Profiling of propolis samples from UK, Indonesian and sub-Saharan Africa and isolation of some pure components

A Thesis presented for the Degree of Doctor of Philosophy in the Strathclyde institute of pharmacy and biomedical science at the University of Strathclyde

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Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

Date:

Acknowledgement

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Table of Contents

List of Figures

List of Tables

LIST OF ABBREVIATIONS

µM Micromolar

C NMR13 Carbon nuclear magnetic resonance

2D NMR Two dimensional nuclear magnetic resonance spectroscopy

CC Column chromatography

CDCl3 Deuterated Chloroform

COSY 1H-1H Correlation Spectroscopy

d Doublet

dd Doublet of a doublet

DEPT Distortionless Enhancement by Polarization Transfer

DMSO Dimethyl sulphoxide

EEP Ethanolic extract of propolis

EI Electrospray ionization

ELSD Evaporative light scattering detection

EtOAc Ethyl acetate

FAB Fast Atom Bombardment

H Hour

H NMR1 Proton nuclear magnetic resonance

HAT Human African Trypanosomiasis

HCA Hierarchical Clustering analysis

HMBC Heteronuclear Multiple Bond Coherence

HMQC Heteronuclear Multiple Quantum Coherence

HPLC High performance liquid chromatography

HR-ESIMS High resolution electrospray ionisation mass spectrometry

Hz Hertz

LC-MS Liquid chromatography mass spectrometry

M Multiple

mg milligram

MHz Megahertz

MIC Minimum inhibitory concentration

ML millilitre

MPLC Medium pressure liquid chromatography

MS Mass Spectroscopy

NMR Nuclear Magnetic Resonance

No. Number

NOESY spectroscopy Nuclear Overhauser effect

^{o}C Celsius

S Singlet

SP straight phase

TTriplet

TLC Thin layer Chromatography

TMS Tetramethylsilane

UV Ultraviolet light

Abstract

Propolis is a resinous material collected by bees as part of the defensive system of the bee hive. It has a widerange of biological activities including anti-protozoal and and microbial activity. Samples of propolis from different parts of the UK, Africa and Indonesia were extracted and profiled by liquid chromatography-high resolution mass spectrometry. The data were aligned and features were extracted into 0.02 amu windows. In order to establish a platform for comparison of the samples the top features by peak intensity, after excluding abundant dimer peaks, were further characterized by MS^2. Putative identities were deduced from accurate masses which were within 3 ppm deviations from the exact mass of the proposed elemental compositions. For the UK propolis samples, over 90% of the peaks could be assigned identities with some level of confidence. The compounds identified fell into the major categories: flavonoid esters and possibly some ethers, phenyl propanoid esters, glycerol esters, flavonoid glycosides and hydroxylated fatty acids. Pinobanksin was the most abundant compound in the samples. The flavonoids pinocembrin, pinobanksin, galangin and chrysin showed a relatively low degree of variation across the nine samples whereas flavonoid esters and glycerol esters were more variable in their response across the nine samples. The propolis samples from hives in three different areas of Scotland yielded hundreds of components, many of which were not identified. In the Aberdeenshire samples principal components analysis (PCA) followed by hierarchical cluster analysis (HCA) grouped nine samples into six groups

according to the abundance of their components. The Aberdeenshire samples were abundant in glycerol esters. Five samples from Fort William could be divided into four groups. These samples had quite a different composition from the Aberdeenshire samples containing an abundance of compounds putatively identified as being sesquiterpene acids. Three samples from Dunblane were different again but had more similarity in composition to the Aberdeenshire samples than the Fort William samples. The propolis sample from Indonesia yielded predominantly phenolic compounds. Although standards were not available to determine the identities and actual amounts of the compounds in the samples, preparative chromatographic separation enabled the identification of some of the compounds by NMR spectroscopy (1D and 2D) and mass spectra as pinobanksin, apigenin, lupeol, gallic acid and quercetin. The role of propolis in warding off infections in the bee hive which is yet established was addressed in this study and the approach provides a potential method for correlating hive health with the composition of the propolis gathered by the bees. Thus this study also confirms local and regional variations in propolis composition over a relatively small areas and over wide geographical regions.

Published work from this research

CHAPTER THREE:

CONSTITUENTS OF PROPOLIS FROM DIFFERENT REGIONS OF THE UNITED KINGDOM BY LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROMETRY AND A METABOLOMICS APPROACH.

Khaled Saleh, Tong Zhang, James Fearnley, David George Watson.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction

Propolis, also known as bee glue is a resinous substance made by honeybees from materials collected from plants particularly from flowers and leaf buds to maintain the hive environment aseptic (Krell 1996). Propolis consists of beeswax and plant-derived secondary metabolites and its composition is highly complex and variable. Bees collect materials from the bark, buds, and flowers of plants which they then partially digest or mix with saliva to form propolis (Wagh, 2013). Propolis fulfils an important function in the hive, where it is used to coat the surfaces of the hive promoting a sterile environment within the hive and thus social immunity. Bees which collect greater amounts of propolis are more hygienic and produce more honey (Nicodema *et al,* 2014). High-propolis producing colonies have been observed to have significantly higher brood viability and greater worker bee longevity (Nicodema *et al,* 2013). Propolis is used in traditional medicine in many countries. (Popova *et al*. 2010) and lately it has become popular as a component of health foods and alternative medicine (Da Silva Frozza *et al*. 2010). For propolis produced in the Pacific region, geranyl flavanones are the characteristic compounds which are also found in Propolis from the African region. The chemical composition of propolis is susceptible to the geographical location, botanical origin, and bee species.

In spite of the growing awareness on the values of propolis, it is mostly wasted by local or traditional bee farmers or honey collectors. After collecting the honey from the hives, the farmers usually abandon the hive in search of other ones. The propolis is thereby abandoned or wasted. If the economic value of propolis is known to the farmers, they would collect and market it to improve their livelihood. Bee propolis is

reputed to have high economic and medicinal values. It is well known for its biological activities such as anti-cancerous (Marcucci 1995), anti-inflammatory and antioxidant (Siripatrawan *et al* 2013), antimicrobial (Bankova 2009) and cytostatic (Shubharani *et al* 2014). Because of these biological activities, it is widely used to prevent and treat colds, wounds and ulcers, rheumatism, sprains, heart disease, diabetes and dental caries (Brito *et al*; 2010).

At elevated temperatures propolis is soft, flexible, and very sticky; however, when cooled, and particularly when frozen or at near freezing, it becomes hard and brittle. It will remain brittle after such treatment even at higher temperatures. Typically, propolis will become liquid at 60 to 70◦C, but for some samples the melting point may be as high as 100◦C (Krell 1996). Raw propolis is typically composed of 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollens and 5% other organic substances (Brito *et al;* 2010). The propolis comes in different colours: yellow, red, and even brown, depending on the plants that materials are collected from, the season of collection, the location of the hive, and the time at which it was made (Fearnley, 2001, Wagh, 2013). The wide application of propolis in modern medicine has drawn growing attention to its chemical composition. Many studies have revealed that the observed effects might be the result of synergistic action of its complex constituents (Brito *et al;* 2010).

1.2 Historical Background

The word “propolis” is derived from a Hellenistic Ancient Greek word which means suburb, which originates from Greek verb (*promalasso*) (Liddell and Scott, 1940). The use of propolis is as old as that of honey, and it has been used by man for ages. There are records suggesting the use of propolis by ancient Egyptians, Persians, and Romans

(Houghton, 1998). Ancient Egyptians depicted propolis-making bees on their paintings, vases and other ornaments and it is used it to alleviate many ailments (Langenheim, 2003). The Egyptians had learnt embalming from the bees, which use propolis as an "embalming" substance. The bees cover the carcass of dead invaders that could not be transported out of the hive, with propolis and wax (Nicolas 1947). In this way the bees restrain the spread of infection caused by the decomposing carcass. In the 1960s, Derevici *et al*. showed that propolis is responsible for the lower incidence of bacteria in hives. The ancient Jews considered *tzori* (the Hebrew word for propolis) as a medicine. *Tzori* and its therapeutic properties are mentioned in some Holy Books. The biblical Balm of Gilead (*tzori Gilead* in Hebrew) is nearly indistinguishable from propolis. It may have been around the Dead Sea for about 1,500 years and achieved fame due to its aroma and medicinal properties. It is made of resin from various poplars, including *P. balsamifera*, *P. nigra*, and *P. gileadensis* (Broadhurst, 1996). Balm of Gilead was one of the several components of the special incense that was used in religious worship. The identification of the balm of Gilead with the Hebrew names *Afarsemon, kataf, nataf*, and *tzori* Gilead can be traced to several sages, including Shimon Ben-Gamliel, Rambam (Maimonides), Saadia Gaon, and the modern biblical botanist Yehuda Feliks (Ben-Yehoshua *et al.*, 2012). The Greeks used propolis as the primary ingredient of *polyanthus*, perfume which combined propolis, olibanum, styrax, and aromatic herbs (Bogdanov 2012). More than 15 Greek and Roman authors reported on the preparation and application of propolis, the so-called third natural product of bees (besides honey and wax). The interest in propolis returned to Europe together with the Renaissance theory of *ad fontes*, which brought back an interest in ancient teaching and medicine. In a famous herbal book, *The History of Plants*, John

Gerard (1597), makes reference to the use of "the resin or clammy substance of the black poplar tree buds" for healing ointments. "The ointment that is made of the poplar buds, is good against all inflammations, bruises, squats, fals, and such like. Propolis was included in pharmacopoeias in England in the seventeenth century as a major ingredient of healing ointments (Murray and Pizzorno, 2005).

1.3 Composition of propolis

Propolis is a honeybee product with a broad spectrum of biological properties. As a resinous substance, propolis is prepared by the honeybees to seal the cracks, smooth walls, and to keep moisture and temperature stable in the hive all year around. Previous reviews have covered the knowledge about the chemical composition and botanical origin of Propolis throughout 20th century. Until the year 2000, over 300 chemical components belonging to the flavonoids, terpenes, and phenolics have been identified in Propolis (Ito *et al* 2001).

Raw propolis is typically composed of 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollens and 5% other organic substances. It has been reported that propolis is collected from resins of poplars, conifers, birch, pine, alder, willow and palm. For propolis produced in the Pacific regions, geranyl flavanones are the characteristic compounds which are also found in propolis from the African regions. The chemical composition of propolis is susceptible to the geographical location, botanical origin, and bee species. The chemical components isolated from propolis from year 2000 onwards have been reviewed in order to help provide a basis for the study of the chemical composition of propolis, the pharmacological activity of propolis compounds and their plant sources. This is also useful for standardization and quality

control of propolis (Popova *et al* 2010). From assessing the yield of flavanoids from propolis obtained from different extraction methods, Niken *et al.* (2014) observed that the highest yield of extract was obtained by using a mixture of ethanol and water (18.33%) and the lowest yield was with olive oil (14.06 %). The ethanol and water extracts were gummy and sticky whereas the oil extract was gummy and oily. The flavonoid content in propolis ranged from 0.2 % to 0.55% in the extracts.

The phytochemical analysis of propolis from different countries or regions has yielded many natural products e.g. a propolis sample from Inner Mongolia yielded (as the major constituents of the volatile oils) bisabolol, 2-methyl-3-buten-2ol and 3-methyl-2-butene-1-ol. Studies on propolis obtained from Ethiopia yielded oxygenated aliphatic hydrocarbons, monoterpenes and sesquiterpenes. Propolis from Libya and Saudi Arabia yielded compounds such as prenylflavanones (propolins A-H) (**1-8**), flavonoids (**9** and **10**), diterpenes (**11-15**), xanthones, lignans (Valcic *et al* 1998, Kardar *et al*, 2014).

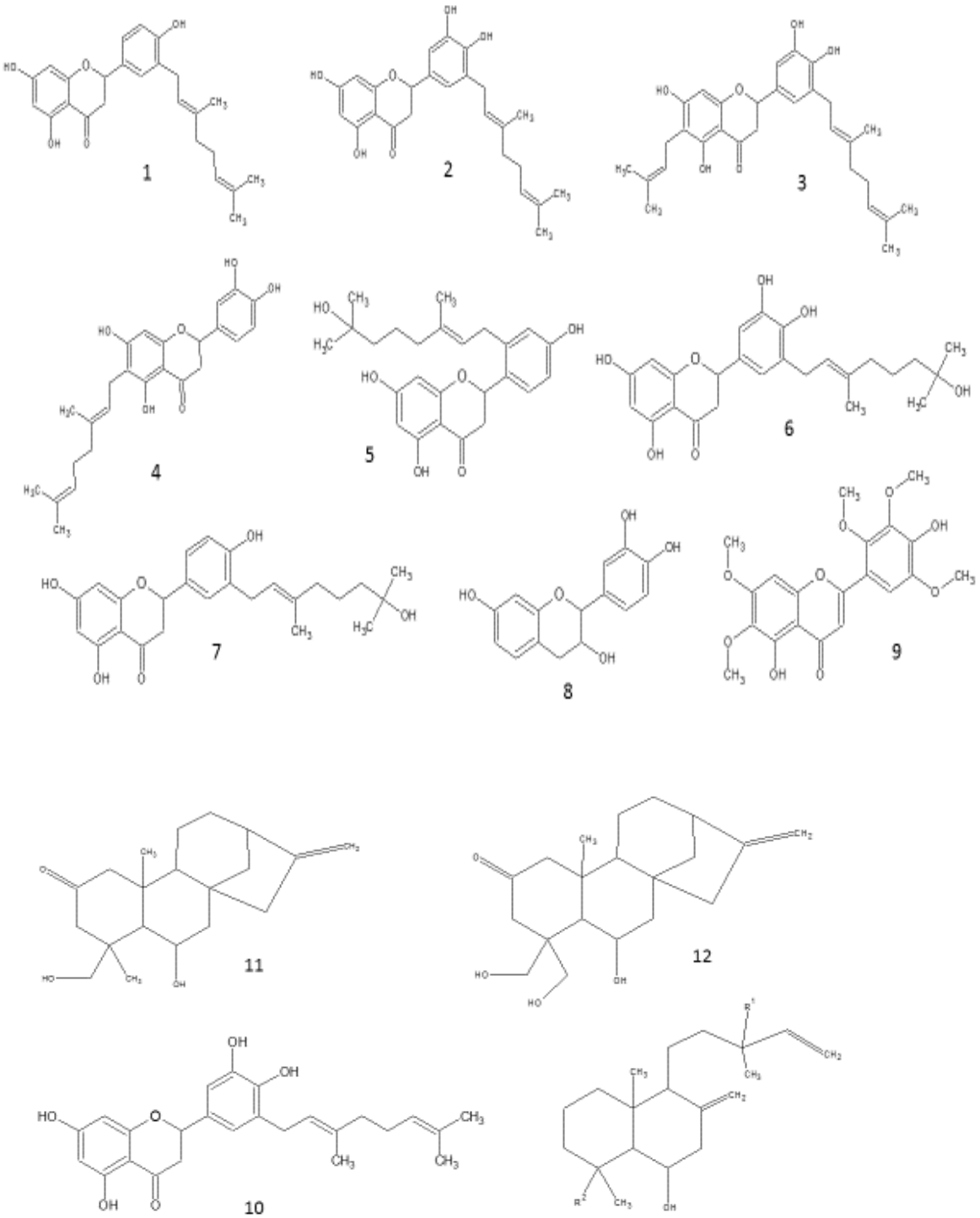


Figure 1.1: Some compounds isolated from propolis from different countries.

Recent studies on propolis from different countries or regions within a country has yielded several known and novel compounds. For example the study on a sample of red propolis from Bonny in Rivers State, Nigeria yielded ten compounds (Figures 1.2 and 1.3) including a novel benzofuran, Riverinol.

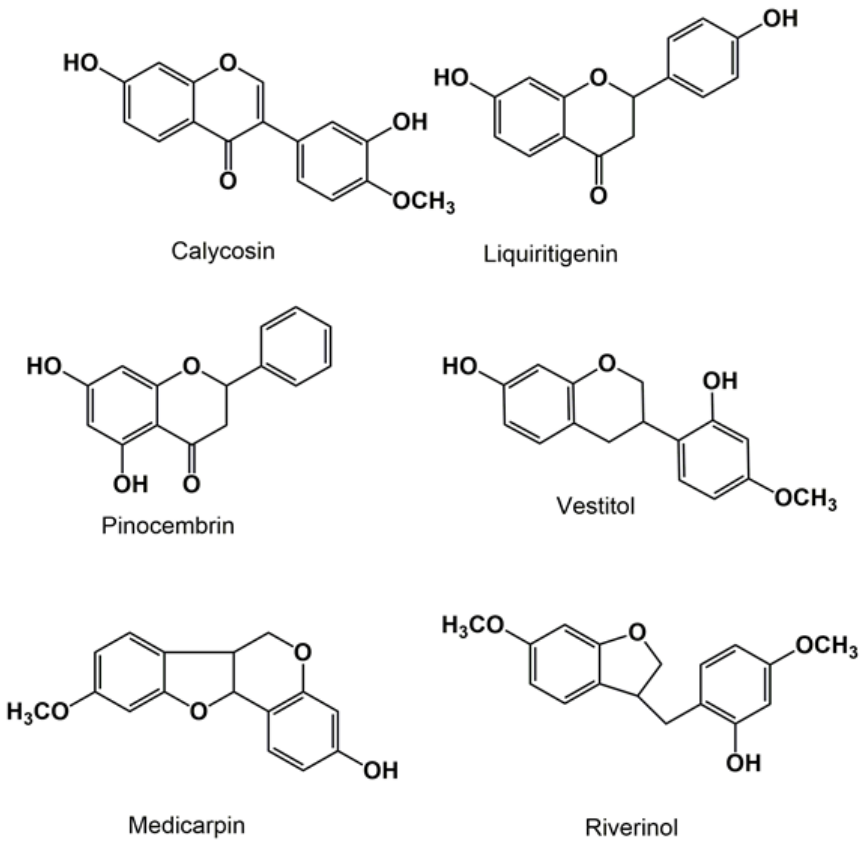


Figure 1.2: Structures of flavonoids, isoflavonoids and benzofurans isolated from Nigerian red propolis

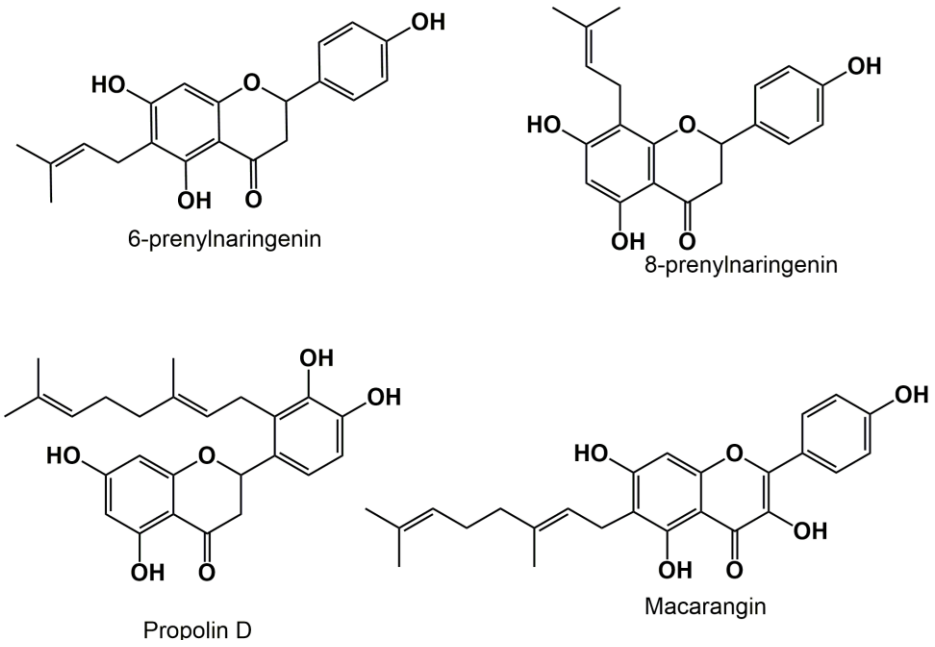


Figure 1.3: Structures of prenylated flavonoids isolated from Nigerian red propolis

Propolis has been used empirically for centuries and has been described as an immunomodulatory agent. In recent years, *in vitro* and *in vivo* assays have provided new information concerning its mechanism of action with respect to the immune system. Recent research on propolis from other parts of the world has mainly focused on its chemical composition and botanical sources and seasonal effects on its composition and biological properties, its immunomodulatory and antitumor properties. Although the constituents of the various types of propolis vary largely depending on plant origin, their biological activities are quite similar. Presently there are two main varieties of propolis in the market: baccharis and poplar and they both possess similar biological activities even though they have different active ingredients. Based on the plant origin where bees collect exudates for the formation of propolis, poplar and baccharis propolis represent propolis from temperate and tropical regions. Propolis is seldom used in its crude form for medicinal purposes but rather as

concentrated ethanolic extracts obtained by extraction with 70% ethanol. Many compounds have been isolated from these types of propolis and notable groups among them are phenolic acids, flavonoids, terpenes, lipids, waxy substances (bee wax) and many other constituents like vitamins proteins, amino acid and sugars. Commercial interest in propolis is growing continuously as it is used as a component of food additives, cosmetics and over the counter preparations. Commercial production requires large quantities or tons of propolis as raw material and there is a high worldwide demand for propolis. Presently the major producers of propolis are China, Russia and USA. The techniques most used for the chemical analysis of propolis are gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC).

Propolis is widely used to prevent and treat colds, wounds and ulcers, rheumatism, sprains, heart disease, diabetes and dental caries due to its diverse biological properties such as anti-inflammatory, antimicrobial, antioxidant, antitumor antiulcer and anti-HIV activities. The wide application of Propolis in modern medicine has drawn growing attention to its chemical composition. Many studies have revealed that the observed effects might be the result of synergistic action of its complex constituents (Brito *et al* 2010). Some breeds of bees collect propolis more than others; for example, the grey mountain Caucasian honey bees have the highest activity in propolis production (Starostensko 1968). Whereas some species and varieties of tropical honey bees such as *Apis cerana*, *Apis florae*, *Apis dorsata* and the African *Apis mellifera* show very little interest in propolis production and almost make no use of it (Kuropatnicki *et al*, 2013) although in practice this does not appear to be completely true since propolis appears to readily available from African *Apis mellifera* and some

species of tropical stingless bees build large structures out of propolis (Nicola Bradbear, talk at Propolis in Human and Bee Health, 2016). Propolis is susceptible to some enzymatic changes from the bees' saliva (Burdock, 1998). This explains why different types of propolis could have different biological activities (Wagh, 2013, Burdock, 1998). The most common class of compounds found in propolis are the flavonoids. Flavonoids of all classes, including chalcones and anthraquinones, have been isolated from propolis samples. The flavonoids vary in complexity and substitution patterns. Several of the flavonoids are methylated or linked through -*O*- to alkyl side chains. These flavonoids have been found to be some of the most active substances in propolis accounting for most of the observed biological activities. The various classes of flavonoids found in propolis are given in Figure 1.4.

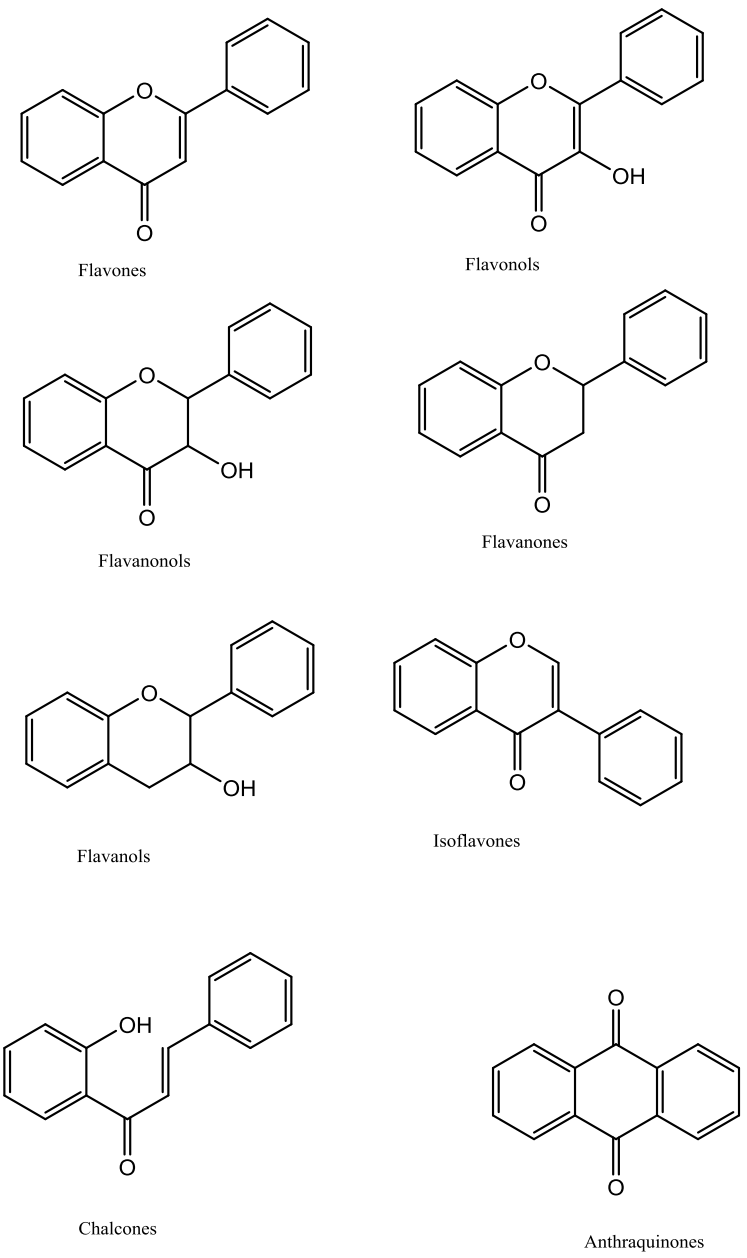
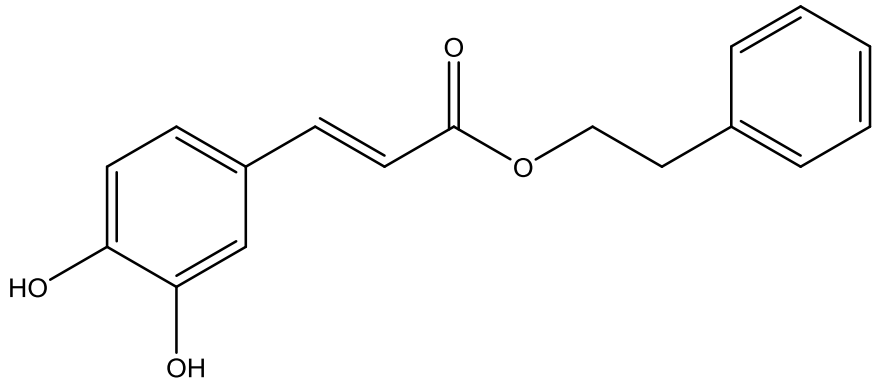


Figure 1.4: Structures of flavonoids and other classes of compounds found in propolis (Kumar and Pandey, 2013).

The effect of climate on the chemical composition of propolis was corroborated by Wagh (2013). For example, propolis collected from tropical countries has more terpenoids, lignans, and isoflavonoids than samples collected from more temperate countries (Piccinelli *et al.*, 2013, Elashry, 2012).

In a study by Seidel *et al*, (2008), samples from different regions with large climatic differences were screened for their antibacterial activity. Some samples showed high antibacterial activity against gram-positive bacteria and less effect on gram-negative bacteria with MIC of 3.9 to 31.25 mg/l). It is assumed that the antibacterial effect is strongly connected to the weather and location from which the propolis was collected; wet tropical areas with rain all the year tend to have stronger anti-microbial activity than tropical areas with less rain seasons and this could be due to wide range of plants species found in the tropical wet areas.

Caffeic acid phenethyl ester (CAPE, Figure 1.5) is a major constituent of temperate propolis with broad biological activities, including inhibition of nuclear factor κ-B; inhibition of cell proliferation; induction of cell cycle arrest and apoptosis. In tropical regions Propolis, especially Brazilian green Propolis, the dominating chemical components are prenylated phenylpropanoids (e.g., artepillin C) and diterpenes. For Propolis produced in the Pacific region, geranyl flavanones are the characteristic compounds which are also found in Propolis from the African region. The chemical composition of propolis is susceptible to the geographical location, botanical origin, and bee species. In order to provide a theoretical basis for studying the chemical composition and pharmacological activity of Propolis and plant sources, and controlling the quality, chemical components that were isolated for the first time from propolis between 2000 and 2012 (Popova *et al* 2010).



Caffeic acid phenethyl ester (CAPE)

Figure 1.5: CAPE

The chemical composition of propolis is strongly influenced by the type of vegetation visited by the bees and by the season of the year (Bankova *et al*., 2000). Though propolis from India has not received much attention like propolis from other parts of the world, but it has become a subject of interest with respect to its chemistry in recent times, (Shubharani *et al*; 2015). For instance, a number of compounds have been identified as constituents of Indonesian propolis since 2000. These include terpene compounds such as patchoulene and several phenolics identified as 5-Pentadecylresorcinol, 5-(8'Z,11'Z-Heptadecadienyl)-resorcinol, 5-(11'Z-Heptadecenyl)-resorcinol, 5-Heptadecylresorcinol, 1,3-Bis(trimethylsilylloxy)-5,5-proylbenzene, 3,4-Dimethylthioquinoline, 4-Oxo-2-thioxo-3-thiazolidinepropionic acid, D-glucofuranuronic acid, Dofuranuronic acid and 3-Quinolinecarboxamine (Wiryowidagdo *et al* 2009, Trusheva 2011).

In a study by Shubharani *et al*; 2015, a total of 93 major compounds belonging to different chemical groups were identified from six different Indian propolis samples. These propolis samples were characterized by the presence of carboxylic acids (20.4%), terpenoids (15.0%), steroids (11.5%), hydrocarbons (9.6%), sugars (6.4%),

alkaloids (6.4%), flavonoids (4.3%), phenols (3.2%), ketones (2.1%), amino acids (2.1%), vitamins (2.1%), volatile oils (2.1%) and other compounds (15.0%). The major compounds present in these propolis samples were carboxylic acids, which are mainly p-hydroxycinnamic acid, p—coumaric acid, 5-heptanoic acid, decanoic acid, octadecanoic acid, hexadecanoic acid, cis-vaccenic acid, sebacic acid, propanoic acid, linoleic acid, oleic acid, ethyl oleate, eicosenoic acid, octacosane and phthalic acid. Variation in these compounds of different origin have also been observed and reported by many scientists (Thirugnanasampandan *et al.* 2012). A similar result was reported from the chemical composition of propolis collected from Gujarat which contained fatty acids and their derivatives as main type of compound (Kumar *et al.*, 2009).

In a report on the properties and flavonoid content in propolis using different extraction methods for raw propolis in Indonesia by Niken *et al* (2014), ethanol produced higher yield values than other solvents in the amount of 18.3% w/w followed by 15.9% for propylene glycol, distilled water was 15.3%, 14.2% for virgin coconut oil and the lowest yield was olive oil at 14.1%. The difference is presumably due to the properties of ethanol as an organic solvent capable of dissolving most of the content of propolis. It also may be due to differences in the origin of propolis, propolis content may be different because of the origin, types of bees, food resources and harvest time. The physical appearance of propolis produced from solvent extraction with ethanol was solid and sticky and with distilled water it was gummy and sticky and a dark brown colour while propolis extracts produced from the solvent VCO (virgin coconut oil), olive oil and propylene glycol were gummy oily and yellowish brown colour. Oily solvents (VCO and Olive oil) produced similar flavonoids content to ethanol and water. The highest levels of flavonoids were derived from using propylene glycol as a

solvent at 0.55% w/v and the lowest was olive oil solvent at 0.2% w/v. Thus VCO and olive oil can be used as solvents to extract propolis and these are more advantageous because they can be used directly in formulations without removing the solvent.

Ethanolic extracts of propolis from Indonesia/East Java province/Batu City were found to contain 5-Pentadecylresorcinol, 5-(8'z, 11'z Heptadecadienyl)-resorcinal, 5-(11'z-Heptadecenyl)-resorcinol, 5-Heptadecylresorcinal, Propolin D, Propolin C, Propolin and Propolin G (Trusheva *et al.,* 2011).

It is presently common to formulate propolis into various products for both industrial and domestic use. Manufacturers and producers have obtained patents for these products and according to the patents profiles (Figure 1.7 and 1.8), China, Japan, and Russia are those that have most of the patents. This fact can be justified by China and Russia being the largest producers of propolis. Today 42% of patents are Chinese, and the first Chinese patent appeared in 1993 (on "Process for production mouth freshener").The Japanese have 15% of patents, and the first appeared in 1988 (about "Deodorants controlling mouth odor"). The first patent was obtained in 1968 on Russian "Toothpaste" and represented 12% of patents. Brazil deposited its first patent in 1997 on "Dental gel", (Viviane *et al* 2013).

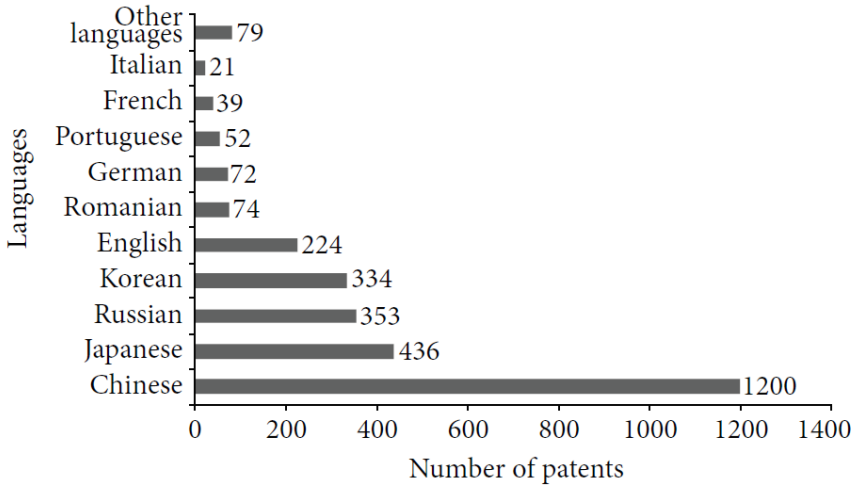


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Figure 1.6: Production of propolis by patents and regions

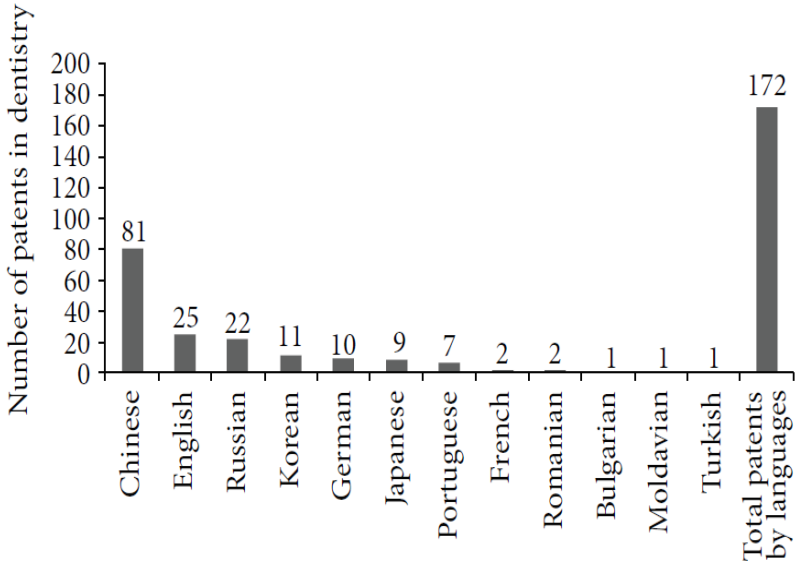


Figure 1.7: Propolis products for dental health by patents and regions

1.4 Poplar Propolis

Populus species are considered to be the main plant origin of propolis all over the world but especially in temperate zones including Scotland. Most propolis collected from Europe, North America, non-tropical regions of Asia, New Zealand (Bankova *et al.,* 2000) and even Africa (mainly the East area of Nile Delta region) (Hegazi *et al.,* 2002) contain the characteristic poplar chemical profile: high level of flavanones, flavones and lower levels of phenolics and their esters (Mohammadzadeh *et al.,* 2007). Annual colony winter losses in Scotland, where beekeeping operations are relatively small scale, are regularly among the highest in Europe (Gray *et al.* 2010, Peterson *et al*., 2009). It is generally agreed that the causes of colony loss are multifactorial. One of the known factors contributing to colony losses is the widespread presence of the ectoparasitic mite *Varroa destructor*.

Potent anti-trypanosomal and anti-leishmanial activity of propolis has been reported, (Almutairi *et al* 2014a, Siheri *et al* 2014, Almutairi *et al* 2014b). This may relate to the fact that bees, like humans, are susceptible to protozoal attack (Schlüns *et al.,* 2010). Protozoal infection of insects is very common and may weaken them rather than kill them (McGhee and Cosgrove 1980) and infection with the protozoa *Crithidia mellificae* has been connected to winter colony loss in a study in Belgium (Ravoet *et al* 2013) and might be a factor in winter colony loss in Scotland.

Temperate region propolis in Scotland can be loosely defined as poplar propolis. Poplar propolis has been found to be active against various bee pathogens and pests including *Varroa* mite (Popova *et al* 2014). It has been observed that bee colonies exposed to *Ascophaera apis* (chalkbrood fungus) increased their foraging for poplar propolis and that increased propolis levels in the hive reduced the intensity of infection

(Simone-Finstrom *et al.*, 2010). A recent paper examined differences between French colonies tolerant to *Varroa destructor*, compared with colonies from the same apiary which were non-tolerant to the mites. The results indicated that non-tolerant colonies collected more poplar propolis than the tolerant ones but the percentage of four compounds, caffeic acid and three pentenyl caffeates, was higher in propolis from tolerant colonies. In a recent publication it has been found that pentenyl caffeates isolated from manuka propolis inhibit quorum sensing in bacteria (Gemiarto,*et al.*, 2015), and pentenyl caffeates are also among the more abundant compounds in poplar propolis (Saleh *et al* 2015). A summary of propolis composition by region is given in Table 1.1 (Toreti *et al*., 2013). The marker compounds were obtained from their ethanol extracts.

Sample	Compounds identified
Bulgarian propolis	3,7-Dihydroxy-5-methoxyflavanone 2,5-dihydroxy-7-methoxyflavanone
North and South Bulgaria	Dihydrocaffeic acid Dihydroferulic acid Dihydroxyacetophenone hydroxymethoxyacetophenone β-Phenethyl alcohol Benzyl alcohol pinobanksin Pinostrobin Dimethyl kaempferol
Brazil/São Paulo state	3-Prenyl-4-dihydrocinnamoloxynnamic acid
Brazil/São Paulo state/Botucatu city	9-E and 9-Z 2,2-Dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyran
Brazil/São Paulo state	Dehydroabietic acid Abietic acid β-Amyrine Triterpenic alcohol of amyrine Lanosterol isomer with 9(11) double bond
Not reported	(E)-2,3-Dihydroconiferyl p-coumarate (E)-3-2,3-Dihydro-2-[2-[(E)-pcoumaroyloxy]-1-methylethyl]-5-benzofuranyl-2-propenoic acid (E)-4-(2,3-Dihydrocinnamoyloxy) cinnamic acid (E)-3-(2,2-Dimethyl-3,4-dihydro-3-hydroxy-2H-1-benzopyran-6-yl)-2-propenoic acid (E)-3-[2,3-Dihydro-2-(1-methylethenyl)-5-benzofuranyl]-2-propenoic acid (E)-3-[2,3-Dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid (E)-3-3-[(E)-4-(2,3-Dihydrocinnamoyloxy)-3-methyl-2-butenyl]-4-hydroxy-5-prenylphenyl-2-propenoic acid Dihydrokaempferol (aromadendrin) 6-Methoxykaempferol 4-Hydroxy-3-prenylbenzoic acid Plicatin B Capillartemisin A
Japan/Okinawa	Prokinawan
Brazilian propolis type 6	Hyperibone A
Mexico/Champoton	1-(3′,4′-Dihydroxy-2′-methoxyphenyl)-3-(phenyl)propane (z)-1-(2′-Methoxy-4′,5′-dihydroxyphenyl)-2-(3-phenyl)propene 3-Hydroxy-5,6-dimethoxyflavan (−)-7-Hidroxyflavanone (−)-Mucronulatol (−)-Arizonicanol a (+)-Vestitol (−)-Melilotocarpan a (−)-Melilotocarpan d (+)-Pinocembrin
Greece (six regions)	18-Hydroxyabieta-8, 11,13-triene Dihydroxyabieta-8,11,13-triene; hydroxydehydroabietic acid 18-Succinyloxyabietadiene 18-Succinyloxyabietadiene (isomer) 18-Succinyloxyhydroxyabietatriene
Kenyan propolis	Tetrahydrojusticidin B 6-Methoxydiphyllin Phyllamyricin C Macarangin Schweinfurthin A Schweinfurthin B

Sample	Compounds identified
Indonesia/East Java province/Batu city	5-Pentadecylresorcinol 5-(8′z, 11′z Heptadecadienyl)-resorcional 5-(11′z-Heptadecenyl)-resorcinol 5-Heptadecylresorcional Propolin d Propolin c Propolin f Propolin g
Jordanian propolis	24(z)-1β-3β-Dihydroxyeupha-7,24-dien-26-oic acid
Honduras	(E, Z)-Cinnamyl cinnamate
Solomon island	Solophenol (A)

Table 1.1: Compounds identified in ethanol extracts of propolis from different regions.

Some of the typical flavanoid compounds found in propolis are shown in Figure1.6

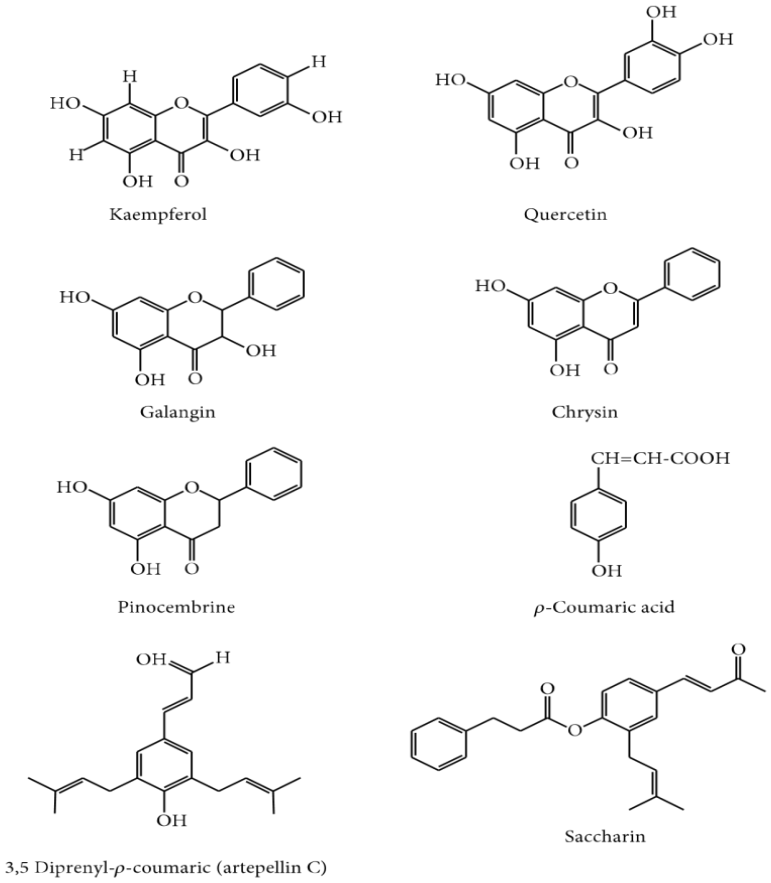


Figure 1.8: Some typical phenolic and flavonoid compounds found in propolis.

1.5 UK Propolis

A metabolomic study of temperate propolis collection by bees in the USA used LC-MS to profile propolis collected by individual bees before the propolis could be mixed in the hive and was able to demonstrate that the main sources of propolis were two poplar species, although many other sources of resin were also used (Wilson *et al*, 2013) .

There have been several studies of temperate propolis compostion utilising LC-MS (Gardana *et al*, 2007, Falcão *et al*, 2010, Falcão *et al* 2013). However, no extensive study has been made of the composition of UK propolis by LC-MS. Chemical components have been isolated from UK propolis in order to screen for potential

immunomodulatory effects, Chrysin, galangin, kaempferol, cinnamic acid and benzyl salicylate were isolated from a sample of UK propolis (Najla Altwaijr MSc Thesis, Universitty of Strathclyde, 2014). Chrysin, galangin and benzyl salicylate were reported to be toxic at concentrations of 100 µg/ml with IC50 of 67.27 µg/ml, 47.66 µg/ml and 55.24 µg/ml respectively. While cinnamic acid and kaempferol were not toxic at the same concentrations. A pro-inflammatory assay showed a remarkable increase of TNF-α concentration in methanolic extract (813.2 pg/ml) and significant increase with the chrysin and galangin with 392.8 pg/ml and 360.4 pg/ml respectively compared with the control value of 297.8 pg/ml. Anti-inflammatory assay using LPS to stimulate the release of TNF-α and then test the samples for their ability to decrease the effect which indicated some potential immunomodulatory activity (Altwaijry, 2014).

1.6 **Indonesian Propolis**

Propolis from different regions of Indonesia have been studied. Propolis collected from Pandeglang Banten, West Java province of Indonesia, Batu City, East Java Province, Indonesia, Lawang (East Java), Indonesia and Cibubur, Jakarta, Indonesia were evaluated for various activities and chemical constituents. Propolis from Batu City was studied for its phytochemical constituents and using 1D and 2D NMR and GC–MS, 11 compounds (Figure 1.9) were isolated and identified. They consisted of four alk(en)ylresorcinols: 5-pentadecylresorcinol (1), 5-(8'-*Z*, 11'-Z-heptadecadienyl)-resorcinol (2), 5-(11'-Z-heptadecenyl)-resorcinol (3) and 5-heptadecylresorcinol (4). 5-(8'-Z,11'-Z-Heptadecadienyl)-resorcinol (2), four propolins, propolin C (7), propolin, F (8) and propolin G (9) and propolin D (6) and three cycloartanes, mangiferolic acid (5), isomangiferolic acid (10) and 27-hydroxyisomangiferolic acid

(11). The propolins or prenylflavanones showed significant radical scavenging activity against diphenylpicrylhydrazyl (DPPH) radicals, while propolin D showed significant antibacterial activity against *Staphylococcus aureus*. The plant sources of the phytochemicals in the Indonesian propolis were identified as *Macaranga tanarius* L. and *Mangifera indica* L. (Trusheva *et al*. 2011). Propolis from Pandeglang Banten gave better yields of phenolics and flavonoids following microwave assisted extraction (MAE) giving 0.4% phenolics and 5.8% flavonoids. The total phenolics were determined as gallic acid equivalents while the total flavonoids were determined as quercetin equivalents (Margeretha *et al.* 2012). An *in vivo* antiplasmodial assay against *Plasmodium berghei* and immunomodulatory activity of propolis from Lawang, East Java, showed that the propolis hydroalcoholic Solution had a strong immunomodulatory activity but weak antiplasmodial activity (Syamsudin *et al*. 2009). Micro and nano-particle encapsulation of propolis samples from Cibubur, Jarkata, gave 94% encapsulation efficiency for flavonoids and 67% efficiency for polyphenols, therefore had higher flavonoid contents than polyphenols. The encapsulated micro and nano-particles showed antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus* (Sahlan and Supardi, 2013).

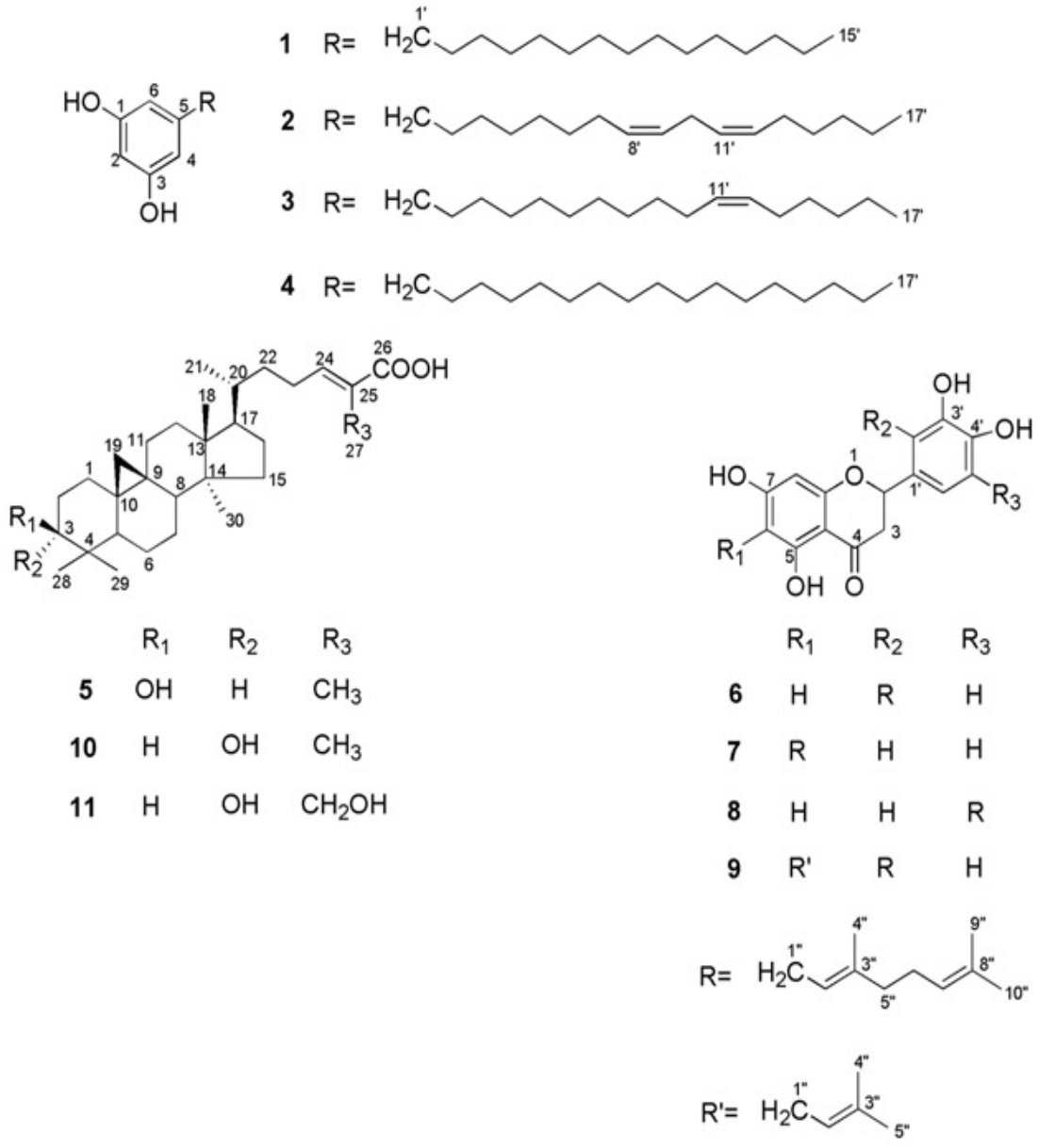


Figure 1.9: Compounds isolated from Indonesian propolis samples.

Ethanolic extracts of three propolis samples from Batang (Central Java), Lawang (East Java) and Sukabumi (West Java) regions in Java, Indonesia were investigated using GC-MS. From the 37 compounds identified, seven of them were identified from a propolis sample for the first time. The extract of propolis from Batang showed the most potent antiproliferative activity against T47D and MCF-7 cell-lines with IC50 34.67±8.3 and 37.8±2.5 μg mL^{-1} while the extract of propolis from Sukabumi showed

the most potent activity against Hela cells with IC50 147.34±8.9. However, none of the propolis extracts showed activity against myeloma and Vero cells. Compounds reported from the study of these Indonesian propolis samples were the sesquiterpene, patchoulene, 1,3-Bis(trimethylsilylloxy)-5,5-proylbenzene, 3,4-Dimethylthioquinoline, 4-Oxo-2-thioxo-3-thiazolidinepropionic acid, D-glucofuranuronic acid, Dofuranuronic acid and 3-Quinolinecarboxamine (Wiryowidagdo *et al*. 2009). The rest of the compounds were identified by GC-MS and their percentage compositions are given in Table 1.2

Compound	Batang	Lawang	Sukabumi
Aliphatic acids			
Hexadecanoic acid	-	-	0.72
Aromatic acids			
Benzoic acid	-	0.41	-
Phenylic acid	94.22	95.62	94.51
D-furanuronic acid	-	-	0.32
D-glucofuranuronic acid	-	0.56	-
1,3-bis(trimethylsilyloxy)-5,5-propilbenzene	2.40	-	-
4-oxo-2-thioxo-3-thiozolidinepropionic acid	-	0.79	-
Terpenes			
Abietic acid	3.76	95.62	-
1-Naphthalenemethanol	-	-	0.27

Patchoulene	-	-	0.27
Quinoline			
3,4-dimethylthioquinoline			
3-quinolinecarboxamine	0.53	-	-
Sugars and alcoholic sugars			
D-mannopyranose	0.31	-	-
D-xylose	0.24	-	-
Arabinofuranose	0.23	-	-
D-Ribose	0.15	-	-
D-Galactose	-	0.51	-
D-Mannitol	-	-	1.62
D-Glucitol	-	-	1.62
Erythritol	0.81	0.86	0.88
Threitol	-	-	0.86
Arabinitol	-	-	0.81
Glycerol	0.81	0.86	0.88

Table 1.2: Chemical composition of ethanolic extracts of Batang, Lawang and Sukabumi propolis samples (percentage of total ion current, GC-MS). (Wiryowidagdo et al. 2009)

The sources of the terpenes are believed to be the *Ferula*, *Pinaceae*, *Cupressaceae* species while the stilbenes and prenylated stilbenes are from *Macaranga* species.

1.7 Biological properties of propolis

Propolis has been used since ancient times for the treatment of many diseases, as well as in food products and cosmetics. In the last three decades, the scientific study of the benefits of propolis began and (Burdock, 1998, Popova *et al*, 2007).

Many biological properties have been attributed to various types of propolis, including anti-inflammatory, antimicrobial, antioxidant, antitumor, wound healing, and immunomodulatory activities Marcio (*et al.,* 2011). The therapeutic indications for propolis are extremely broad. These includes anti-cancer, infection of the urinary tract, swelling of the throat, gout, open wounds, sinus congestion, colds, influenza, bronchitis, gastritis, diseases of the ear, periodontal disease, intestinal infections, ulcers, eczema, pneumonia, arthritis, lung disease, anti-viral, headaches, Parkinson's disease, bile infections, sclerosis, circulation deficiencies, warts, conjunctivitis and hoarseness (Elkins 1996). Propolis has been used in a variety of applications, which include ointments and creams used in wound healing, treatment of burns, skin problems, and ulcers. Various propolis preparations have been applied in treatment of laryngological problems, gynecological diseases, asthma, and diabetes. Propolis has been used in toothpaste and mouthwash preparations to treat gingivitis and stomatitis (Bankova *et al.,* 1983). Antiviral properties of propolis have been known for many years. In studies on *Herpes simplex virus* infection, *in vitro*: 0.5% propolis extract caused 50% inhibition of HSV infection, whereas *in vivo*: as little as 5% propolis prevented the appearance and development of symptoms of HSV-1 infection in animals. Also studies on propolis application in genital herpes infection (HSV type 2) proved its effectiveness. Hassan reported an investigation to evaluate the *in vitro* and *in vivo* antitumor potential of Moroccan propolis extracts where three mammalian tumor cell lines were used for *in vitro* assays: BSR (hamster renal adenocarcinoma), Hep-2 (human laryngeal carcinoma) and P815 (murin mastocytoma). The propolis ethanolic extract as well as the ethyl acetate extract, exerted an *in vitro* cytotoxic activity in dose-dependent manner. The IC50 values ranged from 15 μg/mL to 38

μg/mL (Hassan *et al.,* 2012). This activity depended not only on the chemical composition of the extract (analysed by HPLC/ESI-MS), but also on the target tumor cells. Interestingly, the cytotoxic effect of these extracts on the normal human peripheral blood mononuclear cells (PBMC) was weak when compared to that on tumor cells. On the other hand, oral route treatment of P815 tumor-bearing mice (DBA2/P815) with propolis ethanolic extract (5 mg per mouse every fourth day, five times for group A, and 2.5 mg per mouse every fourth day, five times for group B) significantly reduced the tumor volume (1.2 cm^3 for group A and 2.7 cm^3 for group B at the 22nd day after tumor graft). These effects were statistically significant as compared to those obtained with the control untreated mice (tumor volume 3.5 cm^3 at day 22).

In another study evaluating the anti-inflammatory activity of an ethanol extract of propolis on edema induced by carrageenan, dextran and histamine in mice, Reis (*et al*., 2000) reported that an oral dose of 650 mg/kg significantly inhibited the inflammatory process triggered by carrageenan and antagonized the edematogenic effect produced by histamine, but did not inhibit the inflammatory process induced by dextran. The dose administered had no toxic effects and the authors suggest that the extract exerted an anti-inflammatory effect similar to that of nonsteroidal anti-inflammatory drugs without causing damage to the gastric mucosa or other blood effects. The anti-inflammatory activity of propolis seems to be associated with the presence of flavonoids, especially galangin and quercetin (Shimoi *et al*., 2000).

Several researchers have studied the antibacterial properties of propolis and evidenced its activities against Gram positive and its limited activity against Gram negative bacteria (Tosi *et al.,* 2007; Probst *et al.,* 2011). The susceptibility of a range of Gram-

positive bacteria to ethanol extracts of propolis may vary according to the site of the propolis collected (Gonsales *et al*., 2006; Muli *et al*., 2008).

It has been shown that ethanol extract propolis (EEP) had a greater activity against Gram positive bacteria and less activity against Gram negative bacteria. Above differences might be due to the bacterial wall cell constitution. In fact, Gram positive bacteria have a less complex wall cell and lower lipid contents (Loguercio *et al*., 2005) and thus might have a higher susceptibility to the chemical constituents of propolis.

Takaisi-Kikuni and Schilder (1994) verified that an ethanol extract propolis interfered in the growth of *Streptococcos agalactie* by inhibiting protein synthesis. Koo *et al*., (2002) suggested that propolis and its components may interfere with the enzymatic activity of some bacteria such as *Streptococcos mutans* and *Streptococcos sangui*.

A significant synergy may be verified between clinical antibiotics and propolis from two geographical sources against *Salmonella typhi* (Orsi *et al*., 2006, 2012). Todorov (*et al*., 1968) proved that propolis has an infiltrate action equal to that of procaine. Later on a Bulgarian researcher Tsacov showed that 5% procaine solution of propolis presented a better and quicker action than aqueous alcoholic extract of propolis (Tsacov 1973). Paintz and Metzner in experiments with an ethanol propolis extract and some constituents isolated from propolis tested on the cornea of the rabbit and of the mouse obtained total anesthesia with the total extract as well as with the compounds 5,7-dihydroxyflavanone (pinocembrin), 5-hydroxy- 7-methoxyflavanone (pinostrobin) and with a mixture of caffeic acid esters. Each of these compounds was nearly thrice as potent as the total extract (Paintz and Metzner 1979)

Medium-pressure liquid chromatography (MPLC)

Preparative separation of compounds from a mixture can be achieved via medium pressure liquid chromatography (MPLC). This method can be applied on a large scale. Its primary use is in purifying compounds from natural or other mixtures. It is widely used in purifying pharmaceuticals and has enabled the expansion of the nutraceutical market (Part, 2011). MPLC is operated under a pressure of between 5 and 20 bars at a flow rate that can be adjusted, thus permitting the sample to be eluted more quicker compared to other methods such as open-column chromatography (CC) and low-pressure liquid chromatography (LPLC). Another advantage of MPLC is its flexibility, allowing the use of normal stationary phase, in which silica with different particle sizes is used to fill the columns, or reversed stationary phase (C18). Further purification of fractions from MPLC can be achieved using preparative thin layer chromatography, LPLC or HPLC. Compounds with distinct polarities from crude extracts or semi-purified fractions can be reliably, efficiently and quickly separated with MPLC. As noted by Loibner and Seidl (1997), this method is straightforward, the necessary instruments are widely available, packing materials can be recycled easily and maintenance is inexpensive, which is why it is employed in a broad range of applications. In general optimization of separation conditions is developed prior to transfer to MPLC. As such, TLC experiments are usually conducted first in normal phase mode. However, this could pose problems in reverse phase, as the RP TLC plates available produce Rf data that lack representativeness. This could be solved by using analytical RP HPLC, which can be subsequently scaled up for transfer to MPLC. The key components of an MPLC system are shown in figure 9.

