercetin and luteolin in Australian unifloral *Eucalyptus* honey samples.

Table 4.19 Compounds detected in spotted gum honey

Compound No.	RT (min)^	Compound Name	ESI-MS* $[M-H]^-$	UV (nm)
1	6.49	unknown		249
2	7.41	caffeic acid	179	248, 286, 320
3	10.44	unknown	175	307
4	11.73	unknown	173, 187	
5	22.36	myricetin	317	247,282,316
6	25.86	tricetin	301	253, 353
7	28.81	quercetin	301	255, 364
8	31.86	luteolin	285	255, 350
9	38.04	unknown	269	

^RT Retention Time
*ESI-MS Electrospray ionisation-mass spectrum (negative ionisation)

5. Implications

This project has produced preliminary data on the identity and concentrations of the antioxidant flavonoids and phenolic acids in straightline samples of four Australian floral honeys. Some polyphenols were identified with instrumental techniques such as HPLC-PDA and LC-MS; but there are still many unknown flavonoids and phenolic acids for these four floral types of honey. The implications of this small study is that since only four floral types were studied, a detailed comparison between floral types to determine which Australian honey type has the highest concentrations of the antioxidant flavonoids and phenolic acids, is not possible at this time.

This scientific data on the total concentration of flavonoids and polyphenols in some Australian floral honeys will enable the further marketing of honey as a healthy and nutritious food to the Australian food industry and consumers, in addition to its use as a sweetener

6. Recommendations

The main recommendation is that before any future study of honey antioxidants such as flavonoids and phenolic acids is done, an assay of the total polyphenol content, total flavonoid content, and antioxidant content and capacity using spectrophotometric methods needs to be done on as many samples of as many floral types of Australian honey. Then, those floral types of Australian honey that show high values for these parameters should be analysed using detailed identification methods such as HPLC-PDA, LC-MS and GC-MS. This would mean that the floral types with the highest antioxidant effect can be marketed much like the antimicrobial jellybush honey is marketed by Medihoney.

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Appendix 1

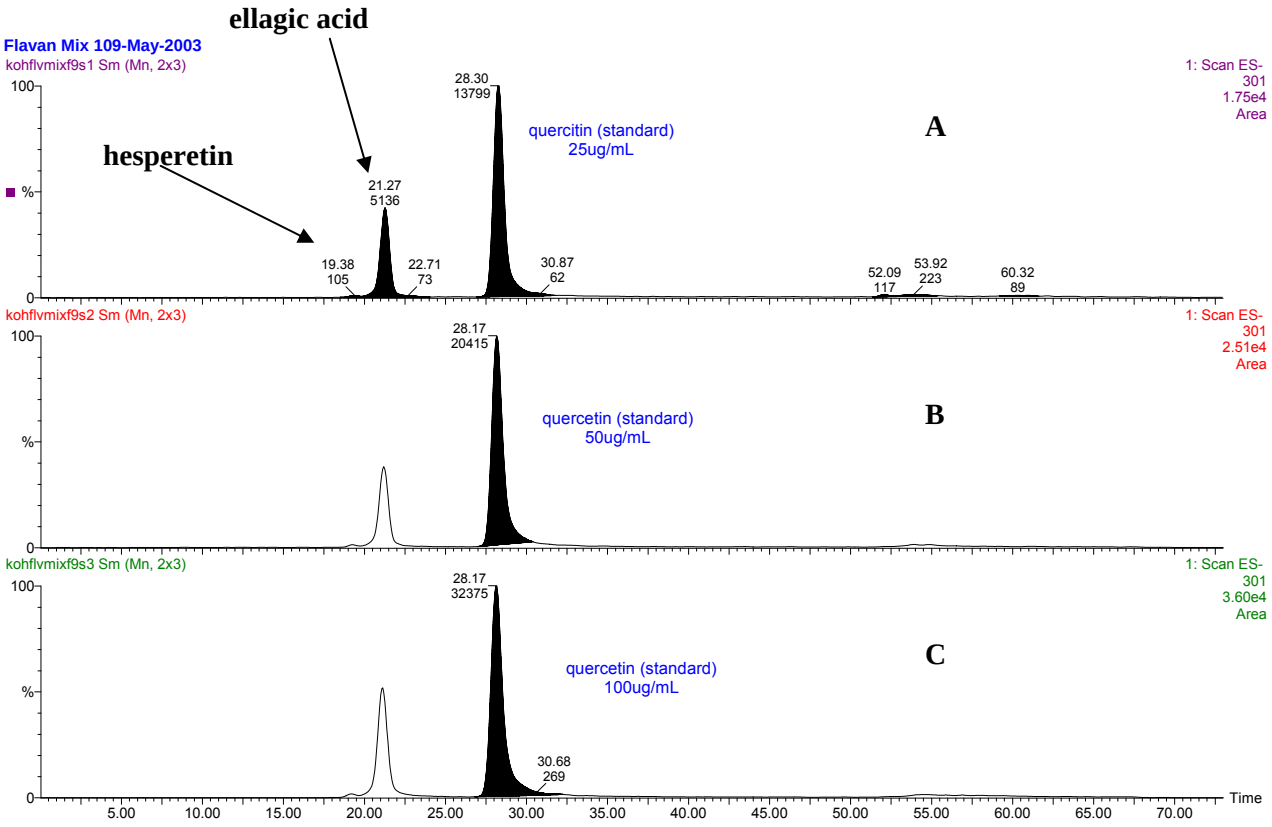

Figure A1.1 **Chromatograms of hesperetin, ellagic acid and quercetin in the mixed standards scanned using negative ionisation, mass spectral selection ion recording (SIR) at 301 at concentrations of 25µg/mL (A), 50µg/mL (B) and 100µg/mL (C)**