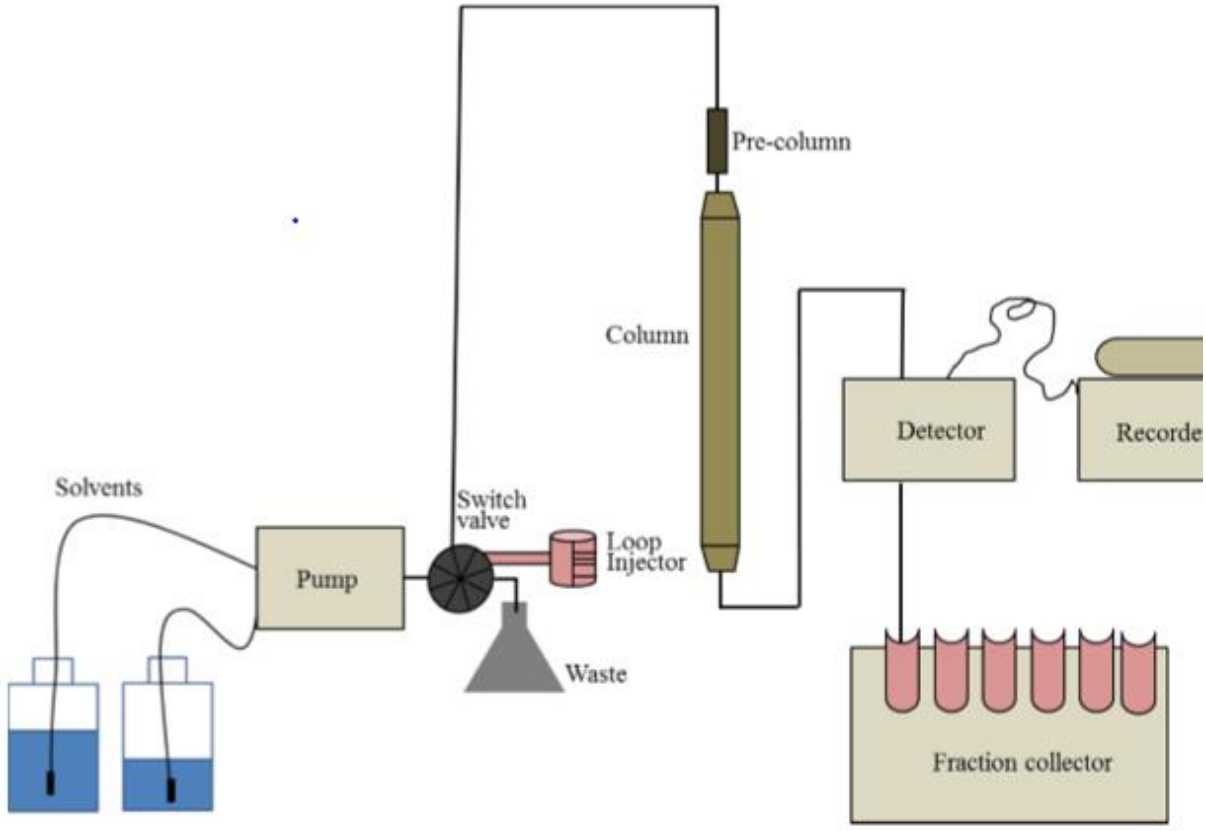


Figure 1.10: The main components of a medium pressure liquid chromatography system (Hostettmann and Terreaux, 1996)

A Gilson semi-preparative chromatography system is a combination of an analytical HPLC system and a preparative LC one. It can be applied to small samples that may not be sufficiently purified by other systems. With an automated system equipped with a UV detector adjustable to five channels, the Gilson HPLC system can inject samples, detect peaks, collect fractions and re-inject collected fractions for further purification. Furthermore, it possesses the strength of both automated and manual fraction collection and has a chromatogram. On the other hand, the Gilson HPLC system does

not have an ELSD detector, which is may be important for certain samples containing non UV absorbing components.

Gas chromatography with mass spectroscopy (GC-MS)

Introduced about 65 years ago, gas chromatography was merged with mass spectrometry (GC-MS) soon after it was developed. It is a method of high power and sensitivity, capable of efficient analysis of compounds with thermal stability and volatility or semi-volatility (e.g. terpenoids, hydrocarbons, short-chain fatty acids and fatty acid esters). The primary function of this method in the analysis of propolis samples is to create a profile of their composition. The method can also be used for dereplication studies, for instance, GC-MS was used to identify 28 new propolis compounds among the 150 that were subjected to analysis (Greenaway *et al*., 1991). For a GC-MS analysis, the sample is dissolved in an organic solvent of high volatility, followed by injection into the GC inlet, it is vaporized and then the carrier gas (usually helium) sweeps it into the GC column. Separation of the compounds occurs according to how the latter interact with the stationary phase and the carrier gas. Subsequently, the column enters a heated transfer line to the ion source, where the method of electron impact ionisation (EI) underpins ion production, and produces ions with positive charges based on the collision between the components and high-speed electrons. Sensitivity is improved by operation of EI at high energy (70 eV) and makes its possible to compare the spectra derived at this level of energy between instruments (Hübschmann, 2008). Figure 1.11 illustrates the components of a GC-MS system.

Compounds are identified on the basis of the molecular weights of their derived fragments, which are examined against compound libraries such as the National Institute of Standards and Technology (NIST) to determine their structures. This

method cannot undertake the analysis of compounds of relative high polarity or low volatility (e.g. flavonoids, phenolic acids and their esters) without their derivatization, as these compounds are not volatile in GC-MS. Furthermore, underestimation of the percentage of compounds with high molecular weights is likely to occur, because transmission of such compounds via a GC column is suboptimal. Derivatisation may not improve the volatility of certain propolis compounds (e.g. some flavonoids and polyphenols) to make them suitable for GC-MS analysis. Hence, the analysis of such compounds is usually undertaken via HPLC.

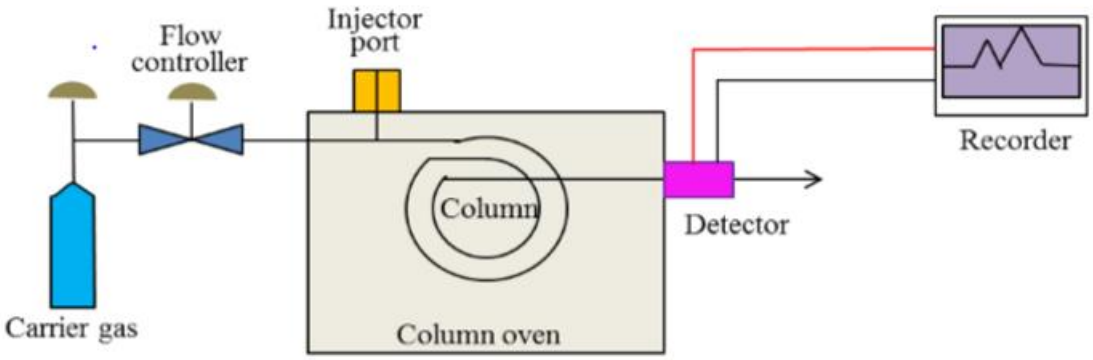


Figure 1.6: Schematic diagram showing the components of a GC-MS system

High performance liquid chromatography (HPLC)

HPLC can be applied to any mixture of compounds, regardless of their volatility or stability. It is presently the chromatographic method of choice. Separation is based on the interaction between the stationary phase and the samples as well as with the mobile phase which dictates the extent to which the compounds travel through the column and separate. For instance, elution of samples that interact more strongly with the stationary phase than the mobile phase is slower, hence they have longer retention

times. By contrast, elution from the column of samples that interact more strongly with the mobile phase compared to the stationary phase is quicker, reducing the retention time. The composition of the mobile phase and the stationary phase type determines how separation occurs. Meanwhile, the type of stationary phase (e.g. liquid-solid adsorption, liquid-liquid separation, size exclusion or ion exchange) also determines how retention occurs. Polarity underpins the manner in which the analyte and the stationary phase interact in the context of adsorption chromatography. For instance, by comparison to compounds with lower polarity, stronger adherence to the stationary phase will be observed in normal phase HPLC for compounds with functional groups capable of robust hydrogen bonding and compounds with high polarity will be eluted slower compared to compounds with lower polarities. The columns used do not differ much in size compared to the columns employed in other pressurized methods, changes are made to the silica through the attachment of long hydrocarbon chains (C-8 or C-18) to ensure that the surface lacks polarity. Furthermore, the polar (aqueous) mobile phase used in this method causes adsorption of the hydrophobic molecules in the polar mobile phase to the hydrophobic stationary phase, while hydrophilic molecules in the mobile phase are the first to undergo elution as they go through the column. To separate compounds of different polarities, and hence facilitating the analysis of the majority of propolis compounds, many applications in natural products employ the reverse phase separation with gradient elution. HPLC can be linked to different detectors according to the features of the compounds making HPLC highly advantageous. Among the most popular detectors are refractive index (RI), fluorescent, radiochemical, electrochemical, near-infrared (Near-IR), evaporative light

scattering detector (ELSD), ultraviolet (UV), nuclear magnetic resonance (NMR), and mass spectrometry (MS).

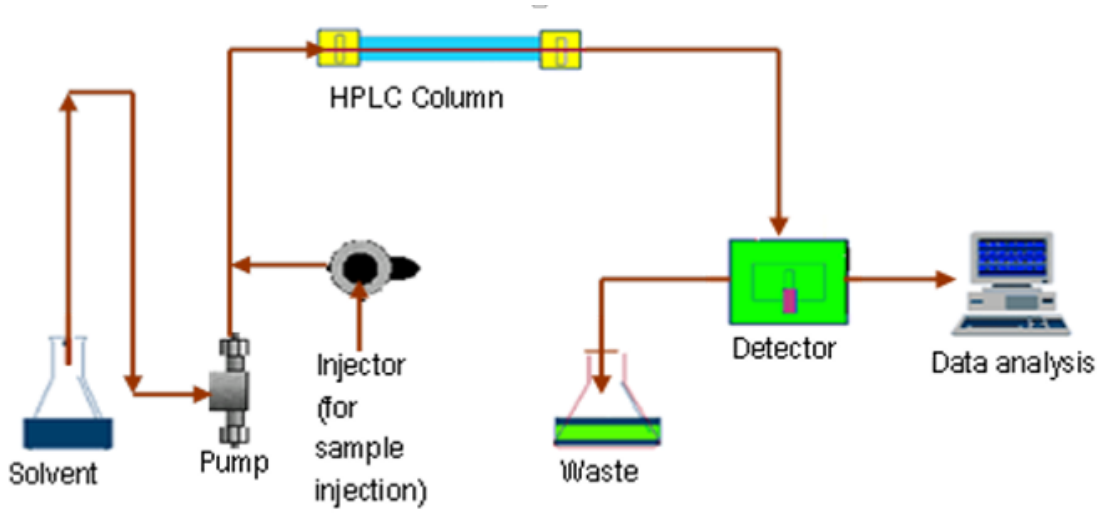


Figure 1.7: Schematic diagram shows the main component of hplc

Table 1.3 provides additional information regarding the experimental conditions between HPLC and other chromatographic techniques.

Technique	Pressure (bar)	Sample amount (g)	Solvent	SP particle size (µm)	Flow rate (ml/min)	General
Column chromatography	Atm	1.00-5.00	General solvent used	63-200	1-5	Self-packing
Vacuum liquid Chromatography	1-2	5.00-30.00	General solvents	40-63	5-15	Self-packing
Low pressure LC	1-5	1-5	More solvent required	40-63	1-4	Pre-packed columns
Flash chromatography	1-2	1.00-100	General solvent used	40-63	2-10	Self-packing
Medium pressure LC	5-20	0.05-100	More solvent required	15-40	3-16	Pre-packed columns
Preparative HPLC	>20	0.01-1	High purity solvent needed	5-30	2-20	Pre-packed columns

Table 1.3: Differences between chromatographic methods. SP = stationary phase and RP = reversed phase. (Hostettmann and Terreaux, 1996).

HPLC detectors

Evaporative light scattering (ELSD), and ultraviolet (UV) detection

The detectors usually associated with HPLC are ultraviolet/visible (UV/VIS), which, alongside photodiode array (PDA), facilitates the acquisition of spectra for a natural product that is not known. Exhibiting high sensitivity, UV/VIS detectors are capable of detecting numerous compounds; on the downside, they lack specificity and detect only compounds that contain chromophores. Meanwhile, a more general detector is the ELSD, which can detect compounds without chromophores and do not absorb UV effectively (e.g. terpenoids, fatty acids and glycosides of non-UV absorbing compounds). Although its response is not influenced by the solvent, the latter must possess volatility and not contain any additives lacking volatility. ELSD is advantageous because it can use mobile phases capable of light absorption at a wavelength identical to that of the compound(s) in question. Its linear response makes it possible to be employed in a quantitative manner at different analyte concentrations, as direct proportionality exists between the amount of scattered light and the concentration of the desired sample compounds. ELSD is a destructive method and could lead to reduction in sensitivity as the analyte becomes more volatile. The processes underpinning ELSD are mobile phase evaporation and measurement of light scattering from analyte particles. The column effluent undergoes nebulisation under a nitrogen gas stream, while the evaporation of the mobile phase in a drift tube leads to suspension of the analyte particles without volatility (Figure 1-13). A photodetector positioned at a fixed angle from the incident beam detects the light scattered by the particles (Snyder *et al*., 2010).

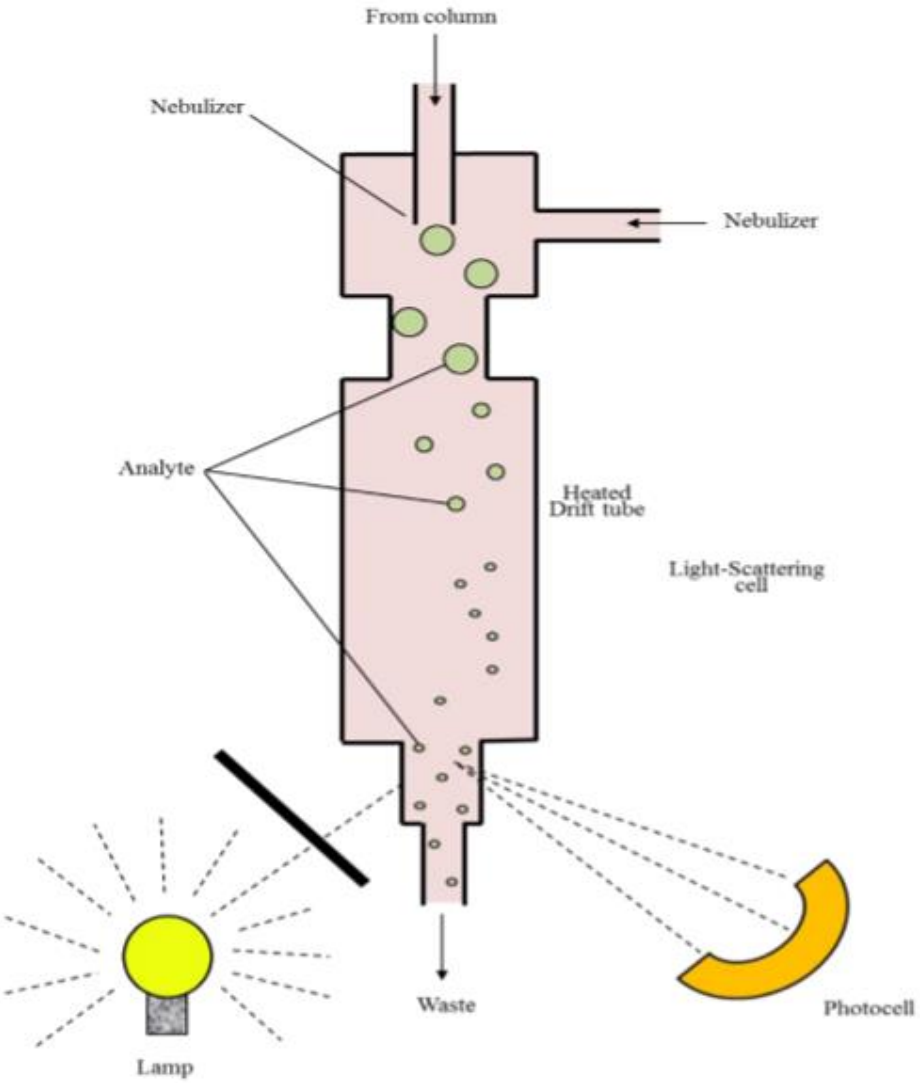


Figure 1.8: Main components of a HPLC-ELSD

HPLC with high-resolution mass spectrometry (HRMS)

Natural product dereplication is usually conducted through HRMS, which is precise and highly sensitive. Characterization can be achieved with this method on the basis of molecular weight, elemental composition and fragmentation patterns. The components of LC-MS are an ion source, mass analyser, detector and computer. After

dissolution in a solvent demonstrating polarity and volatility, the sample is carried through a high-potential needle. Exposure to a warm nitrogen flow causes evaporation of the mobile phase, leading to production and transfer of ions to the high-vacuum area of the mass analyser, where the ratio of mass to charge (m/z) determines the separation of the ions. The detector gathers the generated data and subsequently transforms them into signals for display on a computer monitor.

Size exclusion chromatography

The molecular size of the compounds is the basis of separation in a size exclusion chromatography. Beads exhibiting porosity make up the stationary phases. Thus, compounds of larger sizes are first eluted as they are removed from the internal parts of the beads. Meanwhile, compounds of smaller sizes go into the beads and are trapped. The compounds are then subject to elution based on their ability to exit the pores they have entered. The columns can be silica based or of different chemical components.

Ion exchange chromatography

The selective exchange of sample ions with counter ions in the stationary phase is the basis of ion exchange chromatography. The columns employed in the performance of the ion exchange consist of functional groups that bear charges and are affixed to a polymer matrix. There is a lasting bond between the functional ions with counter ions and the stationary phase. Retention of the sample involves substitutions of the counter ions of the stationary phase with its own ions.

Ionisation methods in LC-MS.

Techniques of ionisation

There are two mechanisms underpinning the various ionisation techniques, namely, ionisation of a neutral molecule via electron elimination or capture, protonation, cationization or deprotonation, or transfer of a molecule bearing a charge from condensed to gas phase (Watson and Sparkman (2007). To profile molecules of small sizes, the techniques usually employed are atmospheric pressure ionisation (API), electrospray ionisation (ESI) and desorption electrospray ionisation (DESI), as well as atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photo ionisation (APPI). Fragmentation is either minimal or completely absent in the case of APCI and APPI, which demonstrate robustness, high buffer concentration tolerance, and efficiency for compounds with thermal stability but without polarity (e.g. lipids) (Bagag *et al*., 2008). Furthermore, matrix-assisted laser desorption ionisation (MALDI), chemical ionisation (CI) and fast atom bombardment (FAB) are methods of ionisation employed for particular applications. Each of the techniques listed above is classified as soft ionisation method. By contrast, electron ionisation (EI) is capable of analyte fragmentation and is therefore classified as hard ionisation. Electrospray ionisation (ESI) method can achieve ionisation of compounds across a broad mass range, which is why it is the type of soft ionisation most frequently employed. It is applicable to compounds with higher polarity, lack of volatility and higher molecular weight, as its capacity is in the range of picomole (10^{-12}) to zeptomole (10^{-21}) level. ESI can be employed both in positive and negative modes, which is what sets it apart from other ionisation methods like electron impact (EI) or chemical ionisation (CI). On the downside, ESI displays sensitivity to matrix effects (e.g. pH, solvent

composition and salt concentration), which could inhibit the signal. Furthermore, structure cannot be determined without fragmentation pattern. Thus, post-ESI, fragmentation patterns are frequently produced through the performance of collision-induced dissociation (CID) MS/MS.

Separation of ions and analysis of mass

Following production, ions are subjected to separation by a mass analyser. The resolving power of a mass spectrometer depends on the spectrometer's capacity to differentiate between similar masses. A variety of methods have been developed for use in the context of HPLC for mass analysis.

Time of Flight (TOF)

In the context of TOF, the masses of the ions determine their velocity, the flight time being shorter the less heavy the ions are. The discrepancies in the kinetic energies of ions result in poor resolution for TOF, which means that mass focusing is inadequate. However, newer instruments can better focus ions as they are equipped with reflectrons. It is possible to apply TOF alongside MALDI ionisation or QTOF MS, in which a quadrupole is attached to TOF. This latter method enhances sensitivity and resolution, and hence it has become quite popular.

Quadrupole-tandem MS

Consisting of four rods arranged in parallel, the charge potential is the same and opposite for every pair. Due to this, to be transported through the quadrupole and to the detector, ions must have m/z in the established range. The quadrupole increases the sensitivity of analysis and enables fragmentation ion formation. Moreover, the use

of a triple quadrupole framework permits performance of experiments like neutral loss, product ion spectra and monitoring of chosen reactions.

Fourier transform ion cyclotron (FT-ICR)

No other MS ion separation method is capable of a higher resolving power and greater mass accuracy than FT-ICR. It involves measuring the frequency of ion oscillation and its performance is time-based. However, this method does present a significant limitation, namely, the fact that the dynamic range of measurement is reduced by interactions between ions and also scanning times are too slow to be compatible with chromatography.

Orbitrap mass spectrometer

The creation of Alexander Makarov during the 1990s, the Orbitrap was incorporated into the hybrid LTQ Orbitrap instrument by Thermo Fisher Scientific in 2005. It is necessary to capture the ions injected in the trap between an external barrel-like electrode(C-Trap) and an internal spindle-like electrode, in order to separate them. There is direct proportionality between the frequency of harmonic oscillation along the electric field axis and the m/z. The ionisation technique employed by Orbitrap is ESI or APCI. Figure 1-20 illustrates how the instrument is structured. Furthermore, it is possible to interface the Orbitrap analyser to a linear ion trap (LTQ Orbitrap family of instruments), quadrupole mass filter (Q Exactive family), or straight to a source of ions (Exactive instrument).

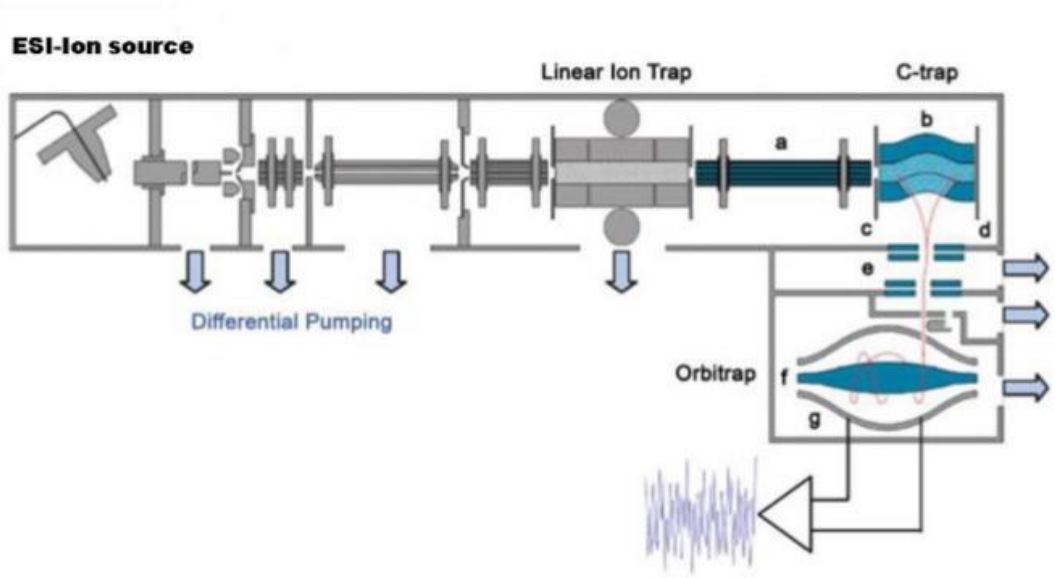


Figure 1.9: Simplified representation of the LTQ-Orbitrap classic model.

(a) transfer octupole; (b) curved RF-only quadrupole (C-trap); (c) gate electrode; d) trap electrode; (e) ion optics; (f) inner orbitrap electrode (central electrode); (g) outer orbitrap electrode (Makarov *et al.*, 2006). The ESI source at the far left is responsible for the production of ions in LTQ Orbitrap. The ions subsequently reach the storage quadrupole via the source, collision quadrupole, and selection quadrupole. The ions aggregate and cluster in the storage quadrupole; when voltage is applied to ions in the C-trap, they speed up in the direction of the internal spindle-shaped electrode, where the application of a special voltage induces their rotation around the electrode, and this movement serves to trap them. The ions are kept trapped by the equilibrium between electrostatic attraction and centrifugal forces. The frequency of transients produced by vibrating ions influences the measurement, but the spatial distribution of the ions and the actual energy of the ions do not. As outlined by Hu et al. (2005), Orbitrap characterisation is based on the performance parameters of resolution, resolving power up to 150,000, 0.5-2 ppm mass accuracy, m/z range of about 6000, and dynamic range of about 10^4.

LTQ Exactive mass analyser

A bench-top Orbitrap instrument, the exactive is particularly relevant for high throughput screening and detection of compounds with rapid polarity. Furthermore, switching is possible, which means that the same experiment can acquire both positive and negative ion data. Mass accuracy is unaffected by the acquisition of two scans in positive and negative ion modes. Aside from mass accuracy, the Exactive demonstrates a resolution of up to 100,000 and a broad dynamic range, which is why it is suitable for many different applications. The introduction of samples through the atmospheric pressure ionisation source occurs via direct infusion or HPLC. This enables the instrument to be used in different applications, such as high throughput screening of natural products, detection of biomarkers, metabolomics, quantitative analysis, as well as precise mass measurements in the context of organic synthesis.

Nuclear magnetic resonance (NMR)

The basic phenomenon of NMR involves excitation of nuclei by a magnetic field followed by absorption of radiofrequency and measurement of the re-emitted electromagnetic radiation. The strength of the magnetic field that is applied and the magnetic properties of the atom isotope determines the resonance frequency of the energy. Intrinsic magnetic moments and angular momentum characterise every isotope with an uneven number of protons or neutrons, whilst every nuclide with an even number of protons or neutrons possess a net spin of zero. Hydrogen-1 (^{1}H) and carbon-13 (^{13}C) isotopes are the nuclei that have attracted the most attention in the development and application of NMR. Figure 1.15 illustrates the component parts of an NMR spectrophotometer. No other method is better at simultaneously identifying compounds from different natural product classes than NMR. Another advantage of

NMR is that, unlike other methods, it is not restricted by conditions of ionizability, chromophore prerequisites or thermal stability. Due to its ability to identify all compounds that have spin active nuclei, NMR is particularly relevant for organic compound analysis. On the downside, the resolution and sensitivity of NMR are suboptimal, but augmenting the magnetic power helps to bring some improvements. In the context of phytochemical analysis, NMR is employed primarily to determine the structure of compounds. This may be achieved by comparison of the acquired NMR spectra and that of a standard sample or spectra from earlier studies. It could be challenging to interpret NMR data particularly in the case of application to unpurified compounds from propolis samples. Hence, to obtain data relevant for profile creation or matching of composition with biological activity or geographic spread of various samples of propolis, chemometric methods like principal component analysis (PCA) and partial least squares (PLS) have been recently adopted in numerous studies (e.g. Gavaghan *et al*., 2002; Stoyanova and Brown, 2001).

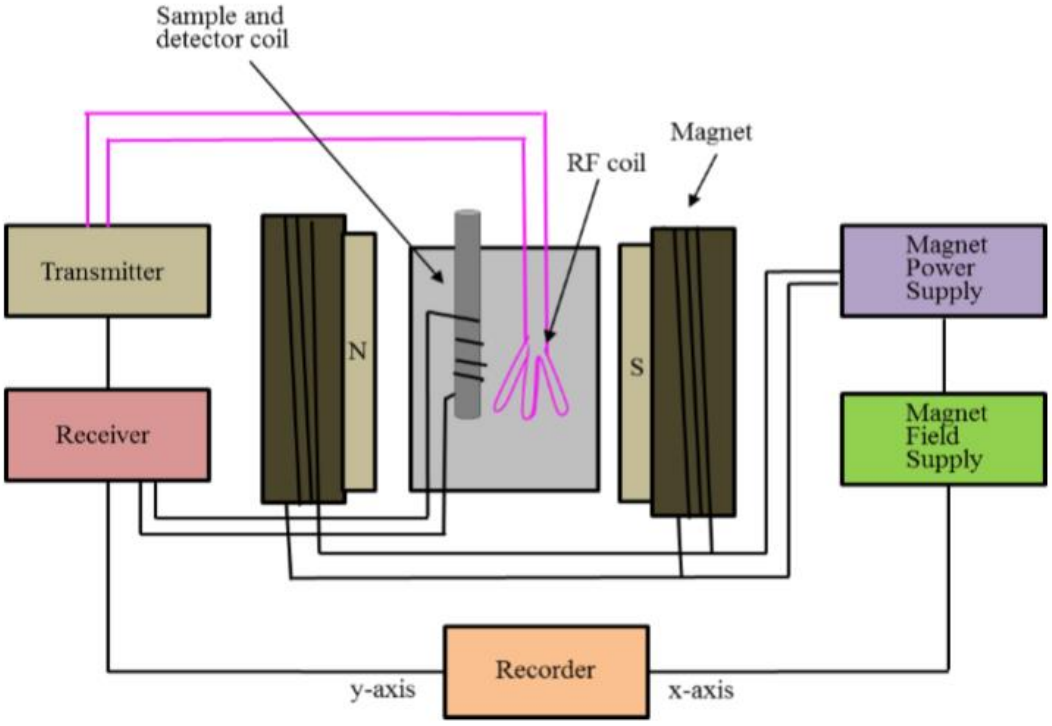


Figure 1.10: A standard NMR spectrophotometer and its major components

^{1}H NMR

^{1}H NMR was informative about the protons in the compounds and their chemical shifts, multiplicity (coupling constants), as well as relative number of protons from the integration. Besides for every isolated compound, ^{1}H NMR was also applied to crude extracts and fractions in this study, in order to facilitate identification. Moreover, the isolated pure compounds were identified based on their acquired spectra (Breitmaier, 2002).

^{13}C NMR

^{13}C NMR is informative about how many and what kind of carbons are present in a compound. There were two types of acquired spectra, namely, broad band-decoupled or *J*-modulated. In the former, ^{13}C acquisition causes irradiation of the 1H nuclei,

resulting in complete decoupling of all protons from the ^{13}C nuclei. When this occurs, a distinct singlet signal is observed for every separate ^{13}C environment in the molecule. The carbons can be differentiated by *J*-modulated experiments based on their proton attachments (C, CH, CH_2 and CH_3). The data of CH signal multiplicity and spin-spin coupling are converted by the pulse-sequenced experiment Distortionless Enhancement by Polarisation Transfer (DEPT) into a phase relationship. Within a DEPT 135 spectrum, the direction of CH_3 and CH is positive phased, whereas the direction of CH_2 is negative phased in the spectrum. Compared to a conventional broad band-decoupled carbon spectrum, a DEPT 135 spectrum is more advantageous because a single experiment is enough to differentiate between C/CH_2 carbons and CH/CH_3 carbons, as well as enhancing sensitivity four fold as it employs ^{1}H-^{13}C polarisation transfer (Breitmaier, 2002; Friebolin, 2011).

Correlation spectroscopy (COSY)

Proton-proton coupling in a molecule can be identified by this two-dimensional experiment, and an appropriate pulse sequence can highlight every coupling relationship in a single experiment. The plotting of the proton chemical shifts is on each axis, the square diagonal representing the contour plot and cross peaks indicating correlations, with the ordinary ^{1}H spectrum being denoted by the diagonal. Hence, the spin-spin coupled protons are represented by the cross peaks. Germinal (2*J*) and vicinal (3*J*) protons cause the observed correlations. Furthermore, the COSY spectrum could include 4*J* and 5*J* couplings or allylic couplings (Breitmaier, 2002).

Heteronuclear single quantum correlation (HSQC)

One-bond, H-C (1*J*) direct correlations are shown by this two-dimensional ^{1}H-^{13}C experiment. The proton spectrum in an HSQC spectrum is along the abscissa, while the ^{13}C spectrum is along the ordinate. Protons directly connected or attached to carbon atoms show cross peaks with their carbon atoms (Claridge, 2006).

Heteronuclear multiple bond correlation (HMBC)

This experiment produces spectra showing 2*J* C-H and 3*J* C-H long-range couplings or correlations. The proton and carbon spectra are plotted on the two axes and the cross peaks indicate the correlations (Claridge, 2006; Breitmaier, 2002).

Data analysis

Processing of data

Large amounts of chromatogram/mass spectrum data of a 2D nature are produced when samples are analysed with LC-MS. The software-based pre-processing of these data involves eliminating noise and extracting pertinent information, typically with techniques such as curve resolution or deconvolution. When the composition of the mobile phase varies, the retention times vary as well, and statistical modelling may be affected by fluctuations in temperature or column variability. This calls for alignment, which can be achieved with software such as MZmatch or Sieve software, which relies on the new algorithm Chrom-Align. However, background interferences are problematic with this software, including false peaks derived from the mobile phase, preparation of samples, column leaching, and plastic tubing, particularly when the temperature used is high. To boost the reliability of the generated peaks, manual checks are often necessary.

MZ-mine 2.10 project is also a commonly used software, being the product of the collaboration between Matej Orešič and Mikko Katajamaa from the Quantitative Biology and Bioinformatics group at VTT Technical Research Centre of Finland and the Computational Systems Biology Research group at Turku Centre for Biotechnology, respectively (Katajamaa & Orešič, 2005). Its introduction in 2005 made it possible to process LC-MS data from more than one MS platform for differential profiling and data visualisation through carrying out several procedures, such as spectral filtering, peak detection, alignment and normalisation.

This software enables the transfer of the produced peak lists to any statistical program to be analysed. Furthermore, a novel function for molecular formula estimation has been recently developed.

Recognition of patterns

Among the unsupervised chemometrics techniques most commonly employed is principal component analysis (PCA). It can differentiate between groups, simplify datasets and diminish their dimensionality, and improve visualisation. Furthermore, besides enabling sample patterns to be visually identified, PCA makes it easier to examine inter-sample discrepancies and determine what causes those discrepancies. PCA also shows how datasets are correlated (Bro & Smilde, 2014). Performance of PCA can be achieved with different software. SIMCA-P 14 was employed in this study to model both PCA and partial least squares (PLS).

Databases

An integral instrument for identifying every metabolite or secondary metabolite according to their precise m/z is yet to be developed. Instead, the process of identification of MS data relies on online or in-house databases such as MarinLit (marine natural products), AntiBase (microbial secondary metabolites), and KEGG (genomes, enzymatic pathways, and biological chemicals). Secondary metabolites in propolis are usually identified based on the NIST (National Institute of Standards and Technology) and Dictionary of Natural Products (DNP) databases.

Aims

- To carry out profiling of temperate propolis collected from hives within the UK in order to assess how variable the composition is from region to region.
- To carry out MS^n experiments in order to characterise the complex mixture of constituents found in UK propolis.
- To carry out LC-MS profiling of propolis collected on the same site in Scotland in order to assess the variations in composition within the same site of collection.
- To carry out LC-MS profiling of propolis from samples of propolis collected from tropical regions including Nigeria, Ghana, Cameroon and Indonesia.
- To isolate and characerise some of the constituents of the different propolis samples.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Chemicals and reagents

The following materials were used: Davisil grade 633 amorphous precipitated silica with a pore size of 60 A and mesh size 200-425 µm for column chromatography, Celite filter agent for sample dry-loading on the Grace system, and deuterated solvents $CDCl_3$ and DMSO-d_6 for NMR analysis were obtained from Sigma-Aldrich (Dorset, UK). Davisil grade 636 column grade silica gel with pore size of 60 A and mesh size 35-60 µm were obtained from Merck (Germany). Fisher Scientific (Loughborough, UK) provided the HPLC grade solvents employed for the extraction procedure, namely, ethyl acetate, methanol, acetonitrile, hexane, and absolute ethanol. BDH-Merck (Dorset, UK) provided the analaR grade formic acid (98%). A Millipore water purification system was used to purify water for the HPLC and LCMS runs.

2.2 Equipment

Fisher Scientific (Loughborough, UK) provided the syringes and Acrodisc filters, rotary evaporator (Buchi, Switzerland), ultrasonic bath (Scientific Laboratory Supplies, Ltd), Erlenmeyer flasks, beakers, and vials. Sigma-Alidrich (Dorset, UK) provided the Gilson automatic pipettes and the NMR tubes (5 mm 300 MHz, 187 mm) respectively from Anachem (UK) and Norell (US). Rotaflo (UK) provided the glass columns for column chromatography, and Alltech (Carnforth, Lancs, UK) provided empty dry-loader cartridges for the loading of samples packing on the Grace system, C18 (12 g) cartridge, and silica cartridge (24 g).

2.3Collection and preparation of propolis samples

James Fearnley from BeeVital Company (Whitby, UK) provided the samples of propolis from the UK and Muhammad Iqbasl provided the samples from Indonesia.

Until needed for analysis, the samples were kept at room temperature in absence of humidity and light. The samples appeared dark brown in a sticky consistency.

2.4 Propolis extraction

A mortar and pestle were used to break off the samples of propolis and grind them into small, coarse fragments. For profiling experiments, 5 ml ethanol was used to extract about 50 mg of the samples by sonication at 40°C for 180 minutes and was repeated twice. A syringe filter (Acrodisc 0.45 μm) was used to filter the extracts and dried under nitrogen. The resulting solvent free extract was stored at -20°C until needed. Isolation of active compounds in fractions was achieved by subjecting the active extracts to bioassay guided fractionation. More amounts of the active samples were subsequently subjected to maceration for 24 h with ethanol. The extracts were filtered and solvents removed using a rotary evaporator. The extracts were kept at -20°C until needed.

2.5 Profiling of the propolis samples

For LC-MS and HPLC-UV-ELSD analyses, 2 mg/ml of extracts were dissolved in ethanol. Extracts that had high hydrophobicity and low solubility were made more soluble by the addition of ethyl acetate.

2.5.1 HPLC-UV-ELSD

Performance of the HPLC-UV-ELSD analysis was undertaken with an Agilent 1100 system made up of a quaternary pump, auto sampler and degasser. Monitoring was conducted by UV channels at 290 and 320 nm and of the ELSD (SEDEX75 model, SEDERE, France) at 30°C. HiChrom (Reading, UK) provided the ACE C18 column (150×3 mm, 3 μm) that was used, while the mobile phase was A: water and B:

acetonitrile at 300µl/min flow rate. The background noise was minimised by omitting the formic acid, which was used in the LC-MS analysis, from A and B during the use of the ELSD detector.

The gradient elution was carried out as follows: linear gradient between 30 and 50% B for 15 minute, followed by holding of the gradient at 50% B for 10 minutes, gradient between 50 and 80% B for 25-40 minutes, 10-minute holding at 80% B, a rise to 100% B for 60 seconds, 9-minute holding at 100% B. The flow rate was subsequently elevated to 500 µl/min in order to wash the column. System re-equilibration was achieved by reverting to 30% B for 10 minutes. Overall, the run time was an hour and 10 minutes. A 10 µl injection volume was employed and the Clarity Software (Data Apex) allowed collection and processing of the data.

2.5.2 Liquid chromatography –High Resolution Mass Spectrometry

Apart from the fact that 0.1% formic acid was added to mobile phase A and B to trigger ionisation under ESI conditions with the purpose of establishing correlations between the previously obtained peaks and the accurate masses from LC-MS, HPLC-UV-ELSD was conducted with identical samples in duplicate, volume of injection and chromatographic conditions. The Accela 600 HPLC system alongside an Exactive (Orbitrap) mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) was used to conduct HRMS. MS detection in the range 100-1500 m/z and ESI polarity switching mode was applied to the performance of scanning. Xcalibur version 2.2 (Thermo Fisher Corporation) controlled the LC-MS system. The experiment was initiated after the conventional Thermo Calmix solution was employed to achieve the

external calibration of the instrument's mass axis, in keeping with the guidelines from the manufacturer. Meanwhile, while the experiment was in progress, an internal calibration was performed on the basis of adequate lock masses, which were m/z 83.06037 and 91.00368 in positive and negative modes for acetonitrile dimer and formic acid dimers respectively. Table 2-2 provides an overview of the major parameters used in the ESI mass analysis.

Attribute	Positive mode	Negative mode
Capillary voltage (V)	35.5	-48.0
Sheath gas flow (bar)	50.0	
Capillary temperature (°C)	275.0	
Auxiliary gas flow	17.0	
Spray voltage (KV)	4.5	-4.0
Tube lens (V)	90.0	-145.0
Source current(µA)	100	

Table 2.1: The parameters used in the ESI mass spectral analysis

2.5.3 Acquisition of data and exploration of databases

ProteoWizard software was employed to separate the peak lists into negative and positive files prior to being transferred to MZ-mine 2.14 for processing, due to the dual polarity mode in which Exactive HRMS functions (Pluskal et al., 2010). In order to conduct PCA to chemically characterise and classify the samples of propolis as well as to identify the main elements underlying the discrepancies in the PCA plot, the obtained peak lists from the positive and negative modes of ESI were transferred individually to SIMCA-P 14 (Umetrics, Sweden). In the context of the MZmine 2.10 software, data were extracted with the following parameters: centroid peaks for mass detection, $1x10^5$ level of noise, m/z tolerance in the range of 0.001-5.0 ppm, $5x10^5$ smallest height of peak, and 100-1500 m/z. Furthermore, retention time (RT) tolerance of 0.2 min, highest charge of 2, and elimination of the isotope with the highest intensity were associated with deisotoping. Weight function was correlated with m/z and RT (20:20) in the case of alignment join aligner, signifying that RT and m/z were equally important, with 5% RT tolerance. Detection of absent peaks was undertaken based on gap filling at 1% intensity tolerance, 0.001-5 ppm m/z tolerance and 0.5 min RT tolerance. This process was followed by elimination of every solvent peak from the data and identification of adducts and complexes peaks. As isolation of compounds with other elements from propolis had never been achieved before, solely compounds comprising C, H and O were subjected to the formula prediction function. For export, the data took the form of CSV files with information about MZ-Mine ID, m/z, RT, name (if there was any), and peak area.

The mean peak area was used to choose the initial 2000 LC-HRMS characteristics from every sample and the accurate masses were searched against the Dictionary of

Natural Products (DNP) (2013 version) to provide a tentative identification. Furthermore, before PCA modelling, univariate scaling and log transformation were applied to the negative ion data.

2.6 NMR analysis

Dissolution of 15 mg of every crude sample into 0.6 ml D-chloroform in conventional 5mm x 187 mm L NMR tubes was undertaken to enable the creation of profiles. A JEOL-LA400-MMR (JEOL Ltd, UK) spectrometer system was employed to obtain ^{1}H NMR data at 400 MHz. The residual solvent peaks constituted the reference points for every spectrum, with the internal standard being TMS. Chemical shifts and coupling constants were respectively expressed in ppm and Hz. The processing of the NMR spectroscopic data was undertaken with the MestReNova 8.1.2 software (Mestrelab Research SL, Spain).

2.7 Chromatography based on silica gel

Silica gel grade 60 with 200–425 µm mesh size was employed for column chromatography (CC). This method of purification was chosen because it enabled bulk separation of compounds sufficient for identification and bioactivity studies. The columns were wet packed and elution started with hexane using 55 x 3 cm glass columns. Ethyl acetate was used to dissolve the propolis extracts. This was then mixed with coarse silica and placed in a fume hood to dry. After loading the adsorbed extracts on to the column, the columns were eluted using a gradient of ethyl acetate in hexane and methanol in ethyl acetate using 200 ml of solvents beginning with hexane. Ethyl acetate was then added 90:10, 80:20, 60:40, 40:60, 20:80 and then 100% ethyl acetate

thereafter, methanol was added to ethyl acetate in 90:10, 70:30, 60:40, 50:50 and then 100% methanol with 200 ml of each solvent mixture being used. After collection, the fractions were concentrated on a rotatory evaporator. Fractions were combined according to HPLC-UV-ELSD profiles. The weights of the fractions were recorded and stored for future use.

2.8 MPLC using a Grace Reveleris® system

Crude extracts and fractions from the column chromatography were purified using a Grace Reveleris® iES Chromatography System (Alltech, Carnforth, Lancs, UK). Ethyl acetate was used to dissolve the samples and mixed with celite (1:2) according to weight of the samples and the mixture was placed in a fume hood to dry. Normal phase and reversed columns were used based on silica gel GraceResolv Silica (24g/32 ml) or C18 (12 g) cartridges. For the reversed system, method optimisation with appropriate isocratic conditions was achieved with HPLC-ELSD-UV, enhancing peak resolution for transfer to the Grace system.

A two-UV channel detector monitoring at 290 and 320 nm in every experiment was incorporated in the Grace system, which also included ELSD capable of identifying compounds from extracts of great complexity, regardless of the presence of chromophores, in just one run. Fractions under the peaks were collected based on identification of slope or threshold by an automatic fraction collector. A suitable trade-off was achieved in the majority of cases and chromatogram noise was reduced by setting a medium sensitivity level in every experiment. The Reveleris® Navigator™ Windows permitted collection and processing of data, which were converted into pdf format for export.

Test tubes numbered and labelled were used for collection of fractions. The fractions pertaining to common peaks were combined in one tube based on instrument-generated chromatograms; they were then left to dry and their weight was measured separately. HPLC-UV-ELSD permitted testing of how pure the fractions were. NMR was applied to determine the structure of fractions more than 80-90% pure and LC-MS^n validated the identified structures.

2.9 Compound identification

HPLC-UV-ELSD was used to ascertain the purity of the isolated compounds and subsequently they were identified using NMR spectroscopy and LC-MS^n was used to confirm the structures. 1D ^1H and ^{13}C NMR experiments were performed on a JEOL (JNM LA400) at 400 MHz instrument in order to identify the isolated compounds. The spectra associated with known compounds were compared against available spectral data for identification purposes and to determine their structure. When required to ensure that compounds were allocated correct proton and carbon chemical shifts, Bruker Avance 600 was used to acquire ^1D 1H and ^{13}C NMR, DEPT 135 and 2D: ^1H-^1H-COSY, HSQC, HMBC. D-chloroform or DMSO-d6 were used as the deuterated solvents to dissolve the samples for the NMR experiments.

2.9.1 LC-MS^n

The column and chromatographic conditions applied earlier for profile creation were used again for running 2 mg sample dissolved in 1 ml methanol on the Orbitrap. The LTQ-Orbitrap system underpinning the MS^n experiments was comprised of a Surveyor HPLC pump hyphenated to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). The instrument supports MS^n, despite functioning in one polarity at a time. Though excluded from the overall profiling, the fragmentation

data provided by MS^n were nevertheless processed to determine or validate purified compound structures based on comparison of the fragmentation patterns against published data. The aim of this was to create a library of propolis-derived compounds of established purity and related fractions. CID negative mode was applied on LTQ-Orbitrap at 35 V for the MS^n analysis, fragmentation was carried out on the MS full-scan signal of the greatest intensity.

2.9.2 Optical rotation determination

A Perkin-Elmer 241 polarimeter with a sodium lamp at 20°C (PerkinElmer Inc., US) was employed for the measurement of the optical rotation of the optically active compounds. 1 mg/ml was obtained through dissolution of 1 mg of every compound into chloroform or methanol. The optical rotation was determined based on the average of ten readings with the formula $[\propto]\lambda\ T = 100 \times \alpha\ l \times c$ ([α] = rotation at wavelength λ, T = temperature at 20℃, α = average of calculated rotation (o), l = path length (dm), c = solution concentration (g/100 mL).

2.9.3 Melting point determination

A Stuart Scientific melting point device (Bibby, UK) was employed for the measurement of the melting points.

CHAPTER THREE:

CONSTITUENTS OF PROPOLIS FROM DIFFERENT REGIONS OF THE UNITED KINGDOM BY LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROMETRY AND A METABOLOMICS APPROACH.

Khaled Saleh, Tong Zhang, James Fearnley, David George Watson

Introduction

High losses of honey bee colonies have been observed in recent years in many countries (van De Zee *et al.,* 2012, Ellis *et al.,* 2010). It is generally accepted that the cause of colony loss are multifactorial. One of the known factors contributing to colony loss is the widespread presence of the ectoparasitic mite *Varroa destructor*. Annual colony winter losses in Scotland, where beekeeping operations are relatively at a small scale, are among the highest in Europe. There is an ongoing concern about the impact of colony losses on bee population because of the threat it poses to food security. There are several products extracted by bees from plants and the most popular is honey. However, one rather mysterious material is propolis or bee glue, often regarded as a bit of nuisance by beekeepers since it literally glues the hive together and makes it difficult to dismantle for bee colony management. The material has been popularised in health and food products such as toothpastes, cold cures and cosmetics. It can be obtained in large quantities using propolis traps which the bees fill. Propolis fulfils an important function in the beehive, where it is used to coat the surfaces of the hive promoting a sterile environment within the hive and thus social immunity. In view of the recent problems with colony collapse it is of some interest to establish whether or not variations in propolis composition might have some role in this. Of course bees depend on the plants in their environment to collect propolis and changes in land usage might affect the composition of the propolis they collect. Propolis has been found to be active against various bee diseases including *Varroa* mite (Simon-Finstrom *et al.,* 2010). It has been observed that bee colonies exposed to *Ascophaera apis* (chalkbrood fungus) increased their foraging for propolis and that increased propolis levels in the hive reduced the intensity of infection (Simon-Finstrom *et al.,* 2012). A recent study

examined differences between French colonies tolerant to *Varroa destructor*, compared with colonies from the same apiary which were non-tolerant to the mites. The results indicated that non-tolerant colonies collected more resin than the tolerant ones but the percentage of four compounds, caffeic acid and three pentenyl caffeates, was higher in propolis from tolerant colonies (Popova *et al.*, 2014) than that from non-tolerant ones. It has been observed that bees which collect large amounts of propolis are more hygienic and produce more honey (Nicodema et al, 2014). High-propolis - producing colonies have been observed to have significantly higher brood viability and greater worker bee longevity (Nicodema *et al*, 2013). There is a substantial body of research on propolis going back several decades and it has been found to have a wide range of biological activities and is potentially a source of compounds for treating human diseases (Sforcin and Bankova, 2011). Propolis in the UK, and other regions of temperate Europe is said to derive from poplar species. "Poplar" propolis thus far has been reported to contain 344 chemical components (de Groot *et al*, 2014). A metabolomic study of propolis collection by bees in the USA profiled propolis collected by individual bees before the propolis could be mixed in the hive and was able to demonstrate that the main sources of propolis were two poplar species, although many other sources of resin were also used (Wilson et al, 2013) . Thus propolis in Northern Europe is regarded as having a particular chemical composition, which includes some 344 compounds including phenolics and their esters, terpenoids and waxes. The have been several studies of propolis compostion utilising LC-MS (Gardana *et al*, 2007, Falcão *et al,* 2010, Falcão *et al.*, 2013). A comprehensive study examined 40 samples of Portuguese propolis and was able to characterise a new type of propolis rich in flavonoid glycosides. There have not been many studies of propolis

profiles utilising high resolution mass spectrometry where it is possible to separate isobaric compounds and thus gain further insight into the complexity of the mixture. Since propolis exhibits such a variety of biological activities it is of interest to try and establish how much its composition varies. On a worldwide basis in previous work we have found very wide variations in propolis composition (Seidel *et al*, 2008, Watson *et al*, 2006). A recent study profiling propolis from Africa using a variety of techniques and metabolomics approach was able to characterise several different types. The present study is focused on the variation in chemical composition of propolis from the UK using a metabolomics approach since there has been no extensive study of the composition and variations within UK propolis.

Materials and Methods

Materials

Absolute ethanol, HPLC grade acetonitrile, methanol, acetonitrile, formic acid were obtained from Fisher Scientific, Loughborough UK. An ACE C_{18} column (3mm x 150mm, 3µm) was from Hichrom, Reading, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK.

Propolis Samples

The nine UK propolis samples were part of collection made by BeeVital Ltd., Whitby, UK. Three 50 mg amounts of propolis were weighed for each sample and the samples were then extracted with 5 ml of ethanol in screw capped test tubes by sonication for 30 minutes. About 0.2 ml of the extracts were transferred to Eppendorf tubes and mixed with 0.8 ml of HPLC grade water and centrifuged for 5 minutes at 3000 rpm. The supernatant was then used for analysis by LC-HRMS.

LC-HRMS (Liquid Chromatography-High resolution mass spectroscopy)

High resolution mass spectra were obtained using an Orbitrap Exactive mass spectrometer (ThermoFisher, Hemel Hempstead, UK) in positive and negative ion switching modes with a needle voltage of -4.0 kV, 4.5 kV positive. Sheath and auxiliary gases were set at 50 and 17 arbitrary units respectively. The separation was performed on an ACE C_{18} column (150 × 3 mm, 3 µm) from HiChrom UK with 0.1% v/v formic acid in water as mobile phase A and 0.1% v/v formic acid in acetonitrile as B at flow rate of 0.300ml/min using the following linear gradient: 10% B (0 min) 100% B (30 min) 100% B (35 min) 10% B (36 min) 10% B 42 min. MS^2 spectra were acquired on an LTQ Orbitrap under the same chromatographic conditions described above. The spectra were acquired in negative ion mode with the same source settings as described above and with collision energy of 35V.

Data Extraction

Peaks were aligned and then extracted in 0.02 amu windows using Sieve 1.2 (ThermoFisher, Hemel Hempstead UK). The peaks areas were transferred to Microsoft Excel for further manipulation. In addition, the extracted data was used for principal components analysis (PCA) modelling using Simca P 13 (Umetrics, Sweden).

Results

Figure 3.1 shows the locations of regions from which the propolis samples were collected.

Figure 3.1 Counties map showing the approximate origin of the propolis samples collected within the UK

The propolis samples analysed by high resolution mass spectrometry and extracted using m/z Mine software contained thousands of features and many of these features are probably genuine compounds. Putative identities were deduced from accurate masses which are within 3 ppm deviation of the exact mass of the proposed elemental composition. Although positive ion data was acquired the focus was on the interpretation of the negative ion data since the predominant phenolic and acidic compounds in propolis all give strong signals in negative ion mode. Thus the reported 344 constituents in propolis summarised in a recent review may probably only a fraction of those which are present. Table 3.1 shows the ten most abundant compounds in the UK propolis samples by intensity.

Leics	**Devon**	**Warwks**	**Berks**	**Norfolk**	**Cornwall**	**Midlands**	**Bucks**	**Wales**
Coumaric acid	Pinobanksin acetate	Pinocembrin methyl ether	Pinobanksin	Caffeic acid pentenyl ester	Pinobanksin	Caffeic acid pentenyl ester	Pinocembrin methyl ether	Caffeic acid pentenyl ester
Pinobanksin	Pinobanksin	Pinobanksin acetate	Pinocembrin	Pinobanksin acetate	Pinocembrin	Coumaric acid	Caffeic acid cinnamyl ester	Pinobanksin
Pinocembrin	Pinocembrin	Coumaric acid	Coumaric acid	Pinocembrin	Pinobanksin acetate	Pinocembrin methyl ether	Hydroxybenzoic acid	Pinobanksin acetate
Galangin isomer	Caffeic acid pentenyl ester	Pinocembrin	Pinobanksin acetate	Pinobanksin	Galangin isomer	Pinobanksin	Pinobanksin	Pinocembrin methyl ether
Methyl pinobanksin	Galangin isomer	Pinobanksin	Pinocembrin methyl ether	Pinocembrin methyl ether	Coumaric acid	Pinocembrin	Pinocembrin	Pinocembrin
Pinobanksin acetate	Pinocembrin methyl ether	Caffeic acid cinnamyl ester	Caffeic acid pentenyl ester	caffeic acid phenacetyl ester	Caffeic acid pentenyl ester	Pinobanksin acetate	Galangin isomer	caffeic acid phenacetyl ester

Chrysin	Caffeic acid cinnamyl ester	Caffeic acid pentenyl ester	Galangin isomer	Galangin isomer	caffeic acid phenacetyl ester	Galangin isomer	Caffeic acid pentenyl ester	Galangin isomer
Caffeic acid cinnamyl ester	caffeic acid phenacetyl ester	Galangin isomer	Caffeic acid cinnamyl ester	Methyl pinobanksin 1	Chrysin	Caffeic acid cinnamyl ester	Pinobanksin acetate	Methyl pinobanksin 2
Methyl pinobanksin 1	Chrysin	caffeic acid phenacetyl ester	Methyl pinobanksin 2	Methyl pinobanksin 2	Pinocembrin methyl ether	caffeic acid phenacetyl ester	Coumaric acid	Caffeic acid cinnamyl ester
Caffeic acid pentenyl ester	Methyl pinobanksin 2	Methyl pinobanksin 2	caffeic acid phenacetyl ester	Caffeic acid cinnamyl ester	Methyl pinobanksin 2	Methyl pinobanksin 2	caffeic acid phenacetyl ester	Chrysin

Table 3.1: The ten most abundant compounds by response in nine samples of UK propolis

In order to make the data processing manageable in the current case the top 125 compounds by average peak intensity, which appeared to be genuine peaks rather than adducts or dimers formed in the mass spectrometry source, were selected for further characterisation. There are many more peaks giving lower signal intensities than the top 125 peaks but for the purposes of establishing a manageable platform for comparison of propolis samples a cut-off point was set.

The complexity of the samples beyond the cut-off point is illustrated in figure 3.2 where around eleven peaks due to dimethylquercetin isomers can be seen. Only peaks 1 and 2 appear in the top 125 components selected for inclusion in table 3.2 and this is also true for many other compounds.

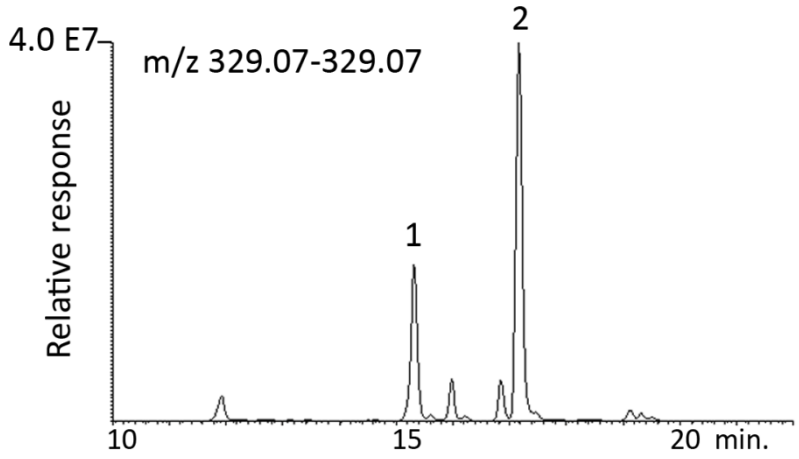


Figure 3.2: Extracted ion trace showing dimethyl quercetin isomers. Only 1 and 2 are among the top 125 metabolites by intensity.

Table 3.2: The 125 most abundant compounds in nine samples of propolis from the UK based on average response across nine samples with each sample analysed in triplicate.

Putative ID	m/z	Rt min	Leics	Devon	Warwks	Berks	Norfolk	Cornwall	Midlands	Bucks	Wales	RSD
Pinobanksin	271.06104	14.9	0.91	0.94	0.75	0.78	1.21	0.93	1.03	0.58	1.45	25.70
Caffeic acid pentenyl ester	247.09781	18.3	0.38	0.72	0.71	0.65	1.51	0.60	1.48	0.54	1.57	48.85
Pinobanksin acetate	313.07202	19.0	0.55	1.11	0.89	0.72	1.58	1.00	1.03	0.58	1.36	33.27
Pinocembrin	255.06615	18.7	0.76	0.88	0.86	0.77	1.42	1.01	1.10	0.65	1.03	23.05
Pinocembrin methyl ether	269.08185	18.1	0.35	0.77	1.17	0.77	1.06	0.60	1.27	0.90	1.32	33.45
Galangin isomer*	269.04553	18.8	0.94	0.95	0.81	0.79	1.25	0.98	1.09	0.75	1.06	15.63

Coumaric acid	163.03992	8.9	1.88	0.38	1.17	0.99	0.60	0.91	1.73	0.65	0.31	54.67
caffeic acid phenacetyl ester	283.09756	18.9	0.44	0.90	0.88	0.70	1.48	0.97	1.06	0.68	1.36	32.77
Caffeic acid cinnamyl ester	295.09744	20.1	0.68	0.98	1.24	0.83	0.99	0.70	1.09	1.13	1.06	18.87
Methyl pinobanksin	285.07672	13.3	1.09	0.84	0.90	0.82	1.17	0.91	0.99	0.50	1.45	25.76
Chrysin	253.05069	18.2	0.94	1.10	0.83	0.82	1.19	1.08	0.78	0.75	1.27	18.65
Caffeic acid	179.03499	7.1	0.65	0.84	0.78	0.86	1.37	0.97	1.31	0.67	0.27	37.00
Methyl pinobanksin isomer methoxy in ring B	285.0769	19.1	1.29	1.16	0.87	0.83	2.20	1.10	1.16	0.53	0.14	51.86
Galangin methyl ether	283.06143	19.5	0.84	1.06	0.89	0.78	1.51	1.00	0.94	0.55	1.25	26.67
Pinobanksin propionate	327.08731	20.6	0.32	0.97	0.63	0.68	2.03	0.90	0.70	0.40	1.50	57.06
Pinobanksin valerate	355.1188	23.9	0.45	1.11	0.61	0.70	1.77	0.96	0.65	0.45	1.72	51.25

Galangin	269.04553	14.1	0.91	1.08	0.86	0.81	1.44	1.12	1.05	0.65	0.85	22.09
Pinobanksin butyrate	341.10327	22.3	0.45	1.08	0.66	0.68	1.73	0.96	0.78	0.51	1.66	46.83
Kaempferol	285.04062	14.5	0.76	0.86	0.74	0.82	1.48	1.07	1.06	0.49	0.77	29.50
Hydroxybenzoic acid	135.04518	9.0	0.61	0.38	2.58	0.89	0.16	0.39	0.71	3.43	0.04	108.46
Caffeic acid hextrieneoate	271.09778	21.6	1.37	0.90	0.90	0.72	2.74	0.69	1.50	0.36	0.03	72.58
Quercetin dimethyl ether	329.06653	17.2	0.70	0.90	0.65	0.77	1.57	1.03	0.82	0.42	1.47	38.57
Kaempferol methyl ether (ring B)	299.05621	15.1	0.62	0.98	0.72	0.77	1.59	0.98	0.92	0.40	1.59	40.12
Coumaric acid cinnamyl ether	279.10284	22.0	2.11	0.47	1.16	1.02	0.79	0.83	1.56	0.58	0.35	53.71

Benzoyl dihydroxyphenylpropionic acid	287.09274	18.1	0.95	0.80	1.11	0.64	3.08	0.59	1.49	0.33	0.00	84.22
Galangin methyl ether	283.06097	16.0	1.13	0.76	0.77	0.79	1.07	1.00	0.80	0.62	2.11	41.80
Acetyl cinnamoyl caffeoyl glycerol	425.12476	17.5	0.03	1.95	0.55	0.76	0.07	1.76	0.18	2.72	0.53	95.30
Galangin methyl ether	283.06146	18.3	1.12	1.02	0.97	0.84	1.40	1.04	0.95	0.68	1.10	18.59
Benzyl coumarate	253.08698	20.1	1.06	0.52	1.11	0.87	0.72	0.37	1.65	0.96	1.00	38.06
Quercetin methyl ether	315.05096	16.5	0.68	0.92	0.71	0.78	1.61	1.09	0.84	0.59	0.75	32.78
Quercetin methyl ether	315.05096	13.2	0.69	0.90	0.60	0.77	1.59	1.01	0.83	0.37	1.50	41.21
Benzoic acid	121.02945	8.4	1.41	1.25	1.34	1.05	0.52	0.81	1.02	1.38	0.29	37.29

Benzoyl hydroxyphenylacetic acid	257.08197	18.5	0.99	0.76	0.56	0.53	3.70	0.79	1.30	0.19	0.07	103.37
Octadecatriol glucoside	463.328	26.9	0.71	1.31	0.91	0.90	1.10	1.13	0.69	0.70	1.20	22.91
Pinobanksin phenyl propionate	403.1189	24.1	0.42	1.28	1.00	0.87	1.30	0.43	1.15	0.69	1.41	36.91
Hydroxypropionate ethyl ester	193.08714	12.4	0.96	0.52	2.43	0.99	0.33	0.61	0.98	2.33	0.01	77.64
Caffeic acid sequiterpene ester	413.19745	22.1	0.42	1.20	0.71	0.77	2.00	1.14	0.76	0.43	0.97	49.08
Prenylated flavonoid	419.15051	20.8	1.70	1.21	1.20	0.95	2.14	0.58	1.34	0.40	0.07	57.28
Dihydroxy eicosenoic acid	343.28558	28.6	0.98	1.42	0.81	0.91	0.87	1.09	0.77	0.80	1.10	20.06
Caffeic acid sesquiterpene ester	397.20239	23.8	2.89	0.35	1.18	1.22	0.34	0.41	1.72	0.32	0.16	89.26
Glucose formate adduct	225.06163	3.0	0.50	0.18	0.59	0.37	0.37	0.82	4.69	0.86	0.31	138.12

Dihydroxylinolenic acid	309.17435	25.2	0.94	0.90	1.01	0.94	0.79	0.90	1.05	0.60	1.27	18.31
Dihydroxyecosanoic acid glucoside	505.3378	29.3	0.06	0.87	0.98	0.62	2.56	0.89	1.31	0.58	0.59	69.93
Dimethyl kaempferol phenethyl ether	417.1347	21.5	1.10	0.89	1.05	0.82	0.77	1.22	1.55	0.63	0.86	26.31
Quercetin methyl ether 3	315.05093	14.7	1.16	0.76	0.72	0.88	1.48	1.13	0.90	0.43	0.80	31.18
Quercetin	301.03543	12.6	0.58	0.91	0.68	0.81	1.61	1.09	0.93	0.56	0.70	35.06
Hydroxybenzoic acid	137.0244	6.5	0.27	0.90	1.05	0.81	0.68	0.73	1.09	1.20	1.18	31.95
Coumaric acid phenacetyl ester	267.10269	20.9	1.76	0.45	1.08	0.90	1.21	1.05	1.30	0.62	0.49	40.45
Dimethyl flavanol	301.10831	21.2	1.13	0.90	1.04	0.70	2.84	0.71	1.26	0.62	0.01	71.15

Caffeic acid monoterpene (geranyl) ester	315.16052	24.6	0.39	0.67	0.39	0.63	1.91	0.53	1.64	0.27	1.52	66.85
quercetin dimethyl ether	329.06693	15.3	0.67	0.91	0.64	0.75	1.55	1.01	0.76	0.35	1.78	45.88
Acetylcoumaroylferuoyl glycerol	455.13538	17.8	0.03	1.43	0.26	0.46	0.13	4.45	0.11	1.92	0.20	137.31
Dihydroxylinoleic acid	311.22321	20.0	0.69	0.92	1.09	0.78	2.42	0.93	1.19	0.18	0.22	66.27
Ethoxy sulphonate detergent	353.20053	26.6	0.98	0.98	1.04	0.96	0.69	0.96	1.17	0.60	1.10	18.49
Methylgalangin hydroxypropionyl ester	431.11401	20.6	0.73	0.74	0.86	0.70	0.61	1.49	1.69	0.75	1.01	37.46
Unknown	433.12964	19.5	1.01	1.07	1.00	0.81	1.02	1.04	1.47	0.79	0.82	19.14

Hydroxy phenyl propionic acid	165.05553	8.7	1.83	0.30	2.15	1.07	0.32	0.47	1.18	1.68	0.08	70.81
Acetylcaffeoylferuoyl glycerol	471.13007	16.4	0.03	1.63	0.31	0.53	0.11	3.91	0.13	1.56	0.67	119.23
Acetylcoumarylcaffeoyl glycerol	441.11957	16.1	0.03	1.71	0.28	0.56	0.08	2.97	0.12	2.01	1.11	99.72
Hydroxy ecosanoic acid glucoside	491.35934	31.0	1.00	1.02	0.64	0.80	0.94	1.07	0.56	0.49	2.26	50.85
Hydroxylinolenic acid	293.1792	28.1	0.92	0.79	0.97	0.84	0.67	0.84	1.07	0.53	1.87	37.89
Kaempferol methyl ether	299.05603	16.4	0.59	1.03	0.68	0.67	1.68	0.94	0.99	0.47	1.70	43.67
Kaempferol methyl ether (ring A)	299.05615	18.8	2.29	0.82	1.02	1.05	0.63	0.89	1.08	1.02	0.34	49.80

Unknown	387.12427	21.3	2.05	0.75	0.81	0.97	1.44	0.82	1.23	0.39	0.65	46.17
Hydroxy phenyl acetyl dihydroxyphenylacetic acid	301.0728	14.9	2.15	0.75	1.43	1.09	0.60	0.69	1.44	0.82	0.19	53.92
Dimethyl pinocembrin benzoate	389.13947	21.2	1.94	1.04	0.55	0.80	2.65	0.58	1.13	0.16	0.08	79.29
Hydroxynonadecanoic acid glucoside	477.34338	29.1	0.97	1.36	0.68	0.98	1.12	1.34	0.67	0.54	0.47	34.60
Dicoumaroyl glycerol	383.11395	14.5	0.04	1.16	0.28	0.50	0.02	0.98	0.06	4.62	0.80	144.87
Unknown	433.12955	20.7	1.62	0.74	1.11	0.88	1.08	0.79	1.15	0.88	0.76	26.38
caffeic acid valeryl ester	249.11308	19.6	0.14	0.31	0.26	0.29	1.04	0.18	2.21	0.49	4.36	129.15
Pinobanksin hexanoate	369.13461	25.3	0.23	0.84	0.45	0.72	2.39	0.66	0.54	0.28	1.79	78.41

Unknown	401.10342	20.4	2.08	0.78	0.71	0.97	1.28	0.98	1.15	0.45	0.31	50.77
Caffeoyl feruoyl acetyl glycerol	471.12924	17.6	0.15	1.68	0.49	0.65	0.11	2.99	0.15	1.95	0.51	98.70
Coumaric acid pentenyl ester	231.10265	20.2	0.39	0.70	0.87	0.75	1.78	0.57	1.73	0.72	0.52	53.66
Methyl methylene dioxy kaempferol hexanoyl ester	435.14517	17.6	1.61	0.84	2.20	1.09	0.75	0.58	1.03	1.16	0.06	55.55
Pinobanksin pentenoate	353.10333	22.3	0.66	1.06	0.67	0.70	1.70	0.70	0.77	0.41	2.54	62.31
Dihydroxypropionyl pinocembrin methyl ether	433.12961	17.7	0.93	0.97	1.07	0.79	0.87	1.26	1.52	0.69	0.97	23.52
Pinocembrin methyl ether	269.08191	21.2	0.19	0.96	0.78	0.64	1.99	0.54	1.79	0.53	1.03	59.67
Hydroxy eicosenoic acid	327.29077	37.5	1.02	1.13	0.69	0.74	0.77	1.09	1.56	0.82	1.12	26.22

Dihydroxy docosahexenoic acid	371.31662	32.5	1.31	1.25	0.74	0.92	0.65	1.04	0.67	0.72	1.47	29.62
Pentenoyl hydroxyphenylpropionic acid	281.10309	8.8	0.07	0.70	0.73	0.66	1.50	0.42	1.70	0.65	1.44	58.96
Unknown	357.30139	30.6	1.35	1.38	0.75	1.01	0.72	1.31	0.67	0.75	0.48	33.86
Hydroxylinolenic acid	293.21246	25.8	1.41	0.59	0.83	0.88	1.52	0.77	1.20	0.37	0.98	37.32
Pinobanksin phenyl propionate	403.11905	18.3	2.17	0.86	0.88	1.02	1.18	0.80	1.25	0.45	0.46	48.01
Trimethyl pinocembrin phenacetyl ether	403.15503	23.7	2.10	1.15	0.88	0.96	2.21	0.59	1.31	0.18	0.02	68.37
Unknown	377.13962	24.2	1.44	1.35	1.40	1.06	0.29	0.60	0.90	1.74	0.04	55.15

Pinobanksin benzoate	375.08765	22.6	1.63	0.57	1.01	0.92	1.10	0.49	1.12	0.38	1.58	43.04
Pinobanksin phenylpropionate	403.11917	19.7	0.77	0.91	0.90	0.82	1.50	0.81	1.32	0.55	0.82	29.77
Unknown	475.14014	21.5	0.20	1.00	1.01	0.64	0.88	1.83	1.29	0.74	1.27	44.28
Methyl methylene dioxy kaempferol hexanoyl ester	435.14526	19.2	1.45	1.02	1.85	1.04	0.78	0.82	1.10	1.17	0.13	42.98
Sesquiterpene caffeic acid ester	399.21796	22.6	0.59	1.06	0.62	0.66	2.97	1.17	0.72	0.39	0.57	76.22
Unknown	449.12445	19.6	1.24	0.47	0.92	0.71	1.09	0.57	0.89	0.47	3.14	73.84
Dihydroxy linolenic acid	309.20712	19.3	0.98	0.81	1.22	0.91	1.21	0.94	0.92	0.87	0.60	19.20
Methyl pinobanksin acetate	327.08768	16.2	0.51	0.84	0.87	0.66	1.74	0.90	0.96	0.43	1.74	46.46
Unknown	337.20593	31.6	0.88	0.82	1.16	0.87	0.56	0.82	1.07	0.68	1.10	20.83

caffeic acid phenylpropionyl ester	297.1134	20.3	0.42	0.62	0.76	0.55	1.07	0.46	2.24	0.80	1.76	61.73
Dihydrokaempferol (dihydroxy ring A)	287.05637	11.1	0.53	1.36	0.71	0.89	1.35	1.00	0.84	0.45	0.70	35.21
sesquiterpene acid	265.14459	17.6	0.46	0.71	0.65	0.66	2.08	0.71	1.45	0.28	1.02	58.87
Pinobanksin caffeate	433.09271	18.1	0.27	1.18	0.39	0.54	2.01	0.86	0.74	0.29	2.45	75.65
Caffeoyl dimethyl pinocembrin	445.12961	21.3	0.78	1.05	0.96	0.80	1.13	0.88	1.28	0.59	0.82	21.27
caffeic acid ethyl ester	207.06645	13.7	0.10	0.08	0.07	0.07	0.10	0.04	0.26	0.07	10.24	259.74
hydroxylinoleic acid	297.24377	25.0	1.06	0.41	0.66	0.91	1.34	0.69	0.75	0.41	1.10	36.63
dicaffeoyl acetyl glycerol	457.11459	14.7	0.03	1.66	0.15	0.46	0.22	3.26	0.16	1.18	2.03	103.24

hydroxyheptadecanoic acid acetate	329.27005	26.5	1.27	1.25	0.81	0.95	1.01	1.26	0.67	0.81	0.32	32.33
methyl chrysin acetate derivative	419.11407	16.1	0.46	0.91	0.65	0.63	1.88	0.56	1.73	0.37	1.74	58.31
prenylated flavonoid	419.15036	22.6	1.52	0.90	1.49	0.97	0.87	0.90	1.11	1.33	0.14	38.65
kaempferol isomer	285.04102	12.5	0.74	1.24	0.73	0.77	1.34	1.12	0.98	0.57	1.26	27.11
Acetyl coumaryl glycerol	279.08734	11.4	0.20	0.73	0.34	0.42	0.08	2.53	0.13	3.18	0.85	112.81
pinobanksin dimethyl cinnamate	429.13437	25.5	0.26	0.83	0.89	0.70	1.23	0.26	1.37	0.63	2.25	62.57
Unknown	361.09039	18.0	1.01	1.17	0.98	0.90	1.04	0.85	0.98	0.77	0.84	12.01
coumarate derivative	597.21307	23.0	1.45	1.04	1.77	1.02	0.83	0.65	0.85	1.37	0.02	47.85

Unknown	461.12469	19.8	0.37	0.98	0.90	0.68	1.80	1.13	1.07	0.51	1.01	41.61
chrysin glucoside formate adduct	461.10138	17.6	0.06	1.73	0.65	0.79	0.13	1.66	0.35	2.11	0.73	76.92
galangin glucoside	431.09808	10.9	0.22	0.62	0.43	0.58	1.95	1.21	0.97	0.36	1.70	64.49
caffeic acid butyl ester	235.09758	17.9	0.08	0.17	0.20	0.18	0.74	0.11	3.35	0.44	4.05	139.83
hydroxydocosapentaenoic acid	347.25925	27.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.29	282.63
Pinocembrin methyl ether	269.08243	15.0	0.49	0.76	0.85	0.72	1.48	1.12	0.96	0.60	1.21	32.74
sesquiterpene	269.17572	14.0	0.58	0.73	0.68	0.60	1.87	0.17	3.17	0.42	0.06	101.43
kaempferol methyl ether (ring A)	299.05615	11.7	0.76	0.67	0.53	0.81	1.41	1.20	0.68	0.35	1.50	42.72

Caffeic acid butenyl ester	233.08188	16.8	0.05	0.20	0.19	0.22	1.19	0.25	3.48	0.42	2.87	124.04
hexadieneoyl dimethylpinobanksin	407.11417	16.6	0.23	0.34	0.18	0.30	0.11	0.18	0.22	0.19	8.48	228.71
quercetin dimethyl ether	329.06693	12.0	0.82	0.56	0.43	0.79	1.41	1.03	0.68	0.30	1.74	51.08
dihydroxy linolenic acid	309.20709	17.0	0.90	1.12	0.96	0.82	1.86	0.58	1.56	0.32	0.59	47.51
hydroxydocoahexanoic acid	345.24356	35.3	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.27	282.16
Pinobanksin butyrate	341.10306	17.9	1.66	0.56	1.22	0.96	0.74	1.01	1.50	0.60	0.64	38.44

Figure 3.3 shows a plot for the average intensity of the top 125 components by average peak area across the nine samples which were each sampled three times. It can be presumed that compounds with similar structures will give responses which are not widely different from each other. Although without standards for calibration the estimate of the actual abundance of the compounds within the sample is only semi-quantitative. Standards are not available for the majority of compounds in propolis. It is clear that the abundance for the top ten compounds falls rapidly before the decline in abundance becomes more gradual.

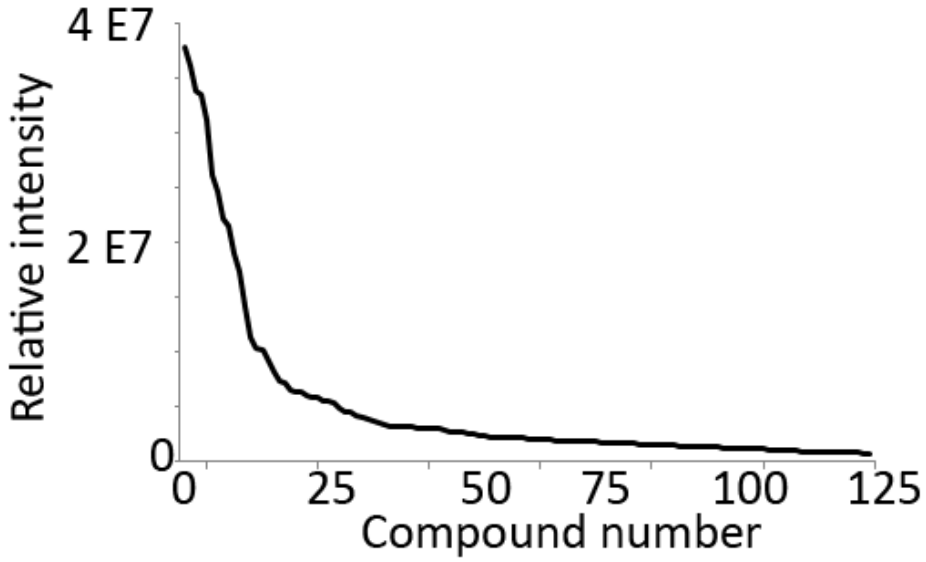


Figure 3.3: Plot showing the fall in average response over the top 125 compounds in UK propolis samples.

Figure 3.4 shows the score plot of the PCA for the UK propolis samples based on the 125 most abundant LC-MS features across all the samples, the data was Pareto scaled before being subjected PCA analysis. The total R2X (cum) is 0.851 and the total Q2 (cum) is 0.65 indicating a good fit of the model to the data. The first and the second principal components represent almost 60% of the 125 compounds which

determine the location of the samples in the plot. It is clear that in some cases the three sample replicates cluster together well but in others the propolis composition is less uniform for the three replicate samples. The degree of uniformity depends on the nature of the sample with some samples containing insoluble plant debris whereas, for example, the densely resinous sample from Wales produced a tight cluster. The best defined clusters for the three sample replicates are for Leicestershire, Buckinghamshire, Devon, Wales and Norfolk. These also represent regions which are geographically far apart.

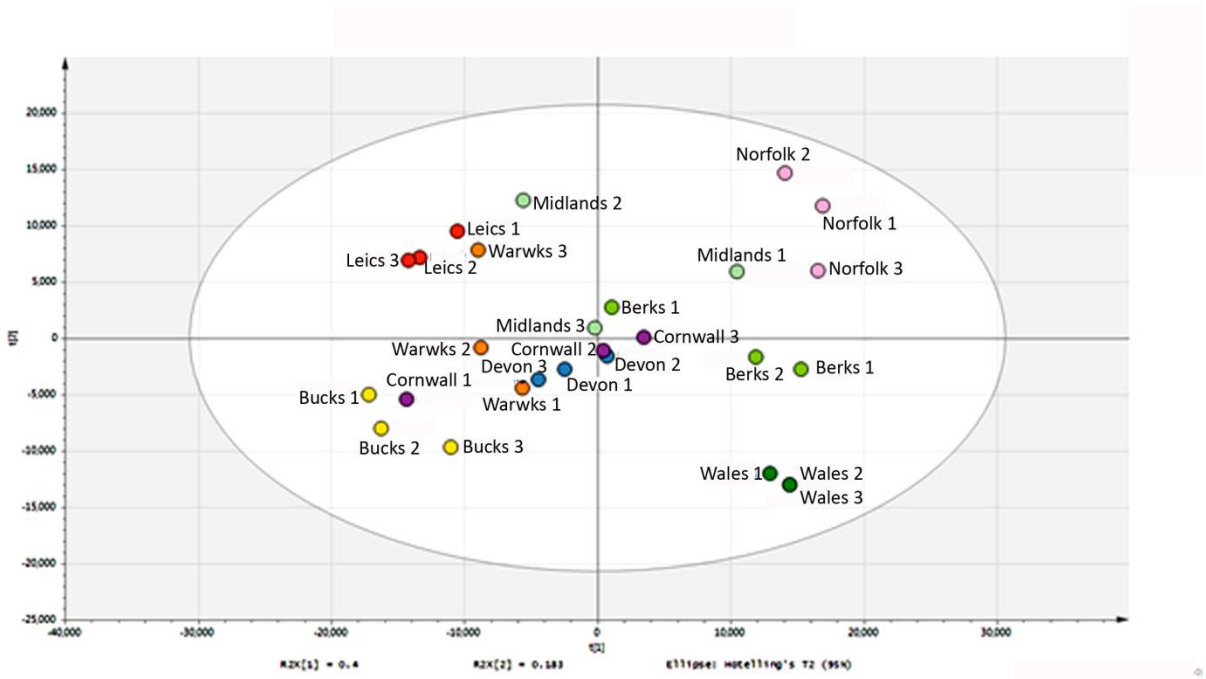


Figure 3.4 PCA plot based on the top 125 features by average abundance in 9 UK propolis samples sampled x 3.

Out of the 125 components pinobanksin has the highest average peak area and as can be seen in figure 3.5 its amounts are consistent across the nine samples. In contrast acetylcinnamoylcaffeoyl glycerol is much more abundant in three of the samples. This compound is one of a series of glycerol derivatives found in propolis and these have

been found to have anti-inflammatory properties (Shi *et al*, 2012). As with other components there are many of these glycerol esters in propolis although many of them are at an abundance below the levels of the compounds shown in Table 3.2. Figure 3.6 shows extracted ion traces corresponding to several putatively identified glycerol esters. The significance of such variations in propolis composition with regard to its biological activity has yet to be fully investigated. As mentioned above there has been some preliminary work on correlating hive resistance to infection with propolis composition (Popova *et al*, 2014).

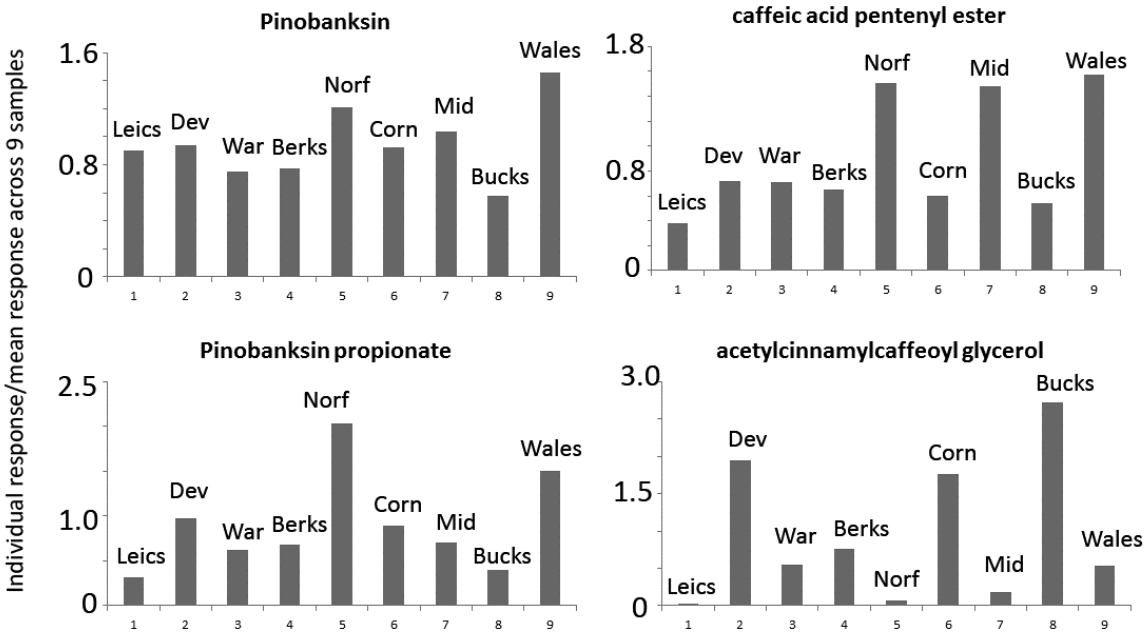


Figure 3.5: Variation in pinobanksin, caffeic acid phenethyl ester, pinobanksin propionate and acetylcinnamylcaffeoylglycerol over nine propolis samples.

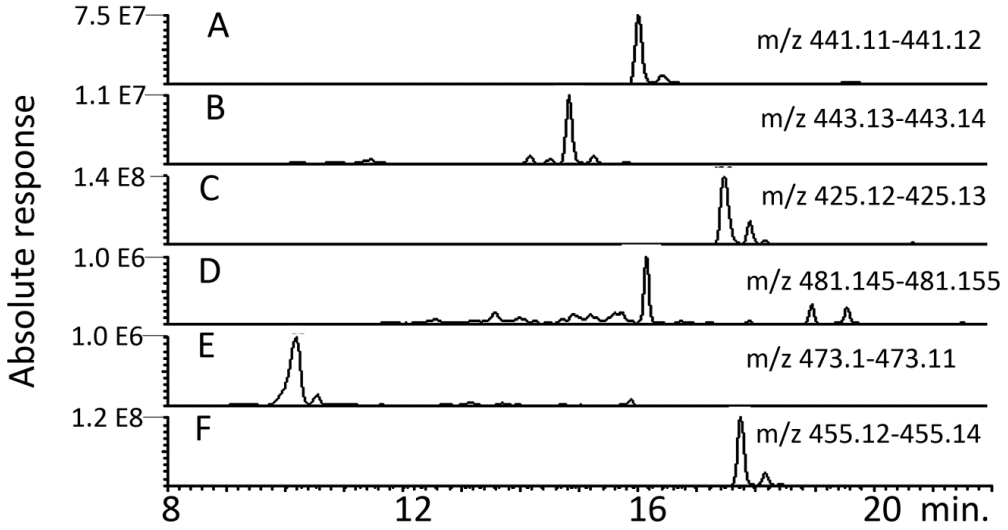


Figure 3.6: Extracted ion traces for glycerol esters from propolis. A Acetyl coumaroyl caffeoyl B Acetylhydroxyphenylpropionylcaffeoyl C Acetylcinnamoylcaffeoyl D Penetenoyl coumaroyl caffeoyl. E. Acetylgalloylcaffeoyl F. Acetylcoumaroylferuoyl.

Figure 3.7 shows the loadings plot of the 125 compounds and in order to differentiate the putatively identified and the unknown compounds the LC-MS features are shaped as circles and crosses respectively. It is possible in Simca P show the weight of each variable (LC-MS feature) contributing to the distribution of the selected observations from the PCA score plot (Figure 3.4). The top 10 significant LC-MS features were selected based on their contribution scores and are correspondingly highlighted in the PCA loading plot (Figure 3.7). For example the green circles in figure 3.7 show the ten compounds (most abundant by relative response) which are most strongly correlated with the position of the Welsh propolis sample in the PCA plot. These compounds are listed in table 3.3 which shows the details of the top 10 compounds correlated with position of the four samples where the three replicates were most closely clustered. As can be seen from figure 3.7 most of the significant LC-MS features associated with these samples were putatively identified and only a few were

unknown. The Leicestershire sample contained the most unknown compounds. As can be seen from the data in table 2 each of the four highlighted samples contains at least ten compounds which clearly define it. Thus for example the sample from Leicestershire is rich in coumaric acid and its esters while the sample from Buckinghamshire is rich in glycerol esters (this can also be observed for one of these compounds in figure 3.5).

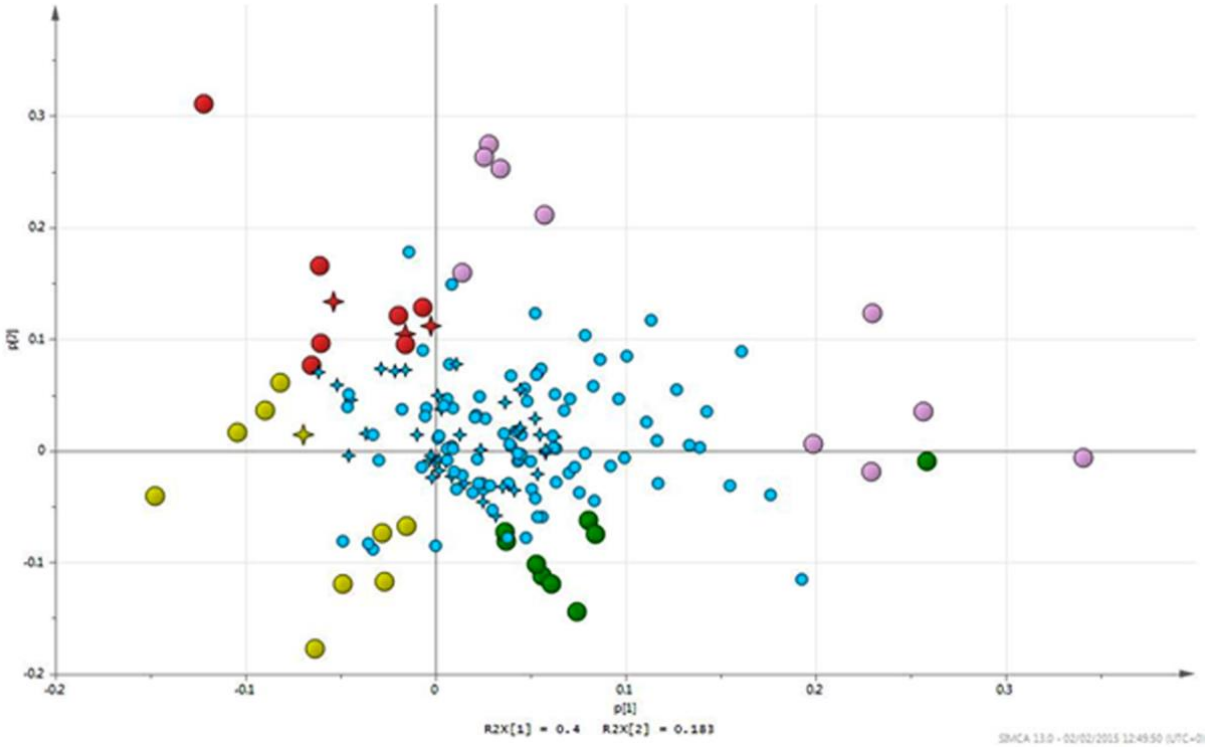


Figure 3.7 Loadings plot for nine UK propolis samples showing the most significant loadings associated with: Welsh sample (green), Norfolk sample (violet), Leicestershire (red) and Buckinghamshire (yellow). Circles are putatively identified metabolites and crosses and unknown. Light blue circles and crosses the rest of the loadings.

Compound Name	**Time**
Wales green circles	
Caffeic acid ethyl ester	13.7
Hydroxydocosahexanoic acid	35.3
Pinobanksin	14.9
Hexadieneoyl dimethylpinobanksin	16.6
Hydroxydocosapentaenoic acid	27.1
Caffeic acid pentenyl ester	18.3

Cinnamoyl caffeoyl acetyl glycerol	19.8
Caffeic acid valeryl ester	19.6
Galangin methyl ether	16.0
Diterpene acid	16.3
Norfolk violet circles	
Methyl pinobanksin isomer methoxy in ring B	19.1
Caffeic acid hextrieneoate	21.6
Benzoyl dihydroxyphenylpropionic acid	18.1
Benzoyl hydroxyphenylacetic acid	18.5
Pinobanksin acetate	19.0
Pinocembrin	18.7
Pinobanksin propionate	20.7
Dimethyl flavanol	21.2
Caffeic acid pentenyl ester	18.2
Caffeic acid phenacetyl ester	18.9
Leicestershire red circles and crosses	
Coumaric acid	8.9
Coumaric acid cinnamyl ether	22.0
Unknown	23.8
Kaempferol methyl ether (ring A)	18.8
Unknown	21.3
Unknown	20.3
Pinobanksin phenyl propionate	18.3
Coumaric acid phenacetyl ester	20.9
Hydroxy phenyl acetyl dihydroxyphenylacetic acid	14.9
Trimethyl pinocembrin phenethyl ether	23.7
Buckinghamshire yellow circles and cross	
Hydroxybenzoic acid	9.0
Cinnamoyl caffeoyl acetyl glycerol	17.5
Dicoumaroyl glycerol	14.5
Hydroxypropionate ethyl ester	12.4
Coumaryl acetyl glycerol	11.4
Unknown	24.2
Benzoic acid	8.4
Coumarylcaffeoyl acetyl glycerol	16.1
Hydroxy phenyl propionic acid	8.7
Chrysin glucoside (formate adduct)	17.6

Table 3.3 The top 10 significant LC-MS features in the samples from Wales, Norfolk, Leicestershire and Buckinghamshire.

The MS^2 data for the compounds listed in table 3.2 is shown in table 3.3. Apart from losses of water, CO, CO_2 and CH_3 which occur commonly, typical fragmentation pathways fall into a few major types illustrated in figure 3.5. Thus it is possible to be

fairly confident of structural identity although the MS^2 spectra in most cases do not give information on positions of hydroxyl groups or chain branching within alkyl chains. In some cases the positions of hydroxyl and methyl groups can be assigned to some extent. Thus the most abundant compounds fall into a few major categories: flavonoid esters and possibly some ethers, phenyl propanoid esters, glycerol esters, flavonoid glycosides and hydroxylated fatty acids. In addition, there were some compounds which are yet to be fully elucidated including esters of sesquiterpenes with phenolics and some unknown compounds whose structures could not be elucidated even with MS^2 spectra. Although many of these types of compounds have been reported previously, there are many variations within the categories of structures which have not been reported before. Arranging compounds in a semi-quantitative manner as in table 3.2 gives an indication of which compounds might be considered important for the biological activities of propolis on the basis of abundance although some minor constituents may be highly active.

The components were identified or partly identified based on their fragmentation patterns shown in Figure 3.8.

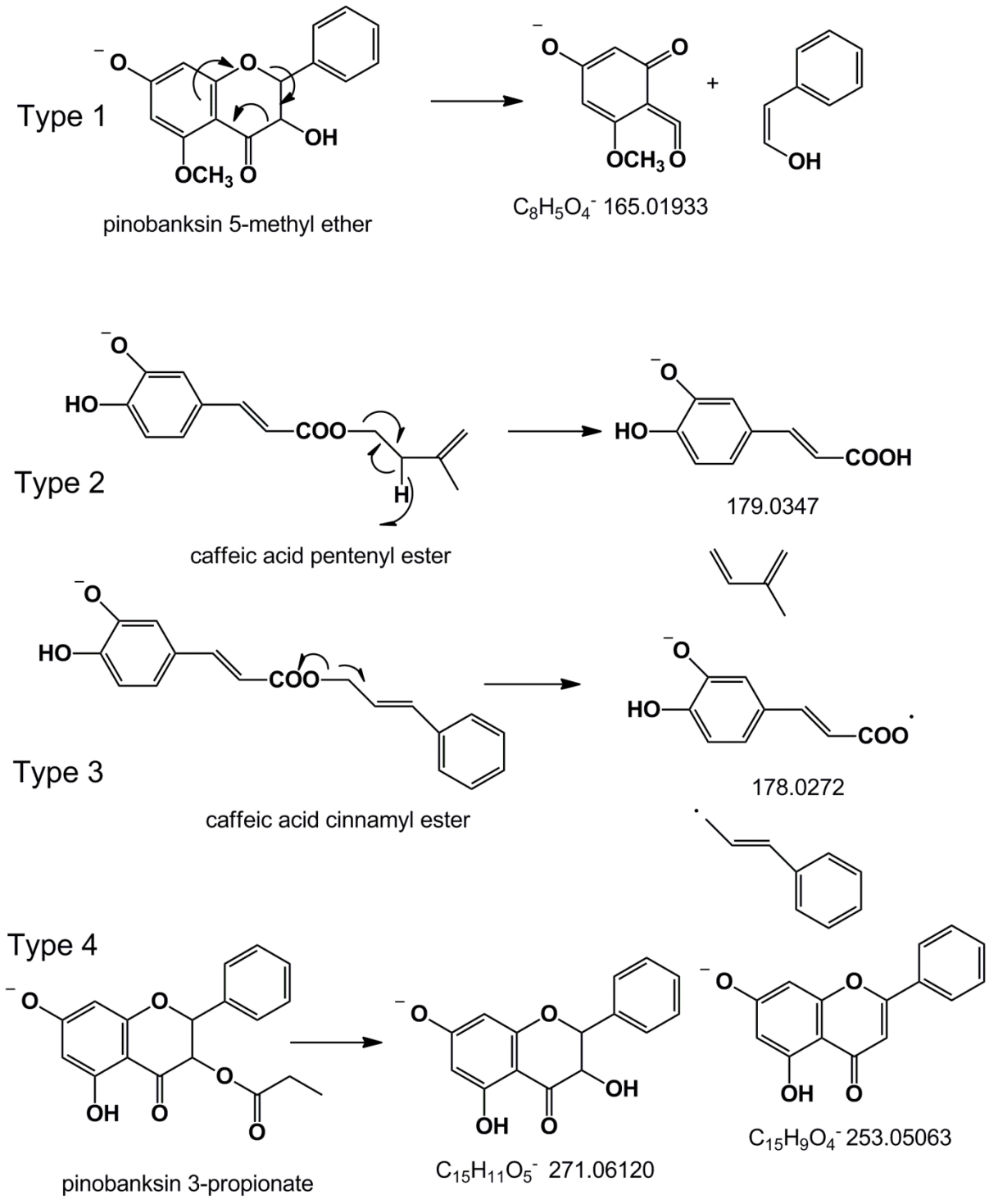


Figure 3.8: Common fragmentation pathways for propolis components

Table 3.4: MS/MS Data for Compounds in Table 3.2

Composition	MZ	Time	MS MS	
$C_{15}H_{11}O_5$	271.06104	14.9	253.0506 ($C_{15}H_9O_4$), 225.0553 ($C_{14}H_9O_5$), 165.0194 ($C_8H_5O_4$) 151.0038 ($C_7H_3O_5$) (Type 1)	Pinobanksin
$C_{14}H_{15}O_4$	247.09781	18.3	179.0347 ($C_9H_7O_4$), 135.0451 ($C_8H_7O_2$) (Type 2)	Caffeic acid pentenyl ester
$C_{17}H_{13}O_6$	313.07202	19.0	271.0612 ($C_{15}H_{11}O_5$) 253.0504 ($C_{15}H_9O_4$) (Type 3)	Pinobanksin acetate
$C_{15}H_{11}O_4$	255.06615	18.7	213.0558 ($C_{13}H_9O_3$) 151.0037 ($C_7H_3O_4$) (Type 1)	Pinocembrin
$C_{16}H_{13}O_4$	269.08185	18.1	225.0923($C_{15}H_{13}O_2$) 178.0272($C_9H_6O_4$) 134.0372 ($C_8H_6O_2$)	Pinocembrin methyl ether
$C_{15}H_9O_5$	269.04553	18.8	241.0509($C_{14}H_9O_4$)227.0351($C_{13}H_7O_4$)213.0559($C_{13}H_9O_4$)137.0245($C_7H_5O_3$)	Galangin isomer*
$C_9H_7O_3$	163.03992	8.9	119.0502(C_8H_7O)	Coumaric acid
$C_{17}H_{15}O_4$	283.09756	18.9	179.0351($C_9H_{27}O_4$)161.0244($C_9H_5O_3$)135.0452($C_8H_7O_2$) (Type 2)	caffeic acid phenacetyl ester

$C_{18}H_{15}O_4$	295.09744	20.1	251.0766($C_{17}H_{15}O_2$)211.0766($C_{14}H_{11}O_2$)$C_9H_6O_4$(178.0272)134.0373($C_8H_6O_2$) (Type 4)	Caffeic acid cinnamyl ester
$C_{16}H_{13}O_5$	285.07672	13.3	267.0662($C_{16}H_{11}O_4$)252.043 ($C_{15}H_8O_4$)239.0714($C_{15}H_{11}O_3$)165.0194($C_8H_5O_4$) (Type 1)	Methyl pinobanksin
$C_{15}H_9O_4$	253.05069	18.2	209.0609 ($C_{14}H_9O_2$) 151.004 ($C_7H_3O_4$) (Type 1)	Chrysin
$C_9H_7O_4$	179.03499	7.1	135.0450($C_8H_7O_2$)	caffeic acid
$C_{16}H_{13}O_5$	285.0769	19.1	270.0535($C_{15}H_{10}O_5$)243.0664($C_{14}H_{11}O_4$)164.0116($C_8H_4O_4$)151.0038($C_7H_3O_4$) (Type 1)	Methyl pinobanksin isomer methoxy in ring B
$C_{16}H_{11}O_5$	283.06143	19.5	268.0377($C_{15}H_8O_5$)239.0348($C_{14}H_7O_4$)211.0402($C_3H_7O_3$) (Type 1)	galangin methyl ether
$C_{18}H_{15}O_6$	327.08731	20.6	271.0613($C_{15}H_{11}O_5$)253.0504($C_{15}H_9O_4$) (Type 3)	Pinobanksin propionate
$C_{20}H_{19}O_6$	355.1188	23.9	271.0606($C_{15}H_{11}O_5$)253.0506($C_{15}H_9O_4$) (Type 3)	Pinobanksin valerate
$C_{16}H_{13}O_4$	269.04553	14.1	225.0558($C_{14}H_9O_3$)201.0558($C_{12}H_9O_3$)183.0453($C_{12}H_7O_2$)151.0037($C_7H_3O_4$) (Type 1)	Galangin*

$C_{19}H_{17}O_6$	341.10327	22.3	271.0613($C_{15}H_{11}O_5$)253.0504($C_{15}H_9O_4$) (Type 2)	Pinobanksin butyrate
$C_{15}H_9O_6$	285.04062	14.5	267.0301($C_{15}H_7O_5$)151.0038($C_7H_3O_4$) (Type 1)	kaempferol
$C_{16}H_{15}O_4$	271.09778	21.6	253.0508($C_{15}H_9O_4$) 161.0246 ($C_9H_5O_3$) (Type 2)	caffeic acid hextrieneoate
$C_{17}H_{13}O_7$	329.06653	17.2	314.0429($C_{15}H_{10}O_7$)165.0197($C_8H_5O_4$) (1 methyl in ring A) (Type 1)	quercetin dimethyl ether
$C_{16}H_{11}O_6$	299.05621	15.1	284.0324($C_{15}H_8O_6$) 151.0034($C_7H_3O_4$) (methyl in ring B) (Type 1)	kaempferol methyl ether
$C_{18}H_5O_3$	279.10284	22.0	235.1127($C_{17}H_{15}O$)195.0813($C_{14}H_{11}O$)162.0321($C_9H_6O_3$) (Type 2)	coumaric acid cinnamyl ether
$C_{16}H_{15}O_5$	287.09274	18.1	193.0506($C_{10}H_9O_4$)181.0505($C_9H_9O_4$) (Type 2)	benzoyl dihydroxyphenylpropionic acid
$C_{16}H_{11}O_5$	283.06097	16.0	268.0377($C_{15}H_8O_5$)239.0349($C_{14}H_7O_4$)211.0403($C_3H_7O_3$)	galangin methyl ether

$C_{23}H_{21}O_8$	425.12476	17.5	365.1030($C_{21}H_{17}O_6$)277.0718($C_{14}H_{13}O_6$)179.0350($C_9H_7O_4$)147.045($C_9H_7O_2$) (Type 2)	cinnamoyl caffeoyl acetyl glycerol
$C_{16}H_{11}O_5$	283.06146	18.3	268.0377($C_{15}H_8O_5$)	galangin methyl ether
$C_{16}H_{13}O_3$	253.08698	20.1	209.0773($C_{15}H_{13}O$)162.0322($C_9H_6O_3$)121.0295($C_7H_5O_2$) (Type 4)	benzyl coumarate
$C_{16}H_{11}O_7$	315.05096	16.5	300.0275($C_{15}H_8O_7$)287.0562($C_{15}H_{11}O_6$)193.0142($C_9H_5O_5$)165.0192($C_8H_5O_4$) (1 methyl ring A) (Type 1)	quercetin methyl ether
$C_{16}H_{11}O_7$	315.05096	13.2	300.0273($C_{15}H_8O_7$)	quercetin methyl ether
$C_{15}H_{13}O_4$	257.08197	18.5	213.0923($C_{14}H_{13}O_2$) 122.0373($C_7H_6O_2$)	benzoyl hydroxyphenylacetic acid
$C_{24}H_{47}O_8$	463.328	26.9	283.2634($C_{18}H_{35}O_2$) MS3 265.2539($C_{18}H_{33}O$)252.2539($C_{17}H_{33}O$)211.2069($C_{14}H_{27}O$)	octadecatriol glucoside
$C_{24}H_{19}O_6$	403.1189	24.1	271.0601($C_{15}H_{11}O_5$) 253.0499 ($C_{15}H_9O_4$) (Type 3)	pinobanksin phenyl propionate
$C_{11}H_{13}O_3$	193.08714	12.4	147.0453($C_9H_7O_2$) (-ethanol)	hydroxypropionate ethyl ester

$C_{24}H_{29}O_6$	413.19745	22.1	369.2073($C_{23}H_{29}O_4$)251.1656($C_{15}H_{23}O_3$)179.0351($C_9H_7O_4$)161.0243($C_9H_5O_3$)	caffeic acid sequiterpene ester
$C_{25}H_{23}O_6$	419.15051	20.8	375.1591($C_{24}H_{23}O_4$)313.1072($C_{18}H_{17}O_5$)299.0916($C_{17}H_{15}O_5$)257.0814($C_{15}H_{13}O_4$)	unknown
$C_{20}H_{39}O_4$	343.28558	28.6	325.2748($C_{20}H_{37}O_3$)283.2643($C_{18}H_{35}O_2$)	dihydroxy eicosenoic acid
$C_{24}H_{29}O_5$	397.20239	23.8	353.2114($C_{23}H_{29}O_3$) 179.0349($C_9H_7O_4$)	Caffeic acid sesquiterpene ester
$C_{18}H_{29}O_4$	309.17435	25.2	291.1967($C_{18}H_{27}O_3$)265.1810($C_{16}H_{25}O_3$)251.1654($C_{15}H_{23}O_3$)185.1184($C_{10}H_{17}O_3$)	dihydroxylinolenic acid
$C_{26}H_{49}O_9$	505.3378	29.3	325.2745($C_{20}H_{37}O_3$)283.2641($C_{18}H_{35}O_2$)	dihydroxyecosanoic acid glucoside
$C_{25}H_{21}O_6$	417.1347	21.5	402.1099($C_{24}H_{18}O_6$)387.0867($C_{23}H_{15}O_6$)298.0475($C_{16}H_{10}O_6$)	dimethyl kaempferol phenethyl ether
$C_{16}H_{11}O_7$	315.05093	14.7	300.0273($C_{15}H_8O_7$)	quercetin methyl ether
$C_{15}H_9O_7$	301.03543	12.6	273.0406($C_{14}H_9O_5$)257.0457($C_{14}H_9O_5$)178.9986($C_8H_3O_5$)151.0037($C_7H_3O_4$) (Type 1)	quercetin
$C_7H_5O_3$	137.0244	6.5	93.0346 (C6H5O)	hydroxybenzoic acid
$C_{17}H_{15}O_3$	267.10269	20.9	163.0399($C_9H_7O_3$) 119.0502 (C_8H_7O) (Type 2)	coumaric acid phenacetyl ester

$C_{17}H_{17}O_5$	301.10831	21.2	286.0848($C_{16}H_{14}O_5$)283.0975($C_{17}H_{15}O_4$)268.0274($C_{16}H_{12}O_4$)152.0115($C_7H_4O_4$)	dimethyl galangin isomer
$C_{19}H_{23}O_4$	315.16052	24.6	271.1707($C_{18}H_{23}O_2$)179.0350($C_9H_7O_4$)135.0451($C_8H_7O_2$) (Type 2)	Caffeic acid monoterpene (geranyl) ester
$C_{17}H_{13}O_7$	329.06693	15.3	314.0429($C_{16}H_{10}O_7$) 299.0200($C_{15}H_7O_7$)	quercetin dimethyl ether
$C_{24}H_{23}O_9$	455.13538	17.8	395.1175($C_{22}H_{19}O_7$)193.0527($C_{10}H_9O_4$)163.0418($C_9H_7O_3$) (Type 2)	coumaroyl feruoyl acetyl glycerol
$C_{18}H_{31}O_4$	311.22321	20.0	293.2123($C_{18}H_{29}O_3$)275.2018($C_{18}H_{27}O_2$)235.1704($C_5H_{23}O_2$)223.1705($C_{14}H_{23}O_2$)	dihydroxylinoleic acid
$C_{25}H_{19}O_7$	431.11401	20.6	401.0664($C_{23}H_{13}O_7$)311.0567($C_{17}H_{11}O_6$)283.0619($C_{16}H_{11}O_5$)255.0669($C_{15}H_{11}O_4$) (Type 3)	Methylgalangin hydroxypropionyl ester
$C_{25}H_{21}O_7$	433.12964	19.5	373.1080($C_{23}H_{17}O_5$)	UK
$C_9H_9O_3$	165.05553	8.7	119.0503(C_8H_7O)	hydroxy phenyl propionic acid
$C_{24}H_{25}O_{10}$	471.13007	16.4	411.1083($C_{22}H_{19}0_8$)295.0825($C_{14}H_{15}O_7$)193.0508($C_{10}H_90_4$)179.0351($C_9H_7O_4$) (Type 2)	acetyl caffeoyl feruoyl glycerol
$C_{23}H_{21}O_9$	441.11957	16.1	381.0974($C_{21}H_{17}O_7$)295.0820($C_{14}H_{15}O_7$)179.0349($C_9H_7O_4$)163.0399($C_9H_7O_3$) (Type 2)	Acetylcaffeoyl acetyl glycerol

$C_{26}H_{21}O_8$	491.35934	31.0	311.2956($C_{20}H_3O2$)	hydroxy ecosanoic acid glucoside
$C_{18}H_{29}O_3$	293.1792	28.1	275.2018($C_{18}H_{27}O_2$)197.1182($C_{11}H_{17}O_3$)185.1182($C_{10}H_{17}O_3$)	hydroxylinolenic acid
$C_{16}H_{11}O_6$	299.05603	16.4	284.0327($C_{15}H_8O_6$)	kaempferol methyl ether
$C_{16}H_{11}O_6$	299.05615	18.8	284.0325($C_{15}H_8O_6$) 151.0034($C_7H_3O_4$) (methyl in ring B) (Type 1)	kaempferol methyl ether
$C_{24}H_{19}O_5$	387.12427	21.3	359.1281 ($C_{23}H_{19}O_4$) 345.1125($C_{22}H_{17}O_4$)293.0814($C_{18}H_{13}O_4$)283.0607($C_{16}H_{11}O_5$)	UK
$C_{16}H_{13}O_6$	301.0728	14.9	165.0193($C_8H_5O_4$)	hydroxy phenyl acetyl dihydroxyphenylacetic acid
$C_{24}H_{21}O_5$	389.13947	21.2	345.1497($C_{23}H_{21}O_3$)283.0976($C_{17}H_{15}O_4$)269.0820($C_{16}H_{13}O_4$)257.0820($C_{15}H_{13}O_4$) (Type 3)	dimethyl pinocembrin benzoate
$C_{25}H_{49}O_8$	477.34338	29.1	297.2795($C_{19}H_{37}O_2$)	hydroxynonadecanoic acid glucoside
$C_{21}H_{19}O_7$	383.11395	14.5	237.0777($C_{12}H_{13}O_5$)219.0670($C_{12}H_{11}O_4$)163.0405($C_9H_7O_3$) (Type 2)	dicoumaroyl glycerol
$C_{25}H_{21}O_7$	433.12955	20.7	373.1080($C_{23}H_{17}O_5$)	UK

$C_{14}H_{17}0_4$	249.11308	19.6	179.0350($C_9H_7O_4$)161.0244($C_9H_5O_3$) (Type 2)	caffeic acid valeryl ester
$C_{21}H_{21}O_6$	369.13461	25.3	271.0609($C_{15}H_1O_5$)253.0501($C_{15}H_9O_5$) (Type 3)	pinobanksin hexanoate
$C_{24}H_{17}O_6$	401.10342	20.4	307.0618($C_{18}H_{11}O_5$)	UK
$C_{24}H_{25}O_{10}$	471.12924	17.6	411.1081($C_{22}H_{19}0_8$)295.0825($C_{14}H_{15}O_7$)193.0507($C_{10}H_9O_4$)179.0351($C_9H_7O_4$) (Type 2)	caffeoyl feruoyl acetyl glycerol
$C_{14}H_{15}O_3$	231.10265	20.2	163.0400($C_9H_7O_3$)119.0502(C_8H_7O) (Type 2)	coumaric acid pentenyl ester
$C_{25}H_{23}O_7$	435.14517	17.6	407.1489($C_{24}H_{23}O_6$)391.1546($C_{24}H_{23}O_5$)311.0557($C_{17}H_{11}O_6$) (Type 3)	Methyl methylene dioxy kaempferol hexanoyl ester
$C_{20}H_{17}O_6$	353.10333	22.3	271.0615($C_{15}H_{11}O_5$)253.0507($C_{15}H_9O_4$) (Type 3)	pinobanksin pentenoate
$C_{25}H_{21}O_7$	433.12961	17.7	267.06921($C_{16}H_{11}O_4$) (Type 3)	dihydroxypropionyl pinocembrin methyl ether
$C_{16}H_{13}O_4$	269.08191	21.2	254.0584($C_{15}H_{10}O_4$)226.0638($C_{14}H_{10}O_5$)178.0273($C_9H_6O_4$)134.0374($C_8H_6O_2$)	pinocembrin methyl ether

$C_{22}H_{41}O_4$	371.31662	32.5	353.3059($C_{22}H_{41}O_5$)311.2951($C_{20}H_{39}O_2$)225.2223($C_{15}H_{29}O$)	dihydroxy docosahexenoic acid
$C_{14}H_{17}O_6$	281.10309	8.8	179.0350($C_9H_7O_4$)161.0243($C_9H_5O_3$)135.0450($C_8H_7O_2$) (Type 2)	Pentenoyl hydroxyphenylpropionic acid
$C_{21}H_{31}O_4$	357.30139	30.6	339.2908($C_{21}H_{39}O_3$)297.2799($C_{19}H_{37}O_2$)	UK
$C_{18}H_{29}O_3$	293.21246	25.8	275.2019($C_{18}H_{27}O_2$)197.1183($C_{11}H_{17}O_3$)185.1182($C_{10}H_{17}O_3$)	hydroxylinolenic acid
$C_{24}H_{19}O_6$	403.11905	18.3	385.1082($C_{24}H_{17}O_5$)293.0820($C_{18}H_{13}O_4$)255.0659($C_{15}H_{11}O_4$) (Type 3)	pinobanksin phenyl propionate
$C_{25}H_{23}O_5$	403.15503	23.7	385.142($C_{25}H_{21}O_4$)373.1069($C_{23}H_{17}O_5$)297.0758 ($C_{17}H_{13}O_5$)	Dimethyl galangin phenacetyl ether
$C_{23}H_{21}O_5$	377.13962	24.2	345.1138($C_{22}H_{19}O_4$)283.0975($C_{17}H_{15}O_4$)271.0974($C_{16}H_{15}O_4$)	UK
$C_{22}H_{15}O_6$	375.08765	22.6	253.0500($C_{22}H_{15}O_6$) (Type 3)	pinobanksin benzoate
$C_{24}H_{19}O_6$	403.11917	19.7	388.0954($C_{23}H_{16}O_6$)373.0719($C_{22}H_{13}O_6$)279.0664($C_{17}H_9O_4$)253.0508($C_{15}H_9O_4$) (Type 3)	pinobanksin phenylpropionate
$C_{27}H_{23}O_8$	475.14014	21.5	415.1178($C_{25}H_{19}O_6$)400.0949($C_{24}H_{16}O_6$)161.0243($C_9H_5O_3$)	UK