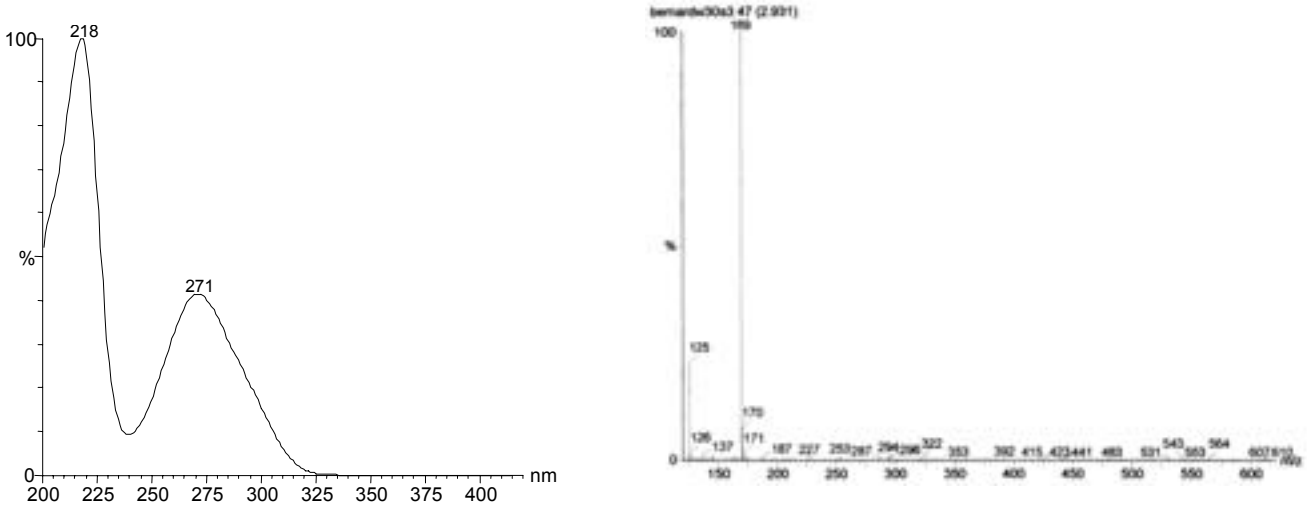

Figure A1.2 UV and electrospray ionisation-mass spectra (negative ionisation) for gallic acid

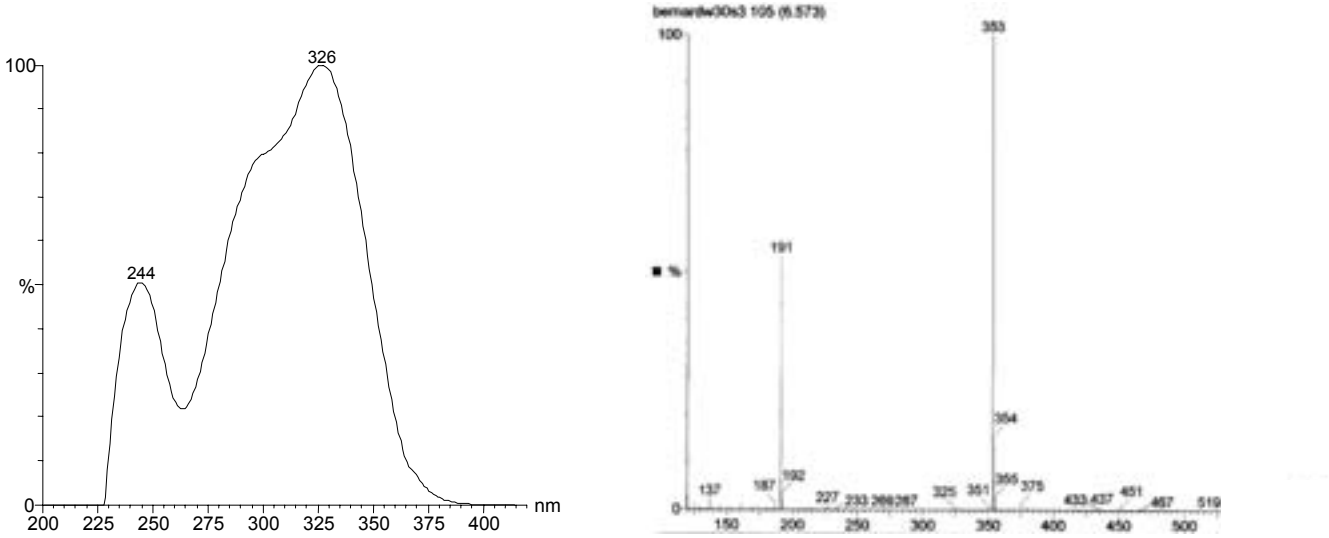

Figure A1.3 UV and electrospray ionisation-mass spectra (negative ionisation) for chlorogenic acid

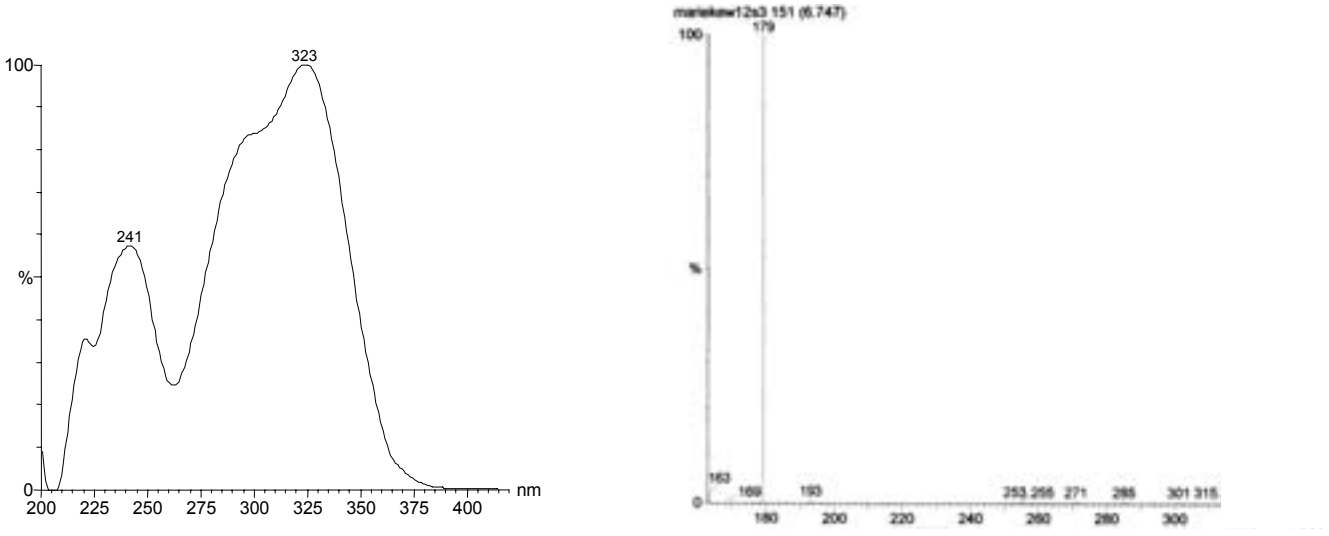

Figure A1.4 UV and electrospray ionisation-mass spectra (negative ionisation) for caffeic acid