$C_{25}H_{23}O_7$	435.14526	19.2	325.1075($C_{19}H_{17}O_5$)311.0917($C_{18}H_{15}O_6$)303.0866($C_{16}H_{15}O_6$)	Methyl methylene dioxy kaempferol hexanoyl ester
$C_{24}H_{31}O_5$	399.21796	22.6	355.2285($C_{23}H_{31}O_3$)179.0353($C_9H_7O_4$)135.0453($C_8H_7O_2$)	Sesquiterpene caffeic acid ester
$C_{25}H_{21}O_8$	449.12445	19.6	339.0873($C_{19}H_{15}O_6$)279.0664($C_{17}H_{11}O_4$)	Unknown
$C_{18}H_{29}O_4$	309.20712	19.3	291.1966($C_{18}H_{27}O_3$)195.1027($C_{11}H_{15}O_3$)171.1027($C_9H_{15}O_3$)	dihydroxy linolenic acid
$C_{18}H_{15}O_6$	327.08768	16.2	285.0767($C_{16}H_{13}O_5$)267.0664($C_{16}H_{11}O_4$)	methyl pinobanksin acetate
$C_{19}H_{29}O_5$	337.20593	31.6	319.1917($C_{19}H_{27}O_4$)251.1290($C_{14}H_{19}O_4$)239.1290($C_{13}H_{19}O_4$)	Unknown
$C_{18}H_{17}O_4$	297.1134	20.3	179.0351($C_9H_{27}O_4$)161.0244($C_9H_5O_3$)135.0452($C_8H_7O_2$) (Type 2)	caffeic acid phenylpropionyl ester
$C_{15}H_{11}O_6$	287.05637	11.1	259.0612($C_{15}H_{11}O_6$)243.0664($C_{14}H_{11}O_4$)178.9987($C_8H_3O_5$)151.0036($C_7H_3O_4$) (Type 1)	dihydrokaempferol
$C_{15}H_{21}O_4$	265.14459	17.6	221.1546($C_{14}H_{21}O_2$)247.1341($C_{15}H_{19}O_3$) 203.1442($C_{14}H_{19}O$)	sesquiterpene acid
$C_{24}H_{17}O_8$	433.09271	18.1	415.08698($C_{24}H_{15}O_7$)271.0645($C_{15}H_{11}O_5$) (Type 3)	pinobanksin caffeate
$C_{26}H_{21}O_7$	445.12961	21.3	430.1047($C_{25}H_{18}O_7$)385.1075($C_{24}H_{17}O_5$)283.0976($C_{17}H_{15}O_4$) (Type 3)	Caffeoyl dimethyl pinocembrin

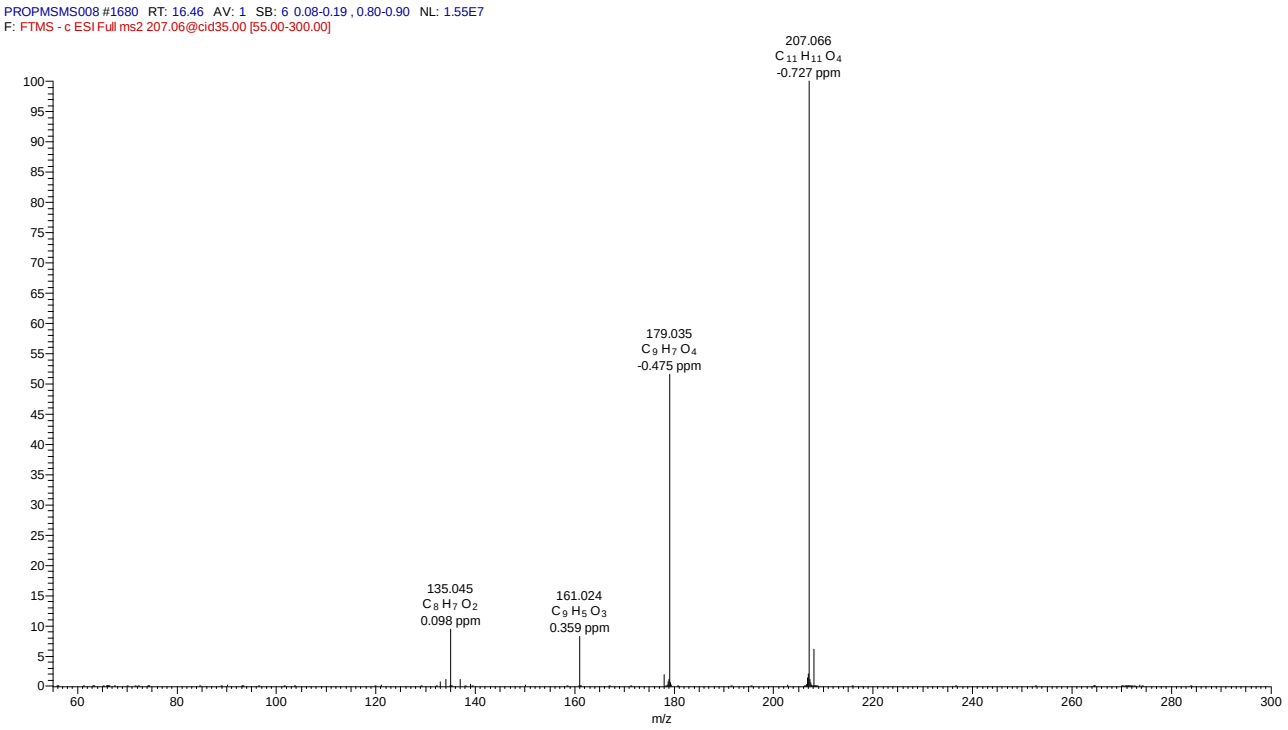
$C_{11}H_{11}O_4$	207.06645	13.7	179.0349($C_9H_7O_4$)161.0245($C_9H_5O_4$)	caffeic acid ethyl ester
$C_{18}H_{33}O_3$	297.24377	25.0	279.2331($C_{18}H_{31}O_2$)	hydroxylinoleic acid
$C_{23}H_{21}O_{10}$	457.11459	14.7	397.0927($C_{21}H_{17}O_8$)295.822($C_{14}H_{15}O_7$)235.0611($C_{12}H_{11}O_5$)179.0350($C_9H_5O_4$) (Type 2)	dicaffeoyl acetyl glycerol
$C_{19}H_{37}O_4$	329.27005	26.5	269.2488($C_{17}H_{33}O_2$)	hydroxyheptadecanoic acid acetate
$C_{24}H_{19}O_7$	419.11407	16.1	375.1243($C_{23}H_{19}O_5$)333.1136($C_{21}H_{17}O_4$)309.0771($C_{18}H_{13}O_5$)295.0616($C_{17}H_{11}O_5$)	methyl chrysin acetate derivative
$C_{25}H_{23}O_6$	419.15036	22.6	401.1404($C_{25}H_{21}O_5$)387.1242($C_{24}H_{19}O_5$)287.0931($C_{16}H_{14}O_5$)255.0667($C_{15}H_{11}O_4$)	prenylated flavonoid
$C_{15}H_9O_6$	285.04102	12.5	257.0457($C_{14}H_9O_5$)241.0511($C_{14}H_9O_4$)	kaempferol isomer
$C_{14}H_{15}O_6$	279.08734	11.4	219.0665($C_{12}H_{11}O_4$)163.0402($C_9H_7O_3$) (Type 2)	Acetylcoumaryl glycerol
$C_{26}H_{21}O_6$	429.13437	25.5	271.0607($C_{18}H_{11}O_5$)253.0503($C_{15}H_90_4$)	pinobanksin dimethyl cinnamate
$C_{24}H_{29}O_5$	597.21307	23.0	163.0398($C_9H_7O_3$)145.0291($C_9H_5O_2$)	coumarate derivative
$C_{26}H_{21}O_8$	461.12469	19.8	443.1132($C_{26}H_{19}O_7$)401.1027($C_{24}H_{17}O_6$)351.0872($C_{20}H_{15}O_6$)	Unknown

$C_{22}H_{21}O_{11}$	461.10138	17.6	414.1036($C_{21}H_{19}O_9$)253.0507($C_{15}H_9O_4$)	chrysin glycoside formate adduct
$C_{21}H_{19}O_{10}$	431.09808	10.9	403.1025($C_{20}H_{19}O_9$)311.0555($C_{17}H_{11}O_6$)260.0370($C_{15}H_8O_5$)	galangin glycoside
$C_{13}H_{14}O_4$	235.09758	17.9	179.0351($C_9H_7O_4$)161.0244($C_9H_5O_3$) (Type 2)	caffeic acid butyl ester
$C_{22}H_{35}O_3$	347.25925	27.1	303.2693($C_{21}H_{35}O$)	hydroxydocosapentaenoic acid
$C_{16}H_{13}O_4$	269.08243	15.0	254.0586($C_{15}H_{10}O_4$)227.0715($C_{14}H_{11}O_3$)165.0194($C_8H_5O_4$) (Type 1)	Pinocembrin methyl ether
$C_{15}H_{25}O_4$	269.17572	14.0	239.1657($C_{14}H_{23}O_3$)211.1339($C_{12}H_{19}O_2$)195.1393($C_{12}H_{19}O_2$)177.1286($C_{12}H_{17}O$)159.1180($C_{12}H_{15}$)	sesquiterpene
$C_{16}H_{11}O_6$	299.05615	11.7	284.0327($C_{15}H_8O_6$) 255.0299($C_{14}H_7O_5$)165.0195($C_8H_5O_4$) (methyl ring A) (Type 1)	kaempferol methyl ether
$C_{13}H_{13}O_4$	233.08188	16.8	189.0921($C_{12}H_{13}O_2$)178.0272($C_9H_6O_4$) (Type 2)	Caffeic acid butenyl ester
$C_{23}H_{19}O_7$	407.11417	16.6	389.1012($C_{23}H_{17}O_6$)297.0755($C_{17}H_{13}O_5$) (Type 3)	hexadieneoyl dimethylpinobanksin

$C_{17}H_{13}O_7$	329.06693	12.0	314.0429($C_{16}H_{10}O_7$) 299.0200($C_{15}H_7O_7$)	quercetin dimethyl ether
$C_{18}H_{29}O_4$	309.20709	17.0	291.1966($C_{18}H_{27}O_3$)265.1810(C16H25O3)185.1184 (C10H17O3)	dihydroxy linolenic acid
$C_{22}H_{33}O_3$	345.24356	35.3	301.2534($C_{21}H_{33}O$)	hydroxydocoahexanoic acid
$C_{19}H_{17}O_6$	341.10306	17.9	271.0613($C_{15}H_{11}O_5$)253.0506($C_{15}H_9O4$) (Type 3)	Pinobanksin butyrate

More Detailed Consideration of some typical MS^2 Spectra

Some examples of typical MS^2 for some the abundant components in the UK propolis are shown below. Caffeic acid ethyl ester exhibits type 2 fragmentation shown above for caffeic acid pentenyl ester. The loss of the acid and the formation of an alkene from the side chain is analogous to the loss of water from from an alcohol.



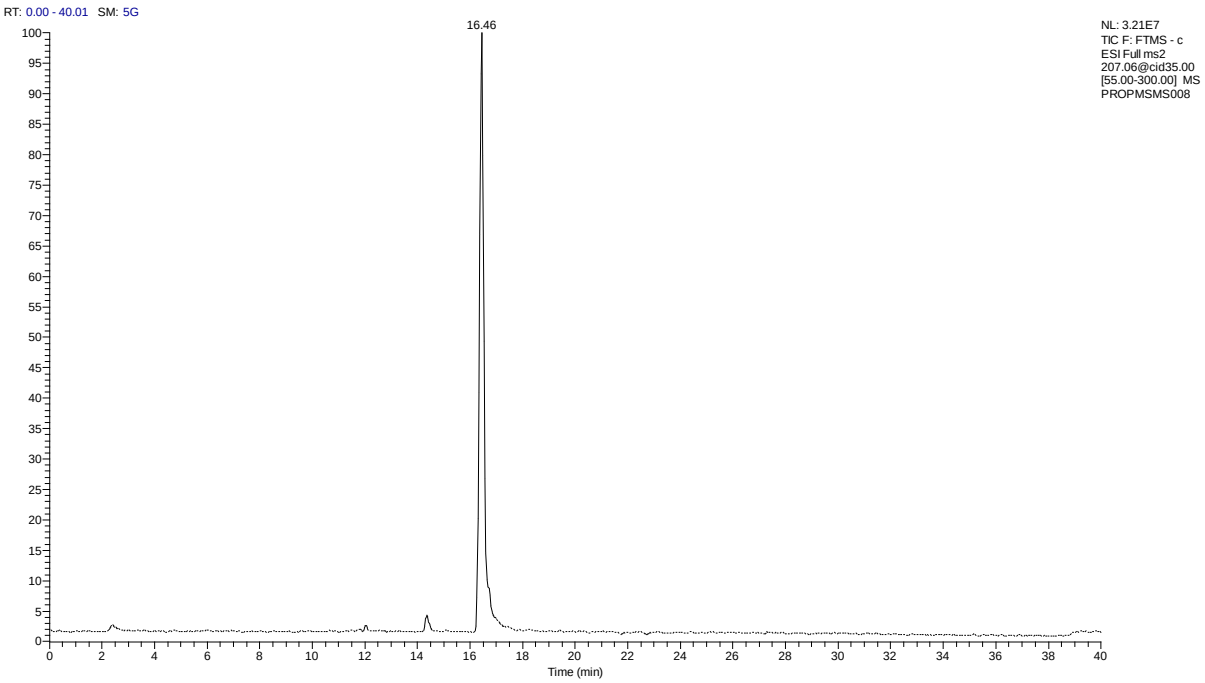


Figure 3.9: MS2 spectrum of caffeic acid ethyl ester and associated extracted ion trace.

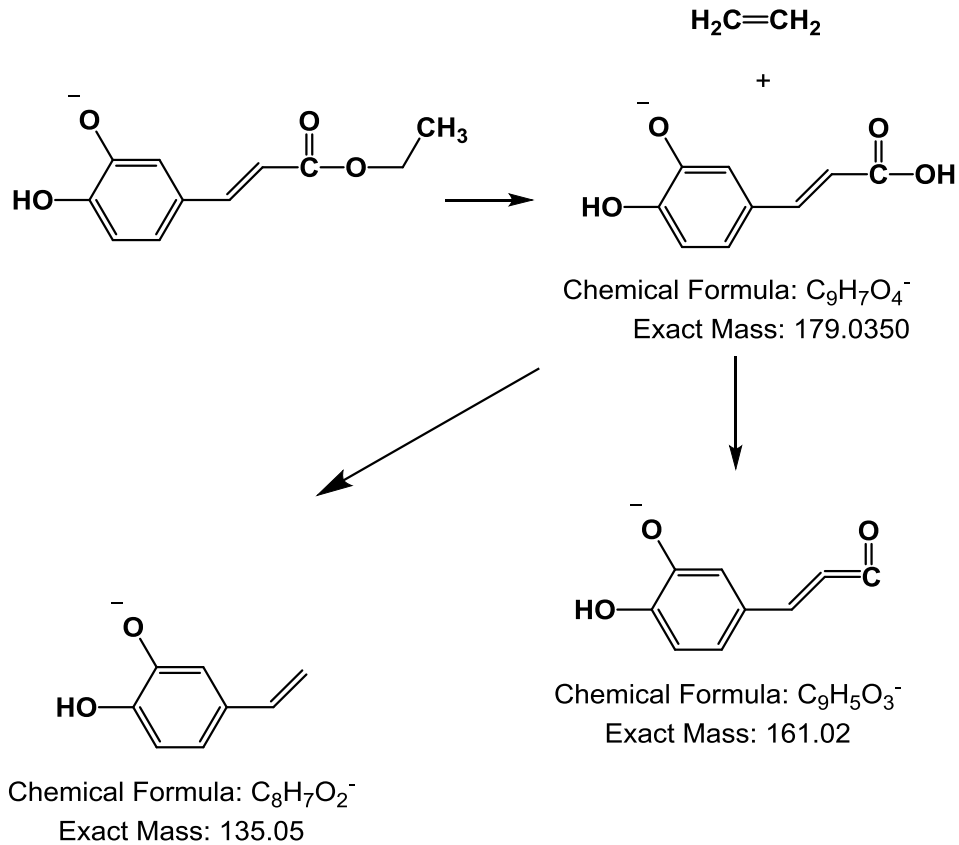


Figure 3.10: Fragementation of caffeic acid ethyl ester.

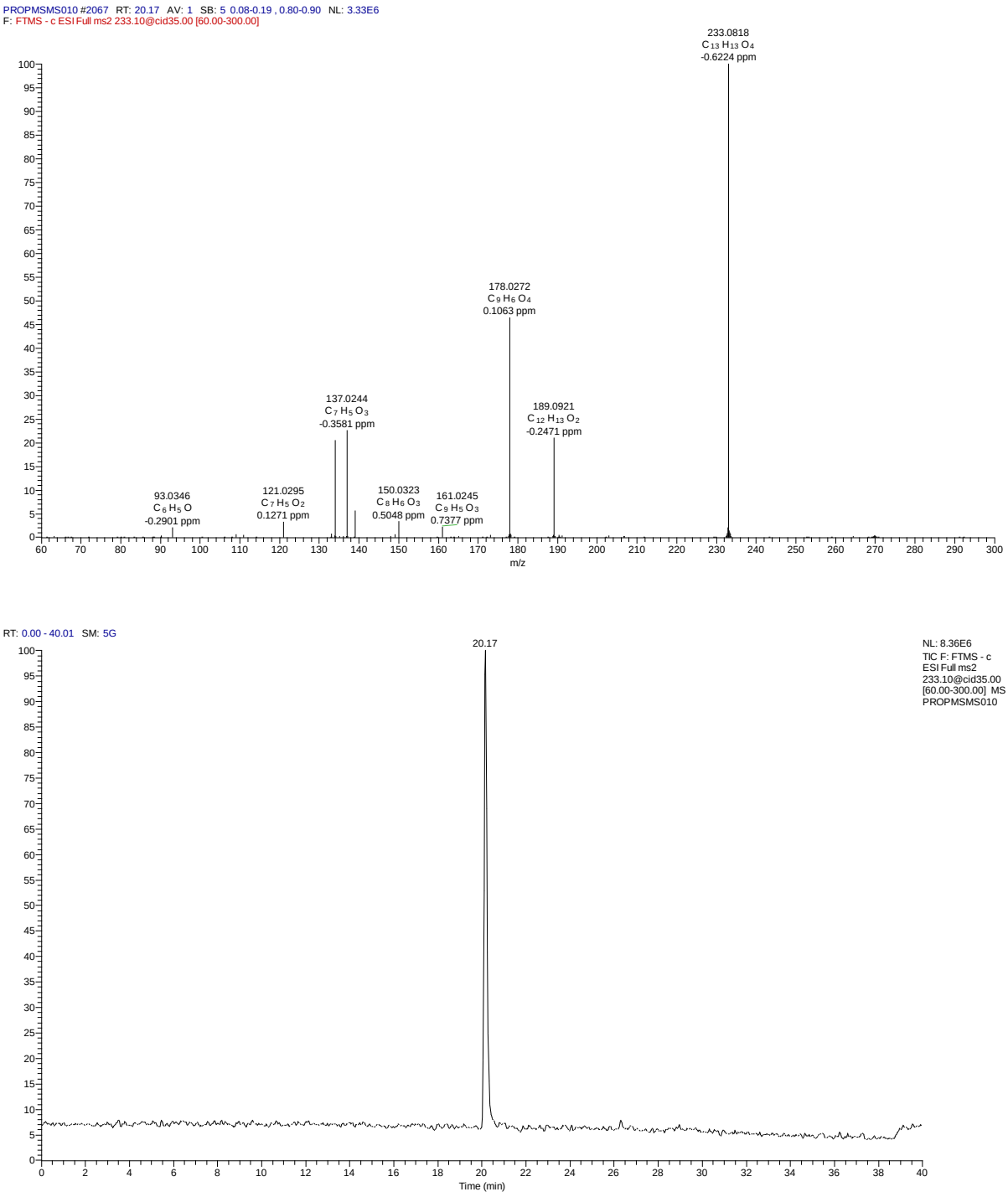


Figure 3.11: Mass spectrum and associated extracted ion trace for caffeic acid butenyl ether.

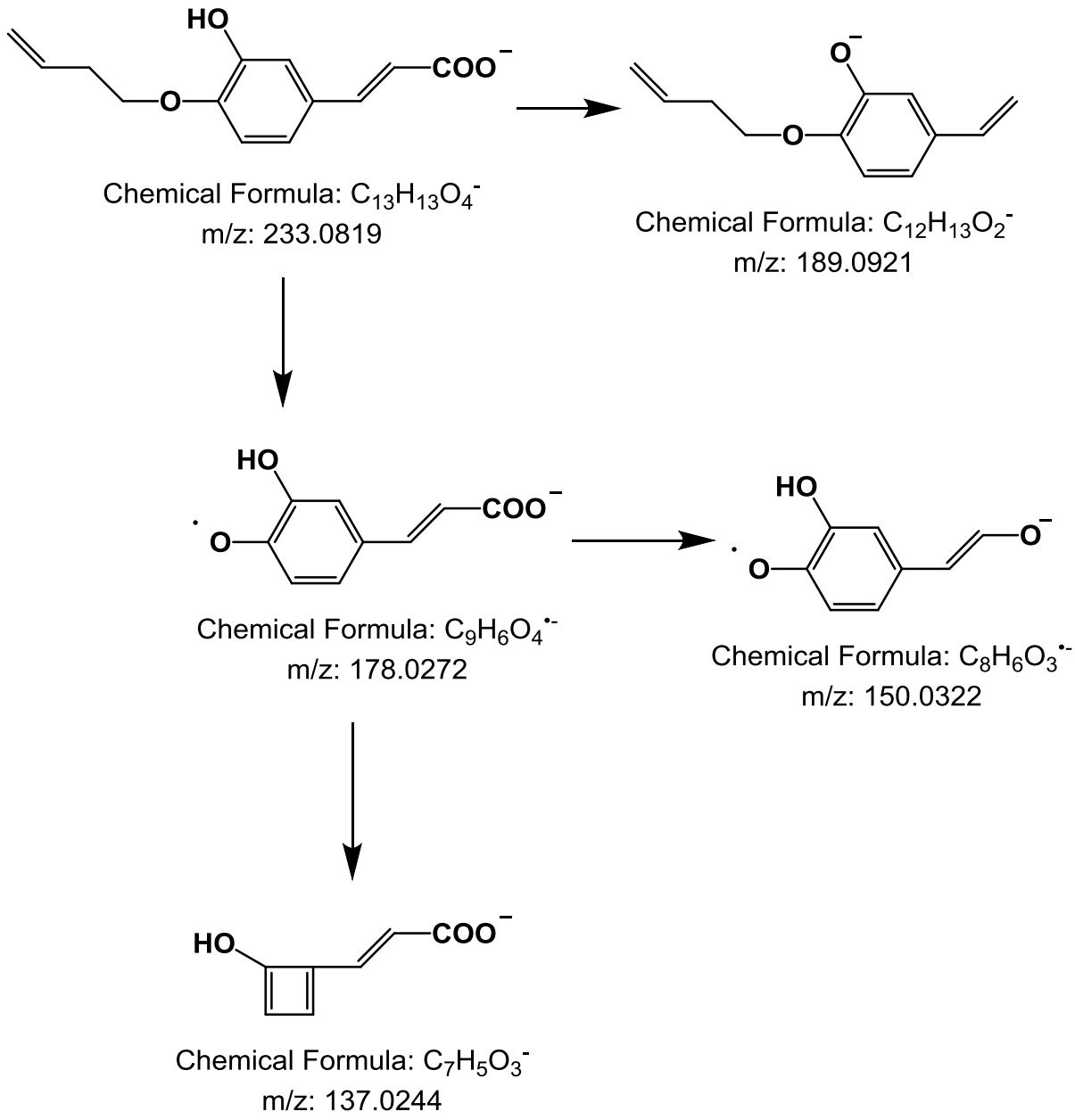


Figure 3.12: Fragmentation of caffeic acid butenyl ether.

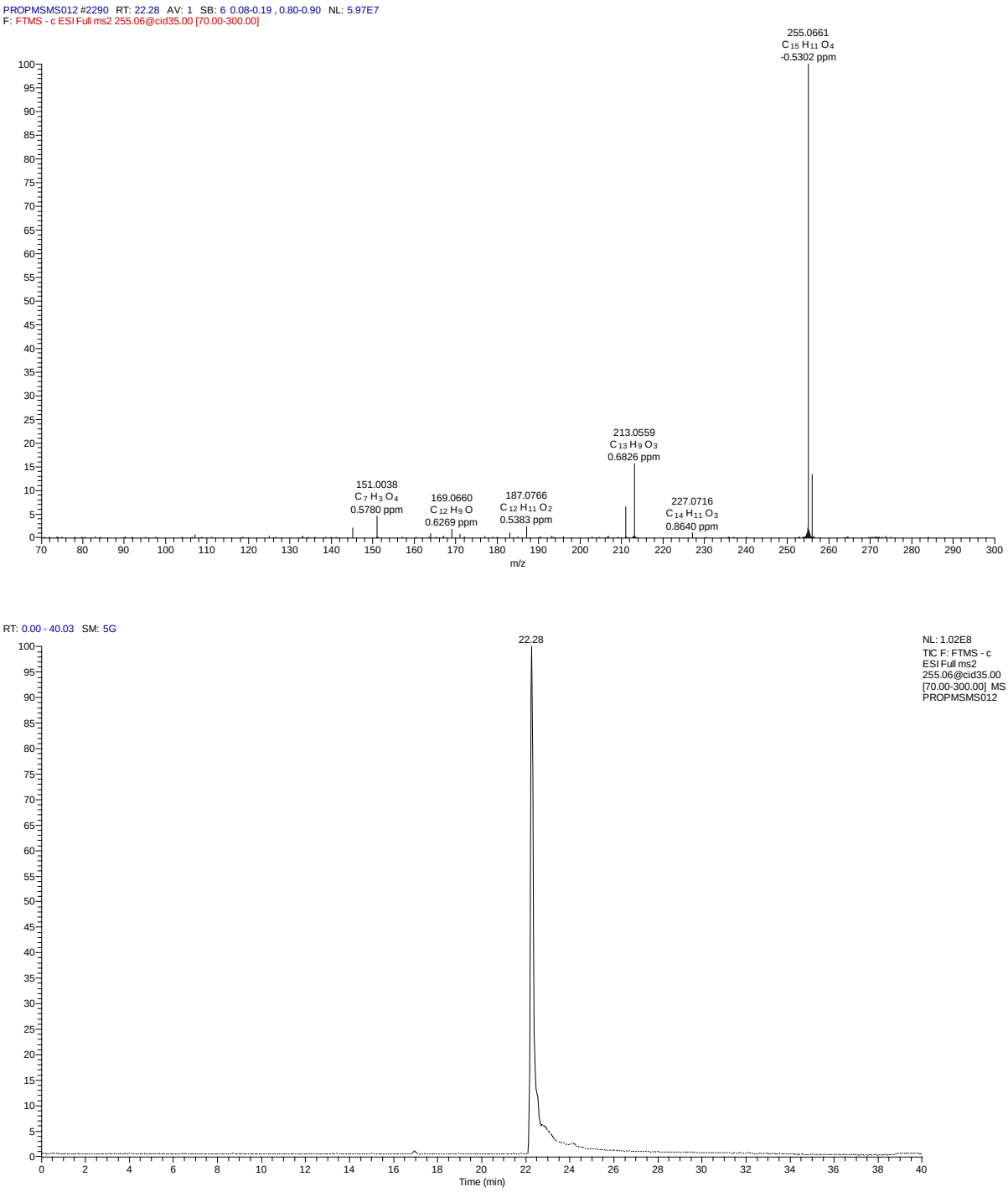


Figure 3.13: MS2 spectrum of pinocembrin with associated extracted ion trace.

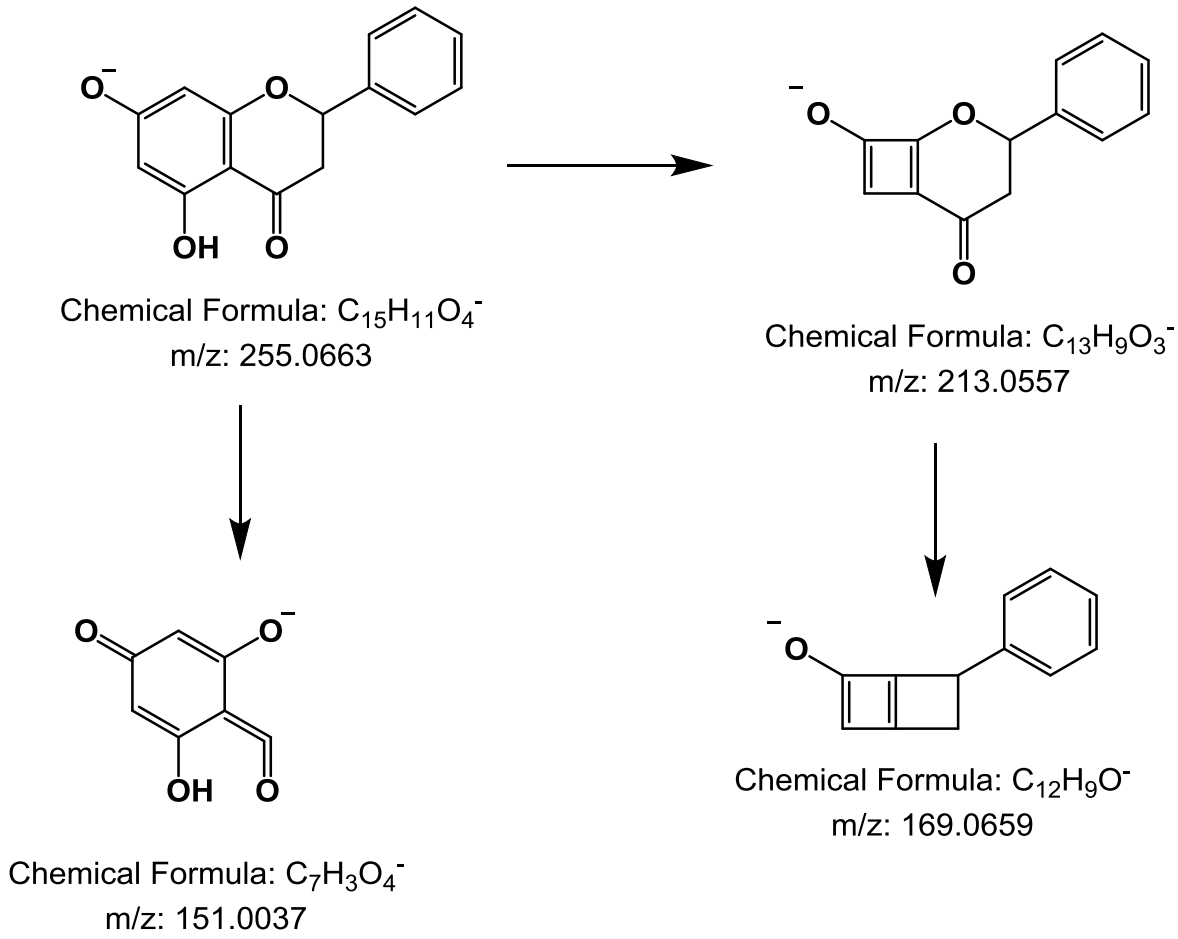


Figure 3.14: Fragmentation of pinocembrin

Conclusion

There have been a number of papers on the analysis of samples of propolis by LC-MS but the relative abundance of the components making up propolis, as judged from response in ESI mode, has not been considered. This approach is in line with the approach used in omics studies which looks at relative levels of expression. Using this approach it is possible to see that, aside from the more familiar flavonoids, there are a number of other less familiar compounds in UK propolis samples which are present in high abundance. The abundant compounds include several unknown

components which are difficult to characterise with MS^2 alone. Thus in this investigation we have been able to develop an approach which could be applied to assessing the significance of variation in propolis composition with regard to geographical location, land use and from hive to hive on a single site. Such an approach could be used to assess the significance of variations in propolis with regard to the effects of changes in patterns of land use in the vicinity of hives and the role that propolis might play in the overall health of bee hives if its composition could be correlated to hive infection rates. The study produced extensive sets of MS^2 some of which has yet to be fully interpreted. It is clear there are many more compoents in temperate propolis than the 344 reported previously.

CHAPTER FOUR: PROFILING PROPOLIS SAMPLES FROM HIVES IN FOUR REGIONS OF SCOTLAND.

Introduction

Honey bee colony collapse has been widespread in recent years in many countries (Van der Zee et al., 2014, Van der Zee et al., 2012, Ellis *et al.,* 2010). There may be many factors contributing to this. One of the known factors contributing to colony losses is infection with the ectoparasitic mite *Varroa destructor*. Annual colony winter losses in Scotland, where beekeeping operations are relatively small scale, are regularly among the highest in Europe (Gray *et al.*, 2010, Peterson *et al*., 2009). Bees manufacture several materials using the plants within their environment, and, based on the chemical complexity, the simplest of these materials is honey and by far the most complex is propolis or bee glue. The material has been popularised in health food products such as toothpaste, cold cures and cosmetics, and has been proposed to be potentially a source of new pharmaceuticals (Salatino *et. al*., 2011, Sforcin *et al*., 2011). It is well established that propolis has interesting specific effects as an anti-infective agent and immunostimulating agent. In our previous work we have observed the potent anti-trypanosomal and anti-leishmanial activity of propolis (Almutairi *et. al*., 2014, Siheri *et. al*., 2014, Almutairi *et. al*., 2014,). This may relate to the fact that bees, like humans, are susceptible to protozoal attack (Schlüns *et.al.,* 2010, McGhee *et.al*, 1980). Protozoal infection of insects is very common and may weaken them rather than kill them (McGhee *et.al*, 1980) and infection with the protozoa *Crithidia mellificae* has been connected to winter colony loss in a study in Belgium (Ravoet *et. al.,* 2013). Thus it is possible that bees collect propolis from plants producing compounds which can protect against protozoa and a range of other infective organisms. In view of the recent problems with colony collapse, it is of interest to establish whether or not variations in propolis composition might have some role in

this and to understand the variations in its composition better. Bees depend on the plants in their environment as sources of propolis, and changes in land usage and botanical species might affect the composition of the propolis that they collect. Propolis in the UK and other regions of temperate Europe is said to derive from poplar species. "Poplar" propolis thus far has been reported to contain 344 chemical components according to a recent review (de Groot *et. al*., 2014), and in our recent paper we were also able to partly characterise around 50 compounds in propolis which had not previously been reported (Saleh *et.al*., 2015). The pioneering work on comprehensive profiling of poplar propolis was carried out by Greenaway *et al* in the early 1990s and they produced profiles by carrying out GC-MS analysis in which up to 150 components were identified (Greenaway *et. al*., 1988, 1990, 1991, 1991). They were able to correlate the propolis composition which they found with the composition of the exudates from poplar buds. In addition they linked variations in the composition of the propolis to the availability of different species of poplar which were accessible to the hive (Greenaway *et. al*., 1988, 1990, 1991, 1991). A metabolomic study of propolis collection by bees in the USA profiled propolis collected by individual bees before the propolis could be mixed in the hive, and was able to demonstrate that the main sources of propolis were two poplar species, although many other sources of resin were also used (Wilson *et.al*., 2013) . The composition of propolis varies greatly according to the surrounding vegetation and there are a number of different types (Seidel *et.al.,* 2008) of propolis, thus it is not possible to generalize with regard to the type of activity which may be found in a particular sample. In this paper we are interested in temperate region propolis in Scotland which can be loosely defined as poplar propolis. Poplar propolis has been found to be active against various bee

pathogens and pests including *Varroa* mite (Popova *et.al.*, 2014). It has been observed that bee colonies exposed to *Ascophaera apis* (chalkbrood fungus) increased their foraging for poplar propolis and that increased propolis levels in the hive reduced the intensity of infection (Simone-Finstrom *et.al*., 2010). A recent paper examined differences between French colonies tolerant to *Varroa destructor*, compared with colonies from the same apiary which were non-tolerant to the mites. The results indicated that non-tolerant colonies collected more poplar propolis than the tolerant ones but the percentage of four compounds, caffeic acid and three pentenyl caffeates, was higher in propolis from tolerant colonies (Simone-Finstrom *et.al*., 2012). In a recent publication it has been found that pentenyl caffeates isolated from manuka propolis inhibit quorum sensing in bacteria (Gemiarto *et.al.*, 2015), and phenethyl caffeates are also among the more abundant compounds in poplar propolis (Saleh *et.al*., 2015). It has been observed that bees which collect large amounts of propolis are also more hygienic and produce more honey (Nicodemo et.al 2013). High-propolis -producing colonies have been observed to have significantly higher brood viability and greater worker bee longevity (Nicodemo *et.al.*, 2014).

In our previous paper we profiled propolis from nine hives from different parts of the Southern UK using liquid chromatography mass spectrometry (LC-MS) (Saleh *et.al*., 2015). It was clear that there was a wide variation in the composition of the propolis from different locations, and also it was apparent that despite many years of research on poplar propolis the compounds making it up are still far from completely characterised. In the current paper we have examined propolis from several hives at three different sites in Scotland in order to determine the degree of variation in propolis composition on each site by using principal component analysis (PCA) based on the

major constituents within the samples. To date there have been no studies of the composition of propolis taken from different hives site at several different locations and given the potential importance of this material in promoting hive immunity this information could be important.

Materials and Methods

Materials

Absolute ethanol, HPLC grade acetonitrile, methanol, acetonitrile and formic acid and were obtained from Fisher Scientific, Loughborough UK. An ACE C_{18} column (3mm x 150mm, 3µm) was obtained from HiChrom, Reading, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK.

Propolis Samples

Small amounts of propolis (*ca* 200 mg) were collected from hives on three different sites in Scotland during July and August 2014. The hives were near Ballater in Aberdeenshire (n = 15), Fort William (n = 5) and Dunblane Stirlingshire (n =3). The samples were stored at room temperature and analysed within one week of receipt. In order to assess the uniformity of the samples, which were scraped from quite large surface areas. Three replicate amounts of propolis (50 mg) were weighed for each sample, and the samples were then extracted with 2 ml of ethanol in screw-capped test tubes by sonication for 30 minutes. An aliquot of the extract (0.2 ml) was then transferred to an Eppendorf tube and a mixture of acetonitrile/water (30:70) (1 ml) was added and the sample was centrifuged for 5 minutes at 3000 rpm. The supernatant was then used for LC-MS analysis.

LC-MS Analysis

High resolution mass spectra were obtained using an Orbitrap Exactive mass spectrometer (ThermoFisher, Hemel Hempstead, UK) in positive/negative ion switching mode with a needle voltage of -4.0 kV, 4.5 kV positive. Sheath and auxiliary gases were set at 50 and 17 arbitrary units respectively. The separation was performed on an ACE C_{18} column (150 × 3 mm, 3 µm) from HiChrom UK with 0.1% v/v formic acid in water as mobile phase A and 0.1% v/v formic acid in acetonitrile as B at flow rate of 0.300ml/min using the following linear gradient: 10% B (0 min) 100% B (30 min) 100% B (35 min) 10% B (36 min) 10% B 42 min. MS^2 spectra were acquired on an LTQ Orbitrap under the same chromatographic conditions described above. The spectra were acquired in negative ion mode with the same source settings as described above and with collision energy of 35V.

Data Extraction

MZMine 2.14 (Pluskal *et.al*., 2010) was used for peak extraction and alignment. Putative identification was also conducted in MZMine by searching the accurate mass against our in-house database compiled from the work reported in chapter 3 (Saleh *et.al*., 2015). SIMCA-P version 14.0 (Umetrics, Sweden) software was used for carrying out PCA and HCA (Hierarchical clustering analysis). The data for the sample components were Pareto-scaled and mean centred prior to using PCA.

Results

As was observed previously for UK propolis (Saleh *et.al*., 2015) there was quite a lot of intra-sample variation in the composition of the propolis samples and this is probably largely caused by variation in the amounts of insoluble plant debris which are embedded within the samples of resin. Figure 4.1 shows the most clearly clustered

samples, which were obtained from nine of the fifteen hives sampled in Aberdeenshire. Figure 4.2 shows the PCA scores indicating that most of the variation in the samples can be explained by seven components. The PCA model was based on 923 components in the propolis extracts, many of which are still unknown components. Some of the replicates are clustered very closely, e.g. KA-C, indicating that the sample of resin had a very uniform composition. The six samples which were excluded from the analysis had widely scattered replicates. HCA was used to group the samples in order to discriminate the composition of the propolis from individual hives. Six groups could be formed without subdividing the sets of three replicates from each hive. The HCA analysis shown in figure 4.3 indicates a strong separation into two groups with a less marked separation for the subgroups. The hives were located on two different sites. Site 1 on the South side of the river Dee was located close to the following plant sources: poplars, aspen, cherry nearby and lime, spruce, fir and pine woods. There were also heather moorlands and wild flower meadows very nearby and village gardens across the river Dee. Site 2 was at 300 m above sea level with access to aspens, poplars, cherry trees growing nearby, as well as spruce/pine/larch forests, birch groves and upland ling heather moors. NT11 and S10 hives were hives on a different site about a mile apart from the rest, but there was not a clear separation based on the site since NT11 clusters with two of the hives on the other site. The extensive work by Greenaway *et al* (Greenaway *et. al*., 1988, 1990, 1991, 1991) examined both propolis samples and exudates of the buds of different poplar species and concluded that there was a correlation between the composition of propolis and the poplars available for collection of propolis in the immediate vicinity of the hive. It was also observed that two hives which were located within 400 yards of each other produced quite different

samples of propolis (Greenaway *et. al*., 1990). This was attributed to the bees from the two hives in the study preferring different poplar trees. Table 4.1 lists the most important marker compounds distinguishing propolis from different hives. The compounds are putatively identified according to accurate mass but also with reference to MS^2 data reported in our previous paper (Saleh *et al*., 2015). Even with MS^2 analysis it is only possible to get and approximate identification since the possibility for structural variations based on a specific elemental composition are large. The most important components distinguishing hives S8 and K (table 4.1) from the average are flavonoids and in particular methylated flavonoids. S10 which is well separated from the K/S8 group has glycerol esters of phenylpropanoid compounds as important components. This class of compounds was first isolated from propolis in 1982 (Popova *et. al.*, 2014) and was recently further characterised (Popravko *et al.,* 1982). PK and RS are characterised by esters of pinobanksin and the PNYC/S7 group (since it is towards the centre of the plot) has important components from the three outlying groups in moderate amounts. Thus it is proposed that the hives in the middle of the PCA plot are tending to use several different sources of propolis, whereas the groups towards the periphery of the plot may focus on more restricted sources. The bar charts shown in figure 4.4 for some of the main components illustrate the same points, with K and S8 being clearly much lower in dicoumaryl glycerol and pinobanksin butyrate than the other samples while being higher in methylkaempferol. There is nothing in the meta-data for the hives shown in table 3.3 that can easily be related to the differences in composition, so it is likely that variations may be due to a preference for a particular source of the propolis.

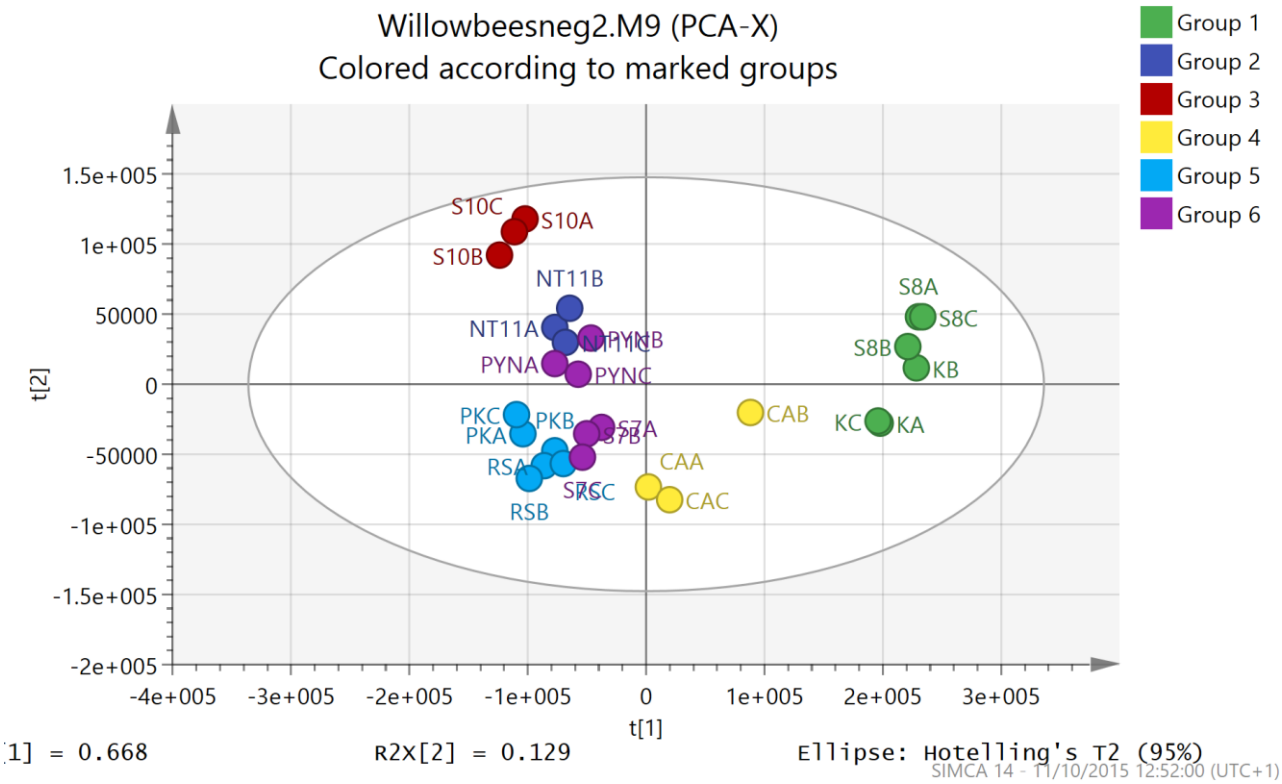


Figure 4.1: Scatter plot of 1st two principal components from PCA of propolis from nine hives on two closely located sites in Aberdeenshire (The hives are named as follows: K=Kayas, S= Smith, CA= Charlie's Angels, PK = Pink Hare, RS=Red Smith). NT and K w

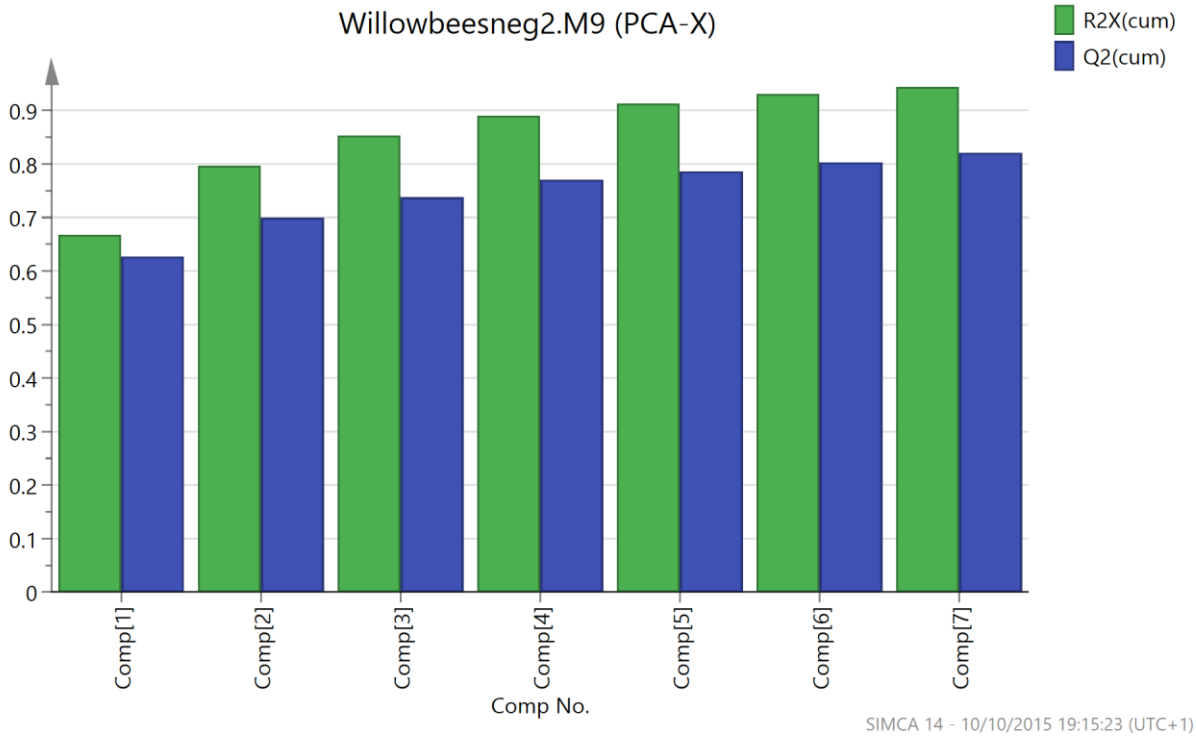


Figure 4.2: Summary of the fit of the PCA model to the 923 variables used to model the propolis samples from 9 Aberdeenshire hives.

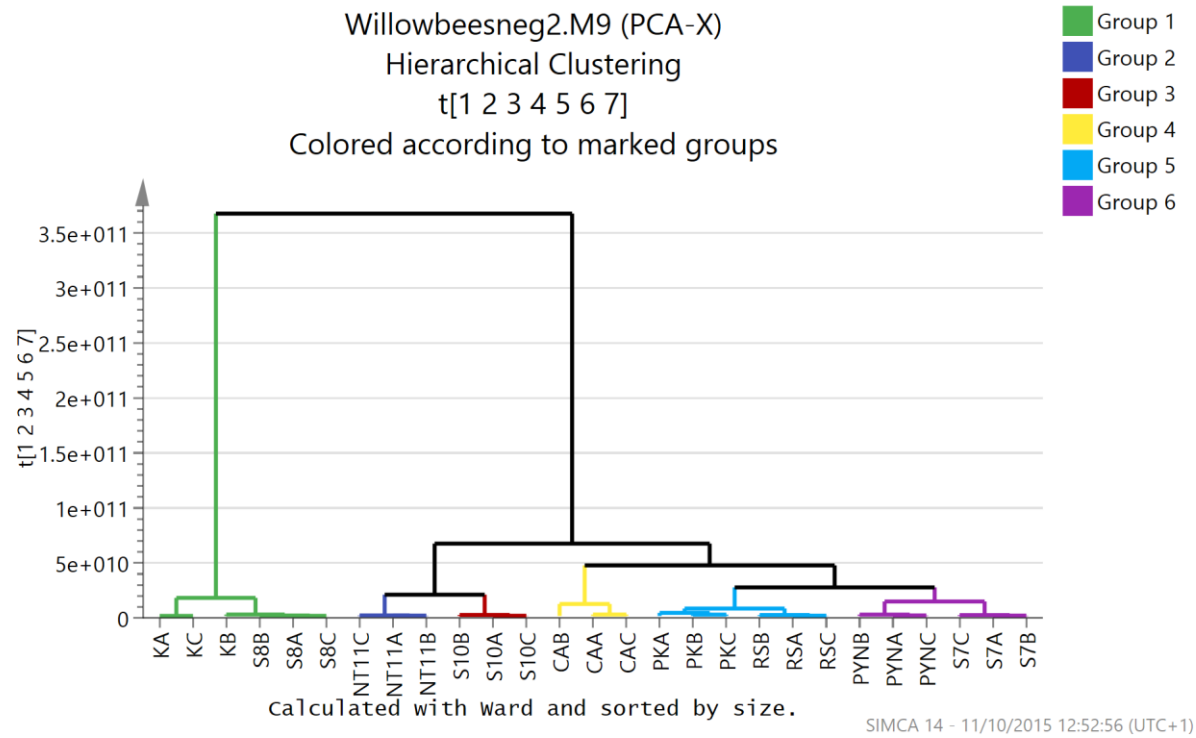


Figure 4.3: Summary of HCA of propolis samples from nine Aberdeenshire hives.

m/z	Rt	Inference	VIP
Kaya/S8			
163.04	9.2	Coumaric acid	21.3
271.061	15.9	Pinobanksin isomer	5.7
403.155	23.6	Pinocembrin phenylethyl trimethyl ether or isomer	4.8
401.14	23.5	Prenylated flavonoid	4.0
417.135	16.4	Kaempferol phenylethyl dimethyl ether or isomer	4.0
299.056	19.1	Kaempferol methyl ether or isomer	3.7
313.072	16.3	Pinobanksin acetate or isomer	2.7
285.077	19.6	Methyl pinobanksin or isomer	2.6
285.041	12.6	Kaempferol isomer	2.3
419.15	21.1	Prenylated flavonoid	2.1
271.061	14.2	Pinobanksin isomer	2.1
S10			
253.087	20.4	Benzyl coumarate	31.0
383.114	14.6	Dicoumaroyl glycerol	10.8
425.125	17.7	Cinnamoyl caffeoyl acetyl glycerol	7.9
441.119	16.3	$C_{23}H_{21}O_9$	7.1
457.114	14.9	Dicaffeoyl acetyl glycerol	6.8

475.176	15.9	$C_{24}H_{27}O_{10}$	5.0
269.082	18.3	Pinocembrin methyl ether or isomer	4.7
399.109	13.3	Quercetin hexanoyl ester or isomer	4.2
285.077	18.7	Methyl pinobanksin or isomer	4.1
403.155	17.3	Pinocembrin phenylethyl trimethyl ether or isomer	3.7
PK/RS			
471.13	16.6	Coumaroyl feruoyl acetyl glycerol	4.0
341.103	16.1	Pinobanksin butyrate or isomer	3.7
485.146	18.2	Diferroylacetyl glycerol	3.5
269.082	18.3	Pinocembrin methyl ether or isomer	3.1
457.114	14.9	Dicaffeoyl acetyl glycerol	3.1
355.119	18.0	Pinobanksin valerate of isomer	2.9
425.125	17.7	Cinnamoyl caffeoyl acetyl glycerol	2.9
371.114	15.4	$C_{20}H_{19}O_7$	2.4
429.119	13.5	Methylchrysin glucoside or isomer	2.1
283.098	20.8	Caffeic acid phenacetyl ester	1.2
PNY/S7			
179.035	7.3	Caffeic acid	4.0
425.125	17.7	Cinnamoyl caffeoyl acetyl glycerol	2.9
341.103	16.1	Pinobanksin butyrate	2.4
295.082	9.9	$C_{14}H_{15}O_7$	2.3
279.087	11.6	Coumaryl acetyl glycerol	2.3

457.114	14.9	Dicaffeoyl acetyl glycerol	2.1
193.051	9.6	Ferulic acid	2.0
471.13	16.6	Coumaroyl feruoyl acetyl glycerol	1.7
383.114	14.6	Dicoumaroyl glycerol	1.5
343.119	11.2	Butanoyl galangin or isomer	1.5
NT			
253.087	20.4	Benzyl coumarate	11.1
425.125	17.7	Cinnamoyl caffeoyl acetyl glycerol	6.3
457.114	14.9	Dicaffeoyl acetyl glycerol	5.5
279.087	11.6	Coumaryl acetyl glycerol	5.4
419.15	21.1	Prenylated flavonoid	3.6
311.093	15.8	Galangin trimethyl ether	3.5
455.135	18.4	Coumaroyl feruoyl acetyl glycerol	2.8
383.114	14.6	Dicoumaroyl glycerol	2.8
287.056	11.3	Dihydrokaempferol or isomer	2.6
471.13	16.6	Coumaroyl feruoyl acetyl glycerol	2.5
CA			
371.114	17.5	$C_{20}H_{19}O_7$	3.5
419.15	21.1	Prenylated flavonoid	2.7
193.087	15.2	Hydroxypropionate ethyl ester	2.3
285.077	18.7	Methyl pinobanksin or isomer	1.9
225.062	2.1	Glucose	1.7
135.045	14.8	Phenylacetic acid	1.4

311.223	21.2	Dihydroxylinoleic acid	1.4
485.146	18.2	Diferroylacetyl glycerol	1.4
285.077	23.5	Methyl pinobanksin or isomer	1.2
299.056	19.1	Kaempferol methyl ether or isomer	1.2

Table 4.1: Top VIPs (Variable influence on projection) for each group in comparison with the mean for propolis from nine Aberdeenshire hives. Each of the 923 variables in theory has a weight of 1 but when the data is projected to make the model some vari

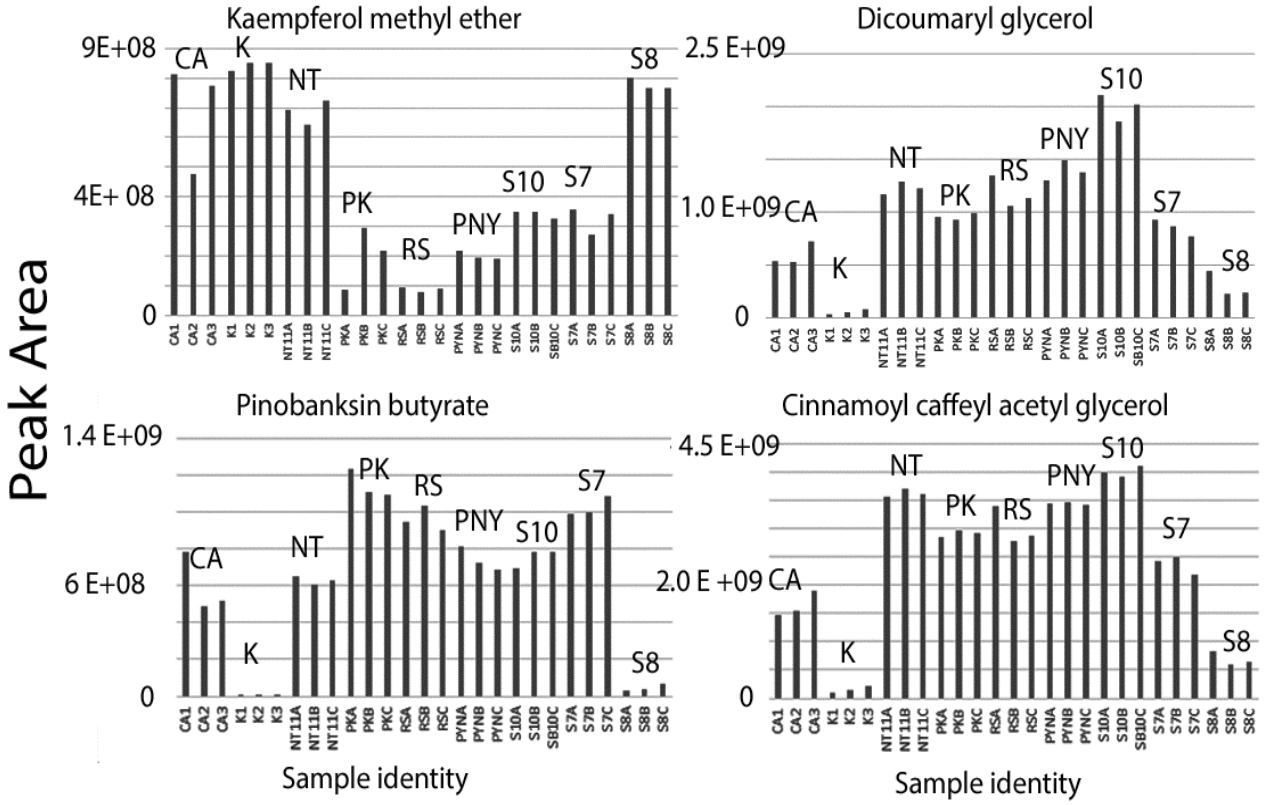


Figure 4.4: Variation in some the VIPs across the Aberdeenshire samples

The PCA of the five samples from Fort William (figure 4.5) explained 97.4% of the variation in four components. The five samples of propolis from Fort William could be classified into four groups corresponding to the four hives in the study by HCA. In

these samples the HCA indicates wide separation of the groups (Figure 4.6). The SK02 propolis, comprised of duplicate samples taken from the same hive, had the most average composition of the four hives. The composition of these propolis samples appears to be fairly different from that of the Aberdeenshire samples. They differ also from each other and overall the components highlighted in Table 4.2 are in many cases not the same as the most significant VIPs in the Aberdeenshire samples. The Fort William samples are rich in compounds putatively identified as sesquiterpene acids. The MS^2 data for the major sesquiterpene acids are shown in table 4.3. The samples from Dunblane are different again but closer in character to the Aberdeenshire samples than the Fort William samples. Tables 4.4, 4.5 and 4.6 compare the 25 components with the greatest mean abundance, as judged from instrument response, for samples from Ballater (Aberdeenshire), Fort William and Dunblane.

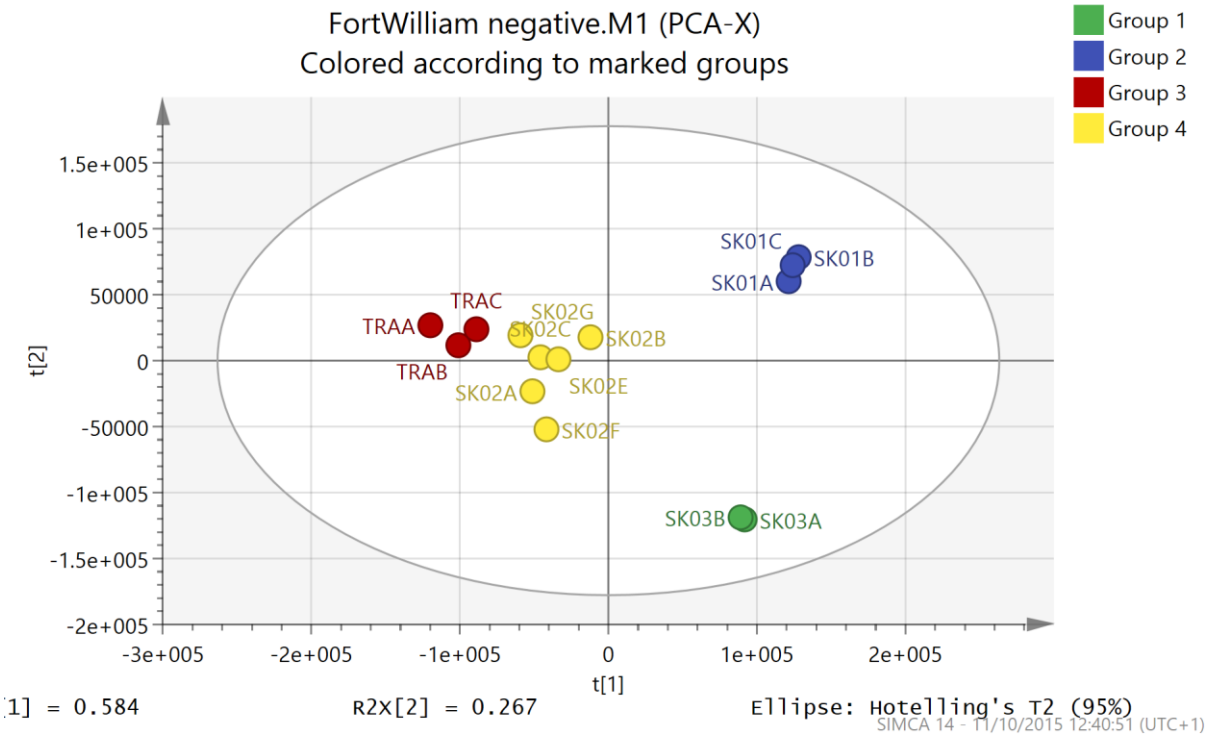


Figure 4.5: PCA analysis of four samples of propolis from near Fort William (two samples were taken from SK02). The model was based on 511 variables.

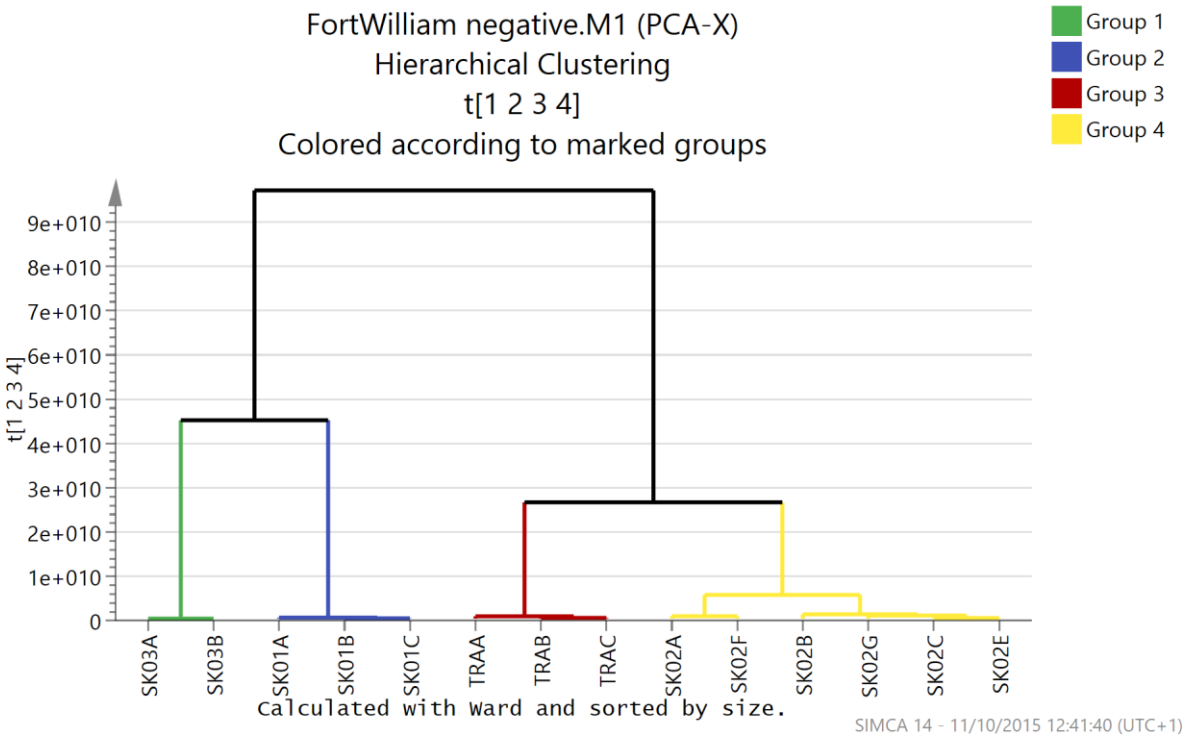


Figure 4.6: Summary of HCA of propolis samples from four Fort William hives.

	m/z	Rt	Compound	VIP
Training				
457.114	15.9	Dicaffeoyl acetyl glycerol		8.2
425.124	18.7	Cinnamoyl caffeoyl acetyl glycerol		7.3
441.12	17.3	Coumaryl caffeoyl acetyl glycerol		6.7
279.088	12.7	Coumaryl acetyl glycerol		6.7
471.13	17.6	Caffeoyl feruoyl acetyl glycerol		5.5
445.166	16.7	$C_{27}H_{25}O_6$		4.7
491.171	16.7	$C_{28}H_{27}O_8$		3.9
475.177	16.9	$C_{28}H_{27}O_7$		3.8
295.098	18.4	Caffeic acid cinnamyl ester		3.7
521.276	21.5	$C_{28}H_{41}O_9$		3.7
SK01				
257.082	19.7	Benzoyl hydroxy phenyl acetic acid		11.1
269.176	15.0	Sesquiterpene*		8.5
419.15	22.0	Prenylated flavonoid		6.9
301.072	16.2	Hydroxy phenyl acetyl dihydroxyphenylacetic acid		5.3
419.15	20.9	Prenylated flavonoid		4.2

285.171	12.8	Sesquiterpene*	4.2
285.171	10.0	Sesquiterpene*	3.8
285.041	15.8	kaempferol	3.5
267.16	15.4	Sesquiterpene*	3.5
301.036	13.9	Quercetin	3.4
SK03			
283.061	19.7	Galangin methyl ether or isomer	8.8
299.056	20.0	Kaempferol methyl ether or isomer	7.2
329.067	16.6	Quercetin dimethyl ether or isomer	6.7
315.051	16.0	Quercetin methyl ether or isomer	5.6
315.16	25.7	Caffeic acid geranyl ester	3.8
445.129	22.3	Caffeoyl pinocembrin dimethyl ether.	0.8
311.093	23.0	Galangin trimethyl ether or isomer isomer	0.6
285.041	15.8	Kaempferol isomer	0.5
535.176	22.3	$C_{33}H_{29}O_7$	0.4
SK02			
455.135	19.0	Coumaroyl feruoyl acetyl glycerol	4.4
383.114	15.7	Dicoumaroyl glycerol	3.7

425.124	18.7	Cinnamoyl caffeoyl acetyl glycerol	3.3
441.12	17.3	Coumaryl caffeoyl acetyl glycerol	3.0
413.125	15.9	Quercetin heptanoyl ester or isomer	2.3
449.161	18.4	$C_{26}H_{25}O_7$	2.3
399.109	14.3	Quercetin hexanoyl ester or isomer	2.2
325.109	18.9	Galangin tetramethyl ether or isomer	1.9
449.276	20.6	$C_{22}H_{41}O_9$	1.7
521.276	21.5	$C_{28}H_{41}O_9$	1.6

Table 4.2: Top VIPs for each group in comparison with the mean. Fort William. Four hives: Training, SK01, SK02, SK03. * MS2 spectrum shown in Table 4.5.

m/z (formula)	
267.1613	249.1501 (-H_2O 100%), 223.1834 (-CO_2, 1.7%),
269.1749 ($C_{15}H_{25}O_4$)	251.1646 (-H_2O 12 %),
273.0775 ($C_{15}H_{13}O_5$)	167.0352 ($C_8H_7O_4$, 100%)
285.1710($C_{15}H_{25}O_5$)	241.1834 (-CO_2, 1.7%), 223.1709((-CO_2, - H_2O) 205.1599 (-CO_2, - 2H_2O, 5.9%), 193.1599 (-CO_2, - H_2O –CH_2O, 7.2%) 175.1493 (-CO_2, - 2H_2O –CH_2O, 11.7%), 171.1023 (-CO_2, -C_5H_{10}, 11.8%), 153.0922 (-CO_2, -$C_5H_{10,}$ - H_2O,), 141.0922 (-CO_2, -$C_5H_{10,}$ - CH_2O, 100%), 123.0816 ((-CO_2, -$C_5H_{10,}$ - CH_2O, H_2O, 36.4%)
285.1709($C_{15}H_{25}O_5$)	241.1834 (-CO_2, 4.4%), 209.1548 (-CO_2,-CH_4O, 41.3%) 205.1599(-CO_2, - 2H_2O, 16.3%), 195.1389 (-CO_2,-C_2H_6O, 100%) 193.1599 (-CO_2, - H_2O –CH_2O, 35.8%), 175.1493 (-CO_2, - 2H_2O –CH_2O, 13.6%),

Table 4.3: MS2 data for the proposed sesquiterpene acids which are abundant in the Fort William samples.

row m/z	Rt min	Name
163.0401	9.2	Coumaric acid
253.0871	20.4	Benzyl coumarate
425.1247	17.7	Cinnamoyl caffeoyl acetyl glycerol
457.1144	14.9	Dicaffeoyl acetyl glycerol
441.1194	16.3	Coumaryl caffeoyl acetyl glycerol
471.1298	16.6	Caffeoyl feruoyl acetyl glycerol
455.1351	18.0	Coumaroyl feruoyl acetyl glycerol
269.082	18.3	Pinocembrin methyl ether or isomer
383.114	14.6	Dicoumaroyl glycerol
419.1503	21.1	Prenylated flavonoid
257.082	18.7	Benzoyl hydroxy phenyl acetic acid
287.0926	18.3	Benzoyl dihydroxyphenylpropionic acid
341.1033	16.1	Pinobanksin butyrate
271.0977	22.0	Caffeic acid hextrieneoate
279.0874	11.6	Coumaryl acetyl glycerol
179.035	7.3	Caffeic acid
285.0769	13.4	Methyl pinobanksin or isomer
135.0452	9.3	Phenylacetic acid
311.223	20.2	Dihydroxylinoleic acid
389.1399	21.5	Dimethyl pinocembrin benzoate
269.0457	14.3	Apigeninin
193.0869	12.6	Hydroxypropionate ethyl ester

269.0456	19.0	Galangin
271.0613	15.1	Pinobanksin

Table 4.4: Top 25 components by intensity of mass spectrometer response in Aberdeenshire propolis. Components which are particularly abundant in this sample set are highlighted in yellow.

row m/z	Rt min	Name
257.0819	19.7	Benzoyl hydroxy phenyl acetic acid
425.1241	18.7	Cinnamoyl caffeoyl acetyl glycerol
441.1195	17.3	Coumaryl caffeoyl acetyl glycerol
455.1346	19.0	Coumaroyl feruoyl acetyl glycerol
419.15	22.0	Prenylated flavonoid
389.1396	22.4	Pinocembrin benzoate dimethyl ether or isomer
383.114	15.7	Dicoumaroyl glycerol
457.1139	15.9	Dicaffeoyl acetyl glycerol
269.082	19.3	Pinocembrin methyl ether or isomer
471.1304	17.6	coumaroyl feruoyl acetyl glycerol
285.0406	15.8	Kaempferol isomer
279.0877	12.7	Coumaryl acetyl glycerol

301.0721	16.2	Hydroxy phenyl acetyl dihydroxyphenylacetic acid
295.098	18.4	Caffeic acid cinnamyl ester
445.166	16.7	$C_{27}H_{25}O_6$
399.1093	14.3	Quercetin hexanoyl ester or isomer
299.0565	20.0	Kaempferol methyl ether or isomer
269.1758	15.0	Sesquiterpene
255.0663	19.2	Pinocembrin
285.1709	12.8	Sesquiterpene acid
285.171	10.0	Sesquiterpene acid
475.1766	16.9	$C_{24}H_{27}O_{10}$
273.0767	15.4	$C_{15}H_{13}O_5$
449.1608	18.4	$C_{26}H_{25}O_7$
521.2757	21.5	$C_{28}H_{41}O_9$

Table 4.5: Top 25 components by intensity of mass spectrometer response in Fort William propolis. Components which are particularly abundant in this sample group are highlighted in yellow.

row m/z	Rt min.	Name
287.0925	18.1	Benzoyl dihydroxyphenylpropionic acid
271.0976	21.7	Caffeic acid hextrieneoate
163.0401	8.9	Coumaric acid
419.1504	20.9	Prenylated flavonoid
287.0926	17.4	Benzoyl dihydroxyphenylpropionic acid
257.0819	18.6	Benzoyl hydroxy phenyl acetic acid
403.1554	23.7	Pinocembrin phenylethyl trimethyl ether or isomer.
269.0456	18.9	Galangin
301.1083	21.2	Flavanol dimethyl ether.
313.0719	19.0	Pinobanksin acetate or isomer.
389.1398	21.3	Pinocembrin benzoate dimethyl ether or isomer.
419.1503	20.1	Prenylated flavonoid
135.0452	9.1	Phenylacetic acid
311.2229	20.0	Dihydroxylinoleic acid
295.0976	19.5	Caffeic acid cinnamyl ester
255.0664	18.8	Pinocembrin
271.0612	14.9	Pinobanksin
425.1247	17.6	Cinnamoyl caffeoyl acetyl glycerol
193.0869	12.4	Hydroxypropionate ethyl ester
253.0508	18.3	Chrysin
247.0976	18.3	Caffeic acid pentenyl ester
253.087	20.2	Benzyl coumarate
279.1026	22.1	Coumaric acid cinnamyl ether
273.0767	15.4	Dihydroxy phenyl acetic acid benzoyl ester
283.0614	19.7	Galangin methyl ether

Table 4.6: Top 25 components by intensity of mass spectrometer response in Dunblane propolis. Top Components which are particularly abundant in this sample group are highlighted in yellow.

Conclusion

Whether or not variations in its composition affects its anti-infective properties remains unknown. Although the current study is on a preliminary scale, it is the first of its type and it is clear from the current study that there are both marked local and regional variations in propolis composition. Although the samples used in the current study were quite small they were scraped from the surfaces of the hives and represent quite a large surface area. Given the current threats to bee health it is clear that further research on the role of this complex material in maintaining bee health is required in order to determine whether or not variations in composition compromise its effectiveness in promoting social immunity. Again the sheer complexity of the data generated by MS and MS^2 means that there is still some interpretation required of the data sets collected.

CHAPTER FIVE: PROFILING AND FRACTIONATION OF SOME PROPOLIS SAMPLES FROM INDONESIA AND SUB-SAHARAN AFRICA AND THE UK.

Introduction

Propolis is a plant-based product collected and used by bees in the construction of their hives and as an antimicrobial agent (Trusheva *et al*., 2011; Simanjuntak, 2012; Hasan *et al*., 2014). The potential antimicrobial, antiviral, and anticancer properties of propolis components such as the aromatic acids and flavonoids have led to increased attention towards the potential use of propolis (Awale *et al*., 2008). However, the components of propolis depend on the local flora, which varies across geographical regions (Bankova, Trusheva, B. and Popova, 2008; Trusheva *et al*., 2011; Nunes *et al.,* 2013). Therefore, to enhance the use of propolis, there is a need to understand the components of the propolis found in different regions and in this study propolis samples from Indonesia (Trusheva *et al*., 2011) were investigated for their chemical constituents. For the work in this section we had established a link with some manufacturers of propolis products from Indonesia. They were interested in us testing two of their raw materials which were obtained from stingless bees. In addition some African samples collected by Hugo Fearnley during a project to examine bee-keeping practices in West Africa became available including some stingless bee propolis from Ghana.

Components of Indonesian propolis

The diversity in the components of the Indonesian propolis increases the potential of wide application in the different areas. The composition and the properties of Indonesian propolis varies based on the location from which the propolis was collected (Trusheva *et al*., 2011, Hasan *et al*., (2014). Propolis collected from five different locations in Indonesia were analysed and the highest amount of flavonoids were found

in the sample collected from Kendal, while the propolis from Pekanbaru had the least amount of flavonoids. The difference in the amounts of flavonoids in the Indonesian propolis samples based on the location has also been reported by Syamsuddin *et al*. (2010) in a similar study of propolis samples from three different locations in Java. Kalsum *et al*., (2016) also noted that the propolis obtained from the different regions in Indonesia contained different bioactive compounds. These findings were based on GC-MS analysis for the presence of various compounds in the ethanol extracts. Trusheva *et al*. (2011) observed that Indonesian propolis from Java had five different alk(en)ylresorcinols with 5-(80 Z,110 Z-Heptadecadienyl)-resorcinol being the most abundant. According to Trusheva *et al*. (2011), Indonesian propolis was observed, to that date, to be the only propolis that has been found to contain alk(en)ylresorcinols. It was also found that Indonesian propolis contained prenylflavanones. The prenylflavanones can be used to inhibit bacterial growth and in the treatment of tumours (Kumazawa *et al*., 2008). Indonesian propolis was also found to contain cycloartane triterpenes, which have also been isolated from Brazilian and Myanmar propolis (Silva *et al.*, 2005; Li *et al*., 2009). Mangiferolic and isomangiferolic acids were the main cycloartane triterpenes found in Indonesian propolis. Based on the isolated compounds, the Indonesian propolis found in Java is mainly formed from plant resins obtained from *Macaranga tanarius* L. and *Mangifera indica* L. The Indonesian propolis was also found to contain phenols and phenolic compounds (Novilla *et al*. 2014). Other components include α–amyrin, quercetin and caffeic acid phenethyl ester (Simanjuntak, 2012; Susanto *et al*., 2017).

Uses of Indonesian propolis

The components of Indonesian propolis have antibacterial ability against some drug resistant bacterial pathogens (Novilla *et al.,* 2014). Phenolic compounds obtained from Indonesian propolis were found to possess antibacterial activity while the ethanol soluble fractions showed antibacterial activity against *S. aureus* and *Bacillus subtilis* and *Escherichia coli.* It was also observed that the phenolic components of Indonesian propolis have free radical scavenging ability, which was found to be higher than the activity of Vitamin C. According to Novilla et al. (2014), the components of Indonesian propolis inhibit the Methicillin-Resistant *S. aureus* by interfering with cell division and by inducing bacteriolysis through the destruction of the bacterial cell membrane and the disruption of the permeability properties. The researchers also argued that the phenol components of Indonesian propolis inhibit the Methicillin-Resistant *S. aureus* by interfering with the bacteria's protein forming ability.

The effect of Indonesian propolis on microbial cell division was also shown by Hasan et al., (2014). Extracts of Indonesian propolis caused a reduction of cell division in *Saccharomyces cerevisiae.* Hasan *et al*., (2014) also observed that the antioxidant activity of the flavonoids obtained from the Indonesian propolis varied based on the locations. Only flavonoid compounds were obtained from the Indonesian propolis collected from Pandeglang and they showed high antioxidant activity based on DPPH scavenging assay. Hasan et al., (2014) also showed that Indonesian propolis has anticancer properties. Indonesian propolis from Pekanbaru enhanced the growth of cancer cells while propolis from Makassar inhibited the growth of cancer cells. Syamsuddin et al. (2010) also noted the difference in the anticancer properties among the Indonesian propolis obtained from the different locations. Based on the work by

Hasan *et al*., (2014) and Syamsuddin *et al*. (2010), it is evident that the composition and the biological activity of Indonesian propolis varies based on the location from which it is collected. Therefore for meaningful documentation and use of Indonesian propolis, there is a need to further analyse Indonesian propolis based on locations. Indonesian propolis contains metabolites that have cytotoxic activity (Syamsuddin *et al*. 2010; Simanjuntak, 2012; Hasan *et al*., (2014). According to Simanjuntak, (2012), the anticancer properties of Indonesian propolis is a linked to the presence of α – amyrin. The researchers noted that Indonesian propolis isolates had cytotoxic effects against MCF-7 cells and T47D cells. Syamsuddin *et al.* (2010) also showed that the ethyl acetate extract of propolis has the ability to induce apoptosis in MCF-7 cells. Hasan *et al*., (2014) also observed that ethanol and ethyl acetate extracts of Indonesian propolis have cytotoxic effects towards MCF-7 line cells.

The study carried out by Susanto *et al*., (2017) showed that the antioxidant effect of Indonesian propolis has the ability to prevent kidney damage. This was found in a study that involved the analysis of the effects of Indonesian propolis extract on malondialdehyde, an end product of oxidative damage of lipids in rats. Susanto *et al*. (2017) noted that quercetin and caffeic acid phenethyl ester present in Indonesian propolis were capable of protecting the kidney from the damaging effects of free radicals as evident in the reduction in the level of malondialdehyde. Al Mukhlas *et al*., (2017) also showed that Indonesian propolis can be used to manage vomiting and nausea. Aqueous extracts of Indonesian propolis had higher antiemetic properties compared to the ethanol extracts. Studies on the use of the antibacterial properties of Indonesian propolis in pharmaceutical and food industry (Sahlan and Supardi, 2013; Sahlan and Rohmatin, 2017) have been carried out. Sahlan and Supardi, (2013)

demonstrated that Indonesian propolis can be encapsulated by casein micelles, which can then be used as antibacterial agents in food processing and the pharmaceutical industry. According to Sahlan and Supardi, (2013), encapsulated Indonesian propolis has similar flavonoid concentration to the free propolis. Sahlan and Rohmatin, (2017) also demonstrated that Indonesian propolis can be incorporated into soap and used in the control of pathogenic bacteria based on analysis of the effect of soap containing varying amounts of Indonesian propolis wax on individuals suffering from *Candida albicans.* Sahlan and Rohmatin, (2017) it was noted that the soap containing as low as 1 to 2 % of Indonesian propolis wax effectively reduced *C. albicans* infections.

Materials and Methods

Materials

Absolute ethanol, HPLC grade acetonitrile, methanol, acetonitrile, formic acid were obtained from Fisher Scientific, Loughborough UK. An ACE C_{18} column (3mm x 150mm, 3µm) was from Hichrom, Reading, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK.

Propolis Samples

The Indonesian propolis samples were part of collection made by BeeVital Ltd., Whitby, UK. Three 50 mg amounts of propolis were weighed for each sample and the samples were then extracted with 5 ml of ethanol in screw capped test tubes by sonication for 30 minutes. About 0.2 ml of the extracts were transferred to Eppendorf tubes and mixed with 0.8 ml of HPLC grade water and centrifuged for 5 minutes at 3000 rpm. The supernatant was then used for analysis by LC-HRMS.

Isolation of Gallic acid, p-coumaric acid and Apigenin from Indonesian propolis

80g of the Indonesian propolis sample was extracted three times with 500 ml ethanol by sonication for one hour. The extracts were combined and after rotary evaporation gave 35 g of solvent free extract. About 8g of this extract was added to 15g of silica gel for adsorption and then dried. The dry adsorbed extract was placed on a 200 g silica gel column and eluted with gradient mixtures of hexane and ethyl acetate resulted in 48 fractions (50 ml) each. Two fractions KIK8 (0.79 g) eluted with 60:40: hexane:ethylacetate, and fraction KIK12 (0.4901 gm) eluted with 50:50 ethyl acetate: hexane were further purified using a Grace Davison Reveleris® flash chromatographic system. After method development with an analytical LC-UV-ELSD system, the fractions were re-chromatographed using the flash system in normal phase mode using a 12 g (C-18) cartridge and a flow rate of 15 ml/min, eluted with gradient mixtures of ethyl acetate in hexane (Fig 5.1). p-Coumaric acid (6mg) was obtained from KIK12 and found in the tube number 14 . While Apigenin (8.1mg) and Gallic acid (5.9mg) were obtained in fraction 9 and 23 resulted from KIK8.

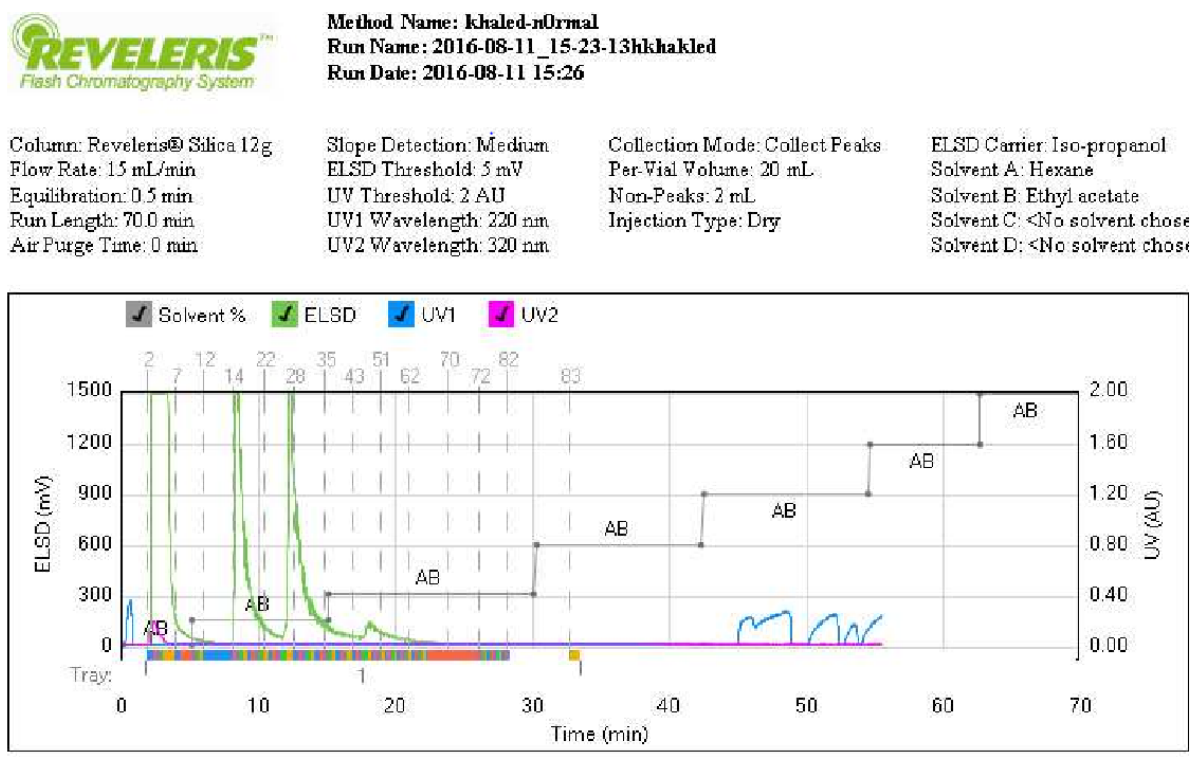


Figure 5.1: MPLC chromatogram obtained for KIK12.

Isolation of lupeol and quercetin from a propolis sample from Ghana

About 30 g of the propolis sample from Ghana was extracted with ethanol using sonication for one hour. The sample was extracted thrice and after evaporation of the extracts, 6 g of a crude ethanol extract of propolis was obtained. The extract was dissolved in a minimum volume of ethyl acetate and then mixed with a small quantity of silica gel. The mixture was allowed to dry under a fume hood for 24 hours. The dry extract was then loaded onto a silica gel (55g of silica gel in a 40x3 cm (id) glass column and eluted using a gradient with increasing amounts of ethyl acetate in hexane from hexane: EtOAc (90:10) to ethyl acetate. Fractions were collected in 50 ml vials and a total of 28 fractions were collected. Using LC-MS profiles similar fractions were combined so that eight combined fractions were obtained. Fraction 5 (650 mg) and

fraction 7 (500 mg) which were not completely pure were subjected to further purification. The two fractions of interest were purified using a Grace Reveleris® iES Flash Chromatography System equipped with a dual-UV wavelength detector set at 210 and 280 nm and an ELSD detector. These fractions were dissolved in a minimum amount of ethyl acetate and pre-adsorbed onto Celite® and were then left to dry under a fume hood. The dry mixtures were put into the solid loader on the top of the Reveleris®24 g/32 ml silica gel column and then eluted isocratic with hexane-ethyl acetate (40:60 v/v) and run for 70 minutes. Components were collected according to peaks in the chromatogram and examined by analytical HPLC with ELSD in order to combine the similar ones. Six sub-fractions were obtained from fraction 5 and lupeol (3.5 mg) was obtained in fraction 2.while 8 subfractions were obtained from fraction 7 and quercetine (4.3mg) was found in fraction number 4.

Isolation of pinobanksin methyl ether from a sample of UK propolis

About 50 g of a propolis sample from the UK was also extracted thrice with ethanol using sonication for one hour. After evaporation of the extracts, 7 g of a crude extract was obtained. The extract was dissolved in a minimum volume of ethyl acetate and adsorbed on to silica gel and allowed to dry. The dry extract was then loaded onto a silica gel column (150 g of silica gel in a 40x3 cm (id) glass column) and eluted using gradient amounts of ethyl acetate in hexane starting with hexane and then ethyl acetate in hexane (20:80) and finally ethyl acetate. Fractions obtained with ethyl acetate: hexane (40:60) were combined to yield 646 mg of a semi purified compound. This was further purified using a Grace Reveleris® iES Flash Chromatography System equipped with a dual-UV wavelength detector set at 210 and 280 nm and an ELSD detector. It was eluted using gradient amounts of ethyl acetate in hexane and

pinobanksin methyl ether was obtained as the only purified compound in fractions found in the tubes number 18-24 with a weight of 13 mg.

LC-HRMS

The high resolution mass spectra were obtained using an Orbitrap Exactive mass spectrometer (ThermoFisher, Hemel Hempstead, UK) in positive and negative ion switching modes with a needle voltage of -4.0 kV, 4.5 kV positive. Sheath and auxiliary gases were set at 50 and 17 arbitrary units respectively. The separation was performed on an ACE C_{18} column (150 × 3 mm, 3 µm) from HiChrom UK with 0.1% v/v formic acid in water as mobile phase A and 0.1% v/v formic acid in acetonitrile as B at flow rate of 0.300ml/min using the following linear gradient: 50% B (0 min) 100% B (30 min) 100% B (44 min) 50% B (45 min) 50% B (50 min).

Data Extraction

Peaks were aligned and then extracted in 0.02 amu windows m/zMine 14.0 and putative identities were assigned by searching against the Dictionary of Natural Products database. The peaks areas were transferred to Microsoft Excel for further manipulation and PCA analysis was carried out in Simca P 14.0.

Results

The propolis extracts were analysed by high resolution mass spectrometry and the ion peaks were extracted using m/zMine software. The chromatogram contained thousands of features and many of these features were probably from genuine compounds. Putative identities were deduced from accurate masses which were within 3 ppm deviations from the exact mass of the proposed elemental composition. The predominant phenolic compounds in the samples gave strong signals in negative ion

mode. The most abundant ions by averaged peak intensity, which appeared to be genuine peaks rather than adducts or dimers, were selected for further characterisation.

It can be presumed that compounds with similar structures will give responses that are not widely different from each other. Although standards were not available to determine the identity and actual amount of compounds in the samples, further preparative chromatographic separation enabled the identification of some of the compounds by NMR spectroscopy. The compounds isolated as column fractions were fully elucidated based on their 1D and 2D and mass spectra.

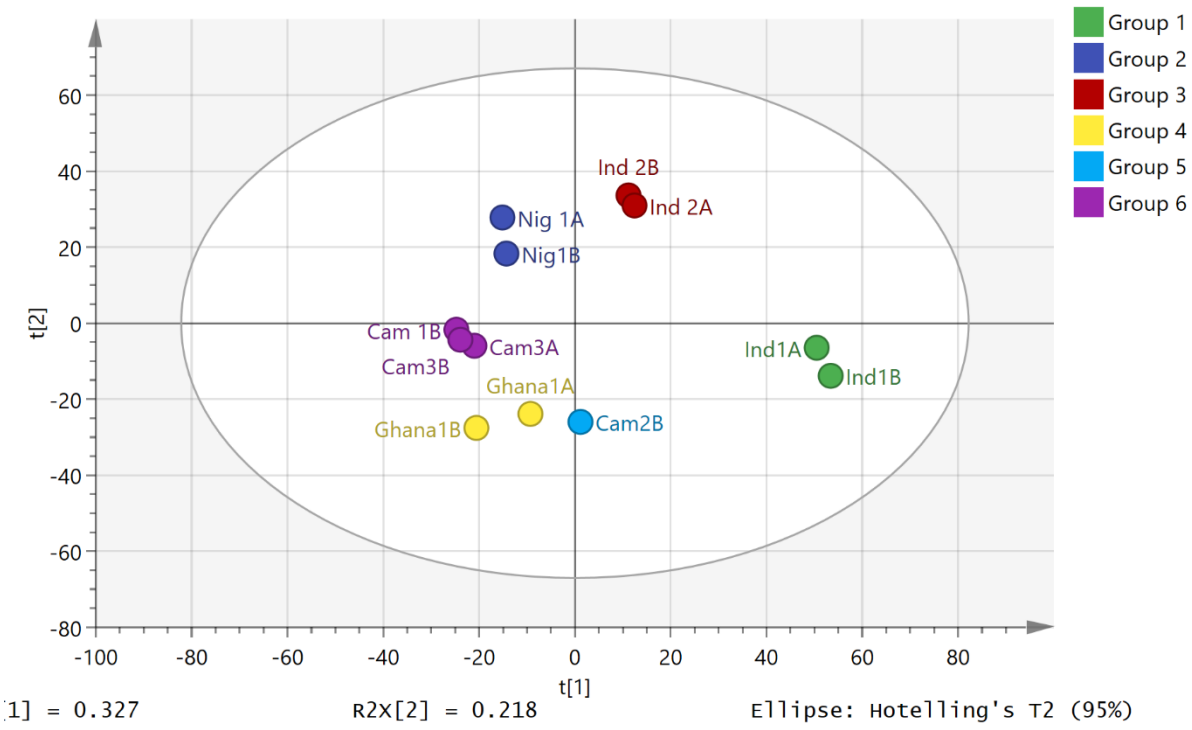


Figure 5.2: PCA separation HCA of propolis samples from Nigeria, Cameroon, Ghana and Indonesia.

Figure 5.2 shows the PCA separation of propolis samples from Indonesia, Cameroon, Ghana and Nigeria. There are clear differences between Indonesian samples 1 and 2

while two of the Cameroon samples cluster quite closely. The profiles of the samples are described below.

Compounds found in Indonesian propolis samples

The most abundant compounds in Indonesian propolis sample 1 appear to be sesterterpene compounds (figure 5.4) these have generally been isolated from marine sponges rather than from plants.

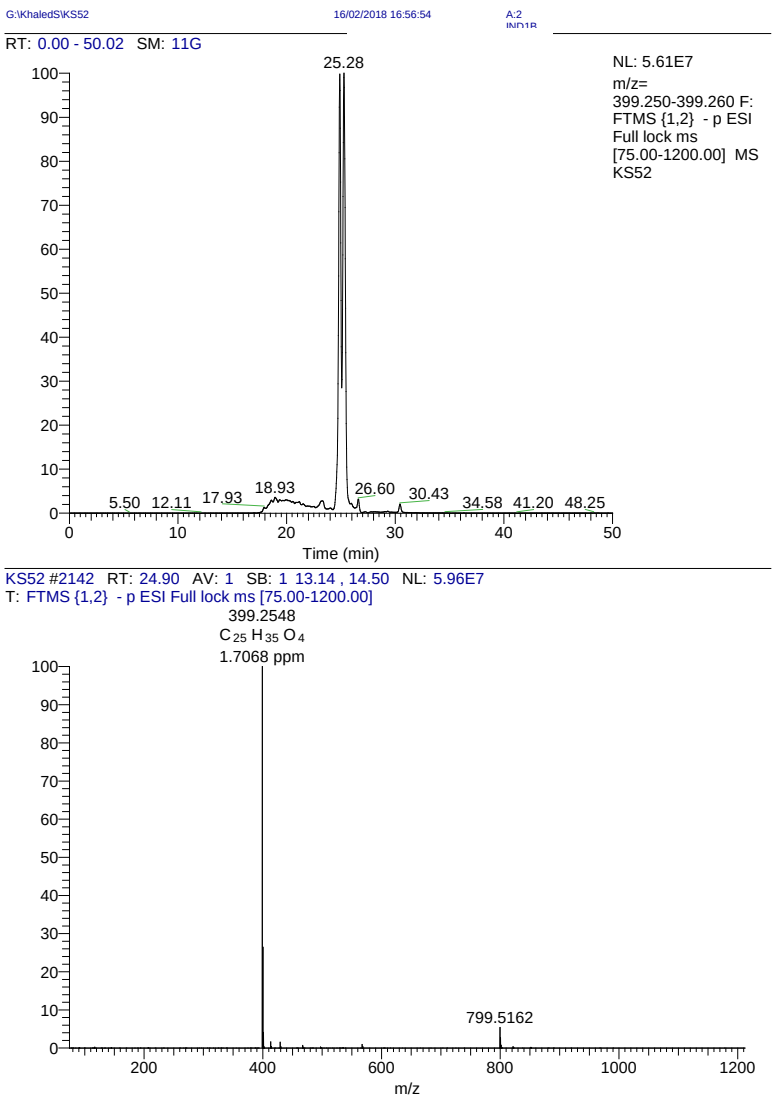


Figure 5.3: Putatively identified sesterterpene compound in Indonesian propolis sample 1.

row m/z	row retention time	Molecular formula	Name
413.2703	27.1	C26H38O4	Actinopyrones; Actinopyrone C
399.2544	25.4	C25H36O4	Actinopyrones; Actinopyrone A
583.4016	39.3	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
481.3332	33.1	C31H46O4	Adlupone
569.3859	37.6	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
583.4016	39.7	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
555.37	35.8	C34H52O6	Colletochin; O-(2R-Hydroxy-3S-methylpentanoyl)
583.4016	39.9	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
569.3858	37.9	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
541.3547	32.1	C33H50O6	Antibiotic BR 050; 2-O-(2R-Hydroxy-3S-methylpentanoyl)
399.2543	19.1	C25H36O4	Actinopyrones; Actinopyrone A
399.2543	19.5	C25H36O4	Actinopyrones; Actinopyrone A
569.3859	36.4	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
399.2543	18.8	C25H36O4	Actinopyrones; Actinopyrone A
531.2756	23.4	C33H40O6	Biyouxanthone C
467.3173	31.4	C30H44O4	Anhydrobelachinal
399.2542	20.1	C25H36O4	Actinopyrones; Actinopyrone A
575.3387	30.1	C36H48O6	Polymaxenolide; ?11-Isomer, 11-deacetoxy
569.3859	36.7	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
555.37	35.1	C34H52O6	Colletochin; O-(2R-Hydroxy-3S-methylpentanoyl)
555.37	33.3	C34H52O6	Colletochin; O-(2R-Hydroxy-3S-methylpentanoyl)
483.3123	30.9	C30H44O5	4(3->2)-Abeo-2-hydroxy-4,7-dioxoergosta-5,22-dien-3-oic acid; (2?,22E,24R)-form, Et ester
515.3387	33.7	C31H48O6	Cabraleahydroxylactone; 17?-Acetoxy, 3-Ac
533.4011	35.5	C36H54O3	Estrone; 3-O-(9Z-Octadecenoyl)
399.2544	18.2	C25H36O4	Actinopyrones; Actinopyrone A
345.2071	20.0	C21H30O4	19(4->18)-Abeo-16,17-dihydroxy-4(18)-kauren-19,3-olide; (ent-3?,16?OH)-form, 16-Me ether
463.2706	7.1	C26H40O7	2,9:3,16-Diepoxy-6-asbestinene-4,11-diol; (4?,6Z,11?)-form, 6?,7?-Epoxide, 11-butanoyl, 4-Ac
501.286	18.0	C29H42O7	Ajugacetalsterone C
669.4176	36.7	C43H58O6	Bronianone
501.3232	32.1	C30H46O6	Acteol
413.2706	32.3	C26H38O4	Actinopyrones; Actinopyrone C
597.4173	41.2	C37H58O6	3-(3,7-Dimethyl-5-oxo-2,6-octadienyl)-2-hydroxy-6-(hydroxymethyl)-4-methoxybenzaldehyde; 1'-Octadecanoyl
399.2542	20.7	C25H36O4	Actinopyrones; Actinopyrone A
447.2757	12.2	C26H40O6	Amphidinolide X
447.2755	13.5	C26H40O6	Amphidinolide X
455.2809	18.0	C28H40O5	4(3->2)-Abeo-2-hydroxy-4,7-dioxoergosta-5,22-dien-3-oic acid
429.2648	25.1	C26H38O5	10-Aromadendranol; (1?,4?,5?,6?,7?,10?)-form, O-[4-O-Angeloyl-D-threo-hex-1-enopyranosid-3-uloside]
431.2805	19.0	C26H40O5	Curvicollide B

429.2648	22.9	C26H38O5	10-Aromadendranol; (1?,4?,5?,6?,7?,10?)-form, O-[4-O-Angeloyl-D-threo-hex-1-enopyranosid-3-uloside]
429.2648	25.4	C26H38O5	10-Aromadendranol; (1?,4?,5?,6?,7?,10?)-form, O-[4-O-Angeloyl-D-threo-hex-1-enopyranosid-3-uloside]
499.344	30.4	C31H48O5	4(3->2)-Abeo-2-hydroxy-4,7-dioxostigmast-5-en-3-oic acid; (2?,24S)-form, Et ester
527.3386	30.3	C32H48O6	Acerinol; 25-Ac
541.3546	31.6	C33H50O6	Antibiotic BR 050; 2-O-(2R-Hydroxy-3S-methylpentanoyl)
583.4016	38.6	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
481.3332	34.0	C31H46O4	Adlupone
447.2755	15.2	C26H40O6	Amphidinolide X
429.2648	19.7	C26H38O5	10-Aromadendranol; (1?,4?,5?,6?,7?,10?)-form, O-[4-O-Angeloyl-D-threo-hex-1-enopyranosid-3-uloside]
429.2648	18.0	C26H38O5	10-Aromadendranol; (1?,4?,5?,6?,7?,10?)-form, O-[4-O-Angeloyl-D-threo-hex-1-enopyranosid-3-uloside]
567.406	24.1	C36H56O5	Cylindrocyclophane A; 14-Deoxy
429.2648	21.1	C26H38O5	10-Aromadendranol; (1?,4?,5?,6?,7?,10?)-form, O-[4-O-Angeloyl-D-threo-hex-1-enopyranosid-3-uloside]

Table 5.1: Most abundant compounds found in Indonesian propolis sample 1.

The second Indonesian propolis sample was quite different from the first sample with the top hits being for diterpene compounds. Figure 5.5 shows the mass spectrum and extracted ion chromatogram for the mose abundant diterpene in the extract. The source of the diterpenes is unclear. This is a quite unusual sample and may represent a new type of propolis.

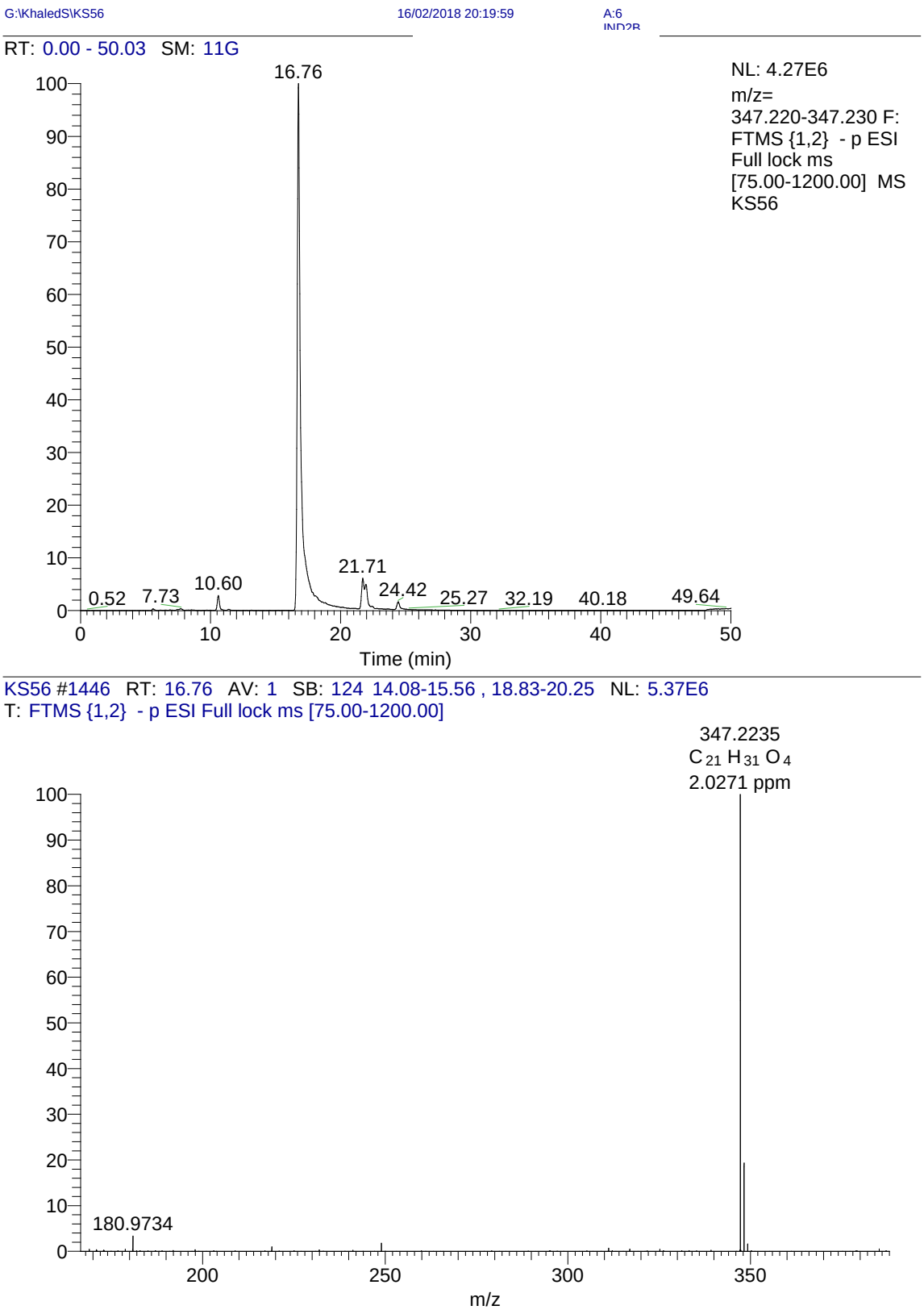


Figure 5.4: Abundant diterpene in Indonesian propolis sample 2.

row m/z	row retention time	Molecular formula	Name
347.2228	16.8	C21H32O4	8,11,13-Abietatriene-3,11,14,19-tetrol; 14-Me ether
351.2177	7.4	C20H32O5	11(15->1)-Abeo-4(20),11-taxadiene-5,9,10,13,15-pentol
379.2128	8.6	C21H32O6	20(10->9)-Abeo-10,16,17-trihydroxy-15-oxo-19-kauranoic acid; (ent-10?,16?OH)-form, Me ester
303.1966	10.7	C19H28O3	Acalycixeniolide K
501.302	31.9	C33H42O4	Clusianone
289.181	7.7	C18H26O3	2-Alkyl-5-hydroxy-4H-1-benzopyran-4-ones; 5-Hydroxy-2-nonyl-4H-1-benzopyran-4-one, 2?,3-Dihydro
347.1865	5.5	C20H28O5	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 1-Angeloyl
317.2123	11.9	C20H30O3	6,8(14),15-Abietatriene-3,11,17-triol
413.2703	27.1	C26H38O4	Actinopyrones; Actinopyrone C
367.2127	9.2	C20H32O6	11(15->1)-Abeo-4(20),11-taxadiene-2,5,9,10,13,15-hexol
363.2177	13.9	C21H32O5	ACTG Toxin A; 1-Hydroxy
395.2076	3.3	C21H32O7	ACRL Toxins II-IV; ACRL toxin IV
365.1972	5.9	C20H30O6	11(15->1)-Abeo-2,5,9,10,15-pentahydroxy-4(20),11-taxadien-13-one
315.197	11.4	C20H28O3	16(15->12)-abeo-l-3-oxo-16-nor-8(14)-pimaren-15,16-olide
393.2287	16.8	C22H34O6	11(15->1)-Abeo-4(20),11-taxadiene-5,9,10,13,15-pentol; (5?,9?,10?,13?)-form, 9-Ac
335.1866	7.7	C19H28O5	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 9-O-(2-Methylpropanoyl)
393.1924	5.5	C21H30O7	Alternaric acid; 10-Deoxy
481.3332	33.1	C31H46O4	Adlupone
381.2285	4.7	C21H34O6	ACTG Toxin A; 2,3-Dihydroxy, 2,3-dihydro
377.197	7.7	C21H30O6	17(15->16)-Abeo-6,7,12,16-tetrahydroxy-8,12-abietadiene-11,14-dione; (6?,7?,16?)-form, 7-Me ether
335.223	4.9	C20H32O4	2(3->4)-Abeo-2-hydroxy-3-oxo-13-cleroden-15-oic acid
501.3014	24.9	C33H42O4	Clusianone
363.1816	4.1	C20H28O6	19(4->3)-Abeo-4,5-epoxy-1,6,7,14-vouacapanetetrol
315.0513	4.0	C16H12O7	2-Acetyl-1,3,6,8-tetrahydroxyanthraquinone; 1'R-Alcohol
293.1759	5.7	C17H26O4	Agglomerin; Agglomerin C
319.1915	5.9	C19H28O4	7(8->9)-Abeo-9-hydroxy-11(13)-eremophilen-8,12-olide; (7?,9?,10?)-form, 2-Methylpropanoyl
409.2235	10.3	C22H34O7	17-Acetoxy-1,6,7-trihydroxymelcantholide
397.2231	3.2	C21H34O7	Alternaric acid; 10-Deoxy, 6R,8,9,19-tetrahydro
349.2022	10.5	C20H30O5	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 1-O-(3-Methylbutanoyl)
347.1865	4.3	C20H28O5	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 1-Angeloyl
517.2966	28.7	C33H42O5	Clusia obdentifolia C33H42O5 Prenylated acylphloroglucinol
363.2181	10.3	C21H32O5	ACTG Toxin A; 1-Hydroxy
499.3436	25.4	C31H48O5	4(3->2)-Abeo-2-hydroxy-4,7-dioxostigmast-5-en-3-oic acid; (2?,24S)-form, Et ester
667.4221	13.4	C40H60O8	Cylindrocyclophane A; 1,14-Di-Ac
301.2173	21.7	C20H30O2	Abeoanticopalic acid
305.1758	4.2	C18H26O4	Acetaldehyde; Dimedone deriv.
651.4274	19.5	C40H60O7	Acacigenin B
381.192	3.3	C20H30O7	11(15->1)-Abeo-5,20:13,15-diepoxy-11-taxene-2,4,7,9,10-pentol
411.2027	3.1	C21H32O8	Abscisic alcohol; 11-O-?-D-Glucopyranoside
263.1289	3.8	C15H20O4	7(8->9)-Abeo-11,12-epoxy-6-hydroxy-1(10)-eremophilen-8,12-olide

467.3174	32.2	C30H44O4	Anhydrobelachinal
303.1966	11.0	C19H28O3	Acalycixeniolide K
333.2074	8.3	C20H30O4	20(10->9)-Abeo-6,16-dihydroxy-19,10-kauranolide
441.1922	5.4	C25H30O7	14(5->6)-Abeo-5,9-eremophiladiene-3,9,14-triol; 3?-form, 14-Angeloyl, 9-propanoyl, 3-Ac
545.3489	25.1	C32H50O7	Amphidinolide B1; 16-Deoxy
321.2071	7.0	C19H30O4	Angepubefurin
277.1445	4.3	C16H22O4	Acrostalidic acid
321.1709	5.6	C18H26O5	Arabinitol; D-form, 1-Benzyl, 2,3:4,5-di-O-isopropylidene
333.2073	8.6	C20H30O4	20(10->9)-Abeo-6,16-dihydroxy-19,10-kauranolide

Table 5.2: Fifty most abundant compounds found in Indonesian propolis sample 2.

However, the extract also contains clusianone which is a polyisoprenolated benzophenone. These compounds are found in various Garcinia species and have previously been found in propolis from Cameroon, Nigeria and Brazil (Al Mutairi *et al* 2014; Omar *et al* 2016) . It can be seen that there are several isomers of clusianone in the extract (figure 5.6). In addition, there are hydroxylated isomers of clusianone present indicating that the bees are collecting propolis from Garcinia mangosteena or a similar plant. These compounds are not present in high abundance in the other Indonesian propolis sample. However, this sample seems to contain some of the sesterpenes which are present in Indonesian propolis sample 1.

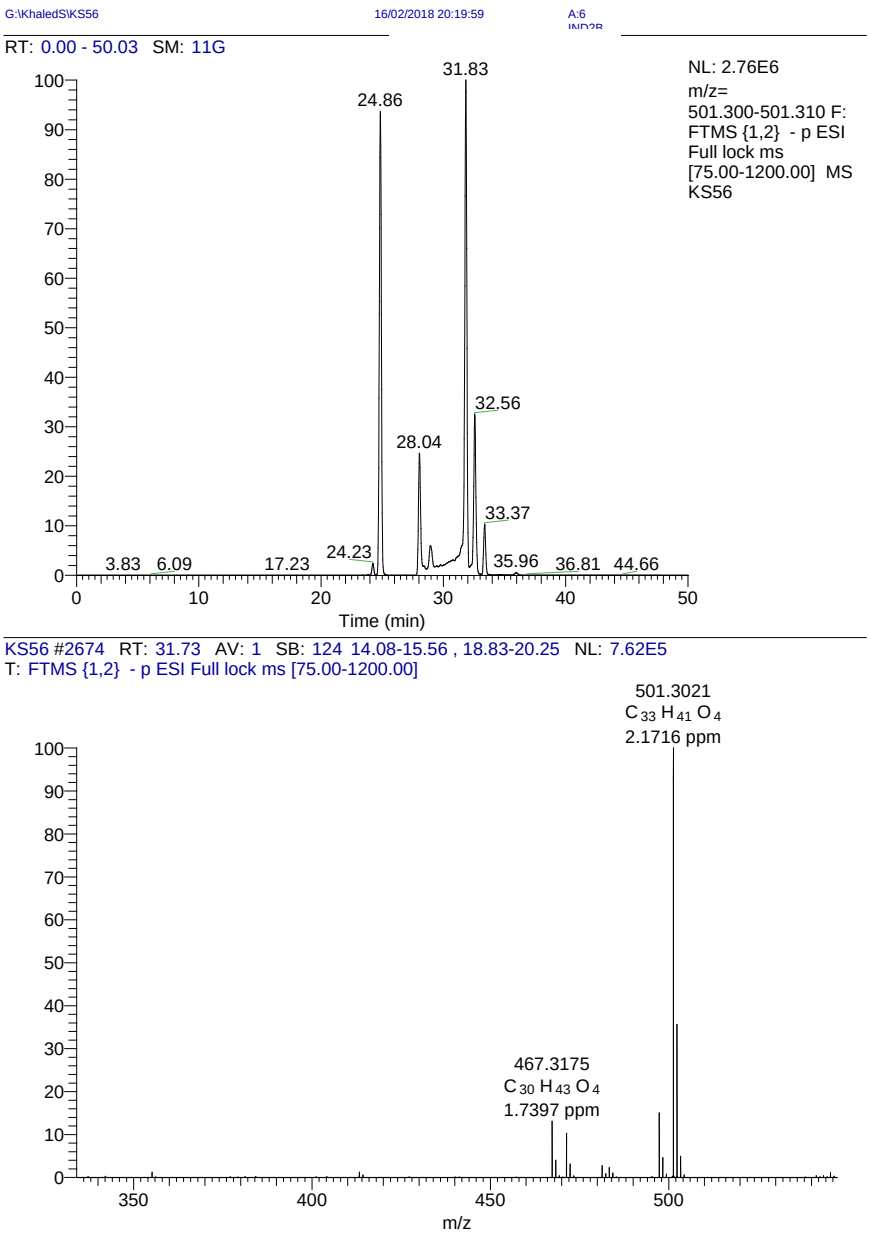


Figure 5.5: Abundant phloroglucinone compounds in Indonesian propolis sample 2.

Propolis from Ghana

The major compounds in the propolis sample appear to prenylated flavonoids. Several prenylated flavonoids were previously isolated from Nigerian propolis and were found to have strong activity against *Trypansoma brucie* (Omar *et al*, 2016). The two top compounds in the Ghanian propolis sample appear to be the same compounds as were isolated from red Nigerian propolis, macarangin and propolin D (Omar *et al*, 2016). In

addition, there are a number of isomers of these compounds present. The red colour in red propolis originates from the legume species Dalbergia but there is no strong evidence for the isoflavone marker compounds for that plant in this sample. There also appear to be sesquiterpenes in this extract which make it different from the Nigerian samples studied previously.