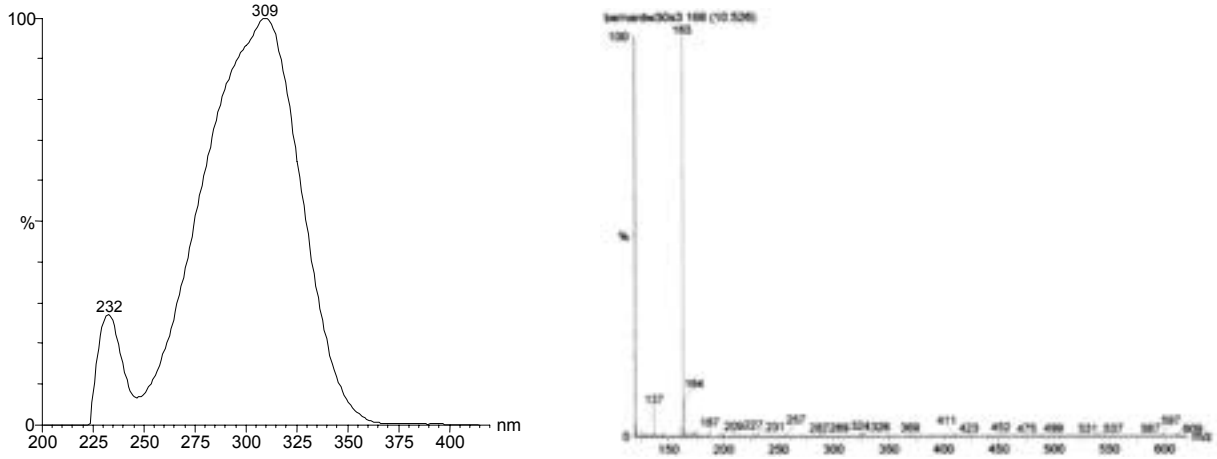

Figure A1.5 UV and electrospray ionisation-mass spectra (negative ionisation) for *p*-coumaric acid

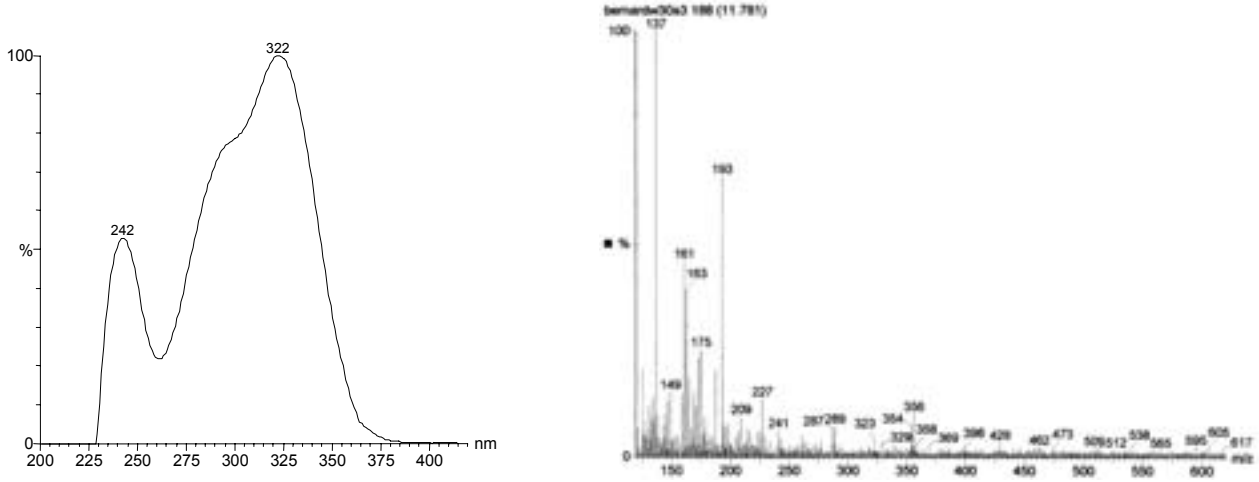

Figure A1.6 UV and electrospray ionisation-mass spectra (negative ionisation) for ferulic acid

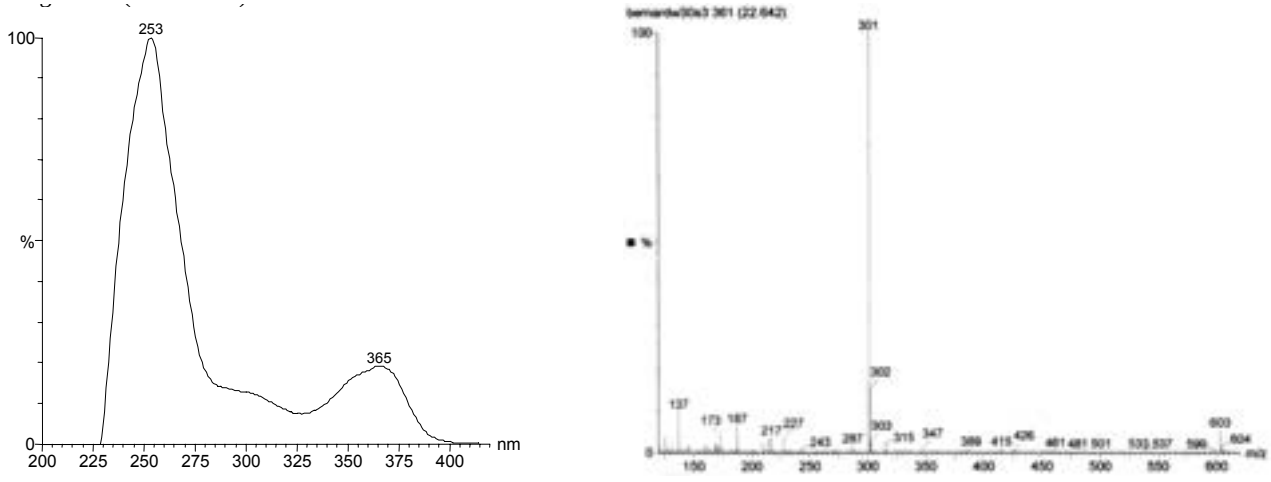

Figure A1.7 UV and electrospray ionisation-mass spectra (negative ionisation) for ellagic acid

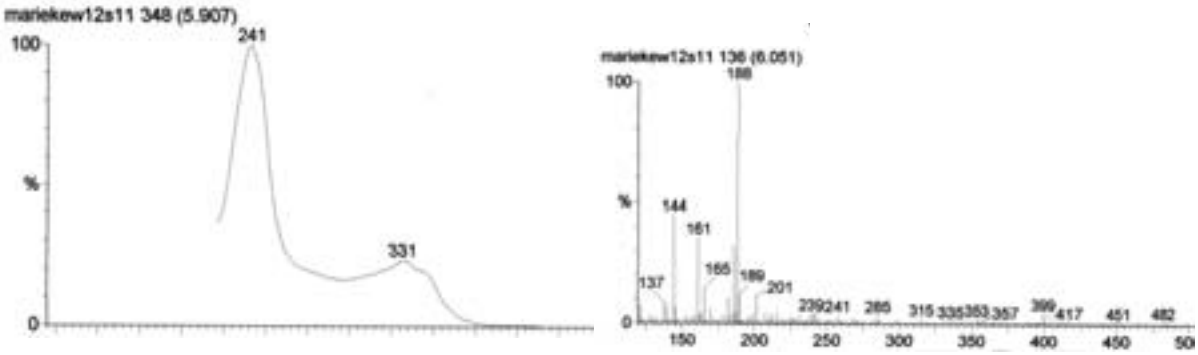

Figure A1.8 **UV and electrospray ionisation-mass spectra (negative ionisation) for α-cyano-4-hydroxycinnamic acid**

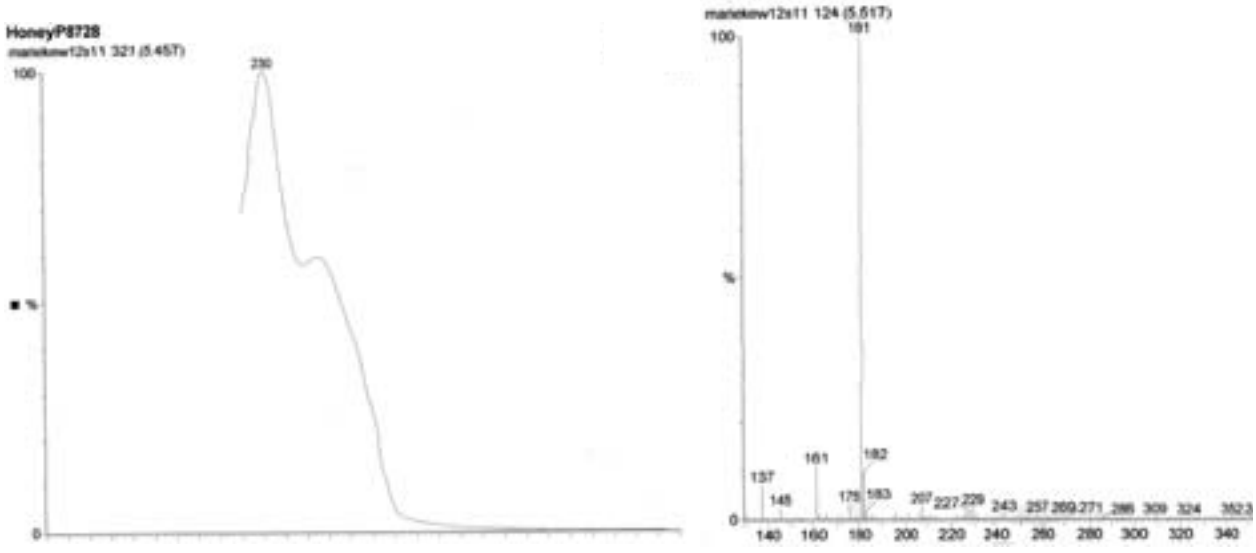

Figure A1.9 **UV and electrospray ionisation-mass spectra (negative ionisation) for 4-hydroxyphenyllactic acid**

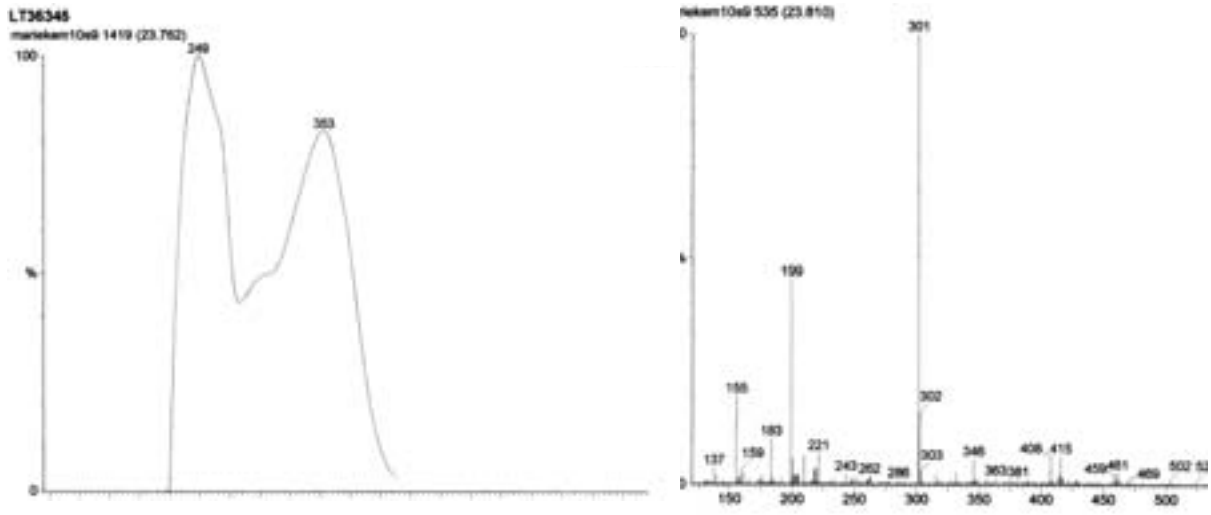

Figure A1.10 UV and electrospray ionisation-mass spectra (negative ionisation) for tricetin

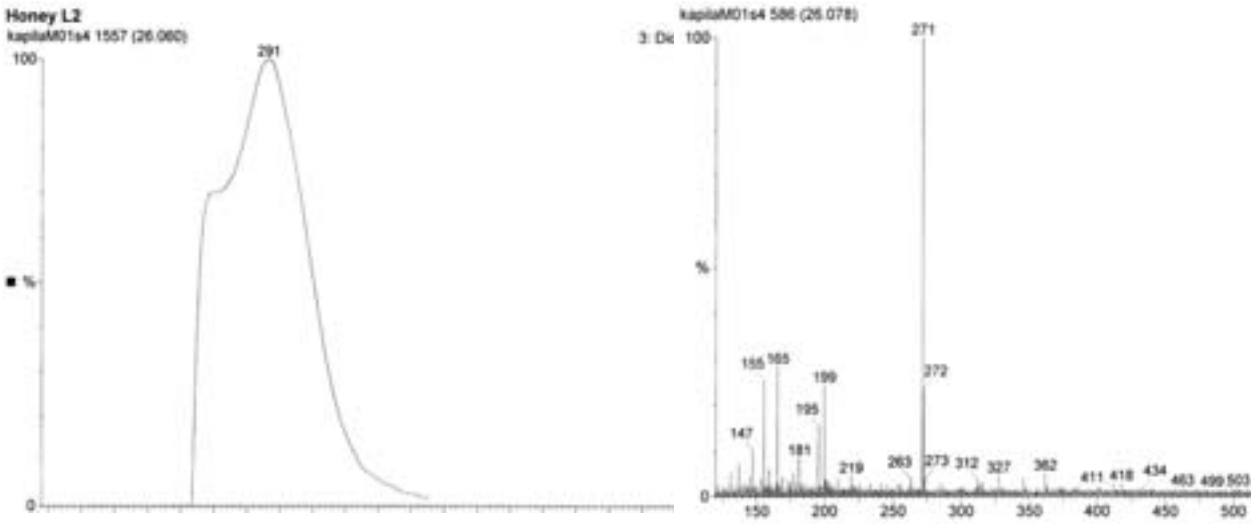

Figure A1.11 UV and electrospray ionisation-mass spectra (negative ionisation) for pinobanksin

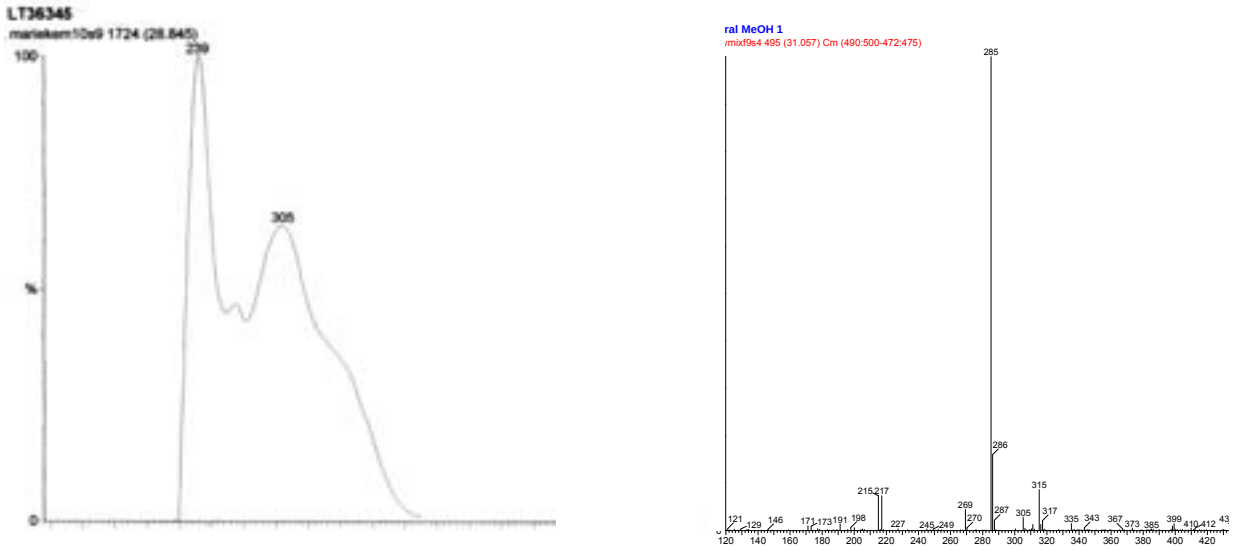

Figure A1.12 UV and electrospray ionisation-mass spectra (negative ionisation) for luteolin

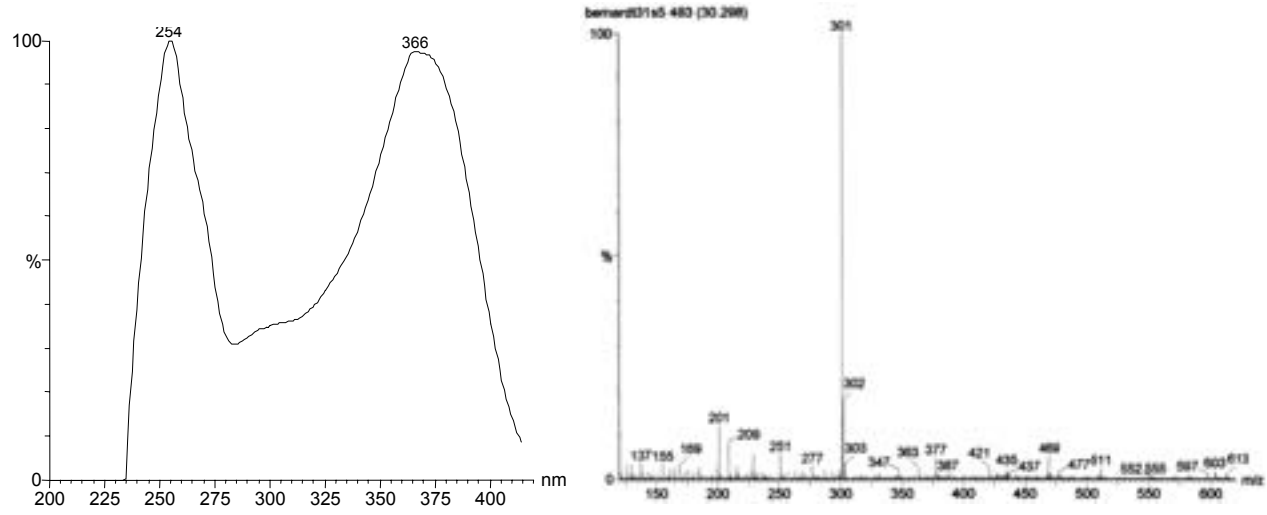

Figure A1.13 UV and electrospray ionisation-mass spectra (negative ionisation) for quercetin

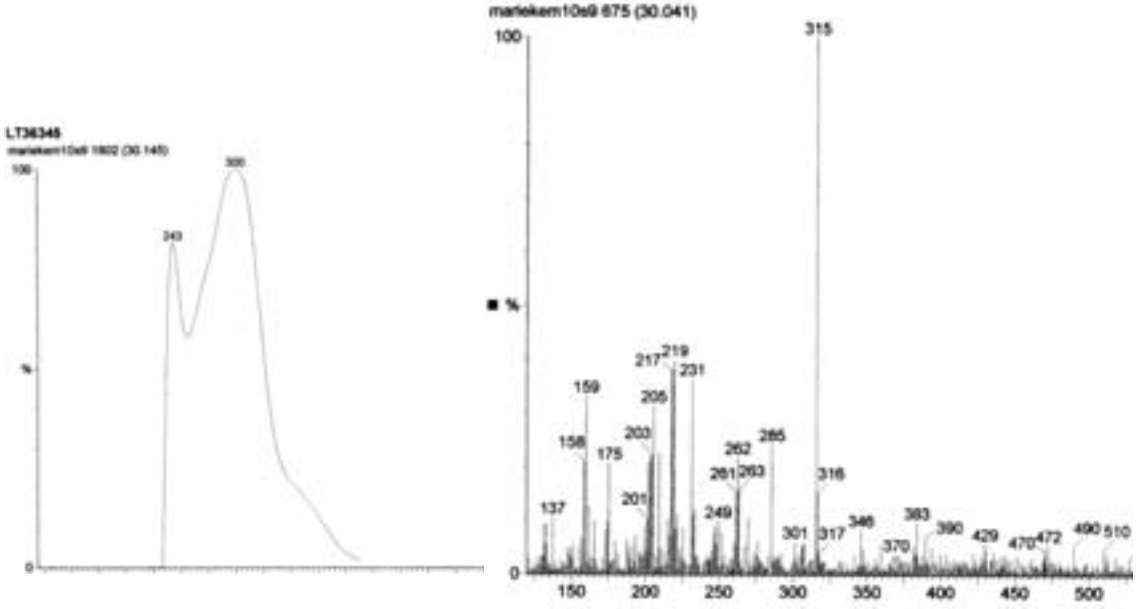

Figure A1.14 UV and electrospray ionisation-mass spectra (negative ionisation) for quercetin-3-methylether

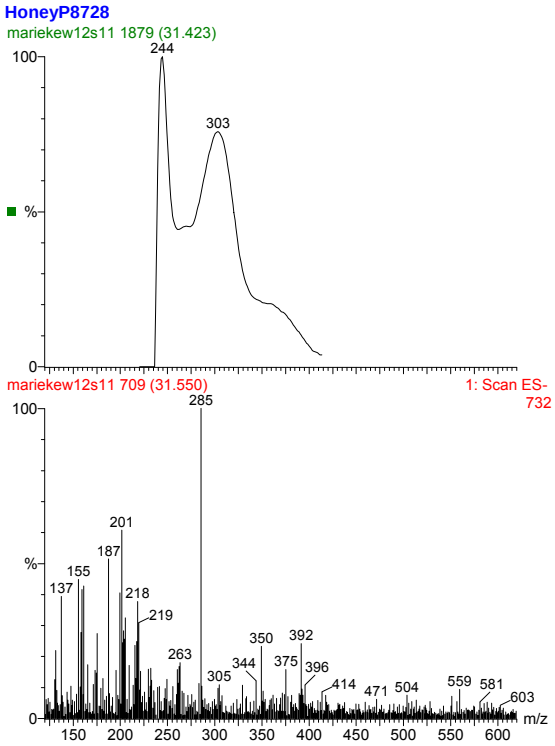

Figure A1.15 UV and electrospray ionisation-mass spectra (negative ionisation) for kaempferol

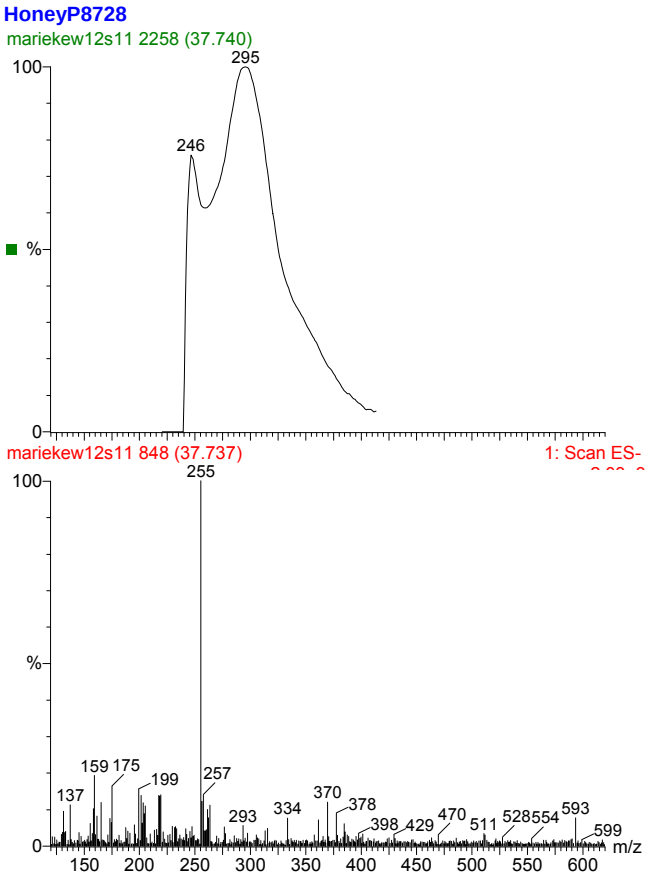

Figure A1.16 UV and electrospray ionisation-mass spectra (negative ionisation) for pinocembrin

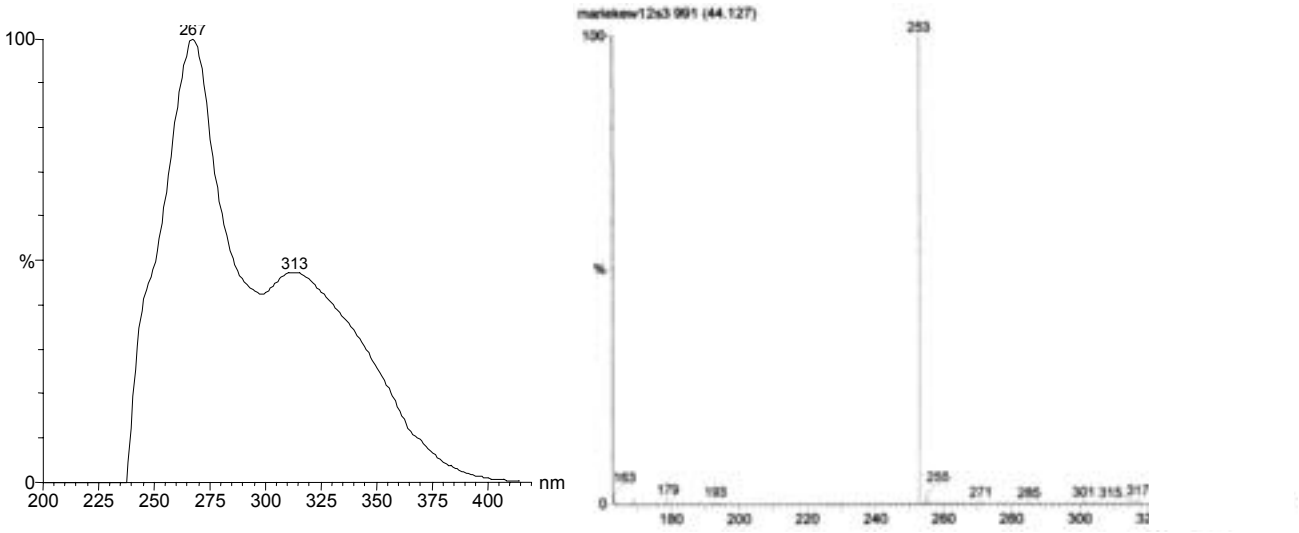

Figure A1.17 UV and electrospray ionisation-mass spectra (negative ionisation) for chrysin

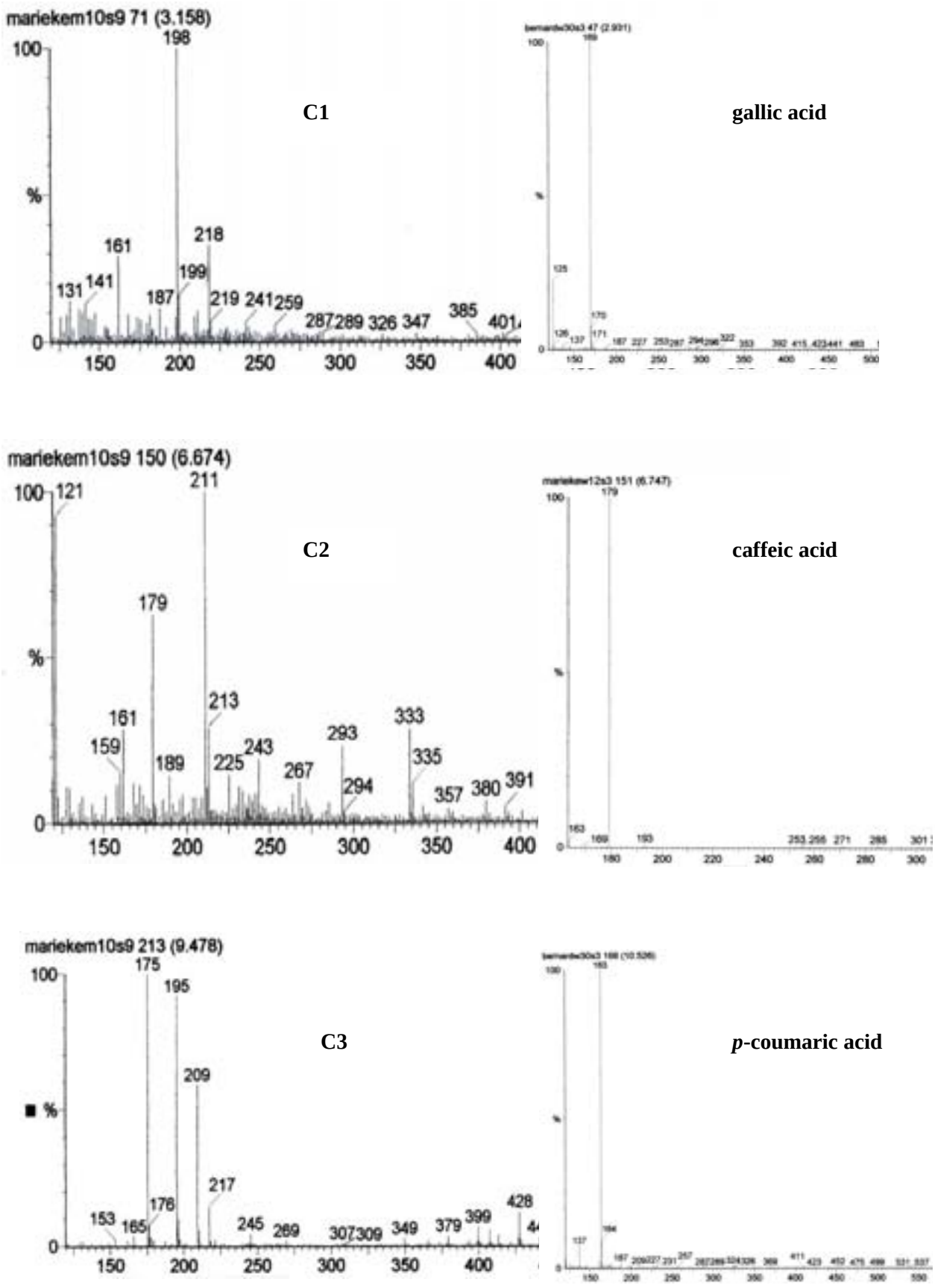

Figure A1.18 Negative ionisation mass spectra for the unknown compounds, C1, C2 and C3 in yapunyah honey, and for gallic acid, caffeic acid and *p*-coumaric acid standards