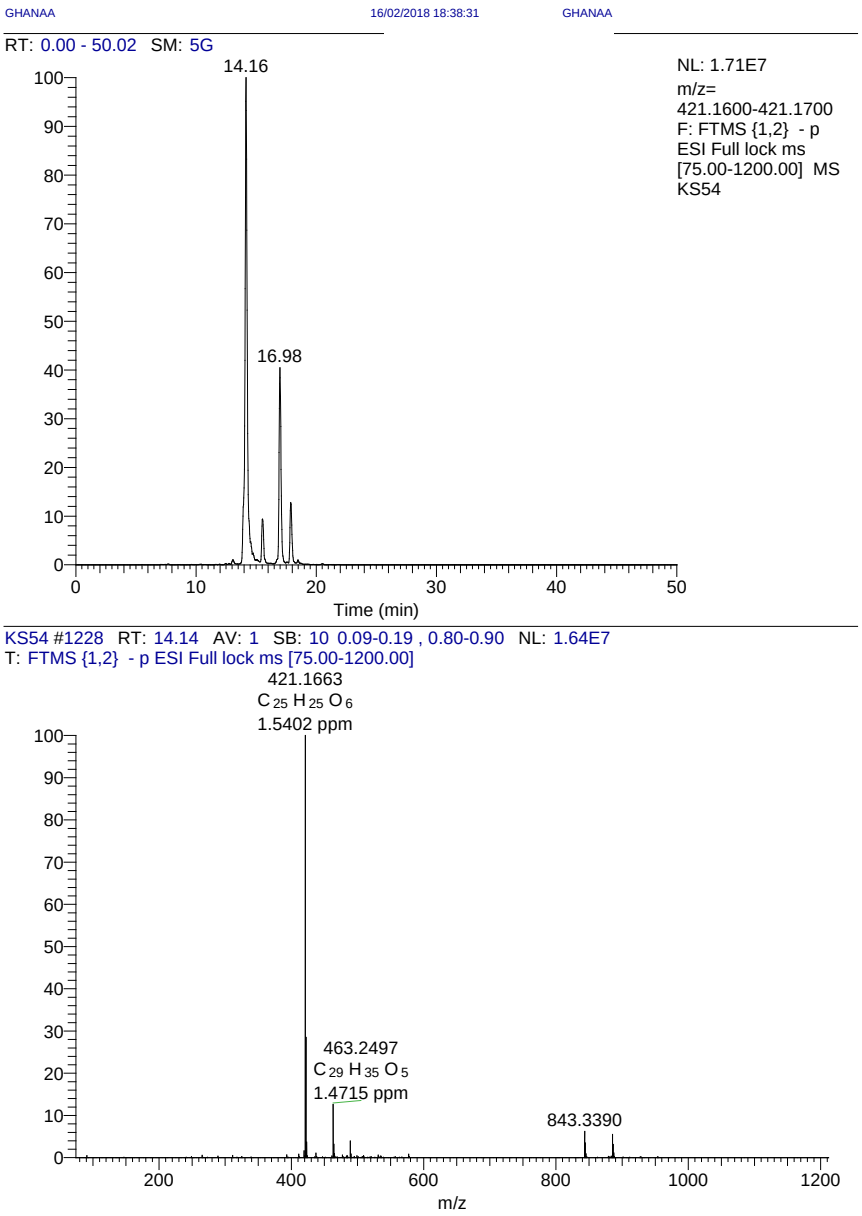


Figure 5.6: Macarangin isomers from Ghanian propolis.

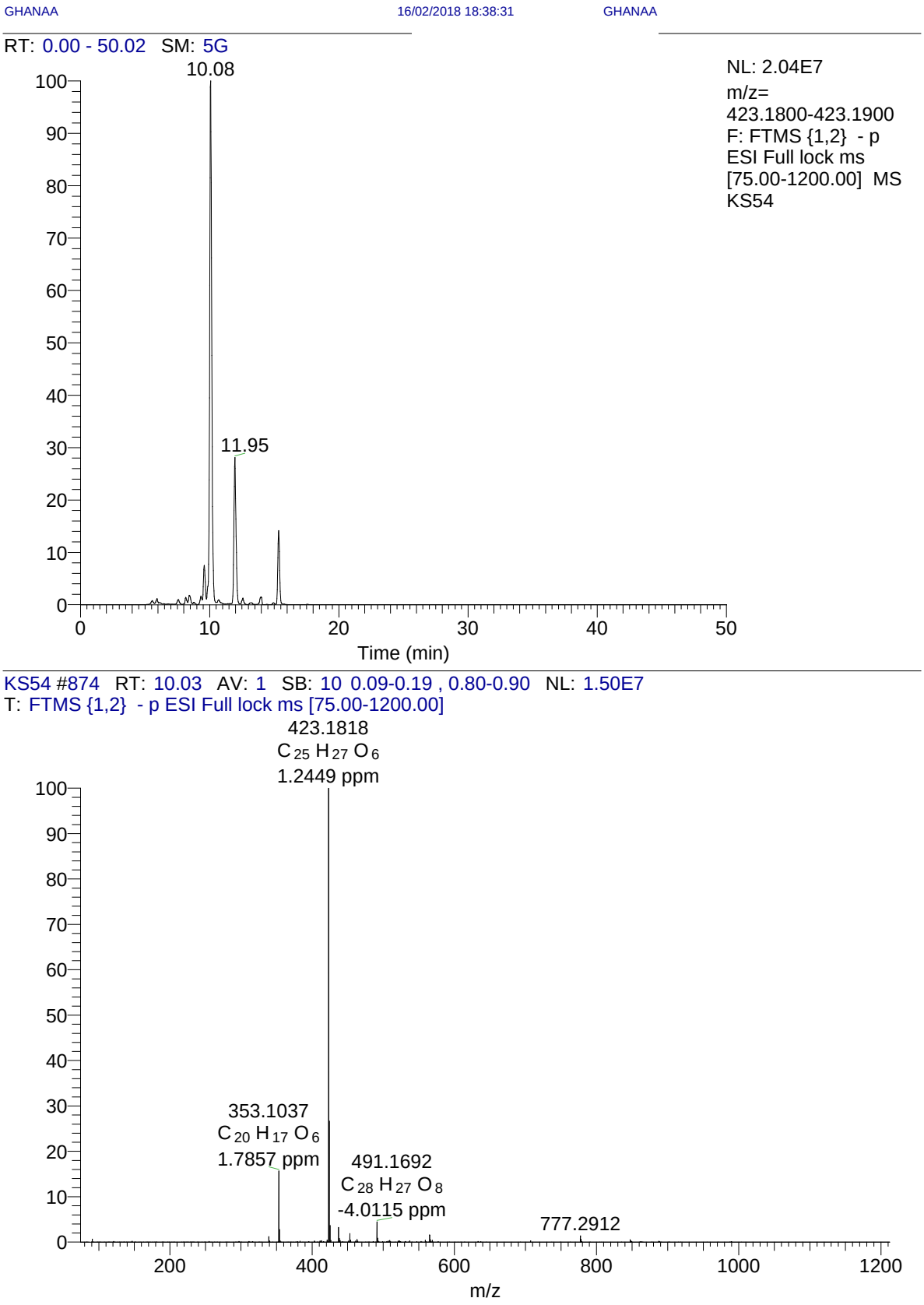


Figure 5.7: Propolin D isomers from a Ghanian propolis sample.

row m/z	row retention time	Molecular formula	Name
421.1661	14.2	C25H26O6	Macarangin
423.1818	10.1	C25H28O6	Propolin D
283.0612	3.2	C16H12O5	Bauhiniastatin 1
413.2703	27.1	C26H38O4	Actinopyrones; Actinopyrone C
255.0662	6.9	C15H12O4	14(5->6)-Abeo-9,13-dihydroxy-1,3,5,9-furanoeremophilatetraen-14-al
447.2544	16.8	C30H32N4	15,17-Butano-3,8-diethyl-2,7,12,18-tetramethylporphyrin; 3-De-Et
379.1561	17.4	C23H24O5	6-Benzoyl-5-hydroxy-2,2,8,8-tetramethyl-2H,8H-benzo[1,2-b:3,4-b']dipyran; 9,10-Dihydro, 9-hydroxy
271.0613	3.9	C15H12O5	14(5->6)-Abeo-9,13-dihydroxy-1,5,7,9,11-furanoeremophilapentaen-14,15-olide
267.0662	4.9	C16H12O4	14(5->6)-Abeo-9,13-dihydroxy-1,3,5,9-furanoeremophilatetraen-14-al; 13-Aldehyde, 9-Me ether
439.1765	6.0	C25H28O7	Hydroxypropolin D
379.1918	10.7	C24H28O4	Angelicolide
339.1239	6.8	C20H20O5	6-Allyl-6-[2-(3,4-dihydroxyphenyl)-1-methylethyl]-3,4-dihydroxy-2,4-cyclohexadien-1-one; Bis(methylene) ether
255.0663	4.4	C15H12O4	14(5->6)-Abeo-9,13-dihydroxy-1,3,5,9-furanoeremophilatetraen-14-al
463.213	19.6	C28H32O6	Biyouxanthone D
383.114	9.3	C21H20O7	2-Acetyl-3-(3,4-dimethoxyphenyl)-3-hydroxy-2-methyl-5,6-methylenedioxyindenone
451.1766	16.0	C26H28O7	Artocarpin; ?3"-Isomer, 2"?-hydroxy
273.1498	13.0	C17H22O3	Algoafuran
447.2543	16.4	C30H32N4	15,17-Butano-3,8-diethyl-2,7,12,18-tetramethylporphyrin; 3-De-Et
283.0611	4.1	C16H12O5	Bauhiniastatin 1
407.1868	13.5	C25H28O5	Adenaflorin A; 2,3-Didehydro
353.1033	10.1	C20H18O6	Acanthotoxin
471.3482	20.1	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
269.082	3.3	C16H14O4	14(5->6)-Abeo-9,13-dihydroxy-1,3,5,9-furanoeremophilatetraen-14-al; 9-Me ether
469.3326	23.5	C30H46O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid; (3?,9?,24E)-form, 22,23-Didehydro (Z-)
423.1819	12.0	C25H28O6	Propolin D isomer
299.0562	4.0	C16H12O6	2-Acetyl-8-hydroxynaphtho[2,3-b]furan-4,9-dione; 7-Methoxy, Me ether
331.0826	4.3	C17H16O7	Abruquinone A; (S)-form, 3',6-Bis(demethoxy), 6',8-dihydroxy
437.1608	14.9	C25H26O7	Oxypropolin D isomer
515.3383	23.6	C31H48O6	Cabraleahydroxylactone; 17?-Acetoxy, 3-Ac
285.0769	3.9	C16H14O5	2-Acetyl-1,8-naphthalenediol; Di-Ac
437.1608	12.4	C25H26O7	Oxypropolin D isomer
503.2079	22.3	C30H32O7	Artelastin; (?)-form, 5'-Hydroxy
479.2441	5.8	C29H36O6	4,18:8,13-Diepoxy-6-hydroxy-15,16-clerodanolide; (ent-4?,6?,8?,13R)-form, 6-E-Cinnamoyl
517.3541	20.1	C31H50O6	Cholest-7-ene-3,4,6,22-tetrol Di-Ac
421.1661	17.0	C25H26O6	Macarangin C isomer
151.0399	3.1	C8H8O3	6-Acetyl-3-methyl-2H-pyran-2-one
355.1189	4.8	C20H20O6	Abyssinone A; 1",2"-Dihydro, 2"?-hydroxy
301.0717	4.4	C16H14O6	5-Acetyl-3,4-dihydro-6,8-dihydroxy-3-(5-oxo-1,3-pentadienyl)-1H-2-benzopyran-1-one
369.1345	9.0	C21H22O6	14(5->6)-Abeo-2,3-epoxy-9,14-dihydroxy-5,9-furanoeremophiladien-1-one; (2?,3?)-form, 9-Me ether, 14-angeloyl
325.1083	14.7	C19H18O5	Ailanthoidol

601.3548	32.0	C38H50O6	Coccinone A
287.0925	4.5	C16H16O5	14(5->6)-Abeo-1,2,6-trihydroxy-1,3,5(10)-furanoeremophilatrien-9-one; 1-Me ether
379.1559	22.6	C23H24O5	6-Benzoyl-5-hydroxy-2,2,8,8-tetramethyl-2H,8H-benzo[1,2-b:3,4-b']dipyran; 9,10-Dihydro, 9-hydroxy
273.0771	4.5	C15H14O5	14(5->6)-Abeo-2,3-epoxy-9,14-dihydroxy-5,9-furanoeremophiladien-1-one
479.2079	16.8	C28H32O7	Bannaxanthone H
423.1819	11.7	C25H28O6	Propolin D isomer
481.3332	33.1	C31H46O4	Adlupone
399.2544	25.4	C25H36O4	Actinopyrones; Actinopyrone A
379.1561	19.5	C23H24O5	6-Benzoyl-5-hydroxy-2,2,8,8-tetramethyl-2H,8H-benzo[1,2-b:3,4-b']dipyran; 9,10-Dihydro, 9-hydroxy
435.1452	14.9	C25H24O7	Prenylated flavonoid

Table 5.3: Fifty most abundant compounds in a Ghanian propolis sample.

Propolis from Cameroon

Cameroon Sample 1

This Cameroon sample appears to be similar to one studied previously in that it contains an abundance of triterpenes (figure 5.9) (Kardar *et al*, 2014). In addition it also contains guttiferone which has been observed to occur in Brazilian red propolis and also macarangin.

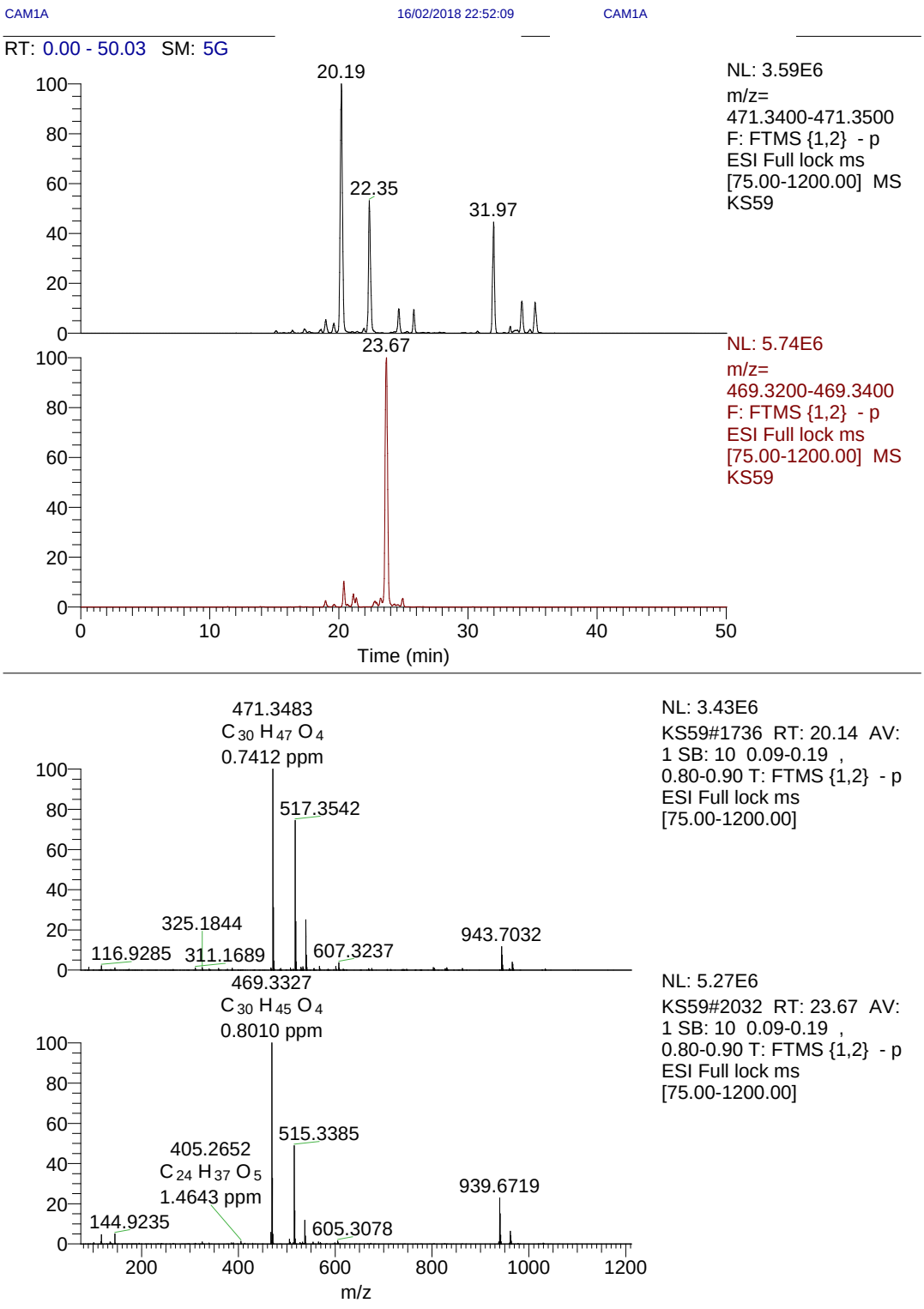


Figure 5.8: Abundant triterpenes in Cameroon propolis sample 1.

row m/z	row retention time	Molecular formula	Name
413.2703	27.1	C26H38O4	Actinopyrones; Actinopyrone C
373.2754	37.2	C24H38O3	Dietrichequinone
471.3482	20.1	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
471.3488	31.8	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
469.3326	23.5	C30H46O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid; (3?,9?,24E)-form, 22,23-Didehydro (Z-)
583.4016	39.3	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
481.3332	33.1	C31H46O4	Adlupone
669.4176	36.7	C43H58O6	Bronianone
401.3066	43.5	C26H42O3	12,16-Dihydroxy-20,24-dimethyl-25-nor-24-scalaranone; (12?,16?)-form, 12-Ketone
515.3381	23.1	C31H48O6	Cabraleahydroxylactone; 17?-Acetoxy, 3-Ac
517.3541	20.1	C31H50O6	Cholest-7-ene-3,4,6,22-tetrol; (3?,4?,5?,6?,22R)-form, 4,6-Di-Ac
515.3383	23.6	C31H48O6	Cabraleahydroxylactone; 17?-Acetoxy, 3-Ac
345.244	32.5	C22H34O3	8,11,13-Abietatriene-12,18-diol; 18-Aldehyde, di-Me acetal
333.1355	9.3	C18H22O6	6-Acetyl-2,3-dihydro-3,5-dihydroxy-2-isopropenylbenzofuran; (2R*,3R*)-form, 2'-Hydroxy, 2'-O-(3-methylbutanoyl)
375.291	44.8	C24H40O3	Amadannulen
533.4011	35.5	C36H54O3	Estrone; 3-O-(9Z-Octadecenoyl)
471.3483	22.3	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
483.3122	30.4	C30H44O5	4(3->2)-Abeo-2-hydroxy-4,7-dioxoergosta-5,22-dien-3-oic acid; (2?,22E,24R)-form, Et ester
583.4016	39.7	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
517.3539	22.3	C31H50O6	Cholest-7-ene-3,4,6,22-tetrol; (3?,4?,5?,6?,22R)-form, 4,6-Di-Ac
399.2544	25.4	C25H36O4	Actinopyrones; Actinopyrone A
343.0826	17.2	C18H16O7	Aflatoxin B1; 8,9-Dihydro, 8?-methoxy
379.1563	16.0	C23H24O5	6-Benzoyl-5-hydroxy-2,2,8,8-tetramethyl-2H,8H-benzo[1,2-b:3,4-b']dipyran; 9,10-Dihydro, 9-hydroxy
421.1659	11.8	C25H26O6	Macarangin
369.244	30.0	C24H34O3	Antibiotic ICM 0301A
555.37	35.8	C34H52O6	Colletochin; O-(2R-Hydroxy-3S-methylpentanoyl)
389.2701	24.0	C24H38O4	Aculeatin F
469.2965	29.1	C29H42O5	Antheridiol
601.3548	32.0	C38H50O6	Coccinone A
371.2598	33.1	C24H36O3	2-Alkyl-5-hydroxy-4H-1-benzopyran-4-ones; 5-Hydroxy-2-pentadecyl-4H-1-benzopyran-4-one
405.2649	15.9	C24H38O5	8(14)-Abietene-7,13,18-triol; (7?,13?)-form, 7,18-Di-Ac
583.4016	39.9	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
407.2805	17.6	C24H40O5	Aculeatol A
379.1563	15.7	C23H24O5	6-Benzoyl-5-hydroxy-2,2,8,8-tetramethyl-2H,8H-benzo[1,2-b:3,4-b']dipyran; 9,10-Dihydro, 9-hydroxy
569.3859	37.6	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
483.3483	21.4	C31H48O4	4(3->2)-Abeo-4-hydroxy-2-oxostigmasta-5,24(28)-dien-3-oic acid; Et ester
531.2756	22.6	C33H40O6	Biyouxanthone C
401.3067	43.7	C26H42O3	12,16-Dihydroxy-20,24-dimethyl-25-nor-24-scalaranone; (12?,16?)-form, 12-Ketone

483.3125	36.9	C30H44O5	4(3->2)-Abeo-2-hydroxy-4,7-dioxoergosta-5,22-dien-3-oic acid; (2?,22E,24R)-form, Et ester
469.2968	28.6	C29H42O5	Antheridiol
429.2649	24.8	C26H38O5	10-Aromadendranol; (1?,4?,5?,6?,7?,10?)-form, O-[4-O-Angeloyl-D-threo-hex-1-enopyranosid-3-uloside]
653.4223	38.7	C43H58O5	Guttiferone I; 5,6-Diepimer, 4"-deoxy
569.3859	36.4	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
469.3326	22.7	C30H46O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid; (3?,9?,24E)-form, 22,23-Didehydro (Z-)
569.3858	37.9	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
471.3488	34.0	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
487.3433	15.6	C30H48O5	Aculeolic acid
483.3482	21.1	C31H48O4	4(3->2)-Abeo-4-hydroxy-2-oxostigmasta-5,24(28)-dien-3-oic acid; Et ester
603.4434	42.6	C40H60O4	Acritopappuslactone A
359.2595	15.8	C23H36O3	8,11,13-Abietatriene-7,11,12-triol; 7?-form, 7-Et, 12-Me ether

Table 5.4: Fifty most abundant compounds in Cameroon propolis sample 1.

Propolis from Cameroon 2

Propolis sample 2 from Cameroon is similar to sample 1 since the most abundant compounds present are triterpenes (figure 5.10). Previous studies have isolated triterpenes, styrenes, alkyl phenol and isoprenylated benzoquinones from Cameroon propolis (Kardar *et al*, 2014; Al Mutairi *et al*, 2014).

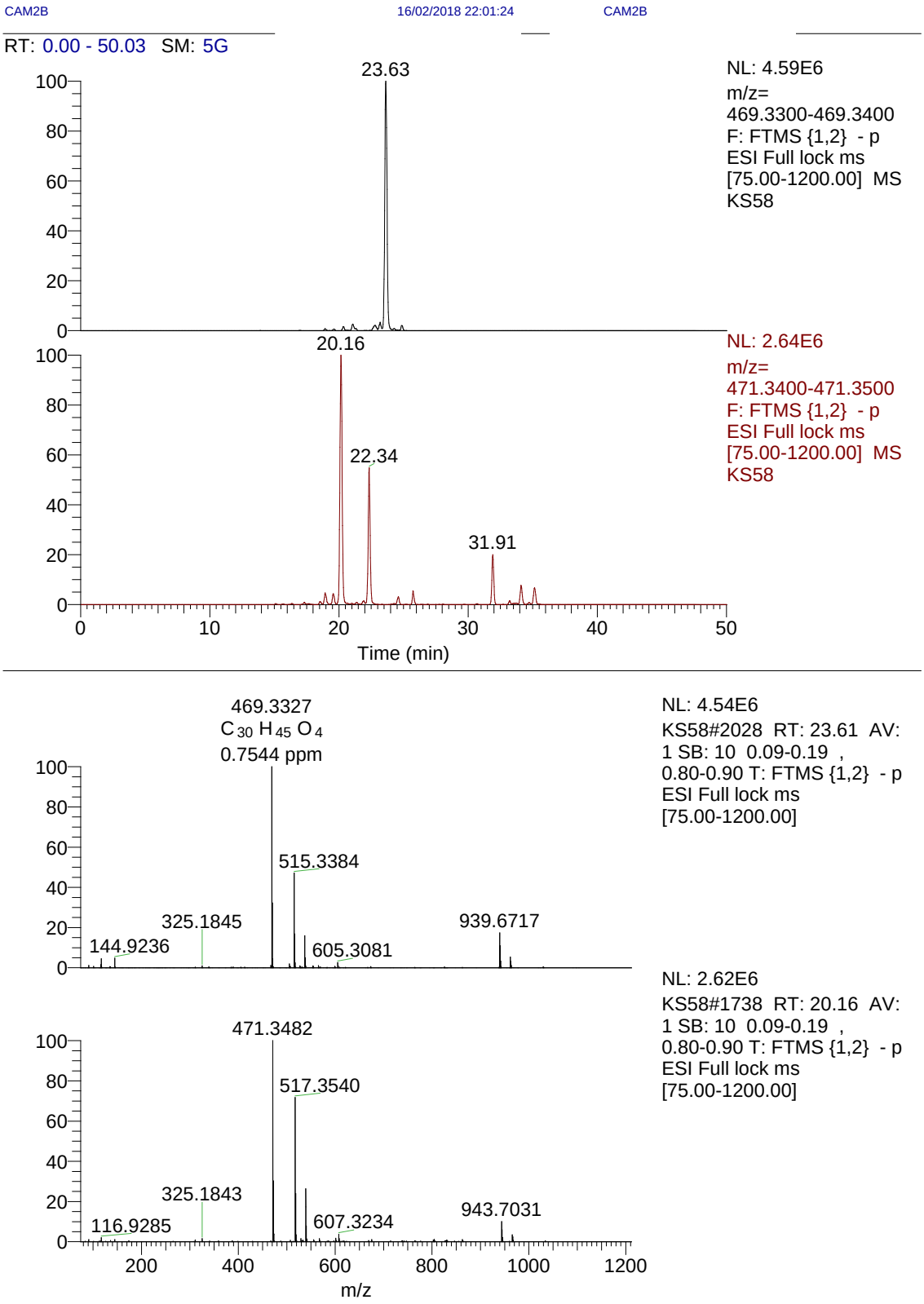


Figure 5.9: Abundant diterpenes isolated from Cameroon propolis sample 2.

row m/z	row retention time	Molecular formula	Name
469.3326	23.5	C30H46O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid; (3?,9?,24E)-form, 22,23-Didehydro (Z-)
669.4176	36.7	C43H58O6	Bronianone
481.3332	33.1	C31H46O4	Adlupone
583.4016	39.3	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
471.3482	20.1	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
515.3383	23.6	C31H48O6	Cabraleahydroxylactone; 17?-Acetoxy, 3-Ac
373.2754	37.2	C24H38O3	Dietrichequinone
517.3541	20.1	C31H50O6	Cholest-7-ene-3,4,6,22-tetrol; (3?,4?,5?,6?,22R)-form, 4,6-Di-Ac
569.3859	37.6	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
617.4588	44.4	C41H62O4	Calcaratarin E
401.3066	43.5	C26H42O3	12,16-Dihydroxy-20,24-dimethyl-25-nor-24-scalaranone; (12?,16?)-form, 12-Ketone
471.3483	22.3	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
583.4016	39.7	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
667.402	36.8	C43H56O6	Garciyunnanin B
555.37	35.8	C34H52O6	Colletochin; O-(2R-Hydroxy-3S-methylpentanoyl)
531.2756	23.4	C33H40O6	Biyouxanthone C
517.3539	22.3	C31H50O6	Cholest-7-ene-3,4,6,22-tetrol; (3?,4?,5?,6?,22R)-form, 4,6-Di-Ac
583.4016	39.9	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
539.3366	19.6	C33H48O6	Antibiotic YM 32890A
539.3363	20.1	C33H48O6	Antibiotic YM 32890A
569.3858	37.9	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
413.2706	32.3	C26H38O4	Actinopyrones; Actinopyrone C
633.454	40.1	C41H62O5	Glycerol 1,2-dialkanoates; Glycerol 1-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoate) 2-(6Z,9Z,12Z,15Z-octadecatetraenoate)
481.3331	32.8	C31H46O4	Adlupone
467.3173	31.4	C30H44O4	Anhydrobelachinal
413.2706	33.9	C26H38O4	Actinopyrones; Actinopyrone C
633.4539	40.4	C41H62O5	Glycerol 1,2-dialkanoates; Glycerol 1-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoate) 2-(6Z,9Z,12Z,15Z-octadecatetraenoate)
465.3379	29.6	C31H46O3	Disidein
345.244	32.5	C22H34O3	8,11,13-Abietatriene-12,18-diol; 18-Aldehyde, di-Me acetal
653.4223	38.7	C43H58O5	Guttiferone I; 5,6-Diepimer, 4"-deoxy
137.0242	11.2	C7H6O3	2,3-Dihydroxybenzaldehyde
413.2706	34.8	C26H38O4	Actinopyrones; Actinopyrone C
569.3859	36.4	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
375.291	44.8	C24H40O3	Amadannulen
555.37	33.3	C34H52O6	Colletochin; O-(2R-Hydroxy-3S-methylpentanoyl)
947.5598	35.6	C48H84O18	Dammarane-3,12,20,24,25-pentol; (3?,12?,20S,24R)-form, 3-O-[?-L-Rhamnopyranosyl-(1->2)-[?-L-rhamnopyranosyl-(1->3)]-?-D-glucopyranoside]
433.2597	18.8	C25H38O6	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 1-O-(2-Methylbutanoyl), 9-O-(3-methylbutanoyl)

667.402	36.5	C43H56O6	Garciyunnanin B
539.3362	22.3	C33H48O6	Antibiotic YM 32890A
429.2648	25.4	C26H38O5	10-Aromadendranol; (1?,4?,5?,6?,7?,10?)-form, O-[4-O-Angeloyl-D-threo-hex-1-enopyranosid-3-uloside]
471.3488	31.8	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
407.2805	17.6	C24H40O5	Aculeatol A
379.1563	15.7	C23H24O5	6-Benzoyl-5-hydroxy-2,2,8,8-tetramethyl-2H,8H-benzo[1,2-b:3,4-b']dipyran; 9,10-Dihydro, 9-hydroxy
483.3482	21.1	C31H48O4	4(3->2)-Abeo-4-hydroxy-2-oxostigmasta-5,24(28)-dien-3-oic acid; Et ester
487.3433	16.0	C30H48O5	Aculeolic acid
483.3483	21.4	C31H48O4	4(3->2)-Abeo-4-hydroxy-2-oxostigmasta-5,24(28)-dien-3-oic acid; Et ester
555.37	35.1	C34H52O6	Colletochin; O-(2R-Hydroxy-3S-methylpentanoyl)
171.0121	26.9	C7H8O3S	2,5-Dihydroxy-3-mercaptobenzyl alcohol
145.0868	7.9	C7H14O3	1,2,4-Butanetriol; (S)-form, 1,2-O-Isopropylidene
487.3433	15.6	C30H48O5	Aculeolic acid

Table 5.5: Fifty most abundant compounds in Cameroon propolis sample 2.

Cameroon Sample 3

Cameroon propolis sample3 is similar but not identical to sample 1 with some of the abundant metabolites being triterpenes (figure 5.11). This sample probably comes from quite similar sources to sample 1,

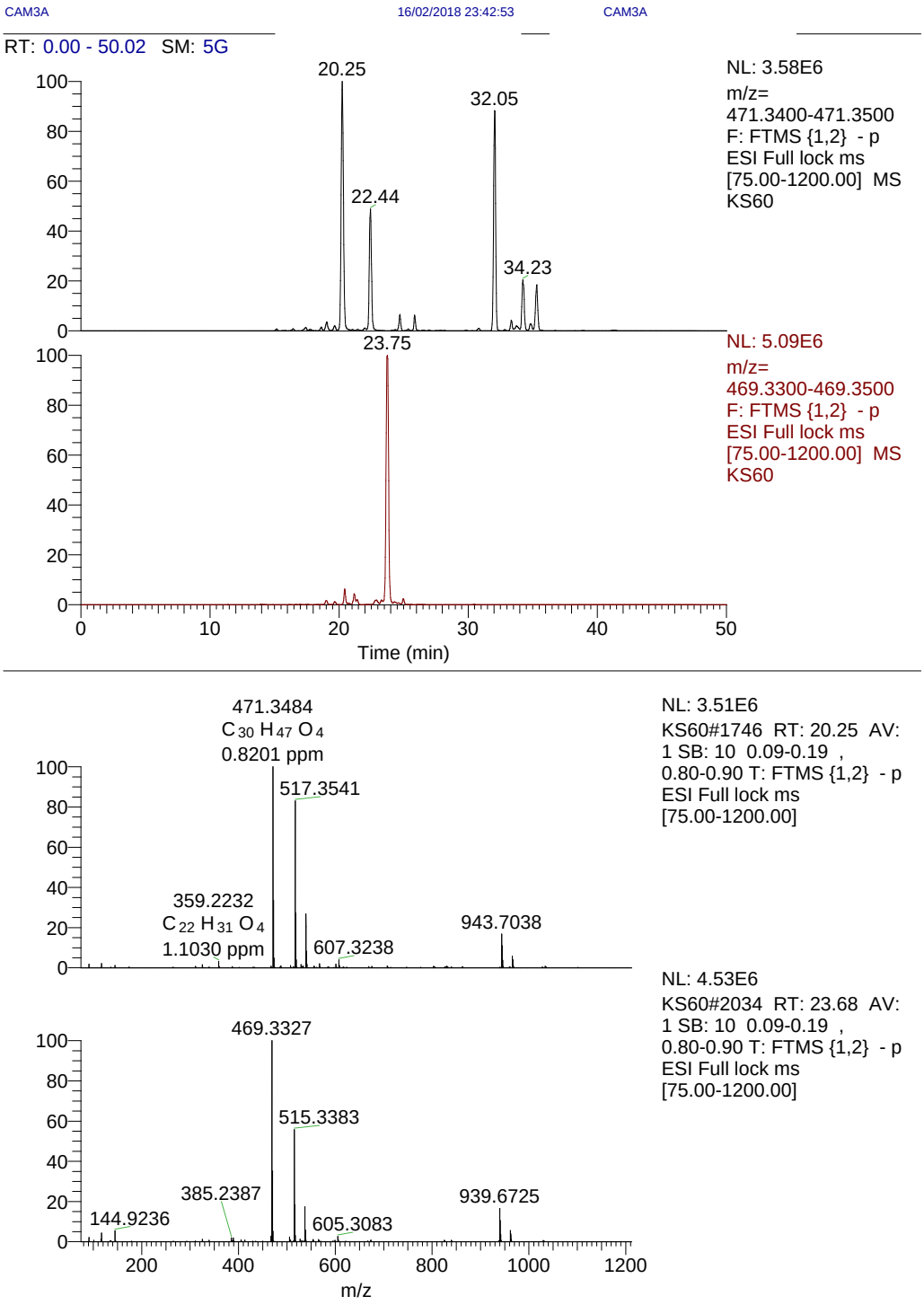


Figure 5.10: Abundant triterpenes in Cameroon propolis sample 3.

row m/z	row retention time	Molecular formula	Name
413.2703	27.1	C26H38O4	Actinopyrones; Actinopyrone C
469.3326	23.5	C30H46O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid; (3?,9?,24E)-form, 22,23-Didehydro (Z-)
669.4176	36.7	C43H58O6	Bronianone
481.3332	33.1	C31H46O4	Adlupone
399.2544	25.4	C25H36O4	Actinopyrones; Actinopyrone A
583.4016	39.3	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
471.3482	20.1	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
515.3383	23.6	C31H48O6	Cabraleahydroxylactone; 17?-Acetoxy, 3-Ac
373.2754	37.2	C24H38O3	Dietrichequinone
517.3541	20.1	C31H50O6	Cholest-7-ene-3,4,6,22-tetrol; (3?,4?,5?,6?,22R)-form, 4,6-Di-Ac
569.3859	37.6	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
617.4588	44.4	C41H62O4	Calcaratarin E
401.3066	43.5	C26H42O3	12,16-Dihydroxy-20,24-dimethyl-25-nor-24-scalaranone; (12?,16?)-form, 12-Ketone
471.3483	22.3	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
583.4016	39.7	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
667.402	36.8	C43H56O6	Garciyunnanin B
555.37	35.8	C34H52O6	Colletochin; O-(2R-Hydroxy-3S-methylpentanoyl)
531.2756	23.4	C33H40O6	Biyouxanthone C
517.3539	22.3	C31H50O6	Cholest-7-ene-3,4,6,22-tetrol; (3?,4?,5?,6?,22R)-form, 4,6-Di-Ac
583.4016	39.9	C36H56O6	14,18-Cyclo-21,23-epoxyapotirucall-25-ene-3,7,21,24-tetrol; (3?,7?,21R,23R,24?)-form, 21-Me ether, 3-O-(3-methyl-2-butenoyl)
539.3366	19.6	C33H48O6	Antibiotic YM 32890A
539.3363	20.1	C33H48O6	Antibiotic YM 32890A
569.3858	37.9	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
413.2706	32.3	C26H38O4	Actinopyrones; Actinopyrone C
633.454	40.1	C41H62O5	Glycerol 1,2-dialkanoates; Glycerol 1-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoate) 2-(6Z,9Z,12Z,15Z-octadecatetraenoate)
481.3331	32.8	C31H46O4	Adlupone
467.3173	31.4	C30H44O4	Anhydrobelachinal
413.2706	33.9	C26H38O4	Actinopyrones; Actinopyrone C
633.4539	40.4	C41H62O5	Glycerol 1,2-dialkanoates; Glycerol 1-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoate) 2-(6Z,9Z,12Z,15Z-octadecatetraenoate)
465.3379	29.6	C31H46O3	Disidein
345.244	32.5	C22H34O3	8,11,13-Abietatriene-12,18-diol; 18-Aldehyde, di-Me acetal
653.4223	38.7	C43H58O5	Guttiferone I; 5,6-Diepimer, 4"-deoxy
137.0242	11.2	C7H6O3	2,3-Dihydroxybenzaldehyde
413.2706	34.8	C26H38O4	Actinopyrones; Actinopyrone C
569.3859	36.4	C35H54O6	3,27-Dihydroxy-20(29)-lupen-28-oic acid; 3?-form, Di-Ac, Me ester
375.291	44.8	C24H40O3	Amadannulen
555.37	33.3	C34H52O6	Colletochin; O-(2R-Hydroxy-3S-methylpentanoyl)
947.5598	35.6	C48H84O18	Dammarane-3,12,20,24,25-pentol; (3?,12?,20S,24R)-form, 3-O-[?-L-Rhamnopyranosyl-(1->2)-[?-L-rhamnopyranosyl-(1->3)]-?-D-glucopyranoside]
433.2597	18.8	C25H38O6	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 1-O-(2-Methylbutanoyl), 9-O-(3-methylbutanoyl)

145.0869	19.6	C7H14O3	1,2,4-Butanetriol; (S)-form, 1,2-O-Isopropylidene
667.402	36.5	C43H56O6	Garciyunnanin B
539.3362	22.3	C33H48O6	Antibiotic YM 32890A
429.2648	25.4	C26H38O5	10-Aromadendranol; (1?,4?,5?,6?,7?,10?)-form, O-[4-O-Angeloyl-D-threo-hex-1-enopyranosid-3-uloside]
471.3488	31.8	C30H48O4	18(13->17)-Abeo-3,9-dihydroxylanosta-12,24-dien-26-oic acid
407.2805	17.6	C24H40O5	Aculeatol A
413.2702	23.7	C26H38O4	Actinopyrones; Actinopyrone C
413.2706	35.4	C26H38O4	Actinopyrones; Actinopyrone C
145.0505	18.9	C6H10O4	3,6-Anhydro-2-deoxyglucose
379.1563	15.7	C23H24O5	6-Benzoyl-5-hydroxy-2,2,8,8-tetramethyl-2H,8H-benzo[1,2-b:3,4-b']dipyran; 9,10-Dihydro, 9-hydroxy
413.2699	12.9	C26H38O4	Actinopyrones; Actinopyrone C

Table 5.6: Fifty most abundant compounds in Cameroon propolis sample 3.

Nigerian Propolis

The Nigerian propolis extract appears to contain several prenylated flavonoids amongst the more abundant compounds and is thus similar to the Ghanian propolis. The most abundant compounds are possibly isoprenylated rather than geranylated flavonoids (figure 5.12) although the genranylated flavonoids macarangin and propolin D are also abundant (figure 5.13).

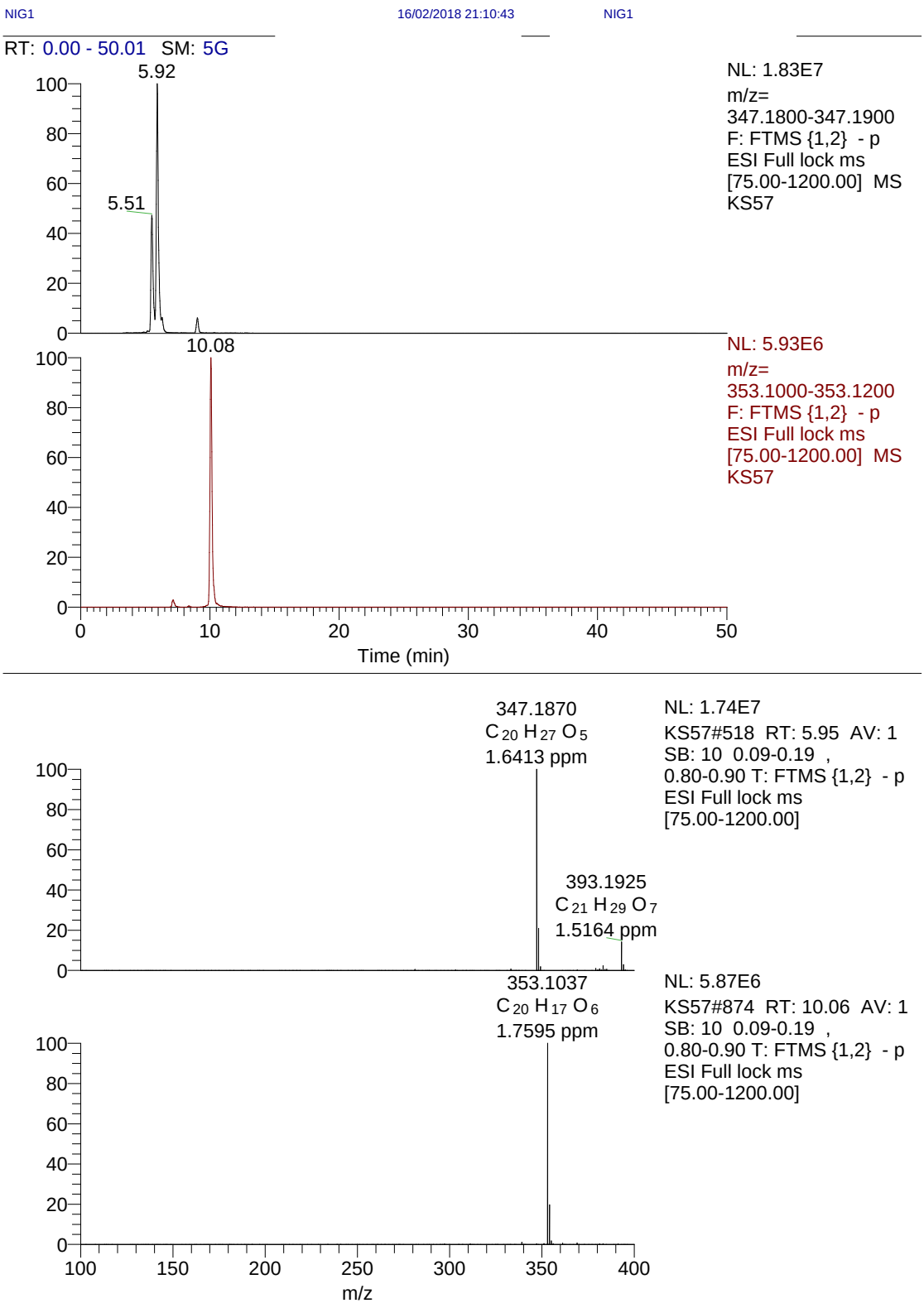


Figure 5.11: Abundant isoprenylated flavonoids in a Nigerian propolis sample.

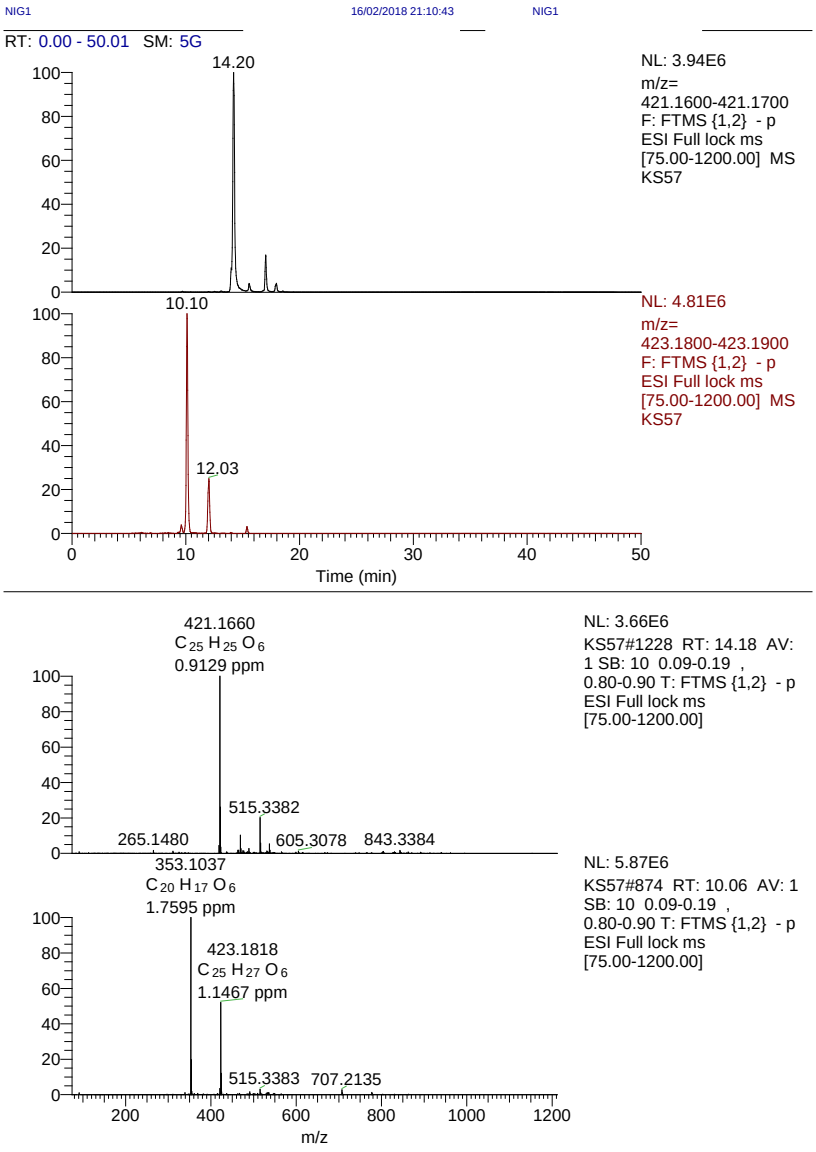


Figure 5.12: Abundant geranlyated flavonoids (macarangin and propolin D) in a Nigerian propolis sample.

row m/z	row retention time	Molecular formula	Name
347.1866	5.9	C20H28O5	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 1-Angeloyl
347.1865	5.5	C20H28O5	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 1-Angeloyl
393.1924	5.5	C21H30O7	Alternaric acid; 10-Deoxy
353.1033	10.1	C20H18O6	Acanthotoxin
421.1661	14.2	C25H26O6	macarangin
413.2703	27.1	C26H38O4	Actinopyrones; Actinopyrone C
423.1818	10.1	C25H28O6	Propolin D
363.1816	4.1	C20H28O6	19(4->3)-Abeo-4,5-epoxy-1,6,7,14-vouacapanetetrol
531.3332	11.1	C31H48O7	Cabraleahydroxylactone; 12?-Acetoxy, 17?-hydroxy, 3-Ac
393.1923	5.9	C21H30O7	Alternaric acid; 10-Deoxy
407.1868	13.5	C25H28O5	Prenylated flavonoid
331.1916	9.5	C20H28O4	19(4->18)-Abeo-16,17-dihydroxy-4(18)-kauren-19,3-olide
367.1187	14.9	C21H20O6	Alpinumisoflavone; 3,4-Dihydro,3?-hydroxy, 4'-Me ether
367.1188	14.5	C21H20O6	Alpinumisoflavone; 3,4-Dihydro,3?-hydroxy, 4'-Me ether
383.114	9.3	C21H20O7	2-Acetyl-3-(3,4-dimethoxyphenyl)-3-hydroxy-2-methyl-5,6-methylenedioxyindenone
381.2286	8.9	C21H34O6	ACTG Toxin A; 2,3-Dihydroxy, 2,3-dihydro
467.3169	16.9	C30H44O4	Anhydrobelachinal
515.3381	14.1	C31H48O6	Cabraleahydroxylactone; 17?-Acetoxy, 3-Ac
423.1819	12.0	C25H28O6	Arugosin A
425.2185	6.3	C22H34O8	Botcinin A
695.3804	5.5	C40H56O10	20(10->9)-Abeo-13,16,17-trihydroxy-15-oxo-19,10-kauranolide; (ent-16?OH)-form, 17-(ent-16?,17-Dihydroxy-15-oxo-19-kauranoyl) ester
551.3598	4.7	C31H52O8	6,13-Epoxy-4,8,9,12-eunicellanetetrol; (4?,6?,8?,9?,12?,13?)-form, 9-Heptanoyl, 4,12-di-Ac
529.3174	10.0	C31H46O7	Amphidinolide A
515.338	11.2	C31H48O6	Cabraleahydroxylactone; 17?-Acetoxy, 3-Ac
347.1865	6.5	C20H28O5	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 1-Angeloyl
565.3386	3.8	C31H50O9	Cholest-7-ene-2,3,5,6,9,11,19-heptol; (2?,3?,5?,6?,11?)-form, 11,19-Di-Ac
347.1858	8.9	C20H28O5	7(8->9)-Abeo-1,9-dihydroxy-11(13)-eremophilen-8,12-olide; (1?,7?,9?)-form, 1-Angeloyl
315.1968	17.6	C20H28O3	16(15->12)-abeo-l-3-oxo-16-nor-8(14)-pimaren-15,16-olide
451.1766	16.0	C26H28O7	Artocarpin; ?3"-Isomer, 2"?-hydroxy
531.3333	15.6	C31H48O7	Cabraleahydroxylactone; 12?-Acetoxy, 17?-hydroxy, 3-Ac
381.2073	5.0	C24H30O4	Ammoresinol
279.0874	7.3	C14H16O6	14(5->6)-Abeo-2,3-epoxy-1,8,9,14-tetrahydroxy-13-nor-5,7,9-eremophilatrien-11-one
333.2073	5.7	C20H30O4	20(10->9)-Abeo-6,16-dihydroxy-19,10-kauranolide
247.1338	7.5	C15H20O3	7(8->9)-Abeo-11,12-epoxy-1(10)-eremophilen-8,12-olide
499.3068	10.3	C30H44O6	Alotaketal B
367.1188	10.7	C21H20O6	Alpinumisoflavone; 3,4-Dihydro,3?-hydroxy, 4'-Me ether
503.3386	9.1	C30H48O6	Alatogenin
363.1817	3.6	C20H28O6	19(4->3)-Abeo-4,5-epoxy-1,6,7,14-vouacapanetetrol
513.3224	17.0	C31H46O6	30(8->9)-Abeo-3,11,16-trihydroxyfusida-7,17(20),24-trien-21-oic acid; (3?,11?,16?,17(20)Z)-form, 16-Ac
339.1239	6.8	C20H20O5	6-Allyl-6-[2-(3,4-dihydroxyphenyl)-1-methylethyl]-3,4-dihydroxy-2,4-cyclohexadien-1-one; Bis(methylene) ether

517.3541	20.1	C31H50O6	Cholest-7-ene-3,4,6,22-tetrol; (3?,4?,5?,6?,22R)-form, 4,6-Di-Ac
379.1561	17.4	C23H24O5	6-Benzoyl-5-hydroxy-2,2,8,8-tetramethyl-2H,8H-benzo[1,2-b:3,4-b']dipyran; 9,10-Dihydro, 9-hydroxy
437.1608	12.4	C25H26O7	Prenylated flavonoid
517.3174	7.2	C30H46O7	Cecropiacic acid
159.0661	4.2	C7H12O4	1-Deoxyxylitol; D-form, 2,4:3,5-Dimethylene
421.1661	17.0	C25H26O6	Macarangin isomer
399.2544	25.4	C25H36O4	Actinopyrones; Actinopyrone A
515.3383	18.7	C31H48O6	Cabraleahydroxylactone; 17?-Acetoxy, 3-Ac
273.1498	13.0	C17H22O3	Algoafuran
669.4176	36.7	C43H58O6	Bronianone

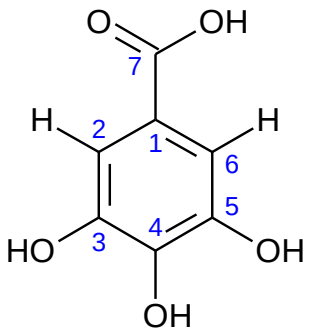
Table 5.7: Fifty most abundant compounds in a Nigerian propolis sample.

Isolation of some compounds from Indonesian propolis sample 1

Some of the compounds in Indonesian sample 1 were isolated and characterized by NMR as described below. The isolated and purified compounds from column chromatography and MPLC were characterized as follows:

Characterisation of fraction GA as Gallic acid (1)

The ^{1}H and ^{13}C NMR (**Table 5.8**) spectrum of the compound showed a signal corresponding to two protons at δ_H 6.92 (2H, *s*) and was attributed to the H-2/6 protons. The carbonyl carbon appeared at δ_C 167.4 (C-7) and three oxygen-bearing quaternary aromatic carbons were observed at δ_C 145.4 (×2) and 137.9 (C-3/5 and C-4, respectively). The structure was further confirmed from its HMBC spectrum which showed ^{3}J correlations from H-2/6 to C-4, C-2/6 and C-7 and ^{2}J correlations to C-1, C-3 and C-5. The spectral data for **GA** were in good agreement with those reported for gallic acid by Owen *et al.*, (2003) and Wang *et al.*, (2007). The high resolution mass spectrum gave an elemental composition $C_7H_5O_5$ in negative ion mode.



Gallic acid (1)

Position	Proton (δ ppm)	Carbon (δ ppm)	HMBC
1	-	120.4	-
2	6.92 (s)	108.7	C-1, C-3, C-4, C-6, C-7
3	-	145.4	-
4	-	137.9	-
5	-	145.4	-
6	6.92 (s)	108.7	C-1, C-2, C-4, C-5, C-7
7	-	167.4	-
3,5-OH	9.16 (s)	-	-
4-OH	8.82 (s)	-	-
-COOH	12.21 (br, s)	-	-

Table 5.8: Chemical shift assignments for gallic acid (1)

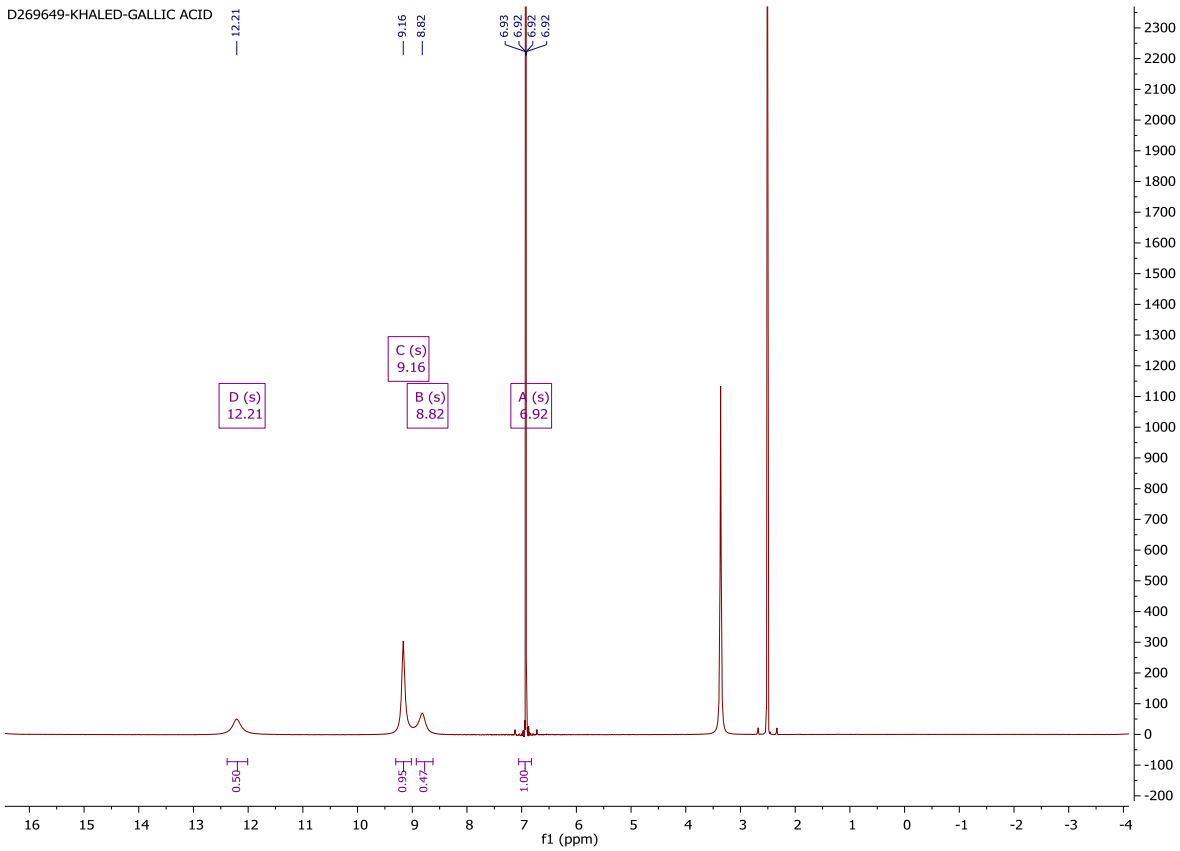


Figure 5.13a: Proton spectrum (DMSO-d6) for gallic acid (1)

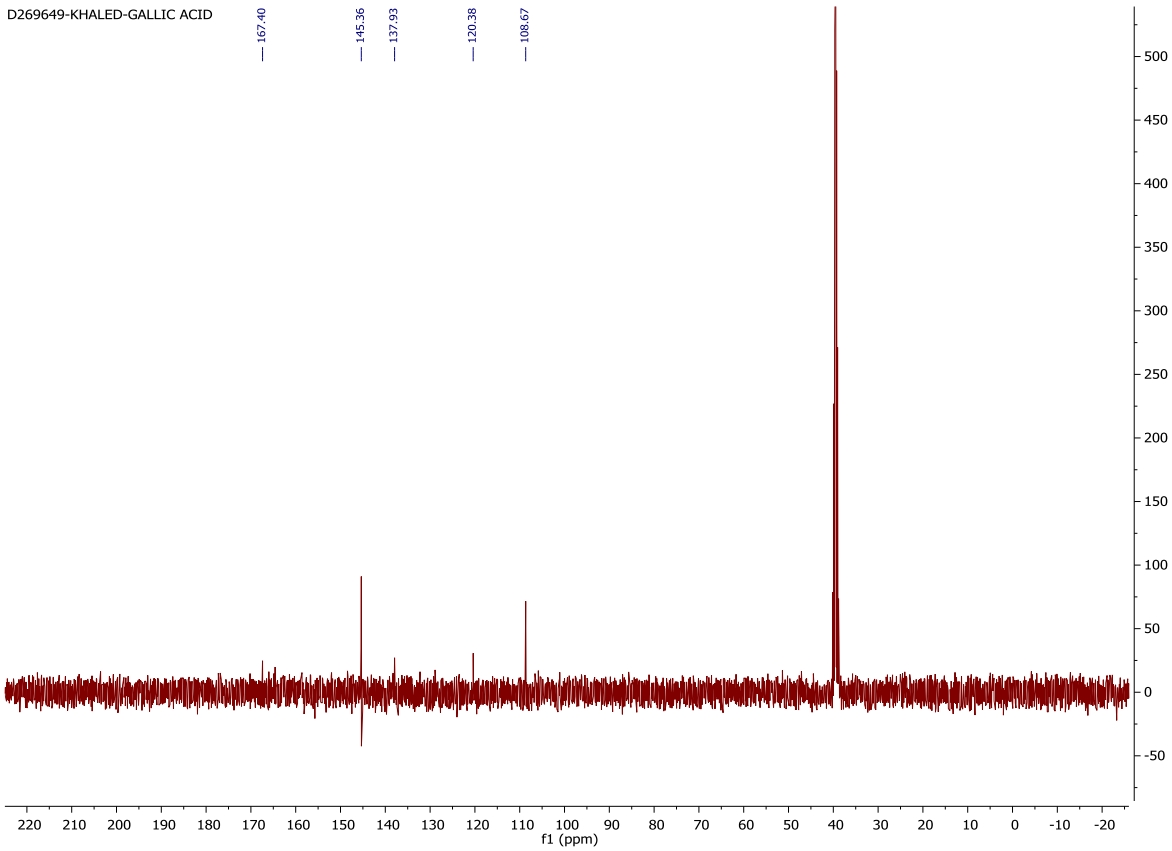


Figure 5.13b: 13Carbon spectrum (DMSO-d6) for gallic acid (1)

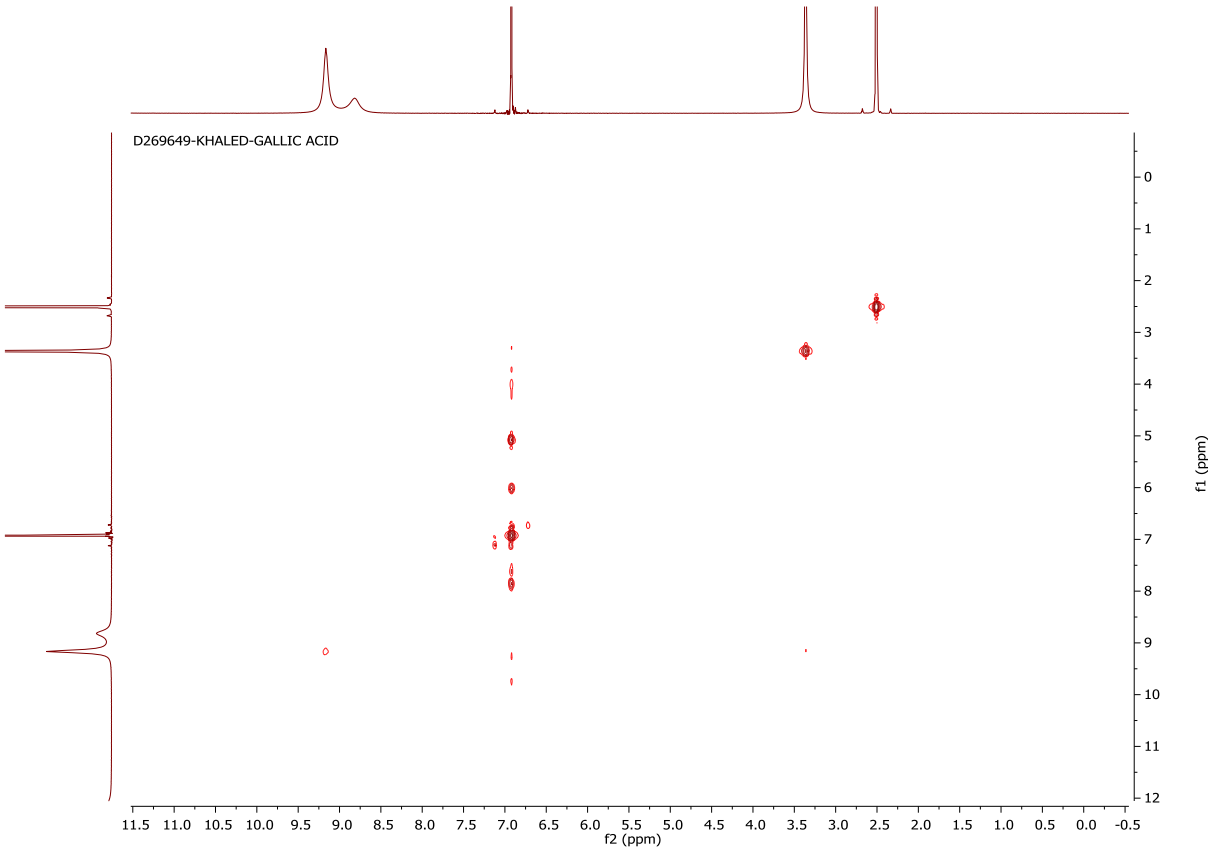


Figure 5.13c: COSY spectrum (DMSO-d6) for gallic acid (1)

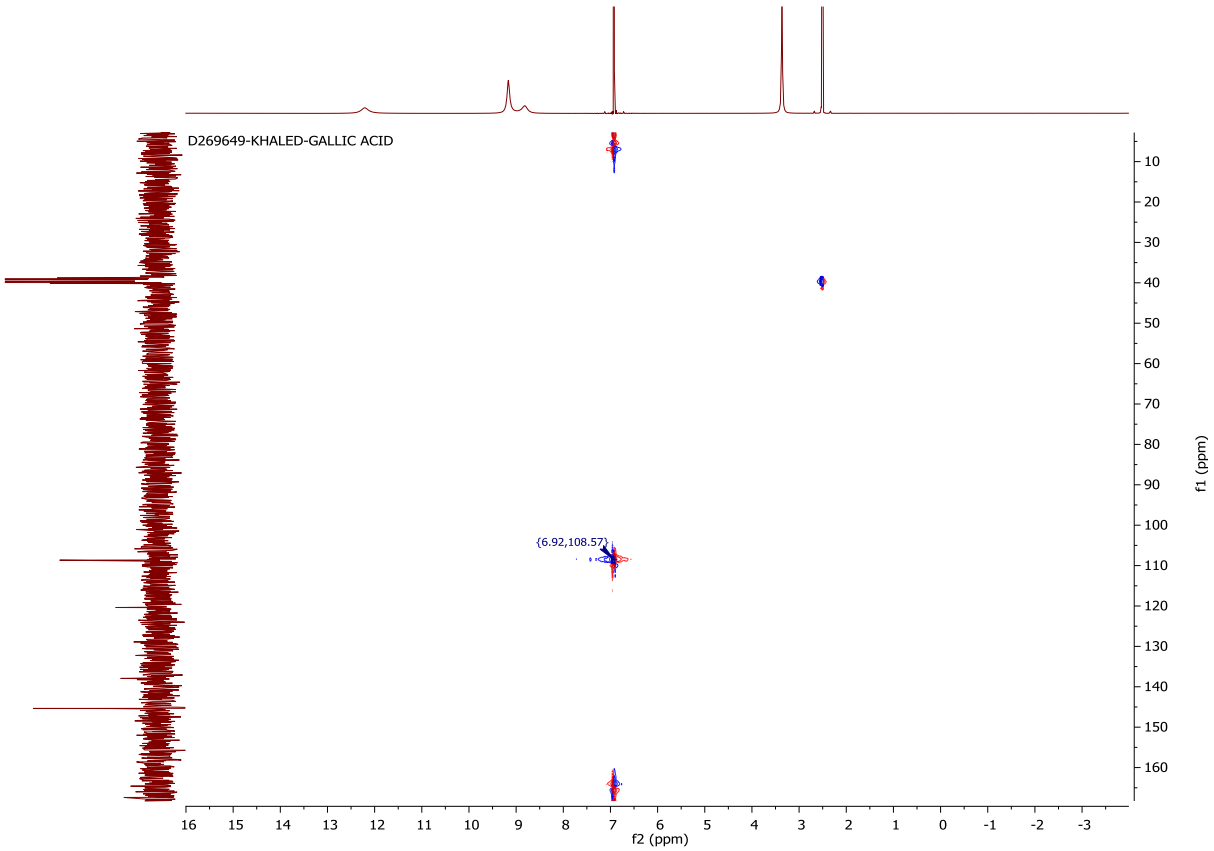


Figure 5.13d: HSQC spectrum (DMSO-d6) for gallic acid (1)

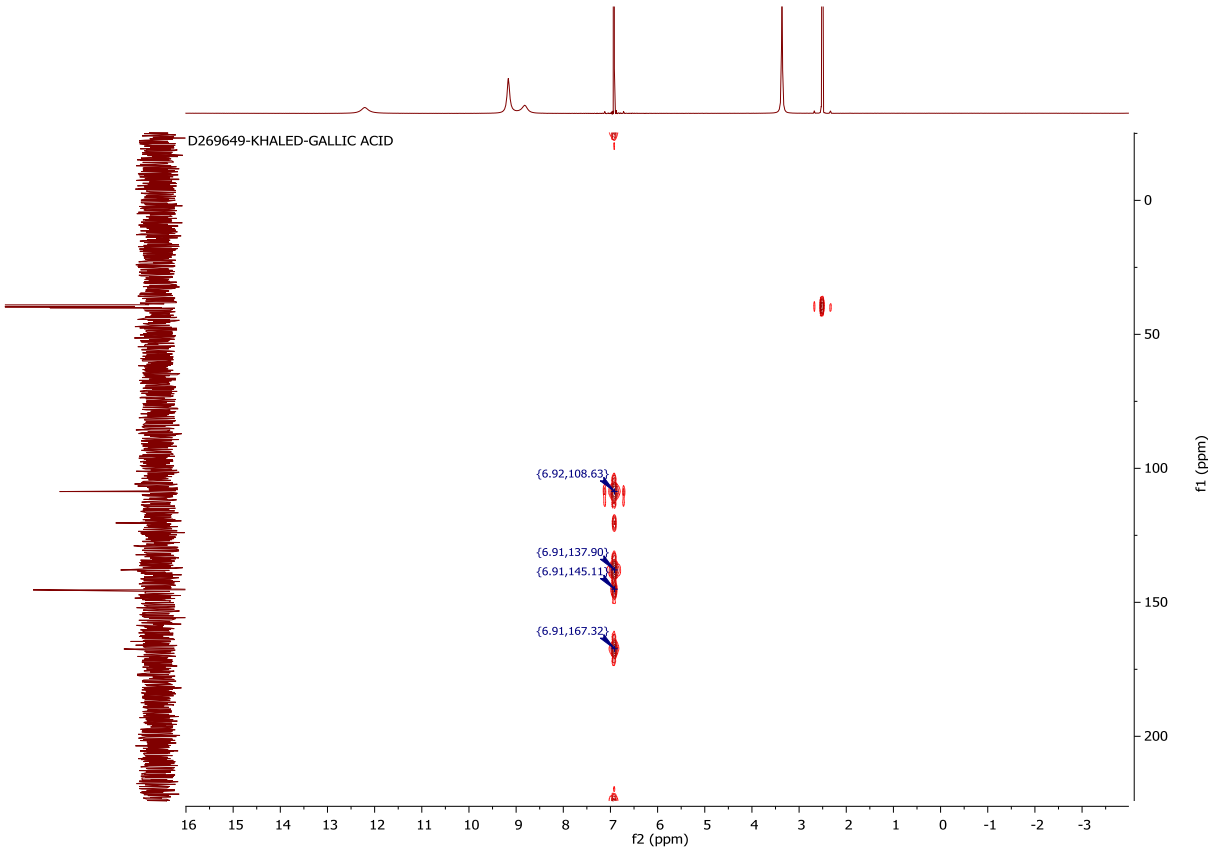


Figure 5.13e: HMBC spectrum (DMSO-d6) for gallic acid (1)

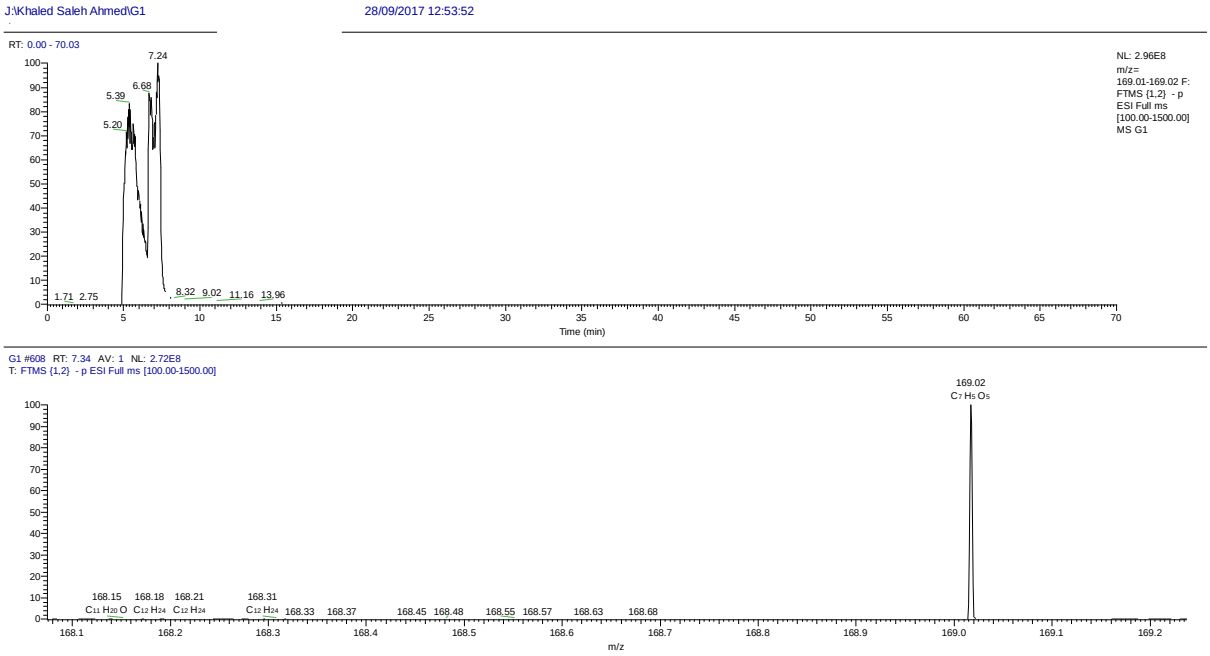
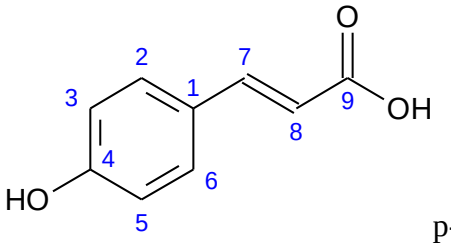


Figure 5.13f: Mass spectrum for gallic acid (1)

Characterization of fraction CO1 as p-Coumaric acid (2)

The ^{1}H-NMR (400 MHz, DMSO-d_6) showed two aromatic doublets at δ_H 6.80 (2H, d, *J* = 8.5, H-3,5) and 7.52 ppm (2H, d, *J* = 8.5, H-2,6). The trans ethylenic protons were observed at 7.49 (1H, d, *J* = 15.9, H-7) and 6.29 ppm (1H, d, *J* = 15.9, H-8). The ^{13}C-NMR spectrum showed a carbonyl carbon (attributed to a carboxylic acid) signal at δ_C 168.38 ppm (C-9), two olefinic carbons at 115.80 and 144.61, two aromatic CH signals at 130.654 (C-2 and C-6), 116.20 (C-3 and C-5) and a quaternary aromatic carbon at 125.73 ppm. The compound was identified as 4-hydroxycinnamic acid (p-coumaric acid) and its NMR spectral data were in agreement with literature reports. (Yi *et al*, 2011; Karthikeyan *et al*. 2015).



p-Coumaric acid (2)

Position	^{1}H (δ ppm)	^{13}C (δ ppm)	HMBC
1	-	125.73	-
2	7.52 (d, *J* = 8.5)	130.54	C-4, C-6, C-7
3	6.80 (d, *J* = 8.5)	116.20	C-1, C-5
4	-	160.04	-
5	6.80 (d, *J* = 8.5)	116.20	C-1, C-3
6	7.52 (d, *J* = 8.5)	130.54	C-2, C-4, C-7
7	7.49 (d, *J* = 15.9)	144.61	C-2, C-6, C-9
8	6.29 (d, *J* = 15.9)	115.80	C-1, C-9
9	-	168.38	-
9-COOH	12.11 (br, s)	-	-
4-OH	9.95 (br, s)	-	-

Table 5.9: 1H (400MHz), 13C (100MHz) assignments for p-coumaric acid

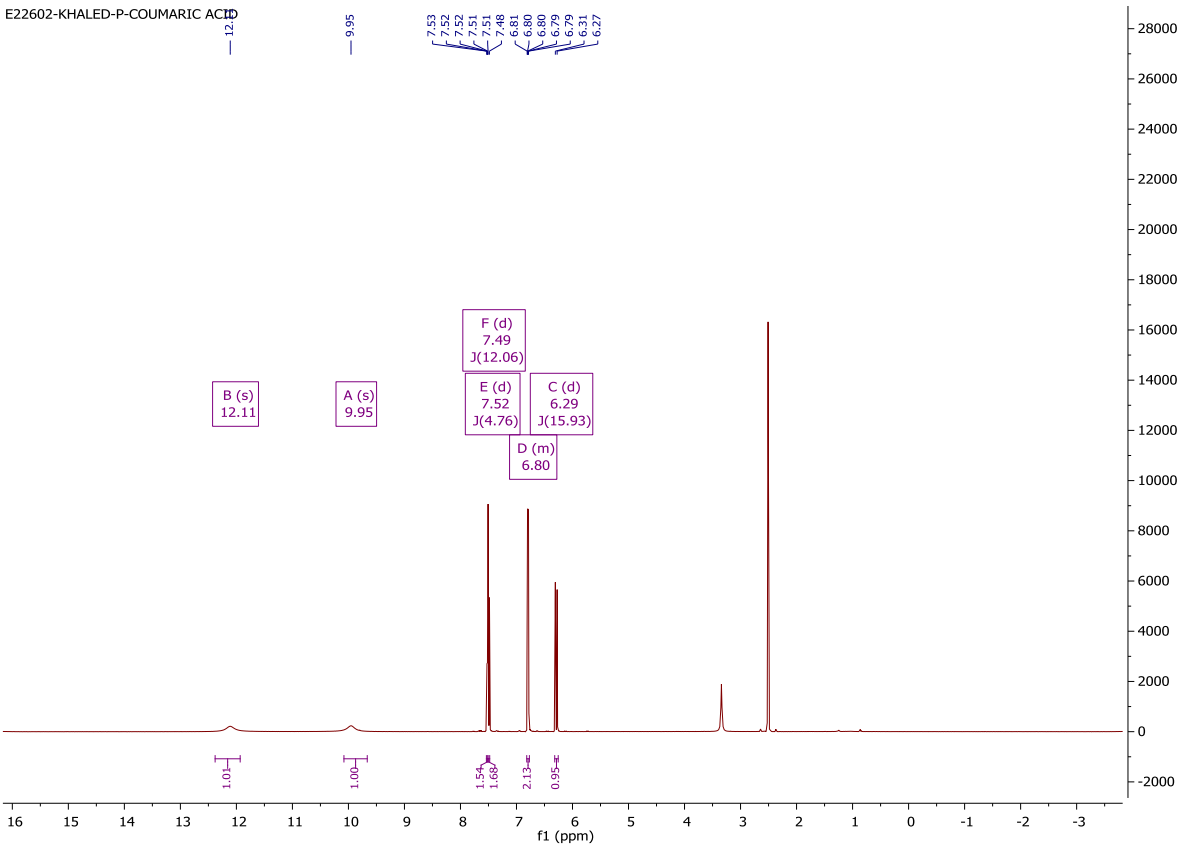


Figure 5.14a: Proton spectrum (DMSO-d6) for p-coumaric acid (2)

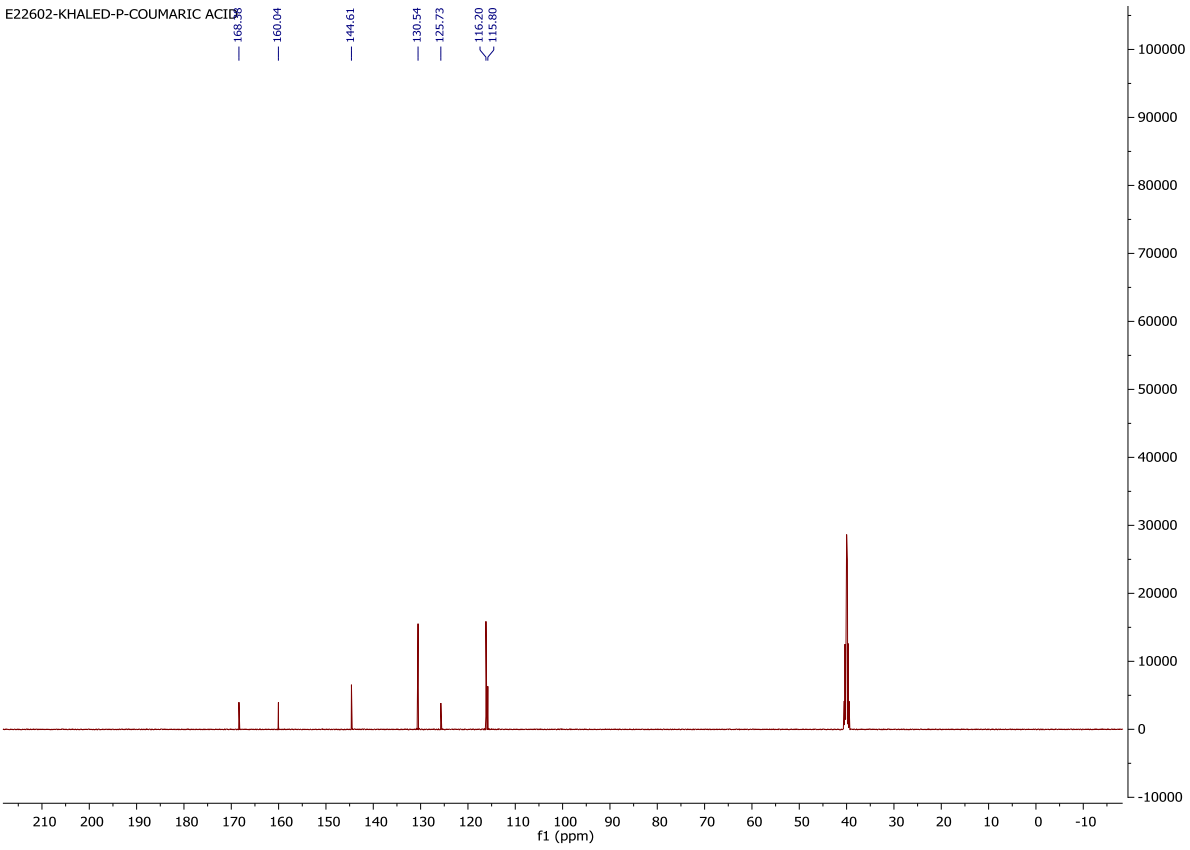


Figure 5.14b: 13Carbon spectrum (DMSO-d6) for p-coumaric acid (2)

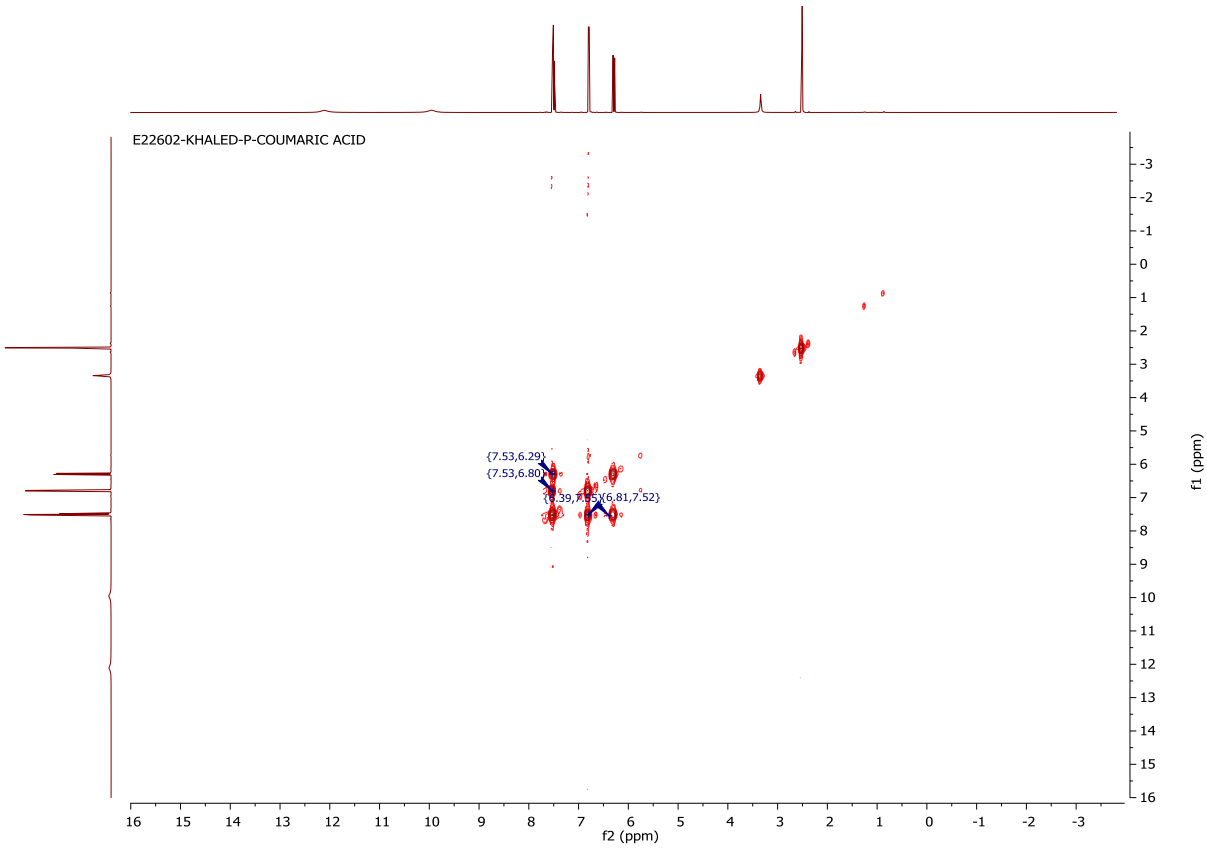


Figure 5.14c: COSY spectrum (DMSO-d6) for p-coumaric acid (2)

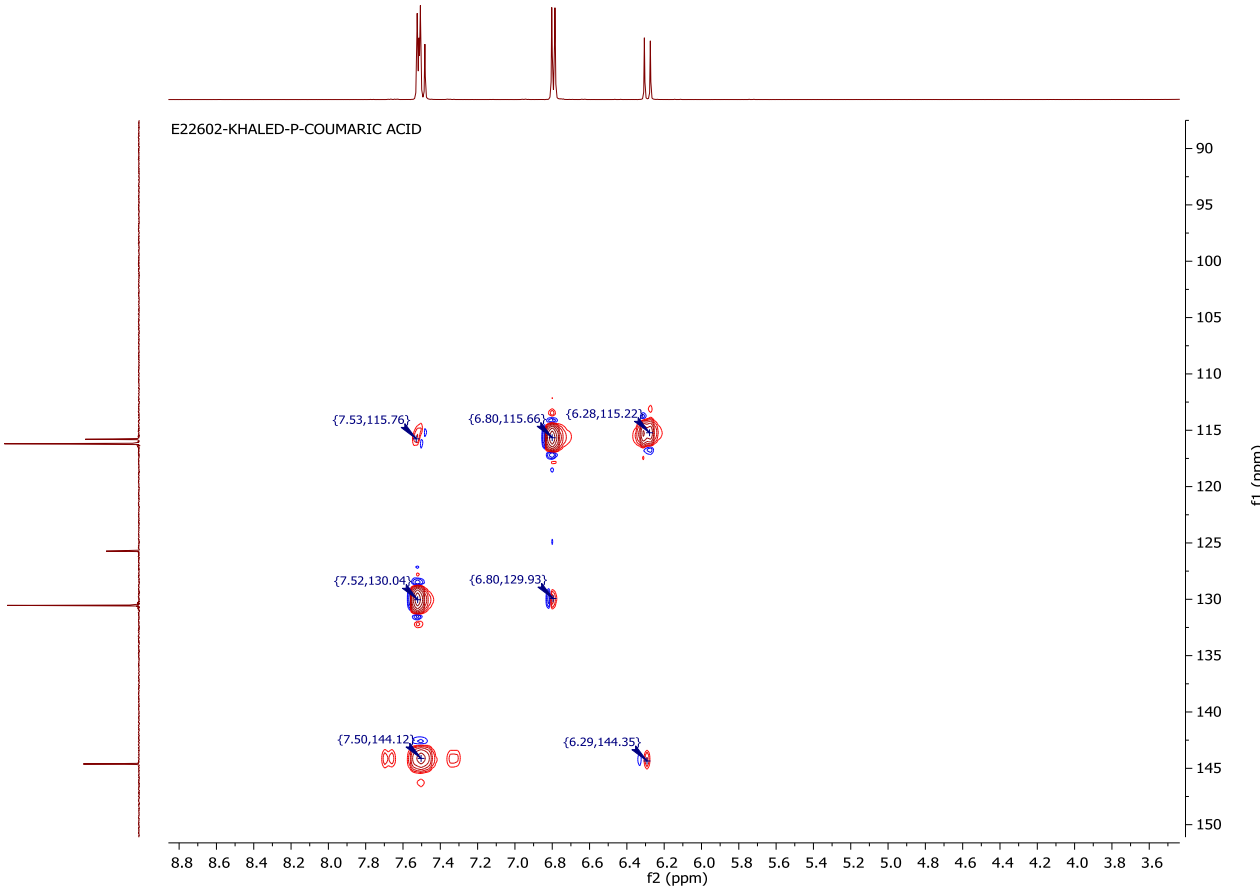


Figure 5.14d: HSQC spectrum (DMSO-d6) for p-coumaric acid (2)

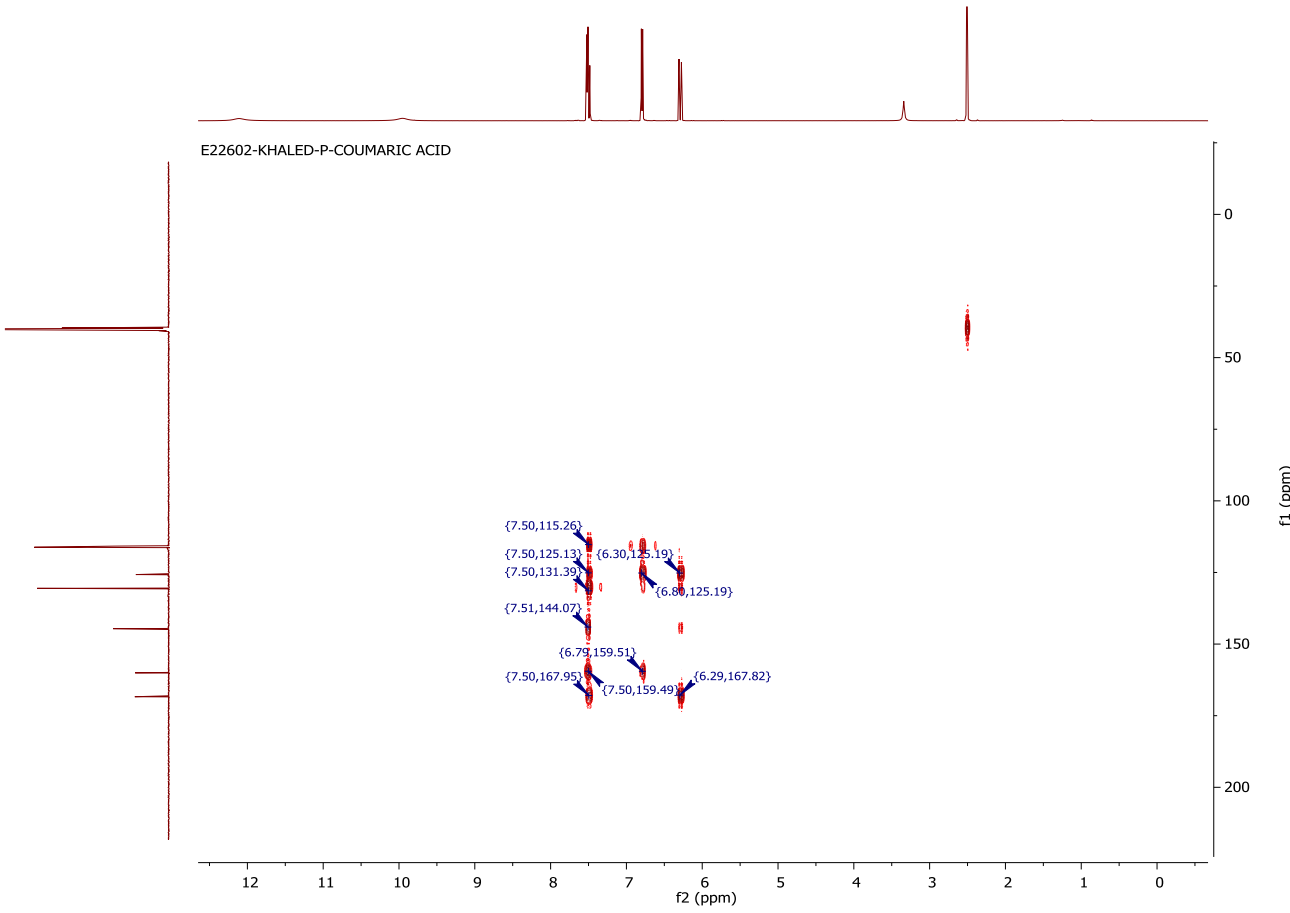


Figure 5.14e: HMBC spectrum (DMSO-d6) for p-coumaric acid (2)

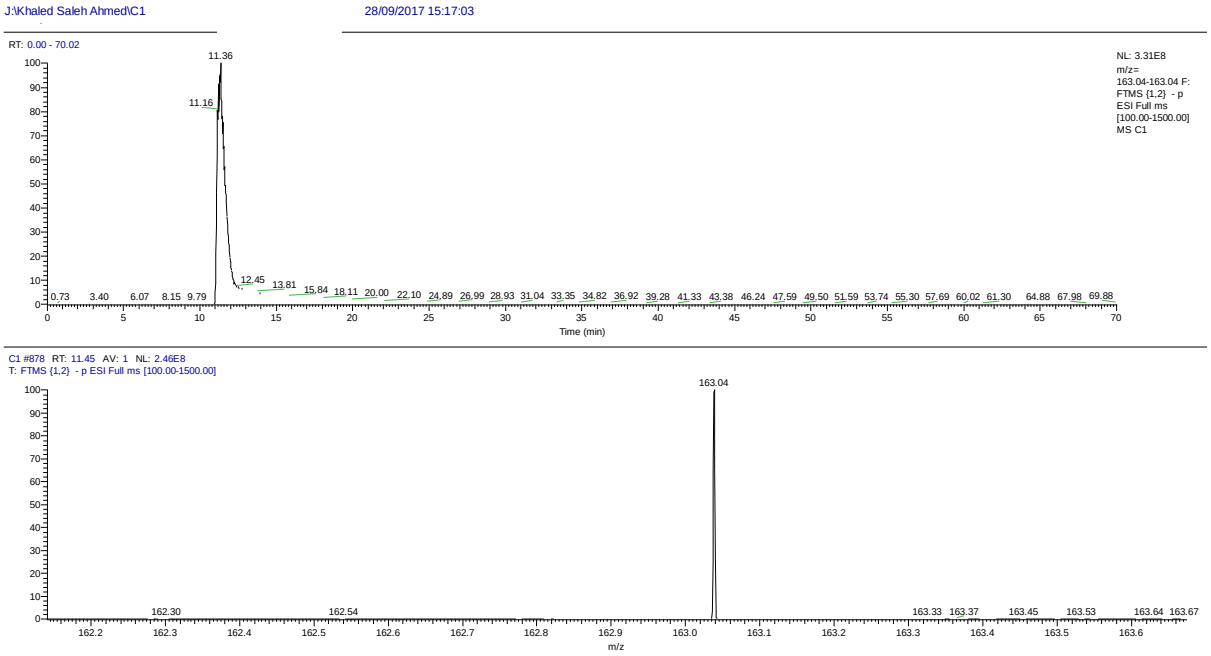
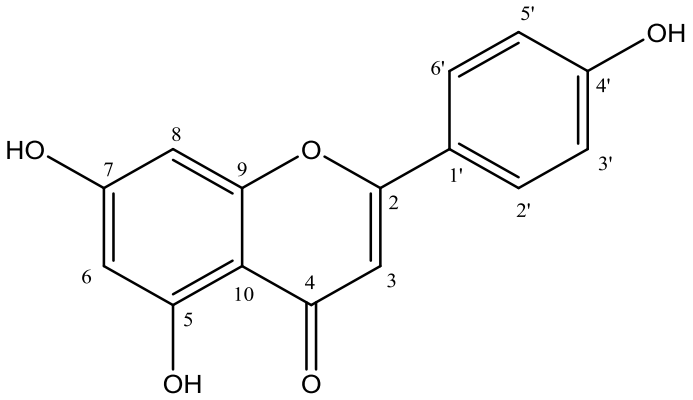


Figure 5.14f: Mass spectrum for p-coumaric acid (2)

Characterization of S22 as Apigenin (3)

The compound in its proton spectrum showed a chelated hydroxyl proton at δ_H 12.81 ppm typical of a 5-OH substituted flavonoid. The presence of two meta coupled protons at δ_H 6.32 and 6.50 completed the ring A substitution as in many 6, 8-dihydroxy substituted flavones. A pair of doublets (integrated for two protons each) indicated a para-substituted benzene ring and this must be on ring B of the flavonoid. The proton singlet observed at δ_H 6.70 is likely from a H-3 of the flavone. From these data, the compound was inferred to be apigenin and from its 2D (COSY, HSQC and HMBC) spectra the structure was confirmed as follows: Long range (HMBC) couplings (^3J and ^2J) from the 5-OH proton identified C-5, C-6 and C-10, while correlations from H-3 identified C-2, C-1' and confirmed C-10. Other correlations and couplings were as expected thus the compound was identified as 4', 5, 7-

trihydroxyflavone, Apigenin and confirmed by comparison of its NMR chemical shift assignments with literature reports (Chaturvedula and Prakash 2013).



Apigenin (3)

Position	Experimental values		Literature values*	
	^{1}H δ ppm	^{13}C δ ppm	^{1}H δ ppm	^{13}C δ ppm
1	-	-	-	-
2	-	163.68	-	164.2
3	6.70 (s)	105.90		106.5
4	-	179.09		180.8
5	-	163.11		164.6
6	6.50 (d, *J* = 2.29 Hz)	94.21		104.8
7	-	162.03		160.0
8	6.32 (d, *J* = 2.23 Hz)	99.48		98.9
9	-	162.40		160.7
10	-	103.82		109.6
1’	-	123.96		123.3
2’	7.92 (d, *J* = 1.47 Hz)	129.11	7.92	129.3
3’	7.54 (d, *J* = 2.37 Hz)	126.94	6.95	117.0
4’	-	164.62		162.6
5’	7.57 (d, *J* = 1.56 Hz)	129.11		117.0
6’	7.90 (d, *J* = 1.86 Hz)	126.32		129.3

Table 5.10: Chemical shift assignments for Apigenin (3)

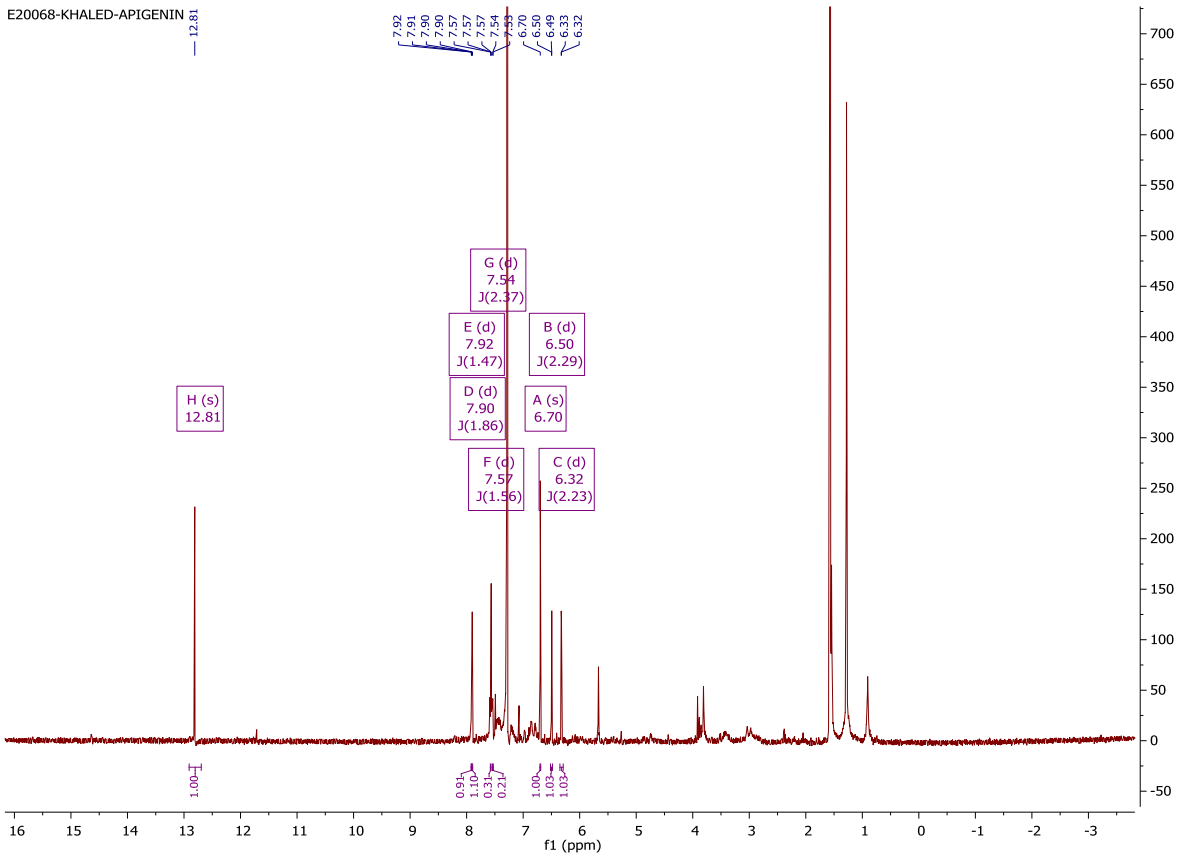


Figure 5.15a: Proton spectrum (CDCl3) for Apigenin (3)

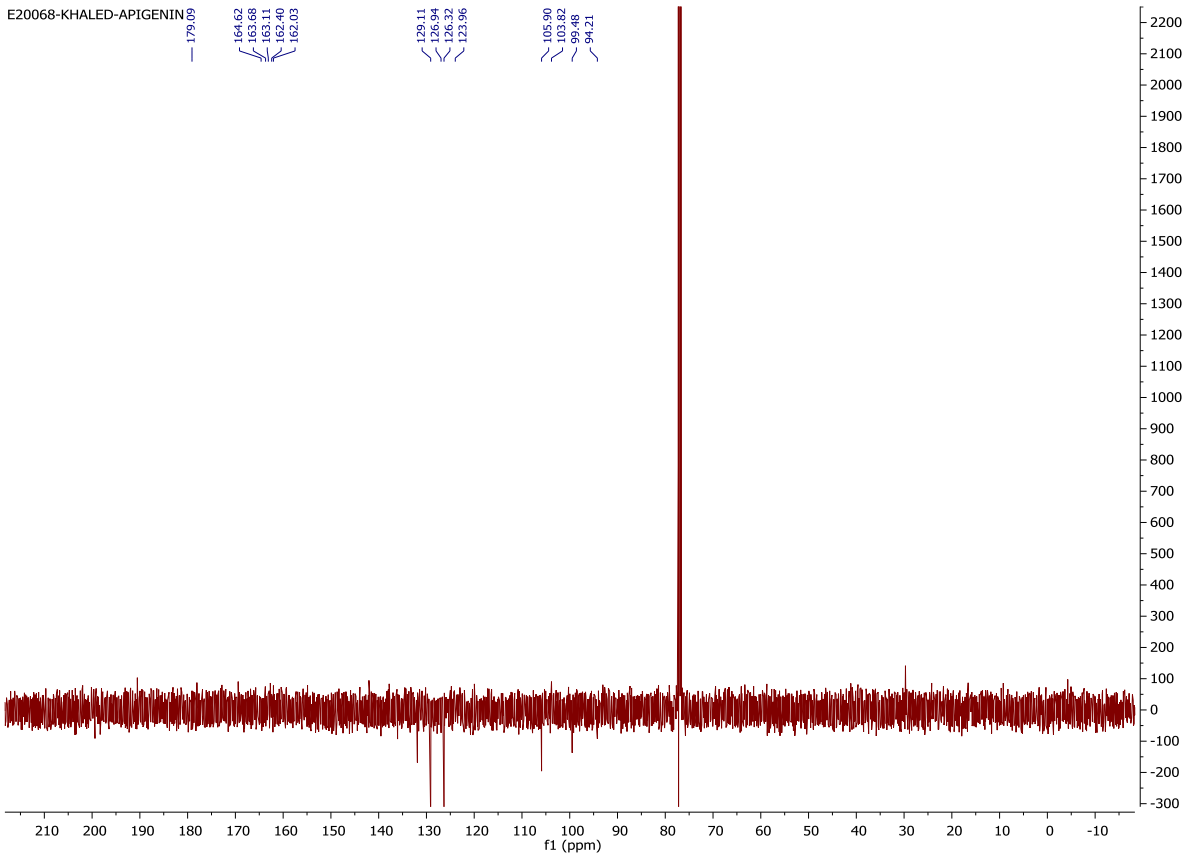


Figure 5.15b: 13Carbon spectrum (CDCl3) for Apigenin (3)

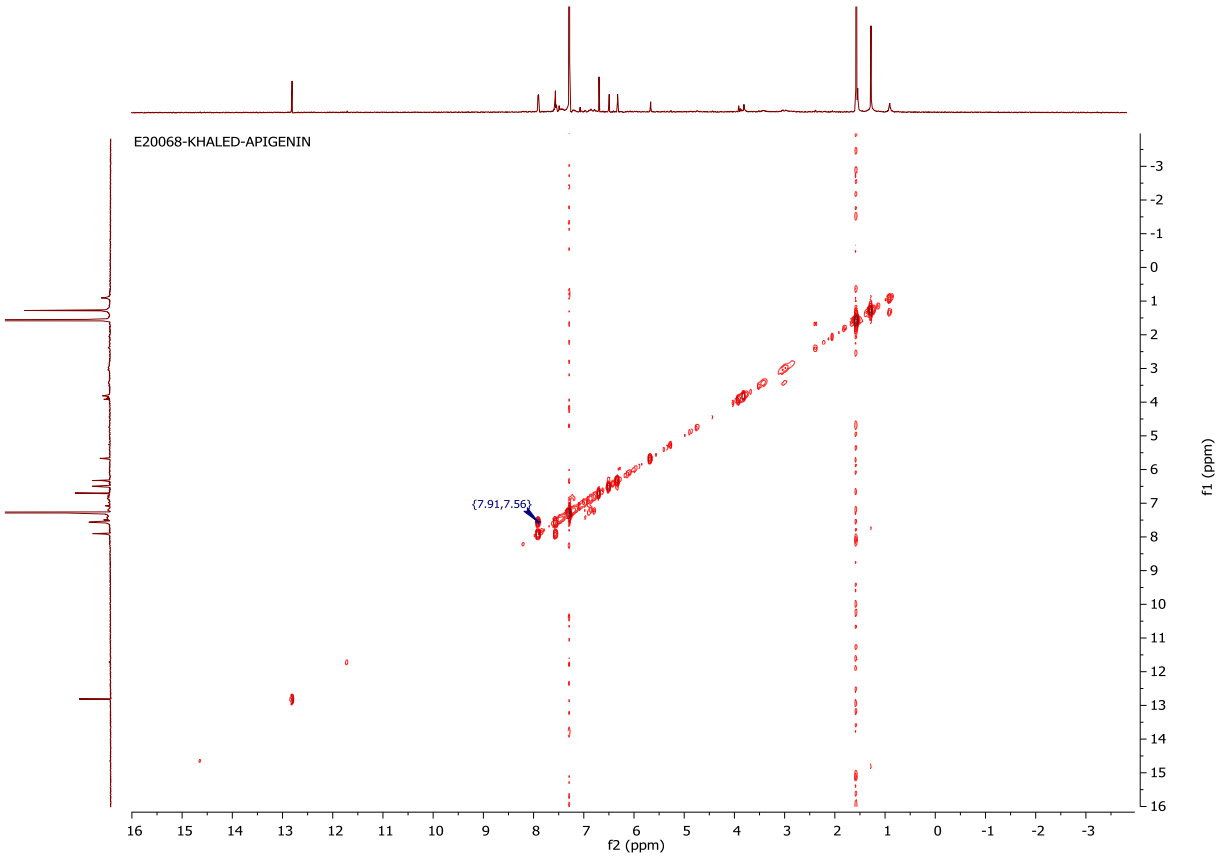


Figure 5.15c: COSY spectrum (CDCl3) for Apigenin(3)

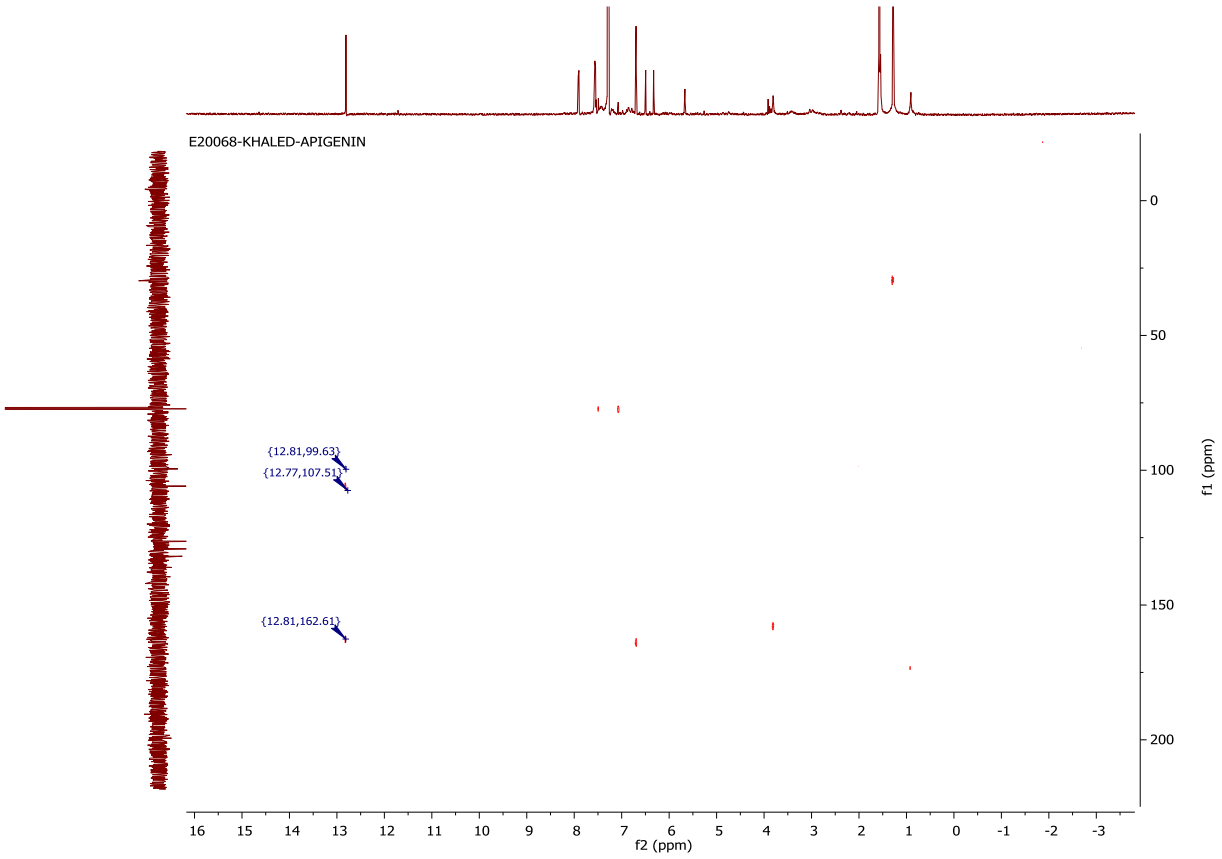


Figure 5.15d: HSQC spectrum (CDCl3) for Apigenin(3)

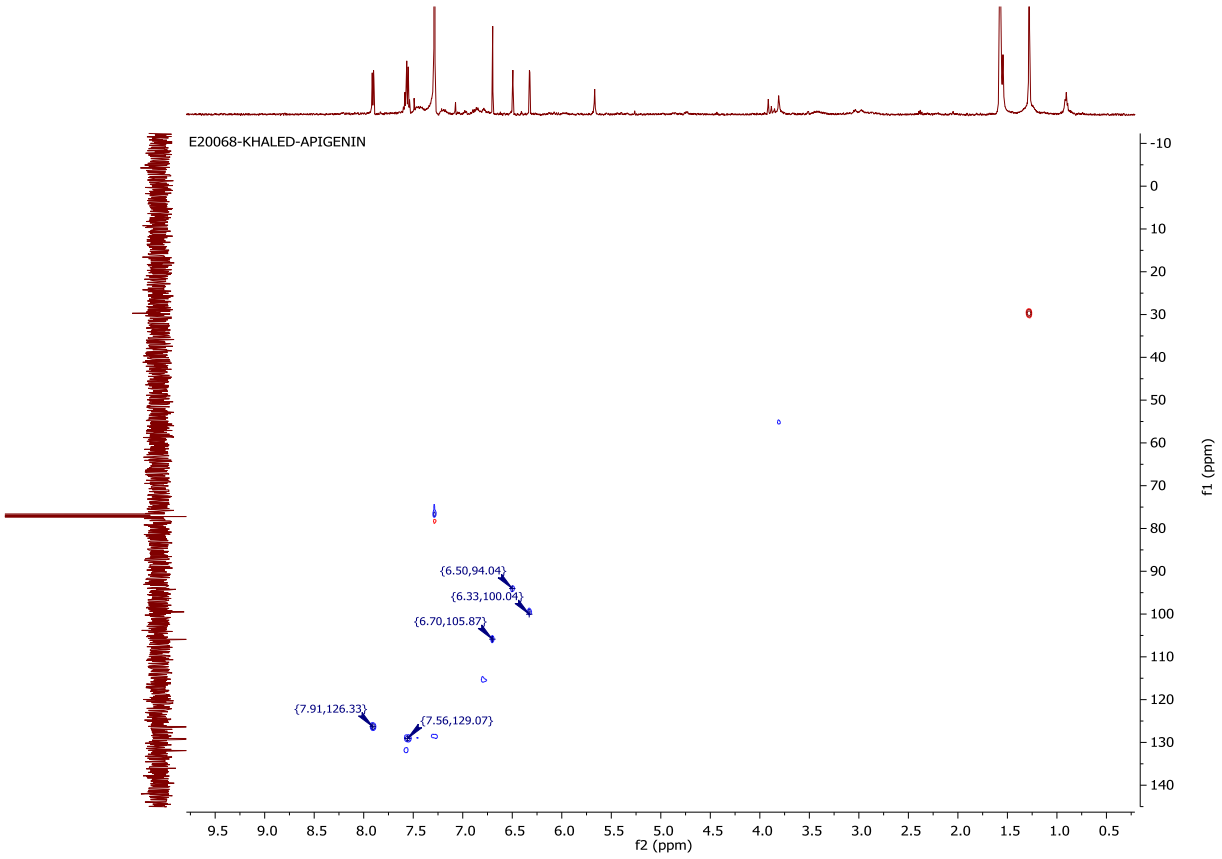


Figure 5.15e: HMBC spectrum (CDCl3) for Apigenin(3)

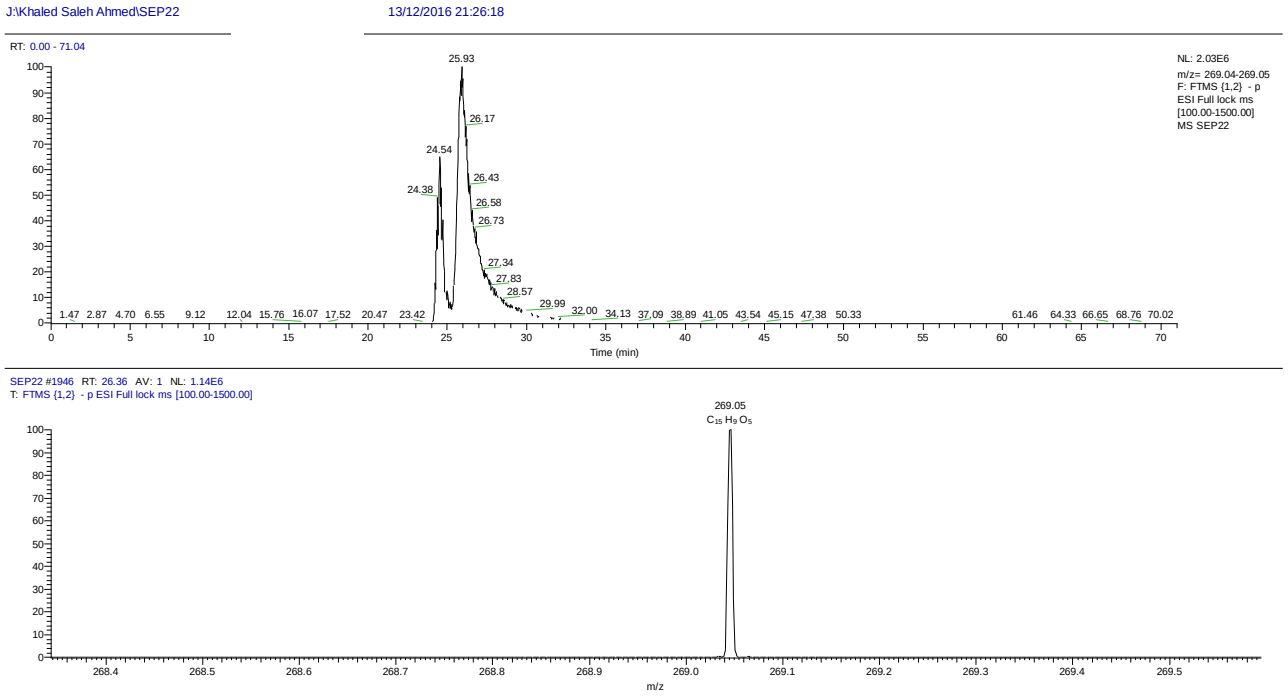


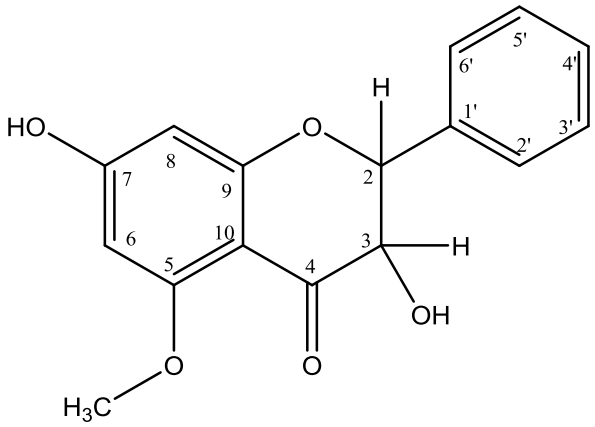
Figure 5.15f: Mass spectrum for Apigenin(3)

The sample from Indonesia did not reveal the compounds earlier identified from samples of Indonesian propolis. None of the resorcinol derivatives were detected or isolated but the flavonoids were obtained. Other compounds which have not been previously isolated from Indonesian propolis samples were also isolated. The compounds isolated were Apigenin (flavonoids), gallic acid (a phenol) and p-coumaric acid (a phenyl propanoid) and their structures comfirmed by NMR and mass spectroscopic analysis. Other compounds were detected but not confirmed.