Table A1.1 Retention times and UV and mass spectral data for the flavonoids and phenolic acids extracted from one yapunyah honey sample

Compound	Chromatogram Source	Retention Time (min)	ESI-MS* $[M-H]^-$	UV Bands (nm)
Gallic acid	**SIR 169**	**2.70**	**169**	**219, 271**
Unknown (C1)	**PDA 290 nm**	**3.40**	**198**	**231, 283**
Chlorogenic acid	PDA 290 nm	6.20	353	244, 300, 326
Unknown (C2)	**PDA 290 nm**	**6.93**	**121**	**222, 265, 285 (sh)**
Caffeic acid	**PDA 290 nm**	**7.17**	**179**	**220 (sh), 242, 295 (sh), 325**
***p*-coumaric acid**	**PDA 290 nm**	**10.20**	**163**	**232, 310**
Unknown (C3)	**PDA 290 nm**	**10.54**	**175, 195**	**230, 286**
Ferulic acid	PDA 340 nm	11.27	193	242, 300 (sh), 323
Ellagic acid	PDA 340 nm	21.27	301	253, 305 (sh), 365
Tricetin	PDA 340 nm	25.34	301	249, 300 (sh), 353
Pinobanksin	PDA 290 nm	27.50	271	250 (sh), 288
Quercetin	PDA 340 nm	28.20	301	254, 270 (sh), 305 (sh), 367
Luteolin	PDA 340 nm	31.20	285	242, 270 (sh), 304, 351
Isorhamnetin	PDA 340 nm	31.77	315	255, 268 (sh), 310 (sh), 328 (sh), 371
Kaempferol	PDA 340 nm	35.57	285	250 (sh), 266, 295 (sh), 320 (sh), 367
Pinocembrin	PDA 340 nm	42.20	255	246 (sh), 292, 329 (sh)
Chrysin	SIR 253	48.30	253	267, 313

*****ESI-MS Electrospray ionisation-mass spectrum (negative ionisation)

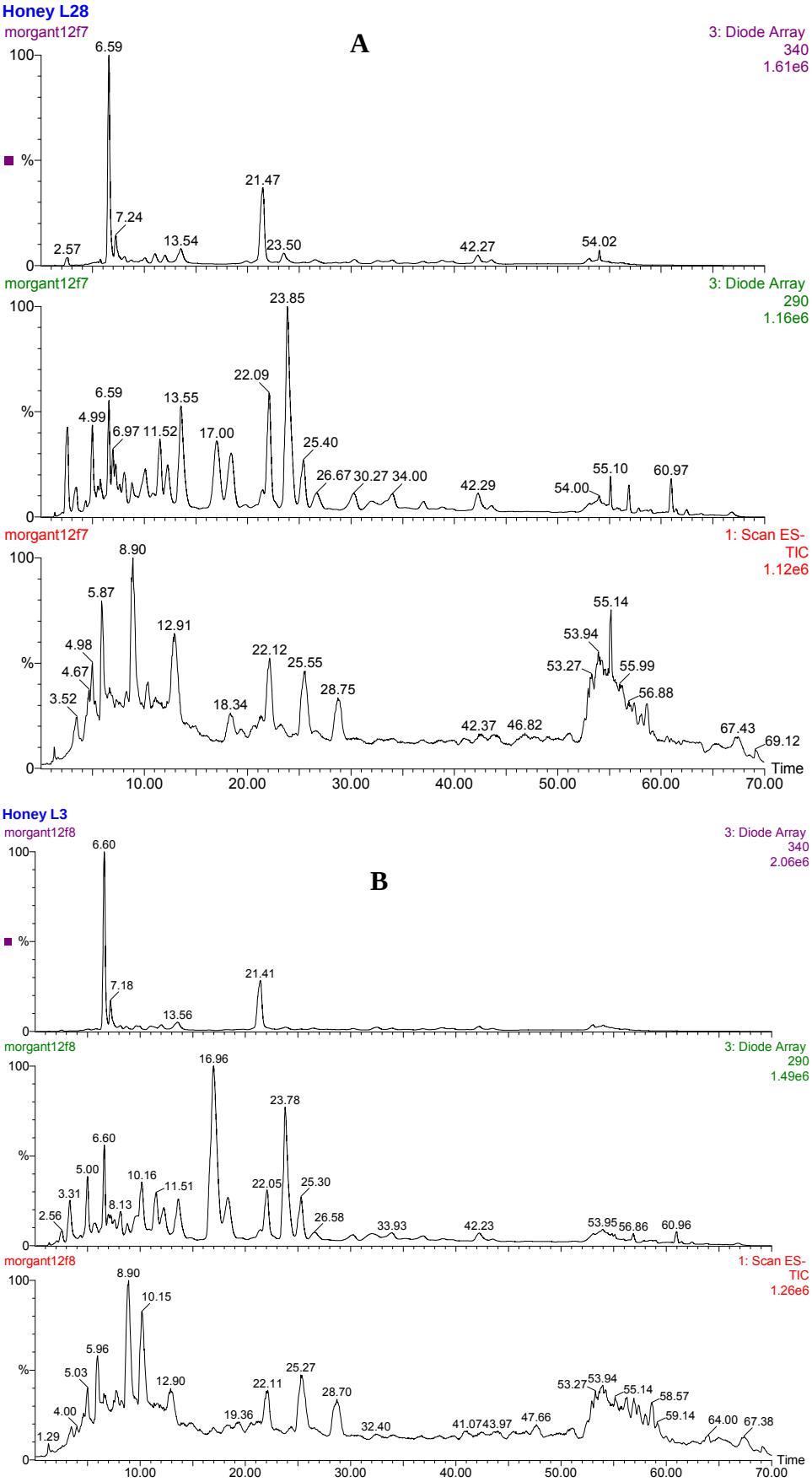

Figure A1.19 Chromatograms for flavonoids and phenolic acids of the leatherwood honey samples L28 (A) and L3 (B) recorded at UV 340 nm, 290 nm and mass spectral total ion current (TIC) mode