Characterization of fraction KH- as Pinobanksin-5-methyl ether (4)

This compound was isolated from a sample of UK propolis. The compound in its proton spectrum showed signals for two oxygenated methine doublets at δ_H 4.26 and 5.00 ppm for H-3 and H-2 respectively. Two meta coupled aromatic protons H-6 and H-8 were observed as doublets at δ 5.88 and 6.03 ppm. Three other aromatic proton signals for an unsubstituted benzene ring were observed at δ 7.42 (H-2',6'), 7.33 (H-

3', H-5') and 7.32 ppm (H-4'). A set of methoxy protons was observed at 3.70 ppm. The absence of a hydrogen bonded -OH proton at above 12.0 ppm in it proton spectrum (Figure 5.17a) indicates there was no -OH at position C-5 but possibly a methyl ether. The ^{13}C-NMR showed signals for 16 carbon atoms including one carbonyl at 190.0 (C-4), two aromatic CH at 95.97 (C-6) and 93.97 (C-8) and oxygenated carbons at 82.82 (C-2) and 73.02 (C-3) and one methoxy carbon at 56.20 ppm (5-OCH_3). The rest of the carbons were for an unsubstituted benzene ring and four quarternary aromatic carbons. Analysis of its 2D spectra identified the compound to be Alpinone (a substituted pinocembrine) and the structure was confirmed using literature reports (Papotti *et al*., 2012). The high resolution mass spectrum confirmed the molecular formula for the compound.



3-Hydroxy-5-methoxypinocembrin(4)

Position	Chemical shifts	
	^{1}H δ ppm (mult, *J* Hz)	^{13}C δ ppm
1	-	-
2	5.00 (d, 11.18)	82.82
3	4.26 (d, 11.25)	73.02
4	-	190.06
5	-	165.31
6	5.88 (d, 2.07)	95.97
7	-	162.60
8	6.03 (d, 2.14)	93.97
9	-	164.06
10	-	102.94
1’	-	138.04
2’	7.42	128.35
3’	7.33	128.62
4’	7.32	129.19
5’	7.33	128.62
6’	7.42	128.35
5-OCH_3	3.70 (s)	56.20

Table 5.11: 1H (400MHz), 13C (100MHz) data for 3-hydroxy-5 methoxypinocembrin in DMSO-d6.

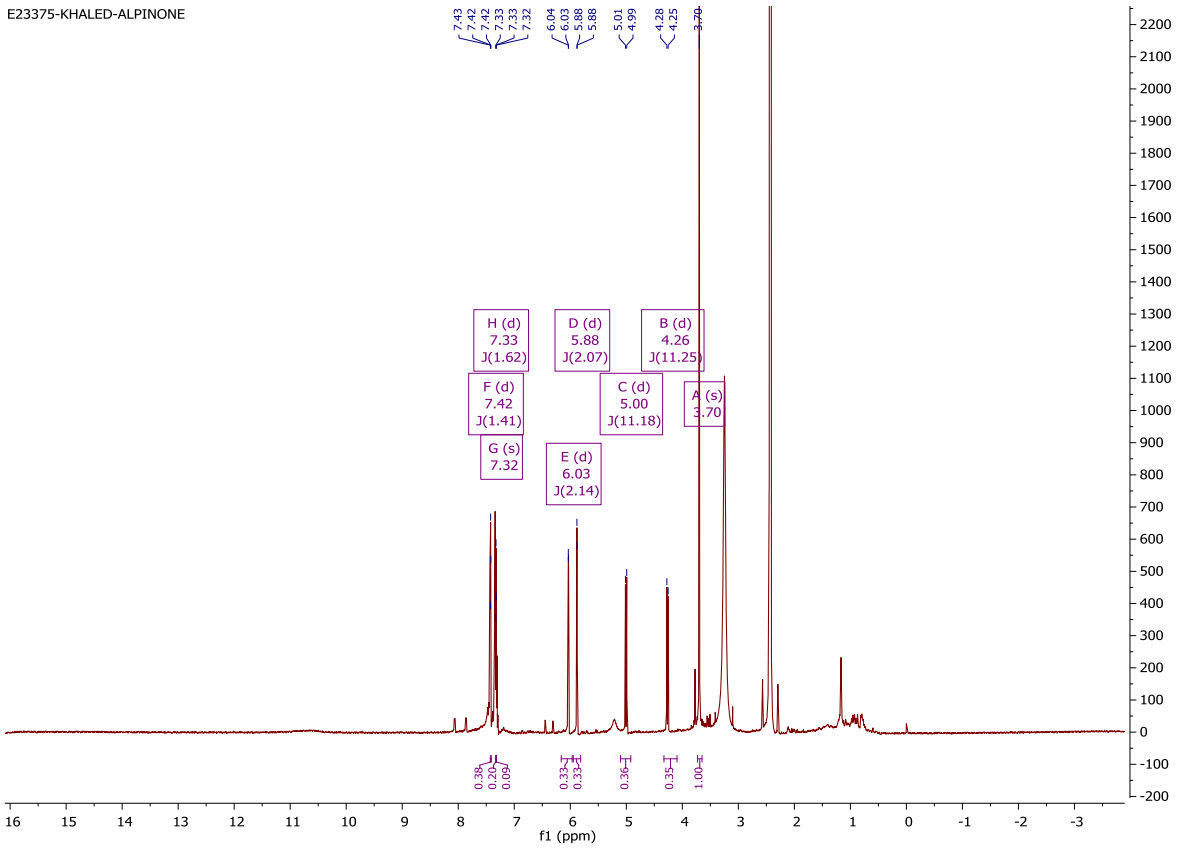


Figure 5.16a: Proton spectrum (DMSO-d6) for pinobanksin (4)

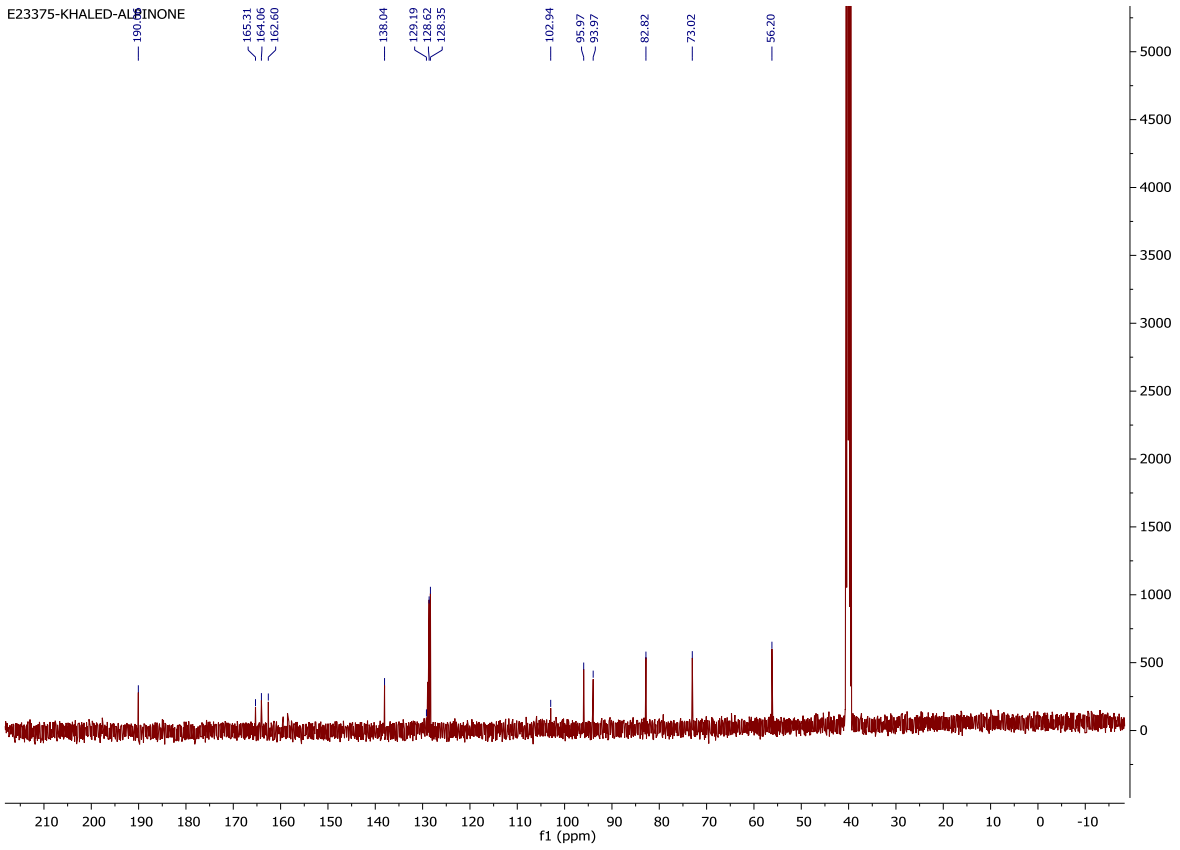


Figure 5.16b: 13Carbon spectrum (DMSO-d6) for pinobanksin (4)

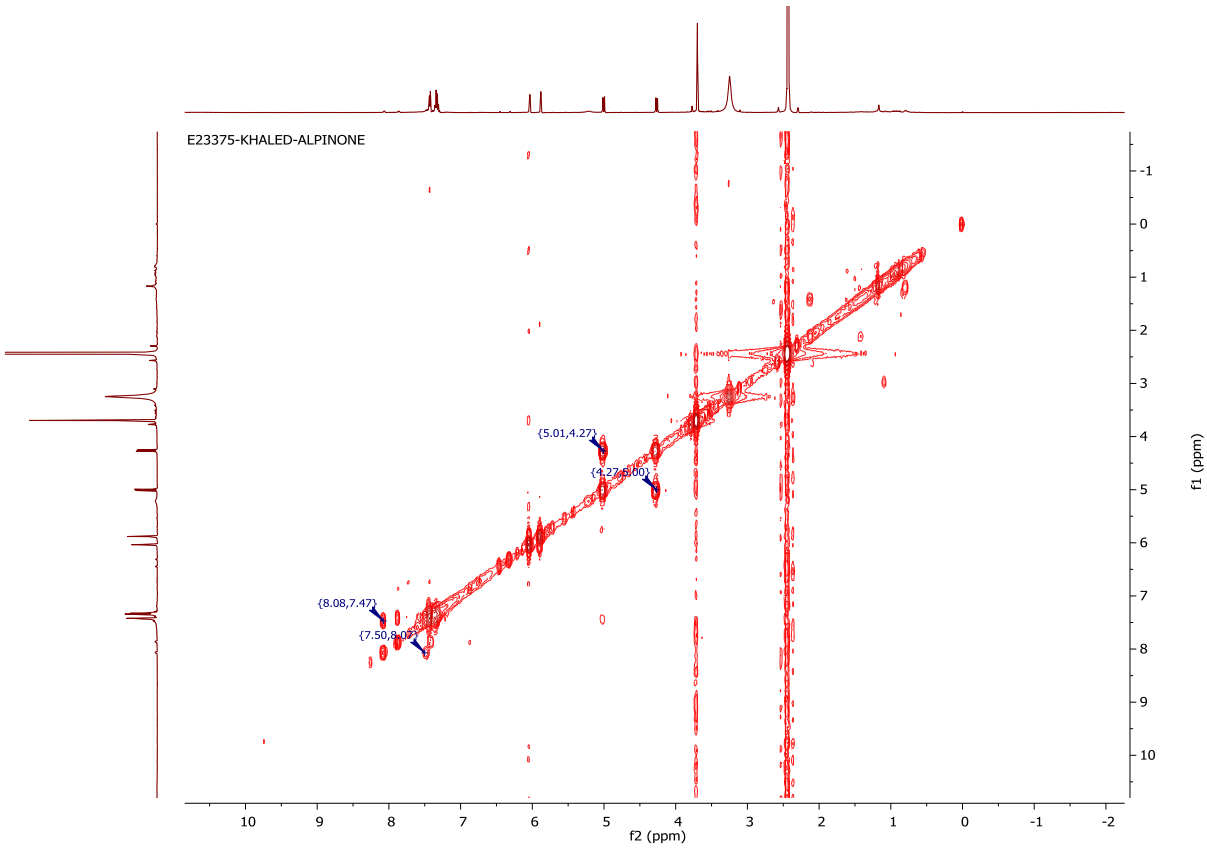


Figure 5.16c: COSY spectrum (DMSO-d6) for pinobanksin (4)

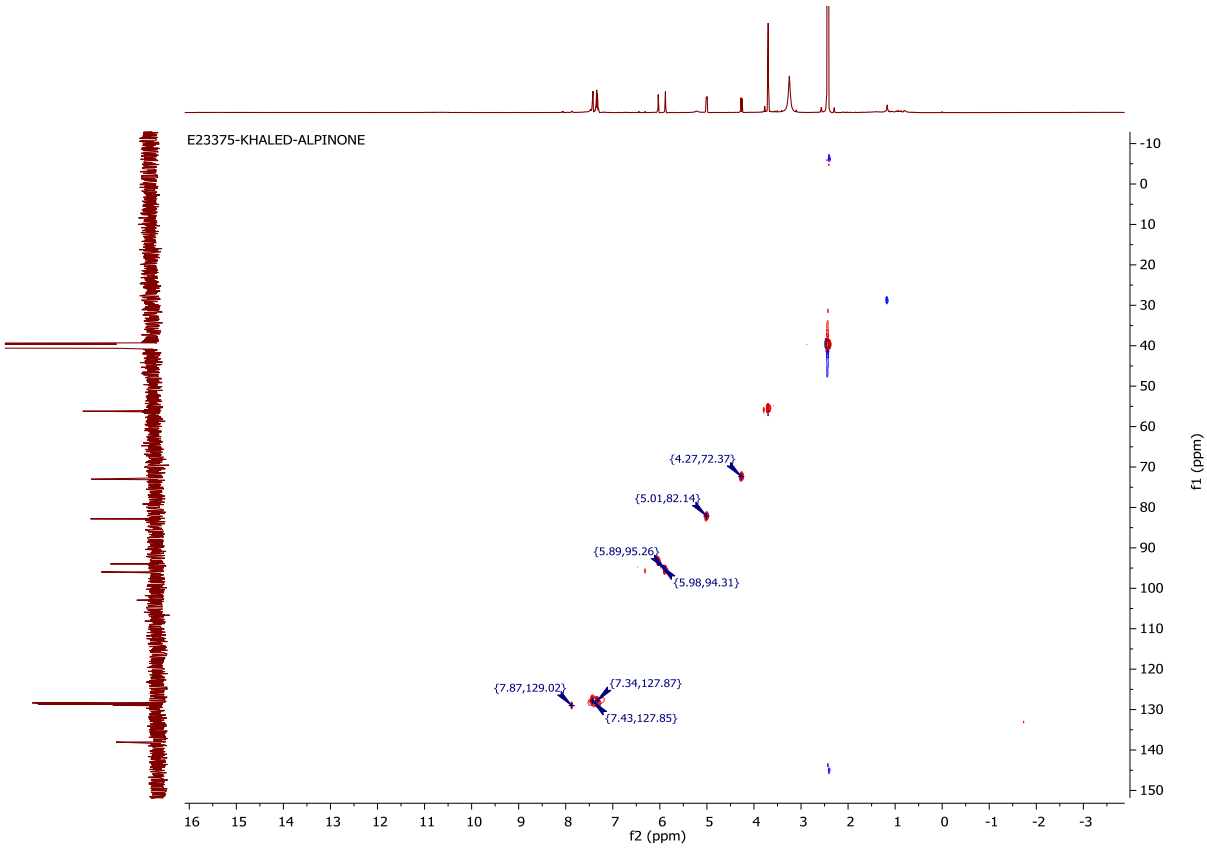


Figure 5.16d: HSQC spectrum (DMSO-d6) for pinobanksin (4)

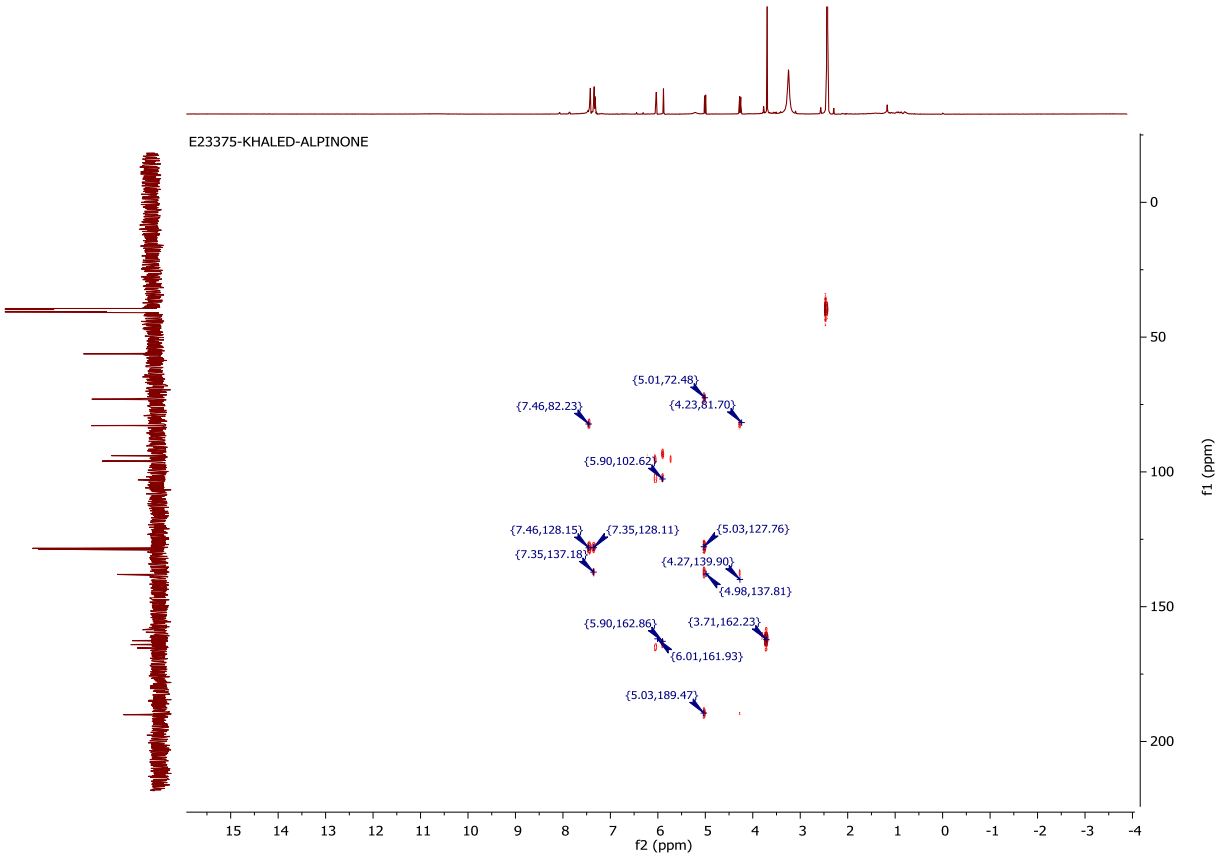


Figure 5.16e: HMBC spectrum (DMSO-d6) for pinobanksin (4)

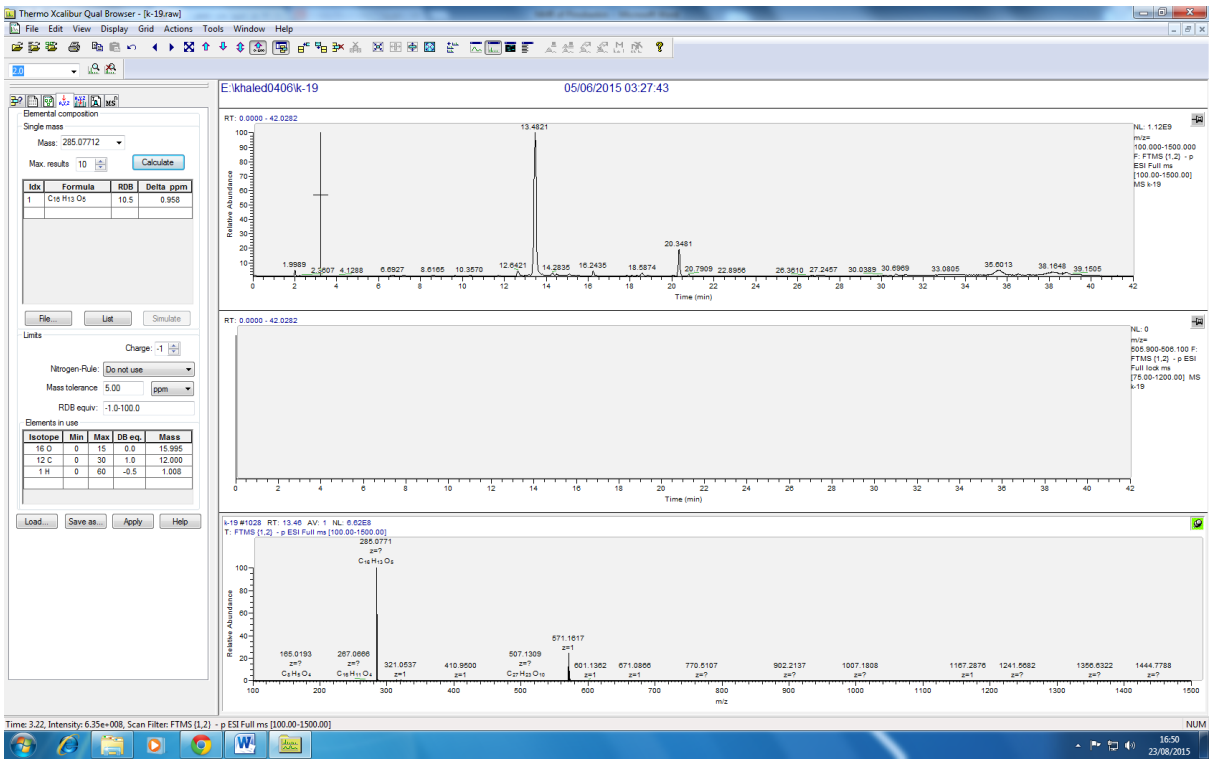
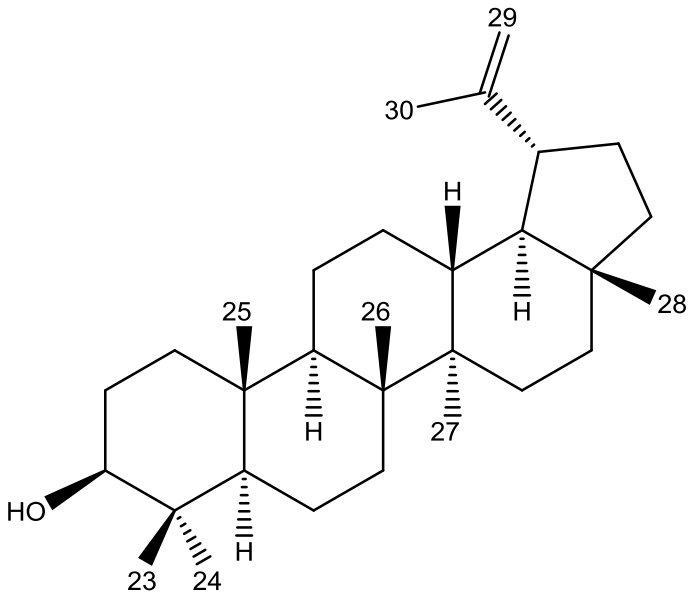


Figure 5.16f: Mass spectrum of 3-hydroxy-5 methoxypinocembrin.(4)

Characterisation of fraction KL-1 as Lupeol Isolated from Ghanian Propolis(5)

The compound showed two exomethylene protons at δ_H (ppm) 4.62 (d, J = 2.5) and 4.55 (d, J = 1.9) for H-29a and H-29b respectively, an oxymethine proton at 3.12 (dd, J = 11.38, 5.03) for proton H-3. Other slightly deshielded protons were H-19 at δ 1.29 (td, J = 11.2, 5.9), H-21 at 1.22 (m). There were signals for seven methyl groups between 1.67 and 0.75 ppm. The carbon spectrum showed signals for 30 carbon atoms including two olefinic carbon signals at δ_C (ppm) 150.8 (C-20) and 110.2 (C-29) and an oxygenated carbon at 80.1 (C-3). Comparison of its spectral data with literature reports (Igoli and Gray, 2008) confirmed the compound to be lupeol.



LUPEOL

	Chemical shifts	
Position	^{1}H δ ppm (mult, *J* Hz)	^{13}C δ ppm
1	0.97, 1.61	38.6
2	1.24	29.7
3	3.12 (dd, *J* =11.4, 5.0)	80.1
4	-	40.1
5	0.70	55.3
6	1.34, 1.50	18.2
7	1.38	34.3
8	-	40.8
9	1.23	50.3
10	-	37.2
11	1.34	20.9
12	1.54	23.7
13	1.58	38.0
14	-	43.0
15	1.5	27.6
16	1.38, 1.44	35.5
17	-	42.8
18	2.26	48.2
19	1.29	47.9
20	-	150.8
21	1.22	30.3
22	1.17, 1.38	40.8
23	0.93 (s)	28.0
24	0.72 (s)	14.4
25	0.84 (s)	16.2
26	1.00 (s)	16.2
27	0.92 (s)	15.7
28	0.77 (s)	17.8
29	4.55, 4.62	110.2
30	1.82	19.4

Table 5.12: Proton and 13Carbon chemical shift assignments for lupeol

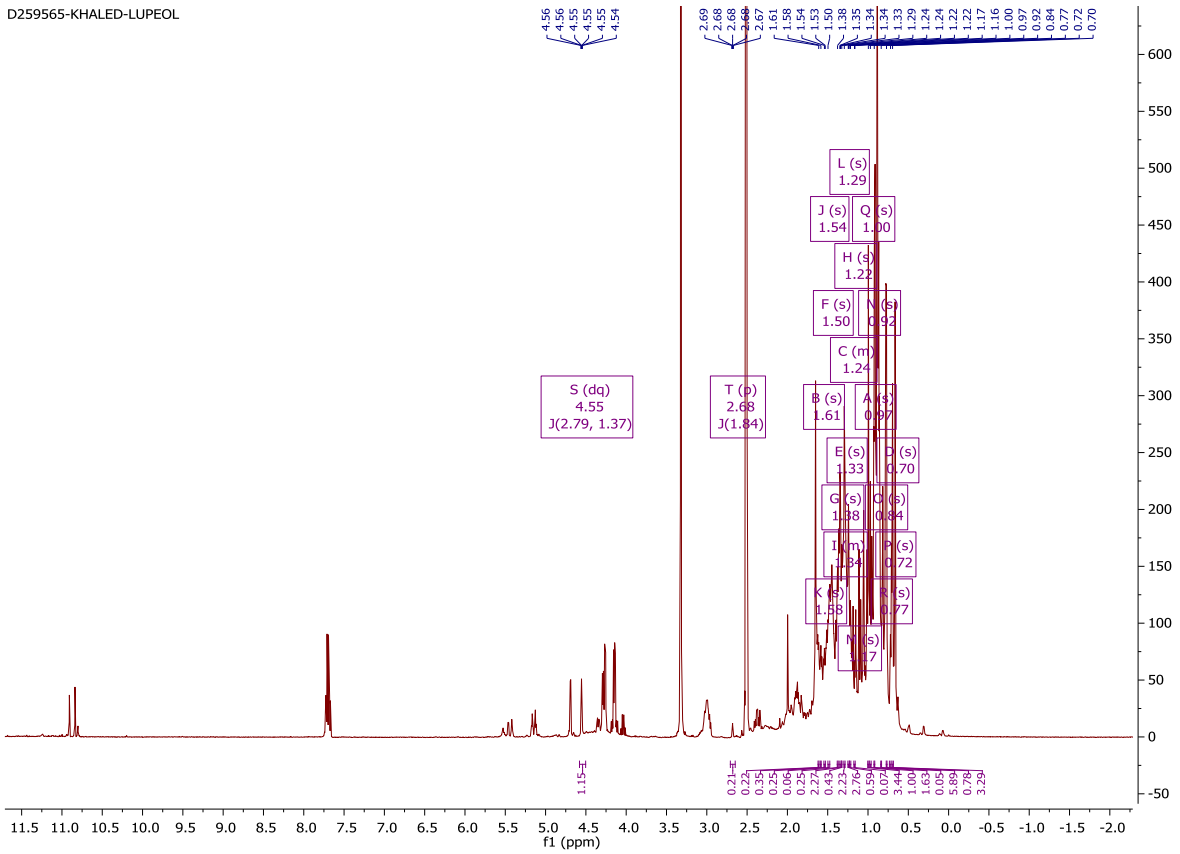


Figure 5.17a: Proton spectrum (DMSO-d6) for lupeol (5)

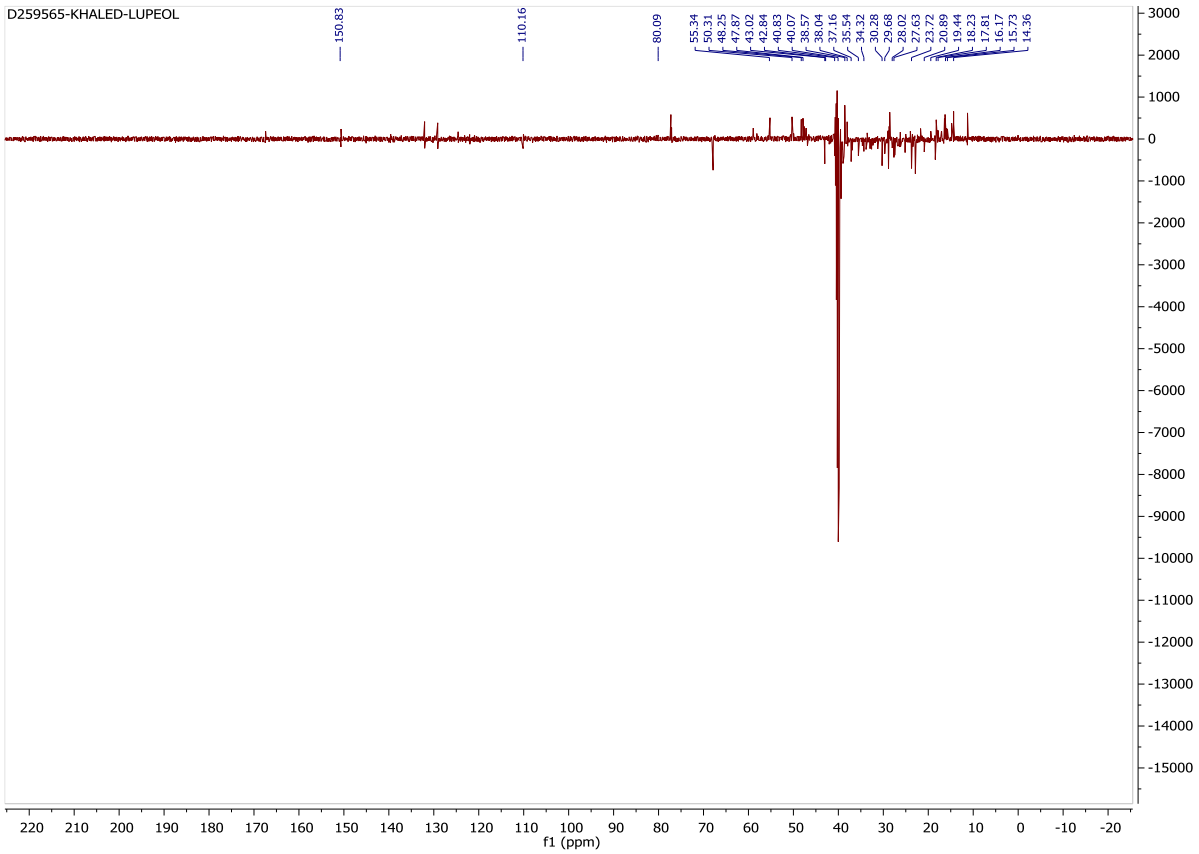
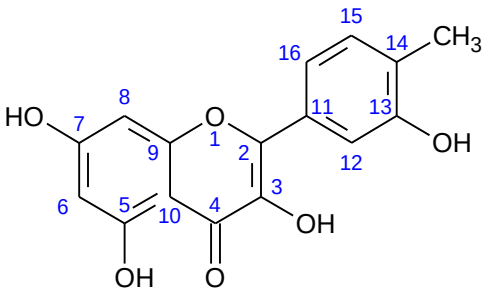


Figure 5.17b: 13Carbon spectrum (DMSO-d6) for lupeol (5)

Characterization of fraction Q1 as Quercetin Isolated from Ghanian Propolis

The proton spectrum of the compound showed a H-bonded hydroxyl proton signal at δ_H 12.49 ppm typical of a 5-OH substituted flavonoid. Two meta coupled proton signals observed at δ_H 6.19 (d, *J* = 2.0) and 6.41 (d, *J* = 1.99) were typical of a 5,7-disubstituted ring A of a flavonoid. Three aromatic proton signals were observed at 7.55 (1H, dd, *J* = 8.47, 2.21, H-6`), 6.89 (1H, d, *J* = 8.52, H-5`) and 7.68 (1H, d, *J* = 2.2, H-2`) for a tri-substituted benzene ring and this must be from the ring B of the flavonoid (figure 5.19a). A proton singlet usually observed for the H-3 of a flavone was not found in the spectrum similarly, no methoxy protons were observed, hence the

compound must be a pentahydroxy substituted flavone and this was confirmed by the five hydroxyl signals observed in the spectrum of the compound. From these data, the compound was inferred to be quercetin and its ^{13}C and 2D (COSY, HSQC and HMBC) NMR spectra were used to confirm the structure as follows: Long range (HMBC) couplings (^{3}J and ^{2}J) from the 5-OH proton identified C-5, C-6 and C-10, while correlations from the 3-OH identified C-2, C-3, C-4. Other correlations and couplings were as expected thus the compound was identified as 3, 4', 5, 5' 7-pentahydroxyflavone (quercetin) and confirmed by comparison of its NMR chemical shift assignments with literature reports (Kyriakou *et al* (2012), Sikorska and Matlawska (2000) The high resolution mass spectrum of the isolated compound gave the elemental composition $C_{15}H_9O_7$ in negative ion mode which corresponds to the elemental composition of quercetin.



Quercetin (**6**)

Position	Experimental values		Literature values*	
	^{1}H δ ppm	^{13}C δ ppm	^{1}H δ ppm	^{13}C δ ppm
1	-	-		
2	-	147.2		
3	-	136.1		
4	-	176.3		
5	-	161.1		
6	6.19 (d, *J* = 2.0 Hz)	98.7		
7	-	164.3		
8	6.41 (d, *J* = 1.99 Hz)	93.8		
9	-	156.5		
10	-	103.6		
1’	-	122.4		
2’	7.68 (d, *J* = 2.2 Hz)	115.5		
3’	-	145.5		
4’	-	148.2		
5’	6.89 (d, *J* = 8.52 Hz)	116.1		
6’	7.55 (dd, *J* = 8.47, 2.21 Hz)	120.4		
3-OH	9.34 (s)	-		
5-OH	12.49 (s)	-		
7-OH	10.77 (s)	-		
3’-OH	9.29 (s)	-		
4’-OH	9.58 (s)	-		

Table 5.13: Chemical shift assignments for Quercetin (6)

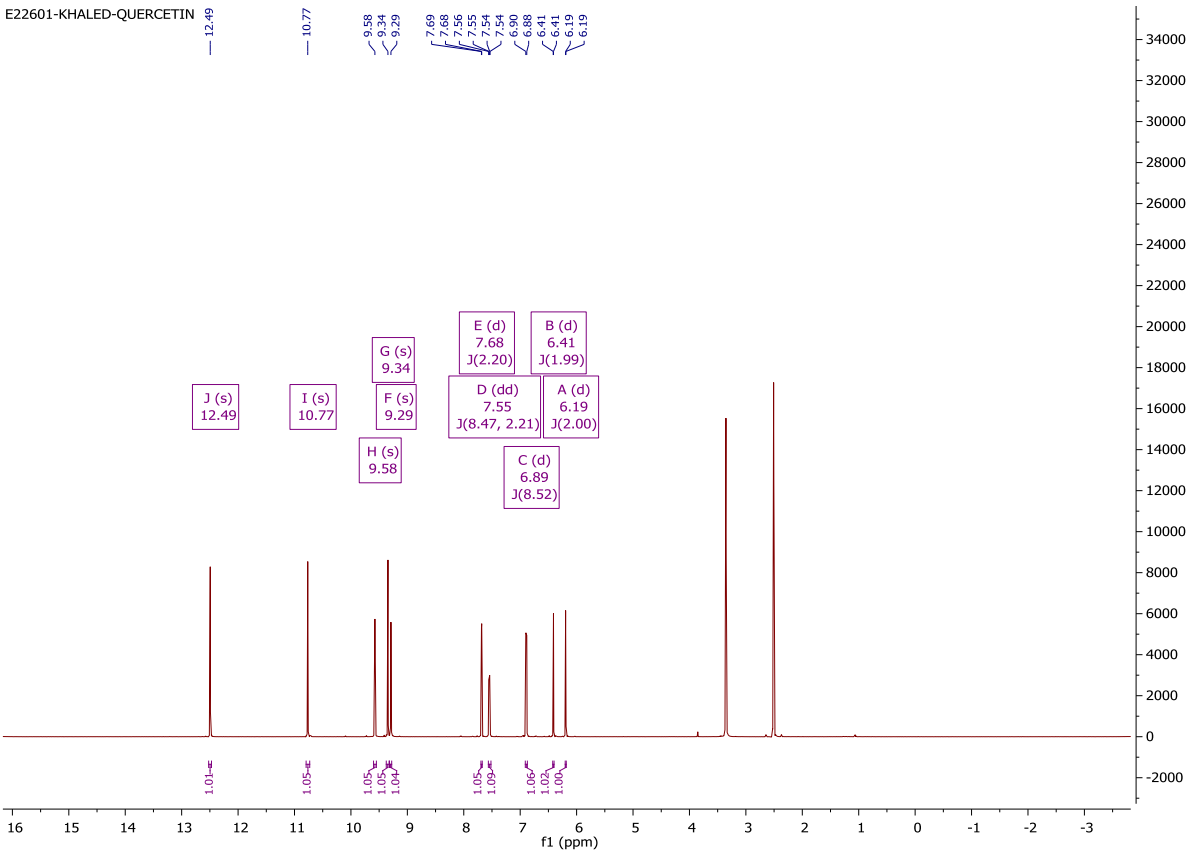


Figure 5.18a: Proton spectrum (DMSO-d6) for quercetin (6)

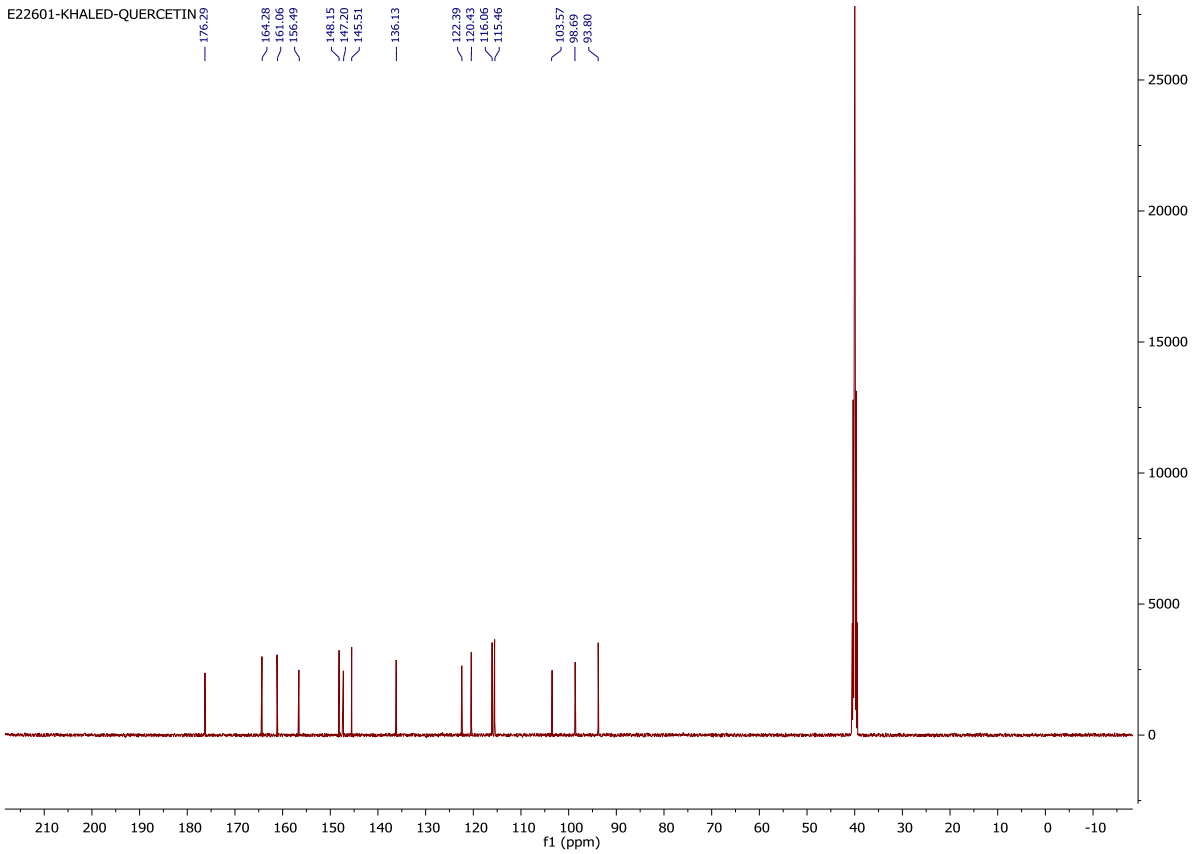


Figure 5.18b: 13Carbon spectrum (DMSO-d6) for quercetin (6)

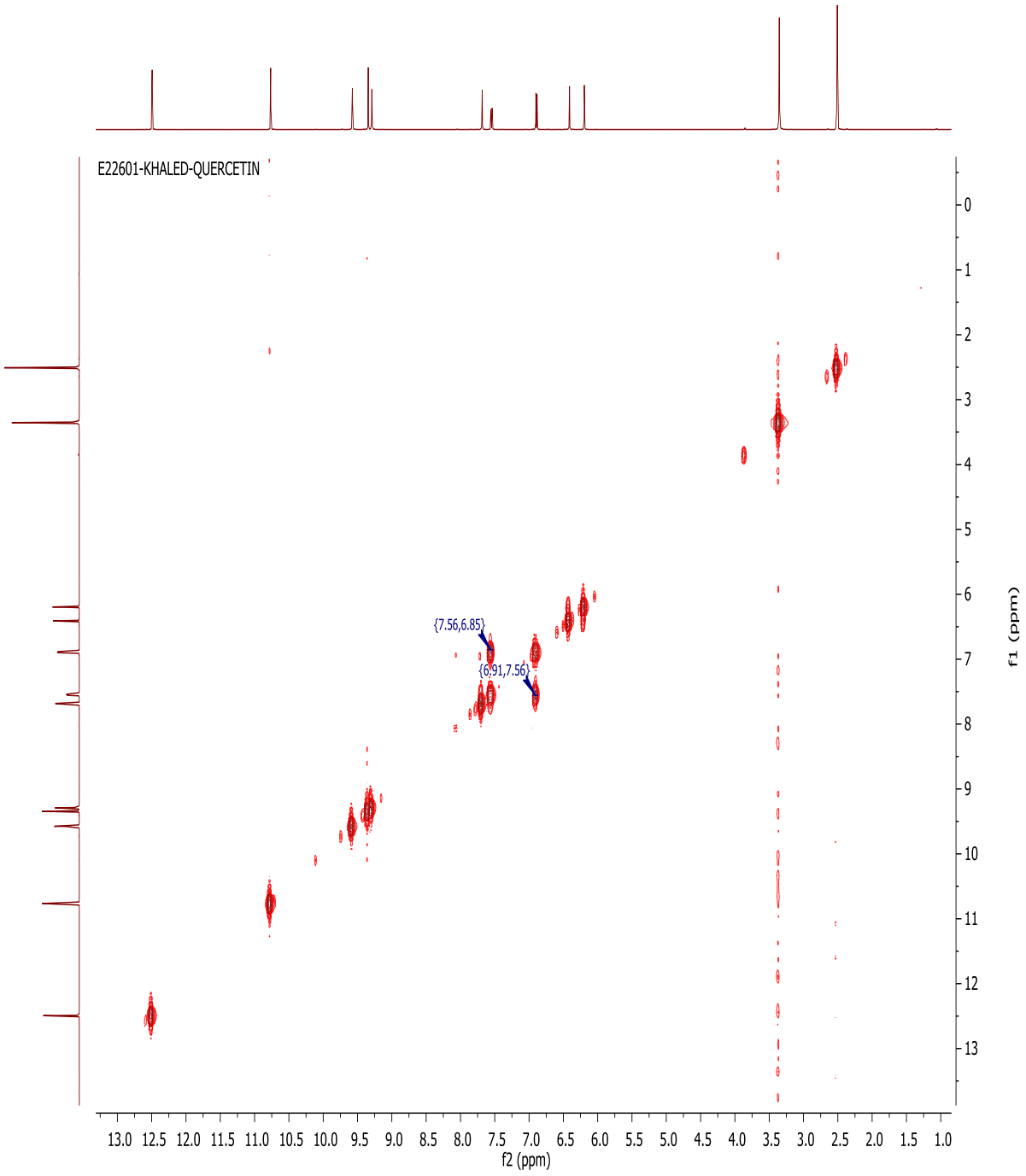


Figure 5.18c: COSY spectrum (DMSO-d6) for quercetin (6)

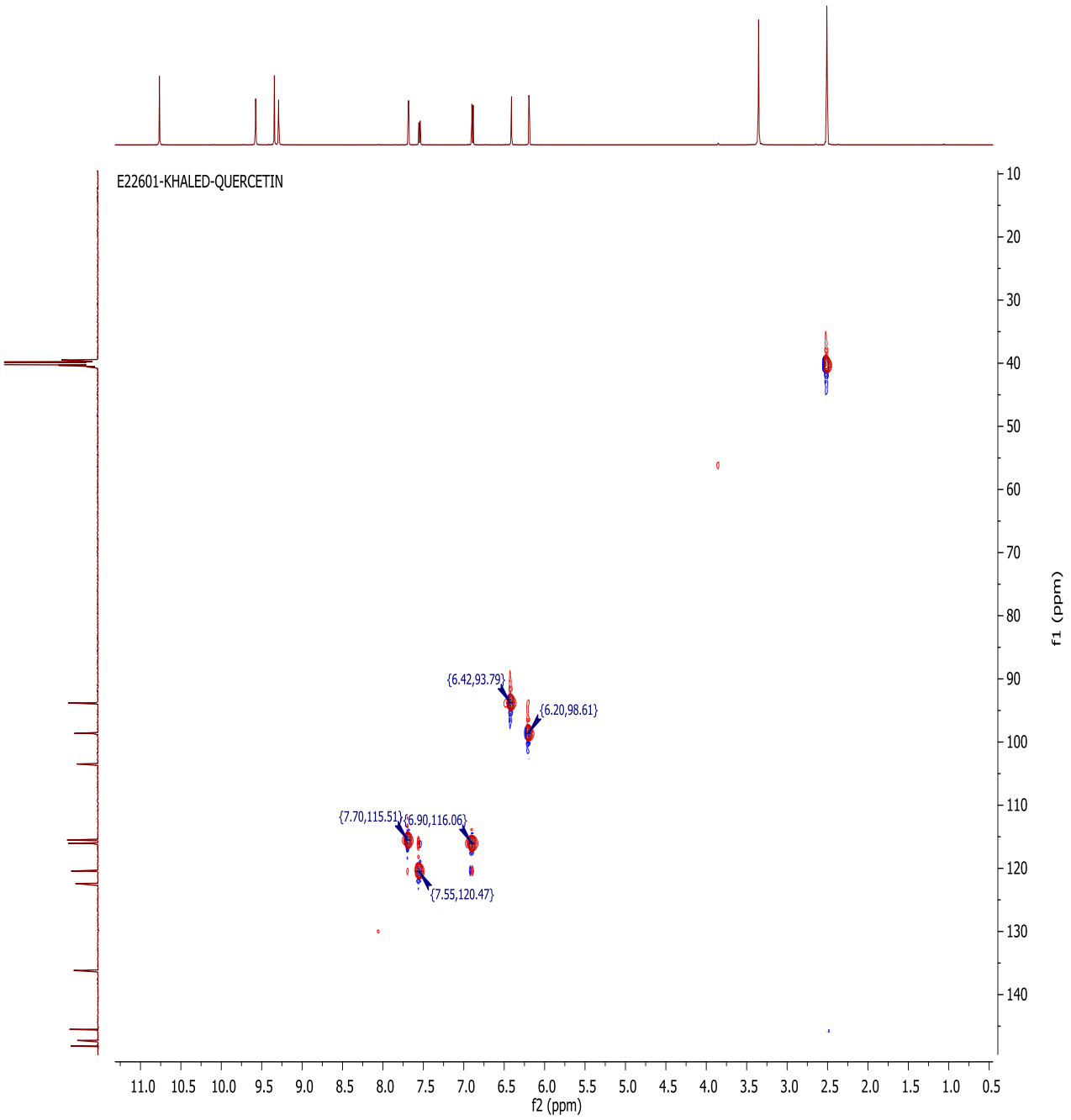


Figure 5.18d: HSQC spectrum (DMSO-d6) for quercetin (6)

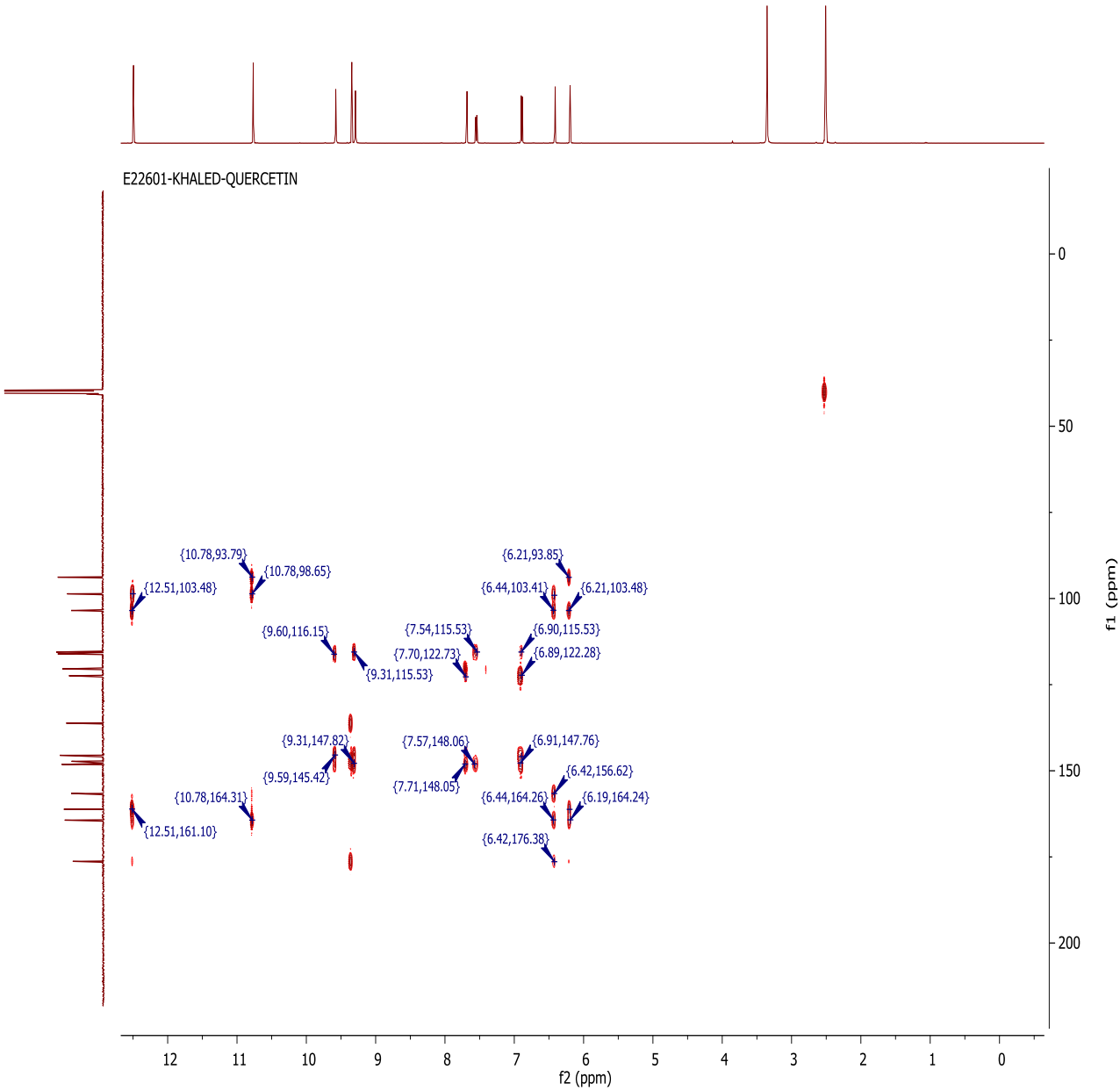


Figure 5.18e: Proton spectrum (DMSO-d6) for quercetin (6)

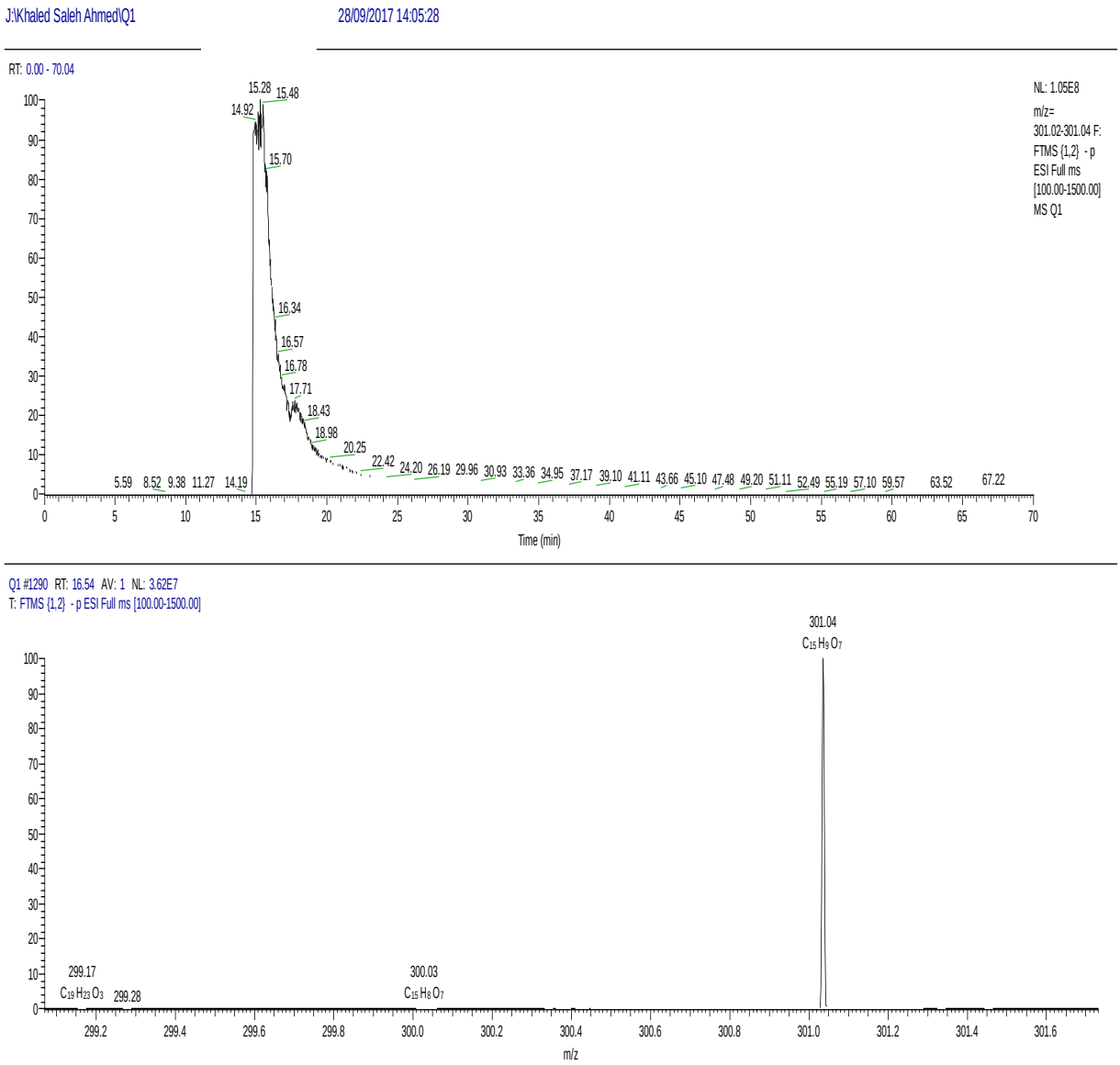


Figure 5.18f: Mass spectrum for quercetin (6)

CHAPTER SIX: DISCUSSION

Evidence abounds that propolis samples from different locations or geographical regions differ in their chemical compositions. This is mainly due to the plant sources the bees visit to collect pollens and exudates to make propolis. This is confirmed in Table 6.1 showing propolis from different geographic regions and their principal plant sources of chemical compounds (Bankova, 2005).

A second reason could be from the particular pressure being faced by the bees in the hive due to microbial and parasitic infestation. As colony collapse can occur if such infestations are not prevented or controlled, the bees have no choice but to search for really active phytochemicals to control such infestation. In this study, propolis from various parts of the UK, Scotland, Indonesia and Africa were investigated for their constituents and variation in the constituents. The samples collected from around the UK showed galangin, caffeic acid phenethyl ester, p-coumaric acid, pinobanksin, pinocembrin methyl ether, pinobanksin acetate, caffeic acid cinnamyl ester,chrysin and methyl pinobanksin to be the major constituents. The samples from Scotland showed similar constituents with some quercetin and ferulic acid derivatives. The samples were grouped into six based on the PCA and HCA analysis. The samples from tropical regions generally were very different from the temperate propolis and included many prenylated flavonoids and triterpenes.

Geographical origin	plant source
Bulgaria	populous nigra,P.italic
Albania	populous nigra
Bulgaria	populous tremula
Mongolia	populous suaveolens
USA (mainland)	populous fremontii
USA (Hawaiian Islands)	plumeria acuminate, plumeria Acutifolia
United Kingdom	populous curamericana
Hungary	Betula, populous, pinus, prunus.
Poland	Betula, Alnus spp.
Equatorial regions	Delchampia spp.
Equatorial regions	clusia spp.
Australia	xanthorroea
North Temperate Zone	poplar, birch,alder,elm,beech and Conifer
Europe, North America, nontropic regions of	poplar spp of section
Asia (poplar propolis)	Aigeirose, most often nigra L
Russia (birch propolis)	Betula verrucosa Ehrh
Brazil (green alecrim propolis) B.dracuculifoia	Bacharis spp.predominantly
Cuba, Venezuela	clusia minor, clusia spp.
South Brazil (typ3).Argentine, and Uruguay	populous Alba
Brazil (type 6 from north-eastern Brazil)	Hyptis divaricate
Brazil (type13 from north-eastern Brazil)	Dalbergia ecastaphyllum

CONCLUSION

Metabolomics and LC-MS/HRMS analysis of propolis samples from several regions of the UK, Scotland and Indonesia showed the variations in chemical constituents. The results were consistent with literature reports and previous studies of a similar nature. The level of confidence for identifying some of the compounds was very high as their spectra MS and NMR compared to those of authentic chemical standards analysed under identical analytical conditions. For the putative and unidentified constituents, the level of confidence was not as high as although they were unidentified and unclassified, these metabolites could still be differentiated and quantified based upon their mass spectral data. Others were putatively identified compounds based upon their spectral similarity with reported spectral data, but without reference to authentic chemical standards. There remains much work to do in mapping the composition of this complex material, understanding its role in the bee hive and testing its properties in the treatment of human diseases.

FUTURE WORK

Chromatographic separation and isolation of the detected but unidentified compounds should be carried out. More samples of propolis from Indonesia and other regions nearby could be screened for their constituents and importantly for their bioactivities.

